Analysis of particle size and lipid composition as determinants of the metabolic clearance of human high density lipoproteins in a rabbit model

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Abstract Hypertriglyceridemia is commonly associated with triglyceride (TG) enrichment of high density lipoprotein (HDL) and reduction in HDL cholesterol and apolipoprotein A-I levels. We have recently reported that lipolytic modification of TG-rich HDL, which reduces particle size, enhances its clearance from the circulation. In the present study, we examined the role of particle size and lipid composition in determining the metabolic clearance of human HDL, in the absence of substantial in vivo modification of the particle by hepatic lipase. The rabbit, which has a very low hepatic lipase activity, was used for this purpose. Plasma fractions d < 1.21 g/ml were first isolated by ultracentrifugation from fasting humans with normal (NTG, n = 6, mean plasma TG concentration = 1.26 ± 0.21 (SEM) mmol/l) or elevated plasma TG levels (HTG, n = 5, TG = 4.49 ± 0.65 mmol/l). Small and large HDL particles were separated by gel filtration chromatography and were labeled with either ^{125}I or ^{131}I. Large HDL were cleared more rapidly than small HDL in 10 out of 11 studies ($P = 0.006$). There was, however, no difference in the fractional catabolic rate (FCR) of large HDL isolated from NTG versus from HTG subjects or in the FCR of small HDL from NTG versus HTG individuals. There was also no correlation between the TG content of HDL and its FCR. In summary, large, lipid-rich human high density lipoproteins (HDL) are cleared more rapidly than small human HDL in rabbits. These results, combined with our previous observation, also support the hypothesis that triglyceride enrichment of HDL, in the absence of substantial lipolytic modification, is not sufficient to enhance its clearance from the circulation.—Lamarche, B., K. D. Uffelmann, G. Steiner, P. H. R. Barrett, and G. F. Lewis. **Analysis of particle size and lipid composition as determinants of the metabolic clearance of human high density lipoproteins in a rabbit model. *J. Lipid Res.* 1998.** 39: **1162–1172.**

Supplementary key words kinetics • hepatic lipase • metabolism • hypertriglyceridemia • rabbits • radioisotopes

The strong inverse relationship between high density lipoprotein (HDL) cholesterol levels and the risk of coronary heart disease (1–5) has stimulated research to identify the mechanisms responsible for HDL cholesterol lowering. Plasma HDL cholesterol concentrations are affected by a number of environmental, physiological, and genetic factors (6, 7). Although the lipid and protein components of HDL may be cleared from the circulation through different mechanisms, production and clearance rates of apolipoproteins (apo) A-I and A-II, the protein moieties of HDL, have a significant impact on the number of particles in the circulation and hence, on plasma HDL cholesterol concentrations.

The clearance of apoA-I and A-II has previously been investigated in human studies as well as in animal models. Brinton Eisenberg and Breslow (8, 9) have shown that the fractional catabolic rate (FCR) of HDL apoA-I and A-II, rather than the production rate, is the most important determinant of HDL cholesterol and apoA-I levels in men, thus confirming earlier observations (10). Particle size may be one important determinant of HDL clearance from the circulation. Brinton and coworkers (8, 11) reported that the HDL cholesterol/A-I + A-II ratio, a crude but not direct measure of particle size, may represent one of the primary determinants of apoA-I FCR, implying that small HDL particles may be cleared more rapidly than large ones. Results from a study in rabbits (12) have shown that human HDL containing apoA-I only (LpAI) of higher density (HDL_3) were cleared at a faster rate than

Abbreviations: apo, apolipoprotein; LpAI, lipoproteins containing apoA-I only; LpAI, AII, lipoproteins containing both apoA-I and apoA-II; TG, triglyceride; FFA, free fatty acid; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; CETP, cholesteryl ester transfer protein; LCAT, lecithin:cholesterol acyltransferase; FCR, fractional catabolic rate; SEM, standard error of the mean; CV, coefficient of variation; EDTA, ethylene diamine tetraacetic acid; SDS, sodium dodecyl sulfate; PAGGE, polyacrylamide gradient gel electrophoresis; PAGE, polyacrylamide gel electrophoresis.

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HDL of lower density (HDL_2). Presuming that HDL_3 particles are smaller than HDL_2, the authors concluded that HDL size may be an important determinant of its catabolic rate, with small particles being cleared faster than large.

Although an increased apoA-I FCR has been documented in hypertriglyceridemic individuals with reduced plasma HDL cholesterol concentrations (11), little is known about the precise mechanisms underlying the accelerated catabolism of HDL apoA-I and A-II in hypertriglyceridemic states. A study by Horowitz et al. (13) has suggested that apoA-I may be loosely bound to triglyceride-rich HDL and that the lipolytic modification of these large, triglyceride-enriched HDL renders the apoA-I even more susceptible to exchange or clearance by the kidney, thus providing one potential mechanism underlying the strong inverse association between plasma HDL cholesterol and triglyceride levels (14–16). In accordance with this hypothesis we have recently reported that small, lipolytically modified human HDL particles were cleared more rapidly than large, triglyceride-rich, non-lipolytically modified HDL when injected into rabbits (17). Taken together, these results emphasize the importance of the lipolytic modification of HDL by circulating lipases (mostly hepatic lipase), particularly in hypertriglyceridemia, in determining the rate at which HDL is cleared from the circulation.

The evidence available, therefore, suggests that a number of processes may be implicated in determining the rate at which HDL is cleared from the circulation. The present study was designed to compare the catabolism of small and large HDL specifically isolated according to size from fasting human subjects, in the absence of significant ongoing in vivo compositional changes attributable to lipolysis of the particles by hepatic lipase. In that respect, the rabbit is an adequate model because it has very low hepatic lipase activity (18). We also investigated the extent to which the triglyceride enrichment of HDL can affect the relationship between particle size and catabolic rate in this model by comparing the FCR of small and large HDL isolated from individuals with varying levels of plasma and HDL triglycerides.

METHODS

Eleven subjects participated in this study (age ranging from 21 to 65 years and body mass index from 23.5 to 37.9 kg/m^2). Participants from whom large and small HDL were isolated were selected to cover a range of plasma triglyceride concentrations. The mean triglyceride level was 2.7 ± 0.6 mmol/l. As shown in **Table 1**, six of the participants had triglyceride levels below 2.0 mmol/l (arbitrarily defined as the normoTG group) and five were characterized as having moderate hypertriglyceridemia (plasma TG levels between 2.0 and 5.5 mmol/l, the hyperTG group). One participant (subject #10) had mild untreated Type 2 diabetes while the remainder did not have diabetes. None of the participants was a smoker nor had been taking any medication known to affect lipid metabolism for at least 6 months prior to entrance into the study. Subjects with systemic illness, malignancy, renal or hepatic disease were excluded from the study. Informed written consent was obtained from all participants in accordance with the guidelines of The Toronto Hospital Human Subjects Review Committee. All human studies were conducted in The Toronto Hospital Clinical Investigation Unit. Animal protocols were reviewed and approved by the Animal Ethics Committee of The Toronto Hospital, University of Toronto.

