Clearance of postprandial and lipolytically modified human HDL in rabbits and rats

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Abstract Triglyceride (TG) enrichment of high density lipoproteins (HDL) in hypertriglyceridemic states renders the particles vulnerable to lipolysis, which reduces their size. In the present study we modified the size and composition of HDL in vivo in hypertriglyceridemic humans by administering a bolus of intravenous heparin, and tested the subsequent clearance of the isolated HDL particles in rabbits and rats. HDL was isolated by ultracentrifugation from 21 moderately hypertriglyceridemic humans, 5 h after ingestion of a high fat meal and then 15 min after an intravenous heparin bolus (60 U/kg). Postprandial large TG-rich preheparin HDL and small, TG-poor postheparin HDL were labeled with either ^{125}I or ^{131}I. The clearance of apoA-I associated with each HDL tracer was determined by injecting the tracers *1*) simultaneously (n = 13) and *2*) sequentially (n = 8) into male New Zealand White rabbits, an hepatic lipase-deficient animal, and *3*) by injecting the tracers simultaneously into male Sprague-Dawley rats (n = 8), an animal that has hepatic lipase. Dieaway curves of each radiolabeled tracer were analyzed using a two-pool model that assumes the existence of an intravascular pool in dynamic equilibrium with an extravascular pool. In the rabbit studies, the fractional catabolic rate (FCR) of small, postheparin TG-poor HDL was greater than the FCR of the larger TG-rich HDL (11% greater in the simultaneous study, $P < 0.001$, and 45% greater in the sequential study, $P < 0.001$). Opposite results were observed in rats as large TG-rich preheparin particles showed a greater FCR (1.8-fold) than smaller TG-poor postheparin HDL ($P < 0.05$). These data suggest that although size and composition of HDL can influence its catabolism, the effect is not always in the same direction and depends on other factors present in vivo.—**Lewis, G. F., B. Lamarche, K. D. Uffelman, A. C. Heatherington, M. A. Honig, L. W. Szeto, and P. H. R. Barrett.** Clearance of postprandial and lipolytically modified human HDL in rabbits and rats. *J. Lipid Res.* 1997. **38:** 1771–1781.

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Levels of HDL cholesterol and the major apolipoprotein component of HDL, apoA-I, are strongly inversely correlated with the plasma triglyceride concentration (1, 2). There is a dynamic interaction between triglyceride-rich lipoproteins (TRL) and HDL in the circulation, with transfer of triglyceride from TRL to HDL, accompanied by a reciprocal transfer of cholesteryl ester from HDL to TRL (3). Hypertriglyceridemia from a variety of causes results in increased mass transfer of triglyceride from TRL to HDL (3). Transfer also increases in the postprandial state when chylomicrons and very low density lipoprotein (VLDL) increase in the circulation, further enriching HDL with triglyceride (4–8).

It has been proposed that triglyceride enrichment of HDL renders the HDL particles vulnerable to lipolysis, which reduces their size (6). Hepatic lipase (HL) is felt to play an important role in this process (9). Studies in animal models where there is HDL triglyceride enrichment have shown an associated decrease in HDL size and increase in HDL catabolic rate. These include studies in monkeys infused with anti-lipoprotein lipase (LPL) antibodies (10), and transgenic mice with co-expression of hypertriglyceridemia (over-expression of the human apoC-III gene), apoA-I and CETP (11). In vitro studies by Clay, Newnham, and Barter (12) have demonstrated the importance of HDL triglyceride-enrichment in causing a marked loss of apoA-I from the HDL fraction when incubated with HL, while Horowitz et al. (13), using a perfused rabbit kidney model, showed that apoA-I is loosely bound to triglyceride-rich

Abbreviations: TG, triglyceride; HDL, high density lipoprotein; apo, apolipoprotein; FCR, fractional catabolic rate; VLDL, very low density lipoprotein; TRL, triglyceride-rich lipoprotein; HL, hepatic lipase; LPL, lipoprotein lipase; CETP, cholesteryl ester transfer protein; THL, tetrahydrolipstatin; PAGE, polyacrylamide gradient gel electrophoresis; BSA, bovine serum albumin; LCAT, lecithin:cholesterol acyltransferase; LpA-I, lipoprotein A-I only; FFA, free fatty acid; EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulfate.

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HDL. Brinton, Eisenberg, and Breslow (14), have demonstrated that HDL cholesterol levels are primarily determined by the fractional catabolic rate (FCR) of apoA-I and A-II in humans and that the FCR correlates with plasma triglyceride concentration and inversely with an estimate of HDL size. Brinton et al. (14) did not, however, directly test the clearance of small versus large HDL particles or of HDL particles of differing composition. The remodelling of triglyceride-rich HDL particles has been postulated ultimately to affect the number of HDL particles in the circulation (3).

At present, the primary mechanism of HDL cholesterol lowering in hypertriglyceridemia is not known and could be due to any one of a number of possibilities. These include: *1*) small HDL particles produced by the lipolysis of triglyceride-rich HDL may be cleared more rapidly from the circulation; *2*) large triglyceride-rich HDL particles may be intrinsically more unstable in the circulation and apoA-I loosely bound, making the particles more vulnerable to apoA-I shedding during lipolysis or during passage through tissues such as the kidney; and *3*) in cases where the hypertriglyceridemia is due to deficient lipoprotein lipase activity, reduced transfer of surface components of TRL to HDL may result in lower HDL concentrations.

The metabolic clearance rate of HDL is ultimately determined by a complex interaction between the particle itself (stability determined primarily by size and composition) and other important in vivo factors (such as the activity of endothelial lipases). As it has been postulated (14), but not directly tested, that small HDL is cleared more rapidly from the circulation, we modified the size and the composition of HDL in vivo in hypertriglyceridemic humans by administrating an intravenous bolus of heparin 5 h after an oral fat load to stimulate the release of endothelial lipases. Large TG-rich preheparin HDL and small lipolytically modified postheparin HDL particles were isolated from the human subjects, radiolabeled, and their clearance rates determined in two species, the rabbit (an animal with very low hepatic lipase activity) and the rat (an animal that expresses hepatic lipase).

METHODS

Subjects

Twenty one men (age 51.8 ± 2.5 years, body mass index 31.2 ± 1.4 kg/m^2, mean ± SEM) with mild to moderate hypertriglyceridemia and low HDL cholesterol participated in the study. Six of these individuals had Type 2 diabetes with moderate glycemic control (mean fasting blood glucose 8.6 ± 0.8, HbA_{1c} 7% to 10%) while the remainder did not have diabetes. In another study, we found that the HDL composition in response to heparin was similar in diabetic and non-diabetic individuals (unpublished data). No subject was taking any medication known to affect lipid metabolism for at least 6 months prior to entrance into the study and all were non-smokers. 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.

Subjects were instructed to consume their regular diet until 1800 h of the evening before the fat-feeding study, and then to fast. At approximately 8:00 an intravenous sampling catheter was inserted into a forearm vein and normal saline was infused into the catheter to maintain patency. After fasting blood samples were drawn, all subjects received a high-fat test meal containing 60 g fat/m^2 body surface area. The meal contained 67.9% calories from fat (polyunsaturated/saturated ratio 0.526), 23.7% from carbohydrate, and 10.4% from protein. It consisted of a high-fat milk shake containing ice cream, cream, peanut butter, and corn oil and a toasted cheese sandwich. After consuming the meal the subjects did not eat again for the duration of the test meal study (5.25 h). Blood samples were drawn prior to ingestion of the meal (baseline) and at 5 h. After the 5-h blood sample was drawn each subject received, by intravenous injection, 60 units heparin sodium/kg body weight (Organon Tieknika, Toronto, Canada). Fifteen minutes after heparin administration (at 5.25 h after the test meal), a final blood sample was obtained. Samples for lipid and lipoprotein analysis were collected into chilled EDTA tubes, on ice, containing 30 μg/ml blood of the lipase inhibitor tetrahydrolipstatin (THL, Hoffman La Roche Limited, Mississauga, Ontario, Canada).

Laboratory measurements

Glucose was analyzed enzymatically using a Glucose Analyzer II (Beckman Instruments Corp., Fullerton, CA). Cholesterol was measured using the CHOD-PAP enzymatic colorimetric kit (Boehringer Mannheim GmbH Diagnostica, Montreal, Canada, Catalogue #236691). Triglycerides were measured as esterified glycerol using an enzymatic colorimetric kit (Boehringer Mannheim GmbH Diagnostica, cat #450032). 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. FFAs were measured by an enzymatic colorimetric method relying on the acylation of CoA by the fatty acids (kit supplied by Wako Chemicals Industries, VA, USA cat #990-75401). Phospholipid was measured using a kit (Boehringer Mannheim GmbH Diagnostica, cat #691844). 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 (15). Free cholesterol was calculated as the difference between total cholesterol and cholesteryl ester. Protein was measured by the technique described by Lowry et al. (16). HDL cholesterol was measured in the supernatant remaining after precipitation of apoB-containing lipoproteins in the plasma with manganese heparin (17). ApoA-I was measured by electro immunoassay using the Sebia Hydragel ApoA1B kit (Gelman Sciences Inc. Toronto, Canada, Cat#4050).

Isolation of HDL

Because of limitations in blood sample volumes from human subjects and the logistics of performing these complicated tracer experiments in animals, HDL from each individual could be used for a maximum of two animal experiments, i.e., usually one experiment in a rabbit and one in a rat. As explained below, HDL isolated from 13 human participants were not pooled but were injected into 13 rabbits and 8 rats (studies 1 and 3, simultaneous injections). Sample from another 8 human participants provided HDL for the second study (sequential injection into rabbits). Blood samples for HDL determination were drawn into tubes containing a final concentration of 1.2 g/l sodium EDTA, 1 mM PMSF, 0.1 g/l sodium azide, 1 mM BHT, 8 mg/l chloramphenicol, 80 ng/l gentamicin sulfate, 10,000 units/l kallikrein inhibitor, 30 μg/ml THL. Pre- and postheparin HDL were separated from plasma by gradient density ultracentrifugal flotation in a discontinuous 3–20% NaBr gradient for 66 h at 38,000 rpm, 15°C in a Beckman SW40 Ti rotor, as previously described (18). Fractions (0.4 ml) were collected through a gradient fractionator with UV monitor at 280 nm. Using this high salt equilibrium centrifugation, we generally find <10% apoA-I in the bottom fraction. As pre- and postheparin HDL are heterogeneous in size, we selected fractions containing the largest of the preheparin HDL particles (fractions corresponding to the upslope of the density gradient preheparin HDL peak) and fractions with the smallest of the postheparin HDL particles (fractions corresponding to the downslope of the density gradient postheparin HDL peak) for the tracer experiments in animals, isolating two fractions with minimal overlap of size (**Fig. 1** and **Fig. 2**). Densitometric scans of the HDL fractions on the gels generally revealed a single major peak with a Gaussian distribution of the HDL tracer. The fractions were dialyzed versus 150 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.4, overnight.