Isolation and labeling of small and large HDL particles

Subjects were instructed to consume their regular diet until 18:00 and then to fast overnight. At approximately 8:00 an intravenous sampling catheter was inserted into a forearm vein and 100 ml of fasting blood was drawn to isolate HDL. Blood samples for HDL were drawn into tubes containing 1.2 g/l sodium EDTA. Whole plasma was first centrifuged at a density of 1.21 g/ml for 48 h at 40,000 rpm, 4°C in a Beckman 70.1 Ti rotor and then dialyzed overnight in a buffer containing 0.15 m NaCl, 0.01 m Tris-base and 5 mm EDTA, pH 8.0 (hereafter referred as Tris buffer). Small and large HDL were then isolated by gel filtration chromatography of the $d < 1.21$ g/ml fraction on Sephacryl 300 using XK 26/70 columns (Pharmacia), at an elution speed of 0.5 ml/min, 1.5 ml/fraction, in a 0.1 m NaH_2PO_4, 0.5 M NaCl, 0.02% NaN_3, pH 7.0, buffer. Using this approach and as shown in **Fig. 1**, which depicts the elution profile of the $d < 1.21$ g/ml plasma fraction of one participant, the apoB-containing very low to low density lipoproteins (VLDL to LDL) and HDL were distinctly separated from one another (top panel). Selected fractions of large and small HDL (indicated by solid circles in Fig. 1) were pooled and dialyzed extensively (i.e., overnight at 4°C) in the Tris buffer. Also shown in Fig. 1 is the densitometric image of the iso-

TABLE 1. Clinical characteristics of the 11 human subjects who participated in this study

Human Subject	Age	Weight	BMI	Triglycerides	Group
	yrs	*kg*	*kg/m²*	*mmol/I*	
1	42	97.3	28.7	1.82	NormoTG
2	65	72.4	26.9	1.61	NormoTG
3	60	88.4	25.8	0.86	NormoTG
4	21	83.4	25.5	0.75	NormoTG
5	27	72.0	23.5	0.78	NormoTG
6	22	80.2	28.4	1.75	NormoTG
7	42	91.8	29.0	5.53	HyperTG
8	41	80.8	37.9	5.46	HyperTG
9	50	90	27.2	3.45	HyperTG
10	57	111.3	31.3	2.44	HyperTG
11	46	66.5	26.0	5.54	HyperTG
Mean ± SEM	43.6 ± 4.5	85.0 ± 3.9	28.2 ± 1.2	2.73 ± 0.59	

BMI, body mass index (weight/height²). NormoTG and hyperTG: individuals with normal or elevated plasma triglyceride levels, respectively, based on an arbitrary cut-point of 2.0 mmol/1.

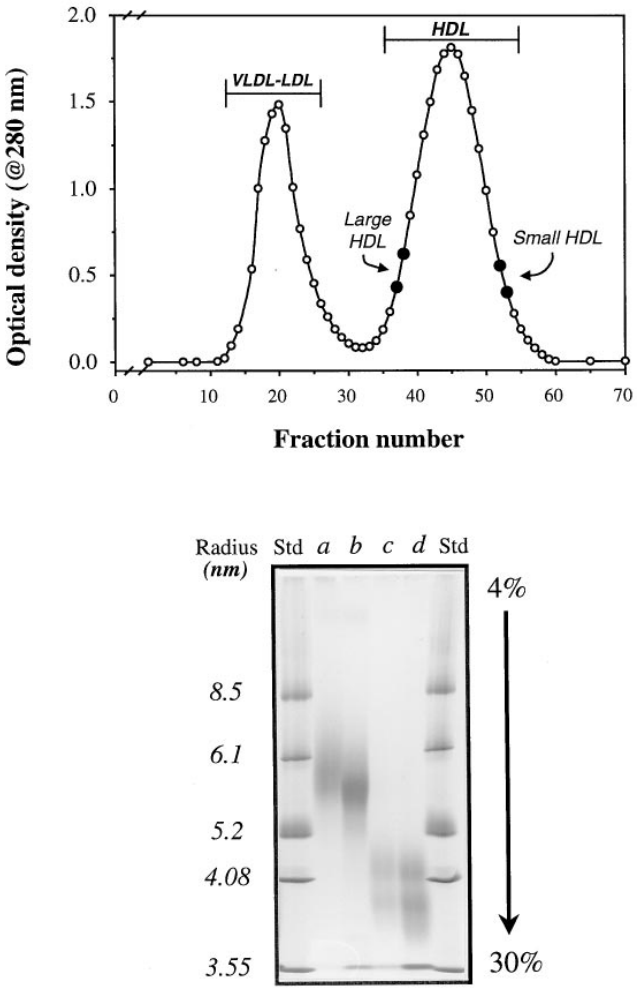

Fig. 1. Gel filtration chromatography of the d < 1.21 g/ml plasma fraction on Sephacryl-300 (top panel). The first peak represents the apoB-containing lipoproteins (VLDL to LDL) and the second peak corresponds to the HDL fraction. The fractions pooled for small and large HDL are indicated by black symbols. The lower panel shows the 4–30% non-denaturing gradient gel electrophoresis of small and large HDL particles. As shown on the figure, there was a clear separation of particles by size, with no overlap between large and small HDL. STD, molecular weight standards. Lanes *a* and *b* represent large HDL (mean radius 5.10 nm). The injectates in lanes *c* and *d* are small HDL particles (mean radius = 4.01 nm). The injectate in lanes *a* and *c* are iodinated while those in lanes *b* and *d* are unlabeled. The albumin found within the small HDL fraction (lane *b* and *d*, 3.55 nm) was completely removed from the injectate after washing the labeled HDL at 1.21 g/ml (lane *d*).

lated large and small HDL particles on 4–30% PAGGE (lower panel). There was no overlap in the size of large and small HDL using this method.

Between 0.5 and 1 mg protein of the small and large HDL fractions were alternately labeled using 500 μCi of ^{125}I and ^{131}I by a modification of the iodine monochloride method of McFarlane (19). Briefly, HDL was first added to a mixture of approximately 20 μmol ICl/μmol HDL protein and 500 μCi of radioactive iodine. The mixture was then eluted through a 0.2 m glycine-Sephadex G-50 column, pH 10 (Pharmacia Biotech) to allow for the iodination of HDL and the separation of the HDL-bound radioactive iodine from the free iodine. The radiolabeled HDL was then eluted through a saline-Sephadex G-50 column to bring the pH of the solution containing the radiolabeled HDL back to physiological range (pH 7.4). An aliquot of unlabeled HDL (1.0 to 2.0 ml, or approximately 300 μg of protein) was added to the radioiodinated HDL fractions as a cold carrier and HDL tracers were washed at d 1.21g/ml, 39,000 rpm, 4°C in a 70.1 Ti rotor for 48 h, followed by an overnight dialysis in Tris buffer at 4°C. The composition of each radiolabeled tracer was measured using commercially available enzymatic assay kits as described below.