HDL iodination and analysis

Protein (0.5–1 mg) of the dialyzed individual HDL samples was iodinated by a modification of the iodine monochloride method of McFarlane (19), using 500 μCi of ^{125}I for the one HDL tracer and ^{131}I for the other (the two isotopes were alternated between the larger pre- and smaller postheparin HDL samples). One to 2.0 ml (≈300 μg) of unlabeled HDL was added as a cold carrier, the HDL was washed at d 1.21 g/ml, 39,000 rpm, 4°C in a 70.1 Ti rotor for 48 h, and dialyzed overnight against 6 litres of dialysate containing 0.15 M NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0, at 4°C. The composition of each injectate was measured using commercially available enzymatic assay kits as described above and HDL components were expressed as percentage composition by weight. As the FFA content of HDL is significantly increased postheparin, the mass of HDL FFA was also considered in the calculation of HDL composition. HDL size was analyzed by electrophoresis in a non-denaturing polyacrylamide gradient (PAGE) system using 4–30% polyacrylamide gels (purchased from Dr. David Rainwater, Southwest Foundation for Biomedical Research, San Antonio, Texas). Thirty micrograms protein was applied as a mixture by volume with three parts sample and one part solution of 40% sucrose with 0.01% bromphenol blue. A mixture of standard molecular weight proteins (HMW Calibration Kit, Phar-

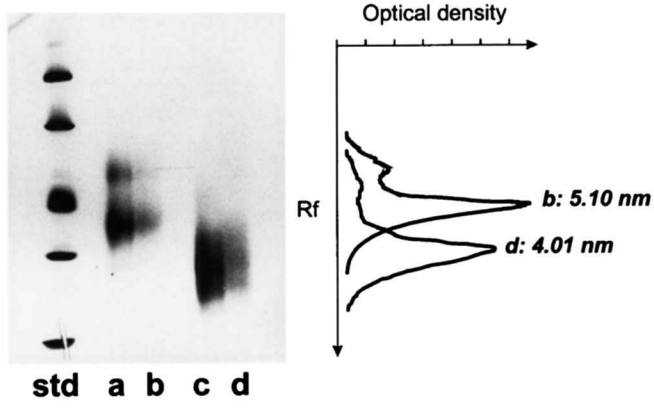

Fig. 1. Pre- and postheparin HDL injectate size. Non-denaturing (4 to 30%) gradient gel electrophoresis and densitometric scan of particle protein concentration of the pre- and postheparin HDL tracers, showing a clear separation of particles by size, with minimal overlap. STD, molecular weight standards. Lanes a and b represent preheparin injectates (mean radius 5.10 nm). The injectates in lanes c and d are postheparin tracers (mean radius = 4.01 nm). The injectate in lanes a and c are unlabeled while those in lanes b and d are iodinated.

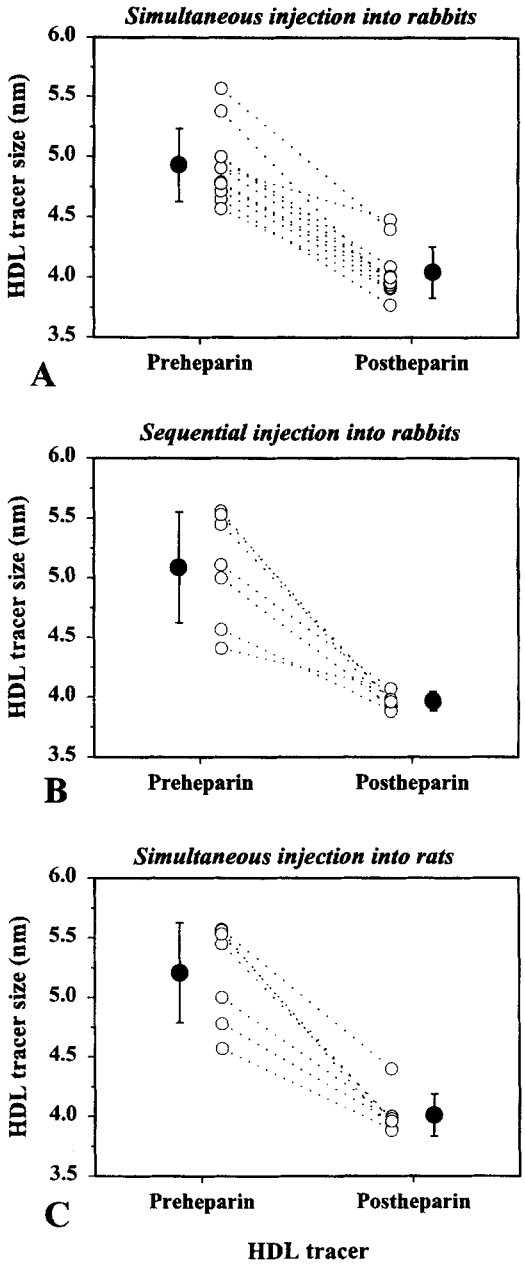

Fig. 2. Size distribution of HDL particles in each study. Each panel presents the distribution of HDL particle size of A, simultaneous injection into rabbits (study 1); B, sequential injection into rabbits (study 2); and C, simultaneous injection into rats (study 3). HDL particle size was obtained by non-denaturing 4–30% gradient gel electrophoresis and was defined as the size of the major peak.

macia, Pistacataway, NT) consisting of thyroglobulin (radius 8.50 nm), ferritin (6.1 nm), catalase (5.20 nm), lactate dehydrogenase (4.08 nm), and bovine serum albumin (3.55 nm) was included in a separate lane in each gel. The gels were stained with Coomassie G250 in perchloric acid (0.1% stain, 5% perchloric acid), destained and stored in 7% acetic acid. The mean radius of the particles was assessed by densitometric scanning using an Image Master DTS densitometer with Imagemaster computer software (Pharmacia LKB, Uppsala, Sweden) based on relative migration distance (R_f) of standards. The integrity of the injectate particles was monitored using 15% SDS and 4–30% PAGE.

Experimental protocol

Large TG-rich preheparin and small postheparin HDL tracers isolated from each human participant (i.e., human HDL samples were not pooled) were injected into different rabbits or rats to determine the metabolic clearance rates as described below.

Study 1. Simultaneous injection of the ^{125}I-labeled HDL and ^{131}I-labeled HDL tracers into rabbits (n = 13)

An aliquot of each of the injectates (2–10 μCi each) was mixed with 0.5 ml of 1% BSA (total volume <4.0 ml) and injected simultaneously into the right marginal ear vein of a 4–5 kg male New Zealand White rabbit, sedated with INNOVAR-VET (0.13–0.17 mg/kg intramuscular, Janssen Pharmaceuticals, Mississauga, Ontario, Canada). Blood samples (2 ml) were drawn from the opposite ear at 10 min and at 1, 2, 3, 4, 6, 24, 30, 48, 54, and 72 h. Plasma was separated and adjusted to a density of 1.063 g/ml with KBr and spun for 5 h at 100,000 rpm at 16°C in an Optima TLX ultracentrifuge (Beckman Instruments, Palo Alto, CA) in a TLA 100.3 rotor. The top fraction was removed and the bottom fraction was adjusted to d 1.21 g/ml with KBr and spun for 5 h at 100,000 rpm, 16°C (Naito, H.K. Application note DS-693, Beckman Instruments, Spinco Division, Palo Alto, CA). The top HDL was washed for another 5 h, sliced, and dialyzed against 6 L of dialysate containing 0.15 M NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0, at 4°C for 24 h. Aliquots (1.0 ml) of dialyzed HDL were delipidated and dissolved in 0.5 M phosphate with 1% SDS, 1% mercaptoethanol, pH 7.2, buffer overnight. 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. The stained bands were sliced from the gel and counted in a Beckman 5500 gamma counter. The apoA-I counts in the gel were taken as a percent of the total gel counts and the apoA-I counts in HDL were calculated from this. The recovery of counts from the SDS gels compared to the counts applied to the gel ranged from 70% to 85%.

Study 2. Sequential tracer injection into rabbits (n = 8)

The rationale for the sequential injection of tracers was to determine whether the difference in clearance of pre- and postheparin tracers observed in the above simultaneous injection study would be greater if we

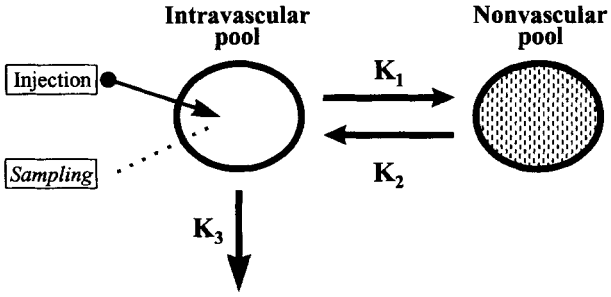

Fig. 3. Schematic model for HDL kinetic analysis. The model consists of two discrete pools, one intravascular pool in dynamic equilibrium with an extravascular pool. This model assumes that all input or exit of radiolabeled material occurs from the intravascular pool. The estimated parameter k_3 corresponds to the elimination rate (fractional catabolic rate, FCR) of radiolabeled material from the system and is essentially the inverse of the area under the activity-time curve.

could avoid the possibility of exchange of label between tracers, either in the syringe immediately prior to injection or in the circulation of the rabbit. Injectates were prepared as above but injected 1 week apart. In alternate studies ^{131}I was used to label the larger preheparin HDL and ^{125}I the smaller postheparin HDL. The ^{131}I-labeled HDL was always injected first. The timed blood samples were treated exactly as those in Study 1.

Study 3. Simultaneous tracer injection into rats (n = 8)

Tracers prepared as in Study 1 were mixed and injected simultaneously into the exposed femoral vein of a 300-g male Sprague-Dawley rat sedated with SOMNOTOL (MTC Pharmaceuticals, Canada) and 0.25-ml blood samples were taken starting at 1.5 min over 1 h from the femoral artery (12 samples at 1.5, 3, 5, 7, 10, 13, 17, 20, 25, 30, 40, and 60 min) and 0.10 ml of saline was injected back into the animal. Plasma was separated and duplicate aliquots of 50 μl were counted in a Beckman 5500 gamma counter. As the radiolabeled tracers were counted from whole plasma rather than from isolated HDL because of limited blood volume in rats, larger blood samples were drawn at six selected time points in three additional rats and apoA-I-associated radioactivity was measured as described above for study 1 in order to validate this alternative approach.

Kinetic modelling and statistical methods

For all studies, curves were analyzed using a two-pool model that assumes the existence of an intravascular pool in dynamic equilibrium with an extravascular pool (**Fig. 3**). This model assumes that new input or exit of radiolabeled HDL occurs from the intravascular pool only. The die-away curves for the larger, TG-rich preheparin HDL and the smaller, TG-poor postheparin HDL were compared as follows. For the rabbit studies, "population" parameters for each tracer were computed by modelling the die-away curves estimated using the POP3CM program (Resource Facility for Kinetic Analysis, University of Washington, Seattle, WA). Population kinetic analysis takes into consideration intersubject variability to estimate kinetic parameters for a group or population of individuals. Mean kinetic parameters obtained from individual fitting of subjects in a group are generally (for data dense situations) similar to population kinetic parameters but they are obtained ignoring the errors associated with the individual parameter estimates. Determining population kinetics is an iterative process of fitting a model to the individual's data and then using at the same time all the available statistical information from all subjects to help generate the parameters of the population, thus reducing the intersubject variability of the estimates. A mean die-away curve within each study and for each radiolabeled tracer was generated using the estimates of the population kinetic parameters and were expressed as fraction of time 0. Although acceptable from a modelling perspective, the fit of the data in the rat studies was not as good as that of the rabbit studies and the population analysis program was not able to handle some of the data from the rat experiments. Die-away curves for rats were therefore individually modelled using the SAAM II program (SAAM Institute, Seattle, WA) and the model described above. Estimates of parameters were obtained for each animal and mean parameter values were determined for each tracer. Subanalyses in rabbits revealed that the estimates of parameters obtained by the population analysis and using the individual fit (SAAM II) were essentially the same. In all three studies, fractional catabolic rates (FCR) were derived directly from the kinetic model as the clearance rate from the intravascular pool (k_3, see Fig. 3). Mean die-away curves in the rat study were computer-generated using mean parameter values. Paired *t*-tests were performed to compare population or mean parameter values between the large preheparin and the small postheparin HDL tracers. A general linear model was used to compare the tracer composition between each of the studies and the post-hoc Duncan test was used when a significant group difference was observed. Results are expressed as mean ± SEM and $P < 0.05$ is regarded as significant.