HDL particle size determination

HDL size was measured by non-denaturing polyacrylamide gradient gel electrophoresis (PAGGE) (gels purchased from Dr. David Rainwater, Southwest Foundation for Biomedical Research, San Antonio, Texas) as previously described (20). Thirty micrograms of protein was applied as a mixture by volume with three parts sample and one part solution of 40% sucrose with 0.01% bromophenol blue. The gels were fixed with 12% trichloroacetic acid, stained with Coomassie G250 in perchloric acid (0.1% stain, 5% perchloric acid), destained overnight, and stored in 7% acetic acid. HDL particle size was determined from the migration of standard molecular weight proteins of known diameter (HMW Calibration Kit, Pharmacia, Pistacataway, NT). Peak and mean radius of small and large HDL particles were determined by densitometric scanning (Image Master DTS densitometer with Imagemaster computer software, Pharmacia LKB, Uppsala, Sweden) based on relative migration distance (R_f) of standards and using two distinct approaches. First, the estimated radius of the major peak in each scan was identified as the HDL peak particle size. A mean (or weighted) HDL particle size was also calculated using a modification of the method descibed by Li et al. (21). The mean HDL particle size for each subject was obtained by multiplying the size of each band by its fractional area. This mean HDL particle radius (in nm) therefore combines the HDL size distribution as well as the relative concentration of each HDL band (21). The integrity of the radiolabeled small and large HDL tracers was monitored on 4–30% PAGGE.

Laboratory measurements

Cholesterol was measured using the CHOD-PAP enzymatic colorimetric kit (Boehringer Mannheim GmbH Diagnostica, Montreal). Cholesteryl ester was measured using the cholesterol kit after degrading free cholesterol in the samples with a mixture of cholesterol oxidase, peroxidase, phenol, Triton-x and phosphate buffer (22). Free cholesterol was calculated as the difference between total cholesterol and cholesteryl ester. Protein was measured by the technique described by Lowry et al. (23). Triglycerides were measured as esterified glycerol using an enzymatic colorimetric kit (Boehringer Mannheim GmbH Diagnostica). Free glycerol was eliminated from the sample in a preliminary reaction followed by enzymatic hydrolysis of triglyceride with subsequent determination of the liberated glycerol by colorimetry. Free fatty acids were measured by an enzymatic colorimetric method relying on the acylation of CoA by the fatty acids (Wako Chemicals Industries, VA). Phospholipids were measured using a kit (Boehringer Mannheim GmbH Diagnostica). Apolipoproteins A-I and A-II were measured in total plasma and in the HDL fractions by electroimmunoassay. The sum of the concentrations of apoA-I and A-II in HDL was used as a crude marker of particle number.

Experimental protocol

Radiolabeled large and small HDL particles isolated from each human participant were injected separately 1 week apart, into a 3.5–5 kg New Zealand White rabbit. The order of injection of small and large HDL was alternated in each experiment. For practical purposes, the fraction labeled with ^{131}I was always in-

jected first. The two tracers were injected sequentially to minimize any potential confounding effect that simultaneous injection may have, such as, for example, exchange of label between large and small HDL. Because the quantity of tracers available for injection into rabbits was limited, we were not able to estimate the inter-assay coefficient of variation of the FCR by injecting the same tracer in consecutive experiments in the same rabbit. However, the inter-assay coefficient of variation was minimized due to the paired nature of the experiment. Data from a previous study suggest that the inter-assay coefficient of variation when using a similar protocol may be less than 10% (12). Between 5 and 10 μCi of the radiolabeled tracers was injected into the right marginal ear vein of a 3.5–5 kg male rabbit, sedated with INNOVAR-VET (0.13–0.17 mg/kg intramuscular, Janssen Pharmaceuticals, Mississauga, Ontario). Mean weight of the rabbits was 4.4 ± 0.2 kg. In the first seven rabbit experiments, 2 ml of blood was drawn from the opposite ear at 10 min and at 1, 2, 3, 4, 6, 24, 30 and 48 h. In the first seven experiments, the clearance of radiolabeled HDL over time was measured in three different fractions: 1, total plasma, 2, the HDL fraction (1.063 g/ml $< d <$ 1.21 g/ml); and 3, apoA-I isolated by SDS-PAGE. To measure the radioactivity in the HDL fraction, rabbit plasma was first separated and then adjusted to a density of 1.063 g/ml with solid KBr and spun for 5 h at 100,000 rpm at 16°C in an Optima TLX ultracentrifuge (Beckman Instruments, Palo Alto, CA) using a TLA 100.3 rotor. The top fraction was removed and the bottom fraction was adjusted to d 1.21 g/ml with solid KBr and spun for 5 h at 100,000 rpm, 16°C. The top HDL was washed for another 5 h, sliced, and dialyzed against 6 L of Tris buffer at 4°C for 24 h. To measure the radioactivity specifically associated with apoA-I, 1.0 ml aliquots of dialyzed HDL were delipidated as previously described (17) and left to dissolve overnight in 0.5 m phosphate buffer containing 1% SDS, 1% mercaptoethanol, pH 7.2. The samples were then run on 15% SDS-PAGE to isolate apoA-I. The apolipoproteins were stained overnight with 0.0025% R250 Coomassie Blue and destained for 2 h with 10% acetic acid and the A-I band was sliced from the gel. Aliquots of total plasma, of the HDL fraction, and the HDL apoA-I band were counted in a Beckman 5500 gamma counter. The apoA-I radioactivity in the gel was taken as a percent of the radioactivity found in that gel and readjusted back to total HDL radioactivity in cpm/ml of plasma. The recovery of radioactivity from the SDS gels compared to that applied to the gel ranged from 70% to 85% and was similar for large and small HDL particles. The radioactivity in the $d < 1.063$ g/ml as well as in the $d > 1.21$ g/ml fraction was also measured in these seven experiments. In four additional experiments, the clearance of radiolabeled HDL was determined using the radioactivity in the plasma only. In these experiments, plasma (1.5 ml) was sampled at 10 min and at 1, 2, 3, 4, 5, 6, 7, 24, 27, 30, 48, 51, and 54 h after the injection of the tracers.