RESULTS

Data from study participants are presented in **Table 1** according to the animal study for which their HDL was used. As expected, all individuals had a significant elevation of plasma triglycerides and FFA postprandially. Postheparin plasma triglycerides decreased significantly, while plasma FFA increased markedly.

TABLE 1. Human plasma fasting, postprandial and postheparin lipids, lipoproteins and apoA-I grouped according to animal study for which HDL was used

	Study 1 (n = 13)	Study 2 (n = 8)	Study 3 (n = 8)
Fasting			
Triglycerides (mM)	3.4 ± 0.6	4.1 ± 0.5	2.7 ± 0.4[a]
FFA (mM)	0.57 ± 0.05	0.70 ± 0.12	0.74 ± 0.13
HDL cholesterol (mM)	0.81 ± 0.05	0.83 ± 0.11	0.72 ± 0.14
ApoA-I (mg/dl)	93.1 ± 5.9	98.8 ± 10.5	91.3 ± 16.1
Cholesterol (mM)	4.6 ± 0.3	5.7 ± 1.3	5.2 ± 1.7
LDL cholesterol (mM)	2.2 ± 1.0	3.0 ± 1.1	3.2 ± 1.3
Postprandial, 5 h			
Triglycerides (mM)	6.8 ± 0.8	8.5 ± 0.9	6.2 ± 1.2[a]
FFA (mM)	0.98 ± 0.11	1.38 ± 0.29	1.44 ± 0.35
Postheparin, 15 min			
Triglycerides (mM)	5.0 ± 1.0	6.7 ± 0.8	4.7 ± 1.3
FFA (mM)	5.25 ± 0.53	4.46 ± 0.37	4.08 ± 0.37

Study 1: simultaneous injection of the tracers into rabbits; Study 2: sequential injection into rabbits; Study 3: simultaneous injection into rats. Values are means ± SEM. HDL, high density lipoprotein; LDL, low density lipoprotein; ApoA-I, apolipoprotein A-I; FFA, free fatty acids.

[a]Significantly different from study 2 ($P < 0.05$).

The mean lipid composition and tracer sizes are presented in **Table 2** and Fig. 2 for pre- and postheparin HDL tracers for each of the three studies. There was no statistical difference in the composition and the size of the tracers among the three studies. Heparin administration resulted in a significant reduction in the proportion of triglycerides and an increase in protein and FFA content of the HDL particles in all three studies. The particles were also significantly smaller following heparin administration. As shown in Fig. 2, there was essentially no overlap in the size of the preheparin and postheparin HDL particles in each of the three studies.

TABLE 2. Mean radius and lipid composition of HDL tracers (% by weight) for the 3 animal studies

	Study 1	Study 2	Study 3
Preheparin HDL			
Radius (nm)	4.93 ± 0.09	5.09 ± 0.17	5.21 ± 0.16
Triglycerides (%)	32.3 ± 5.0	31.0 ± 3.7	31.7 ± 4.0
Cholesteryl ester (%)	10.3 ± 1.2	11.2 ± 1.7	12.2 ± 1.5
Free cholesterol (%)	6.0 ± 1.1	4.7 ± 0.8	4.9 ± 1.1
Phospholipid (%)	22.7 ± 2.5	19.5 ± 1.2	21.4 ± 2.1
Protein (%)	27.5 ± 2.3	32.2 ± 4.7	28.4 ± 2.5
FFA (%)	1.3 ± 0.3	1.5 ± 0.5	1.5 ± 0.5
Postheparin HDL			
Radius (nm)	4.04 ± 0.06	3.97 ± 0.03	4.01 ± 0.07
Triglycerides (%)	11.9 ± 1.4[a]	10.7 ± 1.3[a]	11.5 ± 1.2[a]
Cholesteryl ester (%)	9.0 ± 0.5	8.0 ± 0.8	8.9 ± 0.5
Free cholesterol (%)	2.8 ± 0.3[b]	2.6 ± 0.4[b]	2.4 ± 0.3
Phospholipid (%)	24.7 ± 2.4	17.7 ± 2.4	19.4 ± 2.1
Protein (%)	47.6 ± 3.3[a]	55.7 ± 4.7[c]	54.1 ± 2.4[a]
FFA (%)	3.9 ± 1.1[b]	5.4 ± 1.6[c]	3.9 ± 0.8[b]

Study 1: Simultaneous injection of the tracers into rabbits; Study 2: sequential injection into rabbits; Study 3: simultaneous injection into rats. Values are means ± SEM. No significant difference was observed in HDL particle composition and size among the three studies.

[a] $P < 0.001$; [b] $P < 0.05$; [c] $P < 0.01$, postheparin values were different from the preheparin values.