Analysis of the kinetic data

The two-exponential radioactivity die-away curves were analyzed using a two-pool model that assumed the existence of an intravascular pool in equilibrium with a non-vascular pool as previously described (17). This model assumes that catabolism of radiolabeled HDL occurs from the intravascular pool only. Fractional catabolic rates for large and small HDL were derived directly from the kinetic model as the clearance rate from the intravascular pool using the SAAM II software (SAAM Institute, Seattle, WA). Estimates of parameters were obtained for each animal individually and mean parameter values were determined for each tracer according to their origin (whether they were isolated from normoTG or hyperTG individuals). The estimation of FCR in two of the 11 small HDL experiments was associated with higher coefficients of variation (CV 40% and 31%) whereas other CV associated with the estimation of small and large HDL FCR were all ≤16%. As a result, the mean CV associated with the estimation of the FCR was low (6.8 ± 1.3% and 14.6 ± 3.3% for large and small HDL particles, respectively) which reflects an adequate fit of the model to the data.

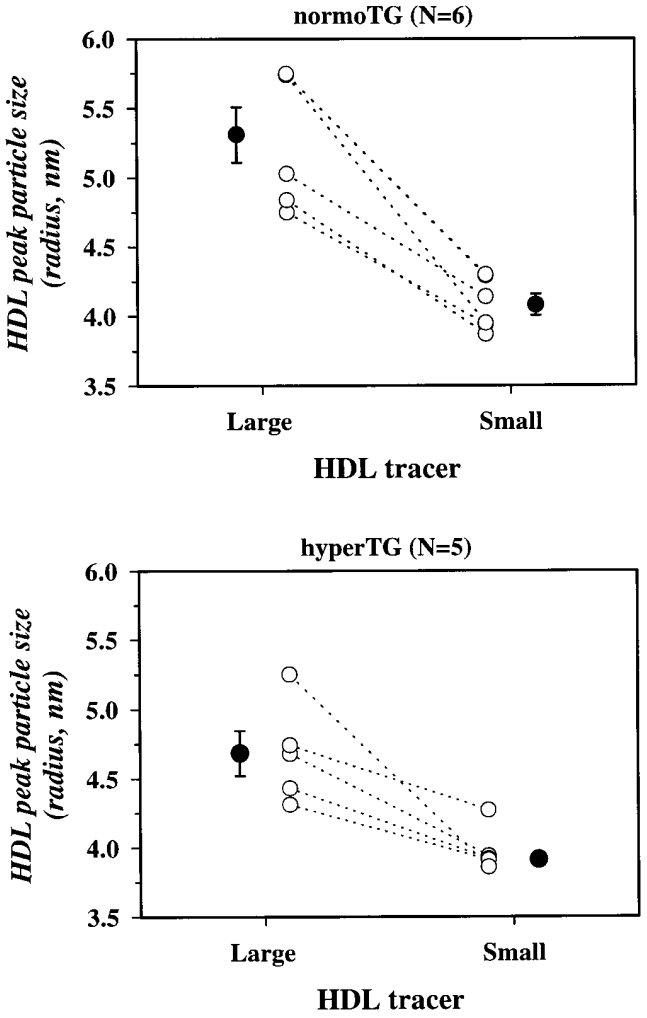

Fig. 2. Mean and individual HDL peak particle size (radius) for the large and the small HDL tracers isolated from subjects with normal (normoTG, top) and elevated (hyperTG, bottom) plasma triglyceride concentrations. There was no overlap between the radius of large (range from 3.86 to 4.30 nm) and small HDL tracers (range 4.31 to 5.75 nm). Mean particle sizes were significantly different between the two tracers in the two groups ($P < 0.001$) and the difference between large and small HDL in hyperTG subjects tended to be smaller than the size difference between the two tracers isolated from normoTG individuals ($P = 0.06$).

Statistical methods

Results are presented as mean ± SEM. Two-way repeated measures analysis of variance was used to test differences in FCR and composition among the various HDL tracers and groups of subjects from whom small and large HDL were isolated. Using this approach one can test the three effects of interest which are: 1) the "between-group" effect, i.e., whether there is a significant difference between HDL isolated from normoTG and from hyperTG individuals after allowing for the HDL size effect (small vs. large), 2) the "within-group" effect, i.e., whether there is a significant difference between large and small HDL particles after

allowing for the HDL origin effect (normoTG vs. hyperTG individuals), and 3) the interaction between the two types of effects, i.e., whether the difference in the composition and FCR of large and small HDL varies significantly according to the origin of the tracer (normoTG or hyperTG individuals) (24). Correlates of HDL particle size and FCR were obtained using Pearson and Spearman correlational analyses for parametric and nonparametric variables, respectively. A stepwise multivariate analysis was used to investigate the best predictors of HDL FCR. Analyses were performed with the Statistical Analysis System (SAS Institute, Cary, NC).

RESULTS

The integrity of the radiolabeled tracers, when monitored on 4–30% PAGGE, was not altered. The iodination procedure had no significant impact on the size of the particles (results not shown) and the two measures of HDL size (peak particle size or as a weighted mean size) were strongly correlated with each other ($r > 0.90$). The impact of HDL particle size on its FCR was investigated using the two measures of HDL particle size but similar results were obtained when HDL peak or mean particle size was used in the analysis. Thus, only the data on the peak particle size are presented. By design and as shown in **Fig. 2**, mean HDL peak particle size of small and large particles were significantly different ($P > 0.001$) both in the normoTG (4.08 ± 0.08 nm vs. 5.31 ± 0.20 nm, respectively, top panel) and hyperTG group (3.98 ± 0.07 nm vs. 4.68 ± 0.16 nm, respectively, lower panel) with no overlap in the size of the two tracers, small and large HDL peak size ranging from 3.86 to 4.30 nm and from 4.31 to 5.75 nm, respectively. The mean intra-individual difference between the size of large and small radiolabeled HDL tended to be more modest in hyperTG (mean difference 0.70 ± 0.18 nm) compared with normoTG individuals (mean difference 1.23 ± 0.16 nm, $P = 0.06$). This was due to the fact that large HDL particles were smaller in hyperTG than in normoTG.