Figure 4 shows the activity-time curves in one representative animal for each study. In 8 of 13 rabbits in which the tracers were injected simultaneously, the small postheparin particles (represented by the study in panel A) were cleared faster than the large preheparin particles, whereas in the other 5 rabbits the clearance was similar or slower. The faster elimination rate of the small, TG-poor postheparin HDL was more apparent when the two tracers were injected sequentially (1 week apart) into rabbits (panel B). Indeed, the clearance rate of the small postheparin HDL was more rapid than the clearance rate of large preheparin HDL in 7 of the 8 animals studied. In rats, opposite results were observed (panel C). In 7 out of 8 rats, the die-away curve of large, TG-rich preheparin HDL was more pronounced than that of the small, TG-poor postheparin HDL particles. In the rabbit studies and as described in the methods, the isotope clearance curves were constructed using the counts in the isolated apoA-I band. Because of limited plasma volume in rats, total plasma counts were used to generate the die-away curves. We performed three additional studies in rats in which the decline in radioactivity was measured both in isolated apoA-I and in total plasma. Although fewer sampling times were used, consistent with the results obtained using total plasma counts, large preheparin HDL apoA-I was systematically cleared more rapidly from the circulation than ApoA-I in the smaller, postheparin HDL in all three validation experiments. Correlational analysis also showed that there was a very close relationship between counts in plasma and in isolated apoA-I bands in rats as well as in rabbits ($0.85 < r^2 < 0.99$). One can therefore assume that the marked differences in the clearance of small and large HDL particles between the rabbit and the rat are unlikely to be attributed to the different strategies of analysis used in each study.

The fraction of the total plasma radioactivity found in the $d > 1.21$ fraction was measured and was found to range from 6% to 18%. The mean proportion of radioactivity in the $d > 1.21$ fraction for each study was 12.1% and 9.6% for the sequential pre- and postheparin studies and 13.4% and 11.6% for the simultaneous injection of the pre- and postheparin tracers, respectively. Within each individual animal experiment, the correlation between the radioactivity counts in plasma and in the $d > 1/21$ fraction was very high, ranging from 0.92 to 1.0. The $d < 1.063$ fraction contained approximately 5–12% of the radioactivity measured in plasma. We found no difference in the relative amount of radioactivity in the $d < 1.063$ fraction between preheparin and postheparin HDL particles in the rabbit studies. Results from the three validation studies in rats

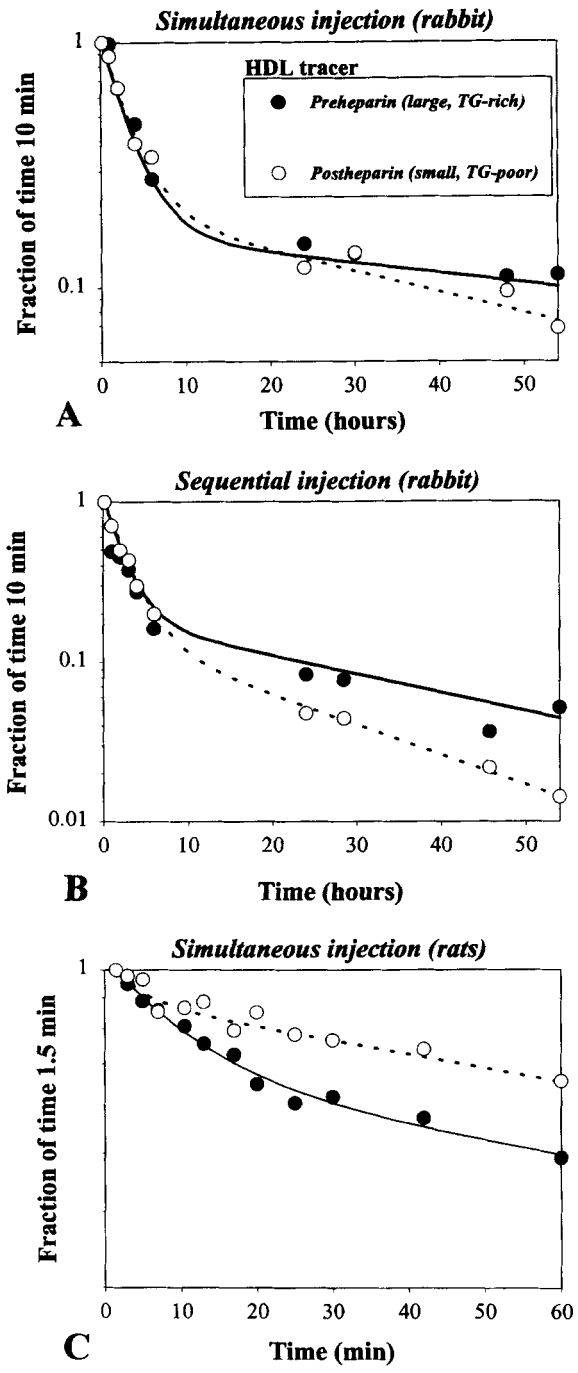

Fig. 4. Plasma clearance curves for large, preheparin and small, postheparin HDL tracers in one representative animal from each of the three studies. Each panel depicts a representative study of A, simultaneous injection into rabbits (study 1); B, sequential injection into rabbits (study 2); and C, simultaneous injection into rats (study 3). Data points are expressed as fraction of 10-min sample in rabbits and 1.5-min sample in rats. The curves are best fit curves for each tracer. Solid line: large, preheparin HDL; dash line: small, postheparin HDL.

suggest that similar proportions of total plasma radioactivity were found in the d < 1.063 fraction of preheparin and postheparin HDL. The amount of radioactivity in plasma and in the d < 1.063 g/ml fraction was tightly correlated. It is therefore believed that such a close relationship among these three measurements may be largely, if not only, related to the ultracentrifugation procedures.

Figure 5 shows the "population" (or mean) die-away curves of radiolabeled HDL for each study. In rabbits (panels A and B), small postheparin HDL were cleared more rapidly from the circulation, most evident when the two tracers were injected sequentially (panel B). The FCR of small postheparin HDL particles was 11% higher than the FCR of large preheparin HDL particles (0.113 ± 0.031 vs. 0.102 ± 0.031 pools/h, $P < 0.0001$) when the two tracers were injected simultaneously and was 45% higher (0.133 ± 0.025 vs. 0.092 ± 0.040 pools/h, $P < 0.0001$) when injected sequentially. In rats, opposite results were obtained as larger, TG-rich preheparin HDL particles were cleared more rapidly than smaller, TG-poor postheparin HDL. As the estimated FCR for the preheparin tracer in one rat study and the FCR for the postheparin tracer in another rat study were associated with a large error (>300%), these two values were not included in the analysis. The difference in the FCR of pre (0.007 ± 0.006 pools/min) and postheparin HDL particles (0.003 ± 0.003 pools/min) was significant ($P = 0.02$).