Small and large HDL in hyperTG and normoTG individuals also showed significant differences in composition (**Table 2**). Although the triglyceride content of small and large HDL was similar when expressed as a percent of total HDL weight (10.0% vs. 11.5% in normoTG group and 17.2% vs 19.9% in the hyperTG group), large HDL particles contained more triglycerides on a "per particle" basis than small HDL (ratio of HDL-TG/(A-I + A-II) = 0.69 vs. 0.33 in normoTG and 0.84 vs. 0.57 in hyperTG, $P < 0.001$). As expected, the triglyceride content of small and large HDL was higher in HDL isolated from hyperTG individuals compared with normoTG individuals ($P = 0.01$). The cholesteryl ester content of large HDL was greater than that of small HDL in both the normoTG (12.2% vs. 8.4%) and hyperTG (8.2% vs. 4.6%) groups ($P = 0.005$) and was reduced in HDL isolated from hyperTG individuals compared with normoTG individuals ($P = 0.004$). The relative protein content was greater in small HDL than in large particles ($P < 0.001$) but similar between the hyperTG and normoTG groups. The concentration of HDL-apoA-I was also greater in small HDL particles compared with large HDL ($P = 0.01$) but differences in the apoA-I content between HDL isolated from normoTG and hyperTG individuals did not reach statistical significance. There was no significant difference in the concentration of apoA-II

TABLE 2. Mean lipid and protein concentrations of small and large HDL particles isolated from normotriglyceridemic (normoTG) and hypertriglyceridemic (hyperTG) human subjects

	NormoTG (n = 6)		HyperTG (n = 5)	
	Small	Large	Small	Large
		% of HDL weight		
Triglycerides[a]	10.0 ± 3.6	11.5 ± 2.9	17.2 ± 2.4	19.9 ± 2.0
Cholesteryl ester[a,b]	8.4 ± 1.3	12.2 ± 1.6	4.6 ± 0.5	8.2 ± 0.7
Free cholesterol	3.7 ± 0.7	4.1 ± 0.7	4.3 ± 0.6	4.4 ± 0.9
Phospholipids[b]	21.3 ± 1.8	31.4 ± 4.5	20.0 ± 2.3	26.2 ± 3.1
Free fatty acids	0.5 ± 0.2	0.6 ± 0.2	0.6 ± 0.3	0.8 ± 0.4
Total proteins[b]	56.2 ± 3.5	40.3 ± 2.8	53.4 ± 3.4	40.3 ± 2.8
		mg/dl		
ApoA-I[b]	203.7 ± 32.0	111.9 ± 14.7	191.2 ± 33.8	162.9 ± 30.6
ApoA-II	69.5 ± 10.2	45.6 ± 5.3	79.0 ± 23.2	67.2 ± 9.4
		ratios		
HDL-TG/(A-I + A-II)[a,b]	0.33 ± 0.12	0.69 ± 0.14	0.57 ± 0.12	0.84 ± 0.12
HDL-CE/(A-I + A-II)[a,b,c]	0.24 ± 0.03	0.72 ± 0.12	0.15 ± 0.02	0.35 ± 0.05
HDL-PL/(A-I + A-II)[a]	0.65 ± 0.09	1.95 ± 0.40	0.61 ± 0.03	1.08 ± 0.10

Values are means ± SEM. TG, triglycerides; CE, cholesteryl ester; PL, phospholipids; NormoTG, HDL isolated from individuals with plasma triglyceride levels <2.0 mmol/1; HyperTG, HDL isolated from individuals with plasma triglyceride levels >2.0 mmol/l. Differences were tested by two-way repeated analysis of variance (see Methods).

[a] Significant difference between normoTG and hyperTG individuals; $P < 0.001$: triglycerides; $P < 0.01$: cholesteryl ester, HDL-TG/(A-I + A-II), HDL-CE/(A-I + A-II).

[b] Significant difference between small and large HDL particles; $P < 0.001$: proteins, HDL-TG/(A-I + A-II), HDL-CE/(A-I + A-II); $P \leq 0.01$: cholesteryl ester, phospholipids, apoA-I, HDL-PL/(A-I + A-II).

[c] Significant interaction between particle size and HDL origin (isolated from normoTG and hyperTG humans); $P = 0.03$: HDL-CE/(A-I + A-II).

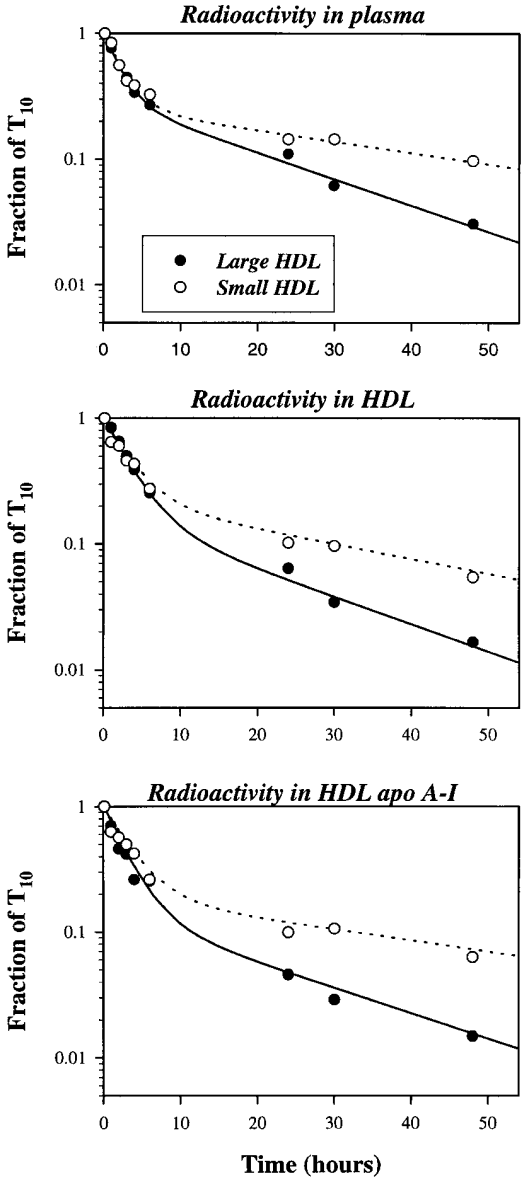

Fig. 3. Die-away radioactivity curves of radiolabeled small (solid line, solid circles) and large (dotted line, open circles) HDL particles measured in the plasma (top panel), HDL (middle panel), and apoA-I (bottom panel) fraction in a representative rabbit study. The disappearance of radioactivity is expressed as a function of the radioactivity measured at the first time interval (10 min). The curves were generated by fitting a two exponential function to the data (see methods).

between large and small HDL nor was there any difference between HDL-apoA-II isolated from hyperTG and normoTG individuals.

Figure 3 illustrates the die-away curve of radioactivity for small and large human HDL in a representative rabbit study (experiment #7, hyperTG individual) as measured in total plasma (top panel), in the total HDL fraction (middle panel), and specifically associated with HDL apoA-I (lower panel). Independent of the method used, large HDL were cleared at a faster rate than small HDL. We have compared the FCR obtained by measuring the disappearance of radioactivity in total plasma, HDL and apoA-I and the values obtained by these three approaches were highly correlated ($0.84 < r < 0.99$). Because the error associated with the estimation of the FCR from the two-pool model was lower with radioactivity measured directly in plasma than with radioactivity measured in the HDL fraction or specifically associated with apoA-I, the radioactivity measured in plasma was only used in four of the 11 experiments. The FCR data presented in the present paper are those obtained from the radioactivity disappearance measured directly in the plasma.

Individual and mean FCR of small and large human HDL in rabbits, stratified according to whether they were isolated from normoTG or hyperTG individuals, are presented in **Table 3.** The two-way repeated measure analysis of variance (size of HDL being the repeated measure and origin of HDL being the second factor) revealed that there was a significant difference between the FCR of small and large HDL ($P = 0.007$) but no difference between normoTG and hyperTG ($P = 0.68$). There was also no significant interaction between HDL particle size and the origin of HDL (normoTG or hyperTG), suggesting that the difference between the FCR of small and large HDL was not statistically different whether HDL were isolated from normoTG or hyperTG individuals (82.4% vs. 40.7%, $P = 0.33$). Analysis of power showed that this dif-

TABLE 3. Fractional catabolic rates of large and small human HDL in rabbits, stratified by HDL origin (from normoTG or hyperTG humans)

Subject	Fractional Catabolic Rate, hour^{-1} (CV%)		Difference (%)[a]
	Small HDL	Large HDL	
NormoTG			
1	0.139 (12%)	0.152 (6%)	8.8
2	0.046 (40%)	0.126 (10%)	172.0
3	0.038 (31%)	0.074 (16%)	97.5
4	0.093 (10%)	0.148 (6%)	59.3
5	0.052 (15%)	0.126 (4%)	143.2
6	0.082 (9%)	0.093 (4%)	13.4
Mean ± SEM	0.075 ± 0.016	0.120 ± 0.012	82.4%
HyperTG			
7	0.077 (10%)	0.122 (6%)	59.6
8	0.089 (5%)	0.139 (3%)	56.1
9	0.069 (11%)	0.106 (14%)	54.2
10	0.046 (6%)	0.077 (3%)	68.4
11	0.128 (13%)	0.084 (4%)	−34.6
Mean ± SEM	0.082 ± 0.014	0.106 ± 0.012	40.7
All			
Mean ± SEM	0.078 ± 0.010	0.113 ± 0.008[b]	63.4 ± 17.7%

CV%: coefficient of variation provided in parentheses; it equals the standard deviation derived from the estimation of the FCR by SAAM II divided by the estimated FCR, expressed in percent. The CV is an estimate of the degree of fit of the model to the data. The lower the CV, the better the fit.

NormoTG: HDL isolated from individuals with plasma triglyceride levels <2.0 mmol/l. HyperTG: HDL isolated from individuals with plasma triglyceride levels >2.0 mmol/l. Differences were tested by two-way repeated analysis of variance (see Methods). Test for a difference between small and large HDL, $P = 0.007$. Test for a difference between normoTG and hyperTG HDL, $P = 0.71$. Test for interaction between particle size and HDL origin (normoTG/hyperTG), $P = 0.34$.

[a] Relative (%) increase (+) or reduction (−) in the clearance of large HDL particles when compared to small HDL particles.

[b] $P = 0.006$ between large and small by paired t-test.

ference would become significant at $p = 0.05$ with a power of 0.80 only if performed on a much larger number of experiments (n = 41). Thus, the lack of significance between normoTG and hyperTG cannot be solely attributable to small sample size. It is also possible that the more trivial difference between the size of small and large HDL in hyperTG subjects compared to that in normoTG individuals may be responsible for the smaller difference in the FCR between large and small HDL in the hyperTG group.

Taken together, large HDL particles were cleared more rapidly compared with small HDL in 10 out of 11 rabbit experiments. As a result, large HDL were cleared from the circulation 63.4% faster than small HDL (FCR for large and small HDL were 0.113 ± 0.008 vs. 0.078 ± 0.010 hr^{-1} respectively, $P = 0.006$).

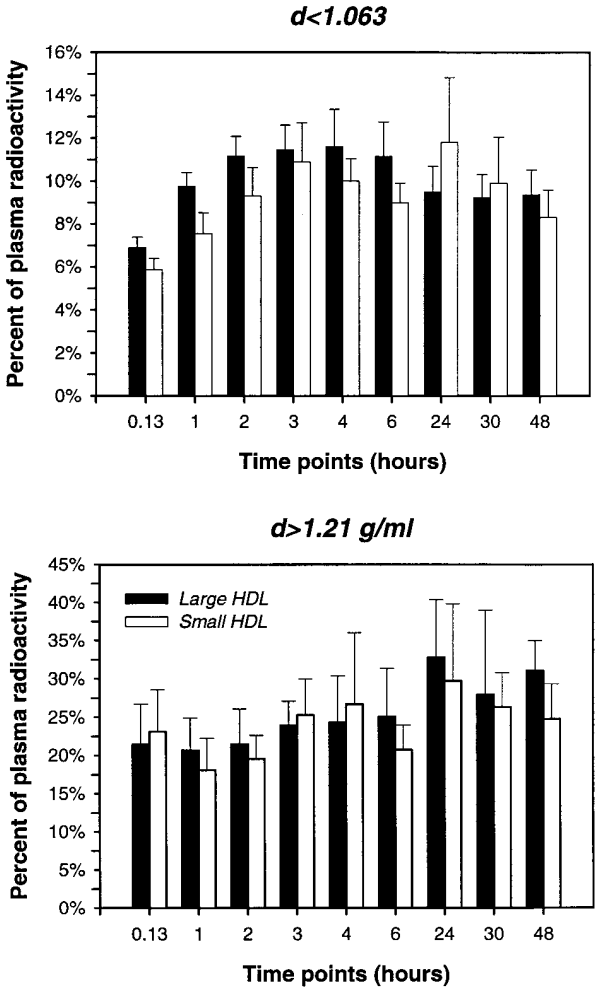

Fig. 4. Percent of plasma radioactivity found in the d < 1.063 g/ml (top) and in the d > 1.21 g/ml (bottom) fractions over time. The solid bars (■) represent the percent of plasma radioactivity transferred from the large HDL tracer and the hollow bars (□) depict the percent of radioactivity transferred from the small HDL tracer. Transfer may reflect true in vivo transfer from tracers to non-HDL fractions or may be attributable to ultracentrifugation artefact. Two-way ANOVA showed that the proportion of plasma radioactivity changed significantly over time only in the d < 1.063 g/ml fraction. (P = 0.01). However the transfer of radioactivity from large and small HDL to these two fractions was similar.

Figure 4 depicts the percent of plasma radioactivity found in the d < 1.063 g/ml (top) and in the d > 1.21 g/ml (bottom) plasma fractions over time. In this analysis, the data from all experiments were pooled into one group because there was no notable difference in the proportion of the label found in the d < 1.063 g/ml and the d > 1.21 g/ml fractions between normoTG HDL and hyperTG HDL. Two-way repeated measure analysis of variance using time as the repeated factor showed that there was no significant change over time in the proportion of radioactivity found in the d > 1.21 g/ml plasma fraction whereas the percent of radioactivity in the d < 1.063 g/ml fraction increased significantly over time ($P = 0.02$). There was, however, no difference between the proportion of label derived from large and small HDL particles ($P = 0.86$). In other words, the amounts of radioactivity "transferred" from large and small HDL to the d < 1.063 g/ml and the d > 1.21 g/ml fractions were comparable.