DISCUSSION

Results of the present study suggest that small, lipolytically modified HDL isolated after intravenous heparin from hypertriglyceridemic individuals is cleared more rapidly than large triglyceride-rich HDL, most evident when injected sequentially into an animal with very low hepatic lipase activity, the rabbit. One theoretical hypothesis would suggest that the more modest difference in the clearance of these HDL particles injected simultaneously into rabbits may have been due to exchange of labeled apoA-I between the pre- and postheparin tracers in the injectate when both tracers are mixed immediately prior to injection into the animals or in the circulation after administration. ApoA-I is known to exchange between lipoproteins in plasma and there is interconversion of HDL particles themselves (20). By administering the two tracers sequentially we were able to attenuate this phenomenon, and by randomizing the order of administration of the pre- and postheparin particles, we were able to avoid any consistent error that may have arisen out of the sequential administration of

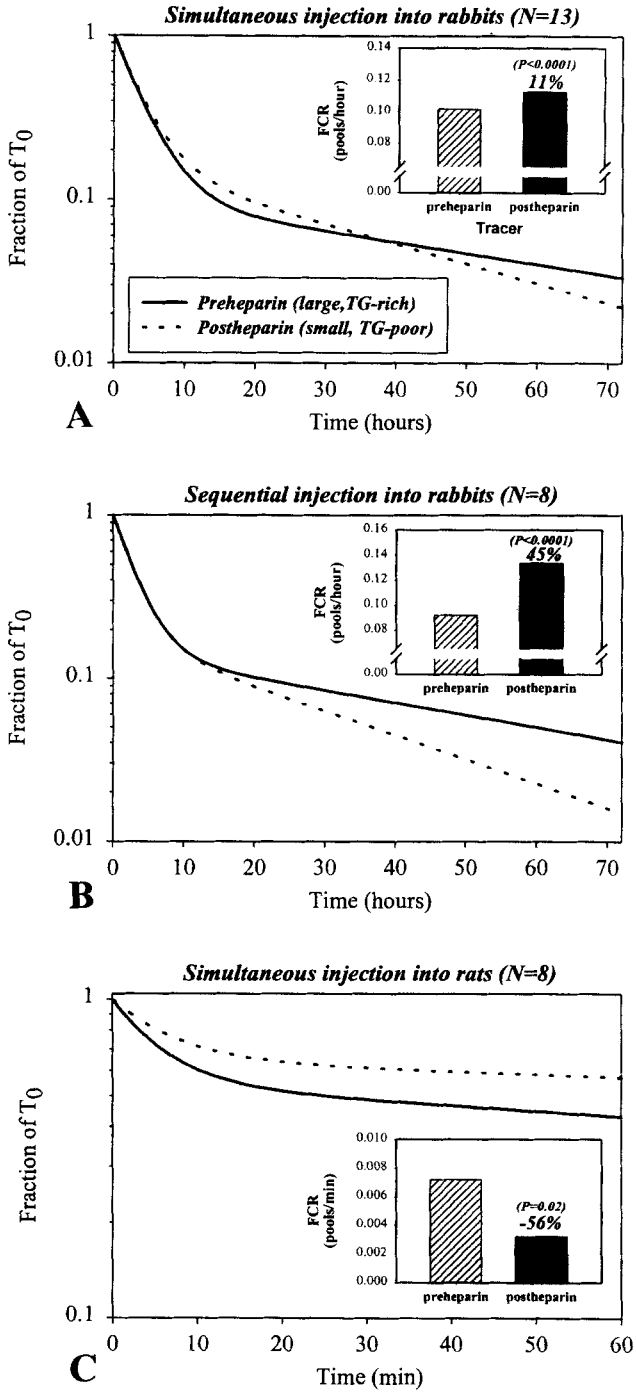

Fig. 5. Simulated plasma clearance curves of radiolabeled tracers over time in the three studies. For the rabbit studies (panels A and B), the die-away curves were constructed using the estimate of the parameters generated by the population analysis. For the rat study (panel C), the die-away curves were generated using the parameters obtained by computing the mean of each individual estimate of parameters. Insets show the fractional catabolic rate (FCR) associated with large preheparin and small postheparin HDL. The relative difference between the FCR of the postheparin compared to the preheparin tracer is also presented. In the rat study, the *P* value was obtained after excluding the calculated FCR value from two tracers (one preheparin and one postheparin in separate animals) in which the parameter estimates were associated with large error (>300%).

the tracers. When the clearance of HDL was examined in a hepatic lipase-sufficient animal, the rat, the opposite was observed as there was a more rapid clearance of large triglyceride-rich HDL particles.

A comparison of tracer clearance between species needs to be made with caution, primarily because there are multiple differences between the rat and the rabbit. Cholesteryl ester transfer protein (CETP) is expressed in rabbits but is deficient in rats (21–23). CETP mediates the heteroexchange of triglyceride and cholesteryl ester between HDL and lower density lipoproteins and has been shown to play a synergistic role with HL in the formation of small HDL particles in vitro (24, 25), and to enhance HDL clearance in CETP transgenic mice (11, 26). Rabbits contain more intermediate density lipoprotein (IDL) than low density lipoprotein (LDL), HDL constitutes the most abundant lipoprotein class and rabbit HDL is enriched in triglycerides (12, 27, 28). Rats, on the other hand, have very low CETP and high LCAT activity, with larger lighter HDL and cholesteryl ester-poor VLDL and LDL (29).

The rabbit has very low HL activity, which is about 1/10th the activity found in the rat (30, 31). A number of lines of evidence suggest that HL plays an important role in the metabolism of HDL. Moderately increased triglyceride-rich HDL is found in individuals with a mutation of the HL gene (32). HDL levels are also dramatically reduced in a rabbit transgenic model with overexpression of the human HL gene (28). In vitro incubation of triglyceride-rich HDL with HL results in the formation of small HDL particles and promotes the loss of apoA-I from HDL (12, 24, 25), and HL plays an important role in the hydrolysis of HDL phospholipids and HDL triglyceride uptake by the perfused liver (33). While the differences in HDL clearance between rabbits and rats may have arisen because of multiple differences between the two animals as discussed above, because of the central role of HL in the metabolism of triglyceride-rich HDL, one may be tempted to speculate that the deficiency of HL in rabbits and its presence in rats could have accounted in large part for the observed differences in HDL clearance between the two species. In addition to the potential contribution of hepatic lipase and the expression of CETP that differs from one species to the other, the marked difference in the endogenous HDL pool size and composition and the difference that may occur in the affinity of human apoA-I for enzymes and receptors also should be considered in explaining the opposite findings in rats and rabbits.

Previous studies in humans have shown no difference in clearance of HDL2 (large particles) and HDL3 (small particles) (34). In those studies, however, the problem of exchange of tracer between particles in the circulation may have obscured real differences in clearance.

Saku et al. (35), demonstrated that pooled human Lp-A-I (HDL containing apoA-I but not apoA-II), separated by ultracentrifugation into a larger size HDL2 fraction and smaller size HDL3 fraction, have a difference in clearance when labeled with ^{125}I and ^{131}I, respectively, and injected simultaneously into rabbits. In the latter study the HDL3 particles were cleared more rapidly than the HDL2 fraction.

The tissue sites of HDL clearance from the circulation have not been fully characterized. In addition to clearance of apoA-I by the kidney (13, 36, 37) and selective uptake of HDL cholesteryl ester by the liver (10), HDL particles equilibrate with the extravascular fluid space (38, 39), and it is theoretically plausible that smaller HDL particles may be more prone to cross the endothelial barrier into the extravascular fluid compartment, contributing to the lowering of HDL cholesterol. In the study by Horowitz et al. (13), apoA-I more readily dissociated from HDL particles that were triglyceride-rich when perfused through a rabbit kidney, but it was only after in vitro lipolysis of the particles by partially purified LPL and HL that the clearance of apoA-I increased dramatically. In an animal with low HL, such as the rabbit, this enhanced renal clearance of triglyceride-rich HDL would not occur, and could explain our finding of a more rapid clearance of small post-lipolysis HDL in this species.

In this study we chose to label HDL in situ with radioactive iodine and we measured apoA-I in rabbit plasma after isolation by SDS-PAGE. An alternate approach has been to reconstitute radiolabeled purified apoA-I with HDL (14, 20, 40). Vega et al. (41) have provided evidence that the kinetic behavior of apoA-I associated with HDL is similar using the two techniques, although labeled purified apoA-I is cleared slightly more rapidly than in situ labeled HDL apoA-I. The concern remains, however, that with the reconstituted purified apoA-I method only exchangeable pools of apoA-I are labeled, and these pools of apoA-I may be more readily dissociated from the HDL particles. There is also some concern that radioiodination of free apoA-I could produce more denatured apoA-I than labeling HDL apoA-I in situ, because the iodination of free apoA-I may modify tyrosine residues in a way that prevents the normal reassociation of apoA-I with HDL (41, 42).

The question of physiological relevance of studying HDL particles modified in vivo by intravenous heparin also needs to be addressed. Heparin administered in this dose causes release of endothelial lipases into the circulation, resulting in supraphysiological levels of circulating lipase and marked lipolysis of plasma lipoproteins. The HDL particles isolated postheparin are not normally measurable in the circulation of healthy normolipidemic humans. However, one could argue that lipolysis occurring in the capillary microcirculation could result in the formation of particles having similar characteristics, which could then be rapidly cleared from the circulation. Be that as it may, this study was designed to compare the clearance of particles that were markedly different in size and composition. Whether the findings of the present study can be extended to a more physiological model will necessitate further investigation. For example, it needs to be determined whether there is a difference in the clearance of fasting HDL and HDL that has undergone physiological lipolysis in the postprandial state. In addition, whether small and large fasting HDL particles are cleared at different rates deserves further investigation in future studies.

Along the same lines, it is possible that the high FFA content of the postheparin particles could have played a role in enhancing the clearance of the particles, perhaps preferentially in the rabbit model. Closer examination of the data presented in Table 2 revealed that the relative proportion of FFA in HDL between the preheparin and the postheparin tracer in study 1 (1.3% vs. 3.9% respectively) was smaller than in study 2 (1.5% vs. 5.4%), and although both studies showed a faster clearance of the postheparin tracer, this difference might explain why there was a greater difference between the clearance rate of the tracers in study 2. The recent demonstration that a scavenger receptor, Class B, Type I, can bind HDL particles (43, 44), raises the intriguing possibility that fatty acids in the postheparin HDL particles could have increased the affinity of the particles for this or some as yet unknown HDL receptor, thus enhancing their clearance.

Results of the present study suggest that in hypertriglyceridemic states a number of mechanisms may be responsible for the lowering of apoA-I associated with HDL and consequently HDL cholesterol, including enhanced clearance of large triglyceride-rich HDL particles, as evidenced by our findings in a hepatic lipase-sufficient species, and a more rapid clearance of small post-lipolysis HDL, as evidenced by our findings in hepatic lipase-deficient species. Future studies will be needed to determine the precise compositional and size properties of HDL that determine its clearance, as well as the in vivo role of enzymes and other factors involved in HDL clearance.

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