Correlational analysis was performed on the data combining the 11 experiments and is presented in **Table 4**. The cholesteryl ester content of the tracers was a significant correlate of HDL FCR ($r = 0.61$, $P = 0.002$) whereas the triglyceride content of the injected HDL ($r = -0.17$, $P = 0.54$) or the human plasma triglyceride concentrations (not shown) were not significantly associated with the FCR of small and large HDL. Peak particle size ($r = 0.47$, $P = 0.03$) and the relative free fatty acid content of the HDL tracers ($r = 0.45$, $P = 0.04$) were also significant predictors of HDL FCR. In multivariate analysis, the cholesteryl ester and free fatty acid content of the tracers accounted for 48% of the variance in HDL FCR ($P = 0.002$) whereas peak particle size did not contribute signicantly to variations in HDL FCR when these two components of HDL composition were included in the model. Variations in HDL peak particle size were significantly correlated with variations in HDL-cholesteryl ester/A-I + A-II ($r =$

TABLE 4. Univariate and multivariate correlates of human HDL fractional catabolic rate in rabbits

	Correlates of HDL FCR	
Variables	R	*P* Value
Univariate		
HDL-TG	−0.17	0.54
HDL-CE	0.61	0.002
HDL-FFA	0.45	0.04
HDL-apoA-I	−0.27	0.22
HDL-apoA-II	−0.21	0.33
HDL particle size	0.47	0.03
	R^2	*P* Value
Multivariate		
HDL-CE	0.39	0.002
HDL-FFA	0.09	0.08
Model	0.48	0.002

TG, triglycerides; CE, cholesteryl ester; FFA, free fatty acids. HDL-TG, HDL-CE, HDL-FFA were expressed as a percent of HDL weight in this analysis. The stepwise multiple regression procedure was used to identify the best model predicting HDL FCR. Only the variables that showed a significant univariate relationship with HDL FCR were entered in the multivariate model, i.e., HDL-CE, HDL-FFA, and HDL particle size.

0.86, $P < 0.001$), HDL-phospholipids/A-I + A-II ($r = 0.86$, $P < 0.001$), and the relative total protein content (in percent, $r = -0.63$, $P = 0.002$) whereas absolute or relative triglyceride and free fatty acid content of HDL showed no relationship with HDL peak particle size.

DISCUSSION

Results of the present study have emphasized two important concepts relevant to our understanding of HDL physiology. First, we found that large, lipid-rich human HDL particles were cleared at a faster rate than smaller HDL when injected into rabbits. Second, the triglyceride enrichment of HDL did not appear to modify the relationship between particle size and HDL-FCR in rabbit, an animal species with low hepatic lipase activity (18). These observations have important physiological implications which will be addressed in the following sections.

Particle size and fractional catabolic rate of HDL

A number of issues must be considered when analyzing the results of the present study with respect to the effect of particle size on HDL-FCR. Small and large human HDL were specifically isolated on the basis of size by gel filtration chromatography of the d < 1.21 g/ml plasma fraction. This method allowed us to achieve a distinct separation of large and small HDL, as there was no overlap in the size of the two tracers. However, not only did the small and large particles differ in their size, but there were also marked compositional differences between them. Clearly this is an issue that cannot be overlooked. Indeed, large HDL particles were enriched in triglycerides (on a "per particle" basis) and cholesteryl ester, contained more phospholipids and also had less apoA-I compared with small HDL. The significant association between the cholesteryl ester and free fatty acid content of the injected HDL and the FCR of these particles suggests that HDL containing relatively more cholesteryl ester and free fatty acids were more susceptible to clearance compared with those having less of these two lipid components. Because size and composition of HDL are so intimately related to each other, it is therefore difficult, perhaps even impossible, to ascribe differences in clearance specifically to size or composition using native HDL particles. In fact, one could argue that size per se had no effect and that the enhanced clearance of large HDL was due only to differences in composition. In that regard, the close relationship between the cholesteryl ester content of HDL and its FCR is interesting and should be investigated more specifically in future studies.

Our results must be interpreted in the context of two previous studies (8, 12), as they would appear to contradict their findings. In the first study (12), large (HDL_2) and small (HDL_3) particles were isolated by density, not specifically by size, and re-injected into rabbits. The investigators' observation that smaller particles were cleared faster than large ones cannot be directly compared with our study, as large and small particles in both studies were likely to differ in a number of respects other than size. Of importance also is the fact that they injected small and large LpAI and not whole HDL as we did in the present study. Finally, in addition to the small number of rabbit experiments in that study (n = 3)(12), the injected HDL_2 and HDL_3 were isolated from one human subject only. This may also limit the generalization of their findings given the fact that, in the present study, there appeared to be some degree of heterogeneity in the difference between small and large HDL FCR (in one individual of the present study, small HDL was indeed cleared more rapidly than large particles).

In the study by Brinton et al. (8) performed in men and women, HDL particle size was estimated using the molar ratio of HDL cholesterol to the sum of apoA-I and A-II. The authors reported a strong inverse correlation between this estimate of particle size and apoA-I FCR ($r = -0.81$). They further suggested that as much as 70% of the variability in apoA-I FCR may be attributable to variations in particle size and density (8), raising the possibility that small and more dense HDL particles may be cleared more rapidly from the circulation in humans. It must be emphasized that they did not directly compare the clearance of small versus large HDL particles but only reported associations.

Why are the results of the present study in rabbits apparently different from observations in humans? Although the rabbit model has been used extensively to investigate lipoprotein metabolism (12, 18, 25–32), one should acknowledge the marked differences between human and rabbit physiology, not least of which relates to expression of hepatic lipase, an enzyme that plays a critical role in HDL metabolism. The very low expression of hepatic lipase in rabbits could, therefore, account for most of the differences between our findings and those of Brinton et al. (8) as will be explained below. Second, recent studies have established the existence of receptors intimately involved in the selective lipid uptake from HDL by tissues, a class B, type 1 scavenger receptor (SR-B1) (33, 34). It has been demonstrated that this novel class of scavenger receptors binds with high affinity to HDL, that the receptors facilitate the selective uptake of cholesteryl ester into cultured cells, and that they are most abundantly expressed in the liver and steroidogenic tissues (34). Recent studies in transgenic and gene knockout mice suggest that the expression of apoA-I and hepatic lipase genes may significantly alter the expression of SR-BI mRNA in adrenal glands (35). Possible inter-species difference in the regulation and expression of SR-B1 may, therefore, explain some of the discrepancies between rabbit and human data.

It could also be argued that dissociation of the "loosely bound" apoA-I from larger HDL particles could lead to the formation of small HDL when the lipid-free apoA-I reassociates with nascent HDL. Due to the limited blood volume of the rabbit, no attempt was made to separate rabbit HDL into large and small HDL, and therefore we have no information on the possible rate at which large HDL may be converted into small HDL particles. There was, how-

ever, no evidence for a precursor–product relationship between large and small HDL in the present study. It is likely, therefore, that newly formed small HDL may represent only a limited proportion of the small HDL tracer, and that the consistent difference between the FCR of large and small HDL particles in our study is unlikely to be attributable to this phenomenon.

Triglyceride-enrichment and fractional catabolic rate of HDL

The second important observation underscored by the present study is the lack of effect of triglyceride enrichment on the clearance of HDL (small or large), and the fact that triglyceride enrichment did not appear to modify the relationship between particle size and FCR of HDL. This must be interpreted in the context of the study, namely, the investigation of the metabolic clearance of large and small HDL in a model where in vivo lipolytic modification of the injected particles was probably minimal due to the low hepatic lipase activity in rabbit (18).

In vitro incubation of triglyceride-rich HDL with hepatic lipase results in the formation of small HDL particles and promotes the loss of apoA-1 from HDL (36–38). Results from ex vivo experiments in animals suggest that the significant increase in renal clearance of apoA-I may be observed only when triglyceride-enriched HDL interacts with lipolytic enzymes (13). Indeed, it has been reported that apoA-I from small, lipolytically modified triglyceride-rich HDL infused through rabbit kidneys accumulate more rapidly in the cortex of the animal's kidney than the apoA-I from triglyceride-rich, unmodified HDL (13). We have recently investigated the potential contribution of in vivo lipolytic modification in determining the rate at which human HDL are cleared in rabbits by comparing the FCR of large, postprandial, triglyceride-rich HDL isolated after a high fat meal to the FCR of small, lipolytically modified HDL, isolated after intravenous heparin in humans (17). It was found that lipolytically modified, small human HDL were cleared at a faster rate from the circulation than postprandial, large triglyceride-enriched HDL particles. By comparing data of the present study and data of the previous one (17), both of which were performed in New Zealand White rabbits using a similar protocol, we found that small, lipolytically modified HDL were cleared at the fastest rate (previous report, FCR = 0.133 ± 0.025 hr^{-1}) compared with the FCR of large fasting HDL (present study, FCR = 0.113 ± 0.008 hr^{-1}) or post-prandial HDL (previous study, FCR = 0.092 ± 0.031 hr^{-1}). In contrast, small fasting HDL tested in the present study showed the slowest catabolic rate of all particles tested (FCR = 0.078 ± 0.010 hr^{-1}). These results underscore the importance of lipolytic modification of HDL, over-and above the size of the particle, in determining the rate at which it is cleared from the circulation. They also support the hypothesis that triglyceride enrichment of HDL may have very little impact on its metabolic clearance if it is not accompanied by substantial simultaneous intravenous lipolysis (13). It must be stressed that small, lipolytically modified HDL were characterized by marked alterations in their composition, not the least of which being a notable 3- to 4-fold increase in the free fatty acid content of the lipolytically modified HDL compared with fasting or postprandial HDL particles (17). One could speculate that such a marked increase in the free fatty acid content of HDL could affect its charge and stability, thereby enhancing its potential for uptake by scavenger receptors such as the SR-BI receptor, and ultimately, its clearance from the circulation. The significant positive correlation between the free fatty acid content of HDL and its FCR in the present study tends to support an important role for this charged lipid in the determination of the particle's clearance rate.

There is evidence to suggest that individuals with reduced HDL cholesterol concentrations, who frequently have elevated plasma triglyceride levels in combination, may have an increased proportion of their HDL apoA-I in a more easily dissociable pool that can be readily cleared from the circulation (39). Results from other studies showing an increased proportion of free apoA-I in plasma or in the $d > 1.21$ g/ml fraction of hypertriglyceridemic individuals support this notion (13). In the present study there was no significant correlation between the triglyceride content of the tracers and HDL-FCR nor was there any difference in the proportion of label in the $d > 1.21$ g/ml fraction between small and large HDL. It is therefore possible that the triglyceride enrichment of large versus small HDL had very little impact in determining its metabolic fate in the rabbit, and that other intrinsic factors such as the cholesteryl ester or the free fatty acid content may become more important in a model where modification of HDL by lipolysis is limited.

Other factors affecting HDL clearance rate

At least two other key enzymes, cholesteryl ester transfer protein (CETP) and lecithin:cholesterol acyltransferase (LCAT), play pivotal roles in the metabolism of HDL. CETP mediates lipid exchange between HDL and apoB-containing lipoproteins (36, 40, 41). LCAT catalyzes the esterification of free cholesterol present in the plasma (42). CETP and LCAT can, therefore, both contribute significantly to modify HDL composition and hence, HDL particle size (40, 42–44). Studies in transgenic animals overexpressing human LCAT (43) or overexpressing CETP in combination with hypertriglyceridemia (45) have reported marked changes in HDL particle size. The extent to which these key enzymes may be directly involved in the determination of small versus large HDL clearance rates from the circulation will require further investigation.

Previous studies have shown that HDL containing both apoA-I and A-II (LpAI, AII) were less rapidly catabolized compared to apoA-I only containing HDL (LpAI) (46). Although we did not measure LpAI and LpAI, AII in the present study, large and small HDL particles had comparable amounts of apoA-II. Because LpAI, AII are more frequently found within smaller HDL subfractions (47), it is possible that differences in the clearance of large and small HDL may be attributable to differences in the pro-

portion of LpAI and LpAI,AII in the subfractions. Large HDL also contain more apoE than small HDL particles (21, 48, 49). We did not measure HDL-apoE concentrations in the present study but it is possible that increasing amounts of apoE in large HDL may also contribute to enhance its removal from the circulation.

Summary

Results from the present study first suggest that lipid-rich human HDL is catabolized more rapidly than small HDL in rabbits. This phenomenon may reflect the influence of particle size per se, but differences in particle composition could have played an equally significant or more significant role. Second, triglyceride enrichment of small and large HDL did not appear to be implicated in this process in the rabbit model. These results, combined with previous observations that have highlighted the importance of the lipolytic processes in determining apoA-I catabolism, suggest that triglyceride enrichment of HDL may not affect its catabolism to a great extent in the absence of substantial in vivo lipolysis by hepatic lipase. The implications of these observations in the pathophysiology of HDL cholesterol-lowering in human hypertriglyceridemic states, where in vivo lipolytic modification of HDL plays an active and significant role in its metabolism, should be determined specifically in future studies.

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