Monoclonal Antibody Inhibition of Cholesteryl Ester Transfer Protein Activity in the Rabbit

Effects on Lipoprotein Composition and High Density Lipoprotein Cholesteryl Ester Metabolism

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Abstract

Cholesteryl ester transfer protein (CETP) promotes in vitro transfer of cholesteryl ester (CE) and triglyceride (TG) between lipoproteins. We studied the function of CETP in vivo in rabbit lipoprotein metabolism using a neutralizing monoclonal antibody (MAb, TP1) to CETP. Rabbits were injected with TP1 (n = 8), or irrelevant MAb or saline (control, n = 8), resulting in an initial 71% inhibition of CETP, which fell to 45% after 48 h. HDL CE rose in the inhibited animals, reaching levels that doubled initial and control values at 48 h ($P < 0.001$). HDL TG fell reciprocally, but HDL protein did not change, suggesting a CE for TG exchange. VLDL CE/TG decreased. Rabbits were also given [^{3}H]cholesteryl ether HDL (a CE analogue). CETP inhibition delayed the initial clearance of radioactivity from HDL (control 6.8 vs. TP1 4.1 pools/d) and plasma (7.8 vs. 5.2 pools/d). We conclude that CETP plays a quantitatively important role in HDL CE catabolism in the rabbit, promoting the exchange of TG for CE and the clearance of CE from plasma.

Introduction

The incubation of human plasma at 37°C results in a net transfer of cholesteryl ester (CE)[1] from HDL and LDL to VLDL, and a reciprocal movement of triglyceride (TG) from VLDL to LDL and HDL (1, 2). The transfer of CE, TG, and phospholipids (PL) among the lipoprotein subclasses is mediated by lipid transfer proteins. One of these, a cholesteryl ester transfer protein of M_r 74,000, has been purified to homogeneity from human plasma (3). Monoclonal antibodies (MAb) have been obtained by immunizing mice with this purified CETP. Through the use of these neutralizing MAbs, the M_r 74,000 has been shown to be responsible for all of the CE and TG, and part of the PL transfer activity, in human plasma (4). In addition, the neutralizing antibodies block the net transfer of lipids in incubated human plasma without affecting the rate of cholesteryl ester formation by lecithin:cholesterol acyltransferase (LCAT) (5). These MAbs were also found to inhibit CE transfer activity in rabbit plasma, indicating conservation of the epitope between rabbits and humans (4).

Although the role of CETP in mediating neutral lipid transfer in incubated plasma is clear, its significance in the distribution and catabolism of lipoprotein neutral lipids in vivo is less certain. Human studies shows a progressive shift of cholesteryl ester from HDL to VLDL as serum triglyceride levels increase (6), perhaps as a result of CETP-mediated transfer of CE from HDL to VLDL (5). In rats, injection of partially purified human CETP resulted in a shift of HDL cholesteryl ester from larger HDL to VLDL and a depletion of VLDL triglyceride (7–9). Interpretation of these studies, however, is limited by the facts that the doses of transfer protein were uncontrolled and that rats naturally lack CETP activity.

In this study, we have examined the role of CETP in the rabbit by injecting neutralizing MAbs, then studying changes in plasma lipoprotein profiles. Based on current knowledge of the actions of CETP, we postulated several effects of CETP inhibition on lipoprotein composition. Primary among these were an increase in HDL CE and a decrease in VLDL CE, with reciprocal changes in TG. We further anticipated that LDL CE might be decreased as a result of depletion of CE in the LDL precursor, VLDL, and that LDL TG would increase as a result of decreased transfer to VLDL. In addition, a decrease in HDL PL was hypothesized, due to the known contribution of CETP to PL transfer. Finally, recent evidence suggests that TG enrichment of HDL, after antibody-inhibition of lipoprotein lipase, triggers increased HDL protein turnover, with a resultant fall in HDL protein (Goldberg, I. J., personal communication). This suggested the possibility of a rise in HDL protein if CETP inhibition did result in HDL TG depletion. In a subset of the rabbits studied, we have also examined the effects of CETP neutralization on clearance and tissue uptake of [^{3}H]-cholesteryl ether HDL. Cholesteryl ether is a cholesteryl ester analogue which is also transferred by CETP. We hypothesized that CETP inhibition might result in decreased uptake of CE by the liver.

The inhibition of CETP in the rabbit caused an increase in HDL CE and a decrease in HDL TG. In addition, VLDL CE/TG fell, suggesting that CETP promotes exchange of HDL CE for VLDL TG. Clearance of [^{3}H]cholesteryl ether from both HDL and plasma was delayed, and there was a trend toward decreased uptake of radioactivity by the liver in inhibited animals.

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1. *Abbreviations used in this paper:* CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; FCR, fractional catabolic rate; LCAT, lecithin:cholesterol acyltransferase; PL, phospholipid; RBC, red blood cell; TG, triglyceride.

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Methods

Materials

Isolation of lipoproteins. VLDL and LDL were isolated by sequential preparative ultracentrifugation at densities 1.006 and 1.063 g/ml. To prepare HDL, 1 ml of 1.063 bottom fraction was raised to density 1.25 g/ml, overlaid with 1.21 g/ml density NaBr and spun at 40,000 rpm for 48 h, at 10°C. A 50.3 Ti rotor and 6-ml sealed polyallomer tubes were used for all lipoprotein preparations.

Preparation of [^{3}H]cholesteryl ether HDL. [^{3}H]Cholesteryl ether HDL was prepared according to the method of Morton and Zilversmit (10) with slight modifications, and was biologically screened (i.e., injected into a donor rabbit and subsequently reisolated) before injection into recipient rabbits. 1 mCi [^{3}H]cholesteryl linoleyl ether (32 Ci/mmol) or [^{3}H]cholesteryl oleyl ether (32 Ci/mmol), 6.25 nmol triolein, and 3.73 μmol egg phosphatidylcholine were dried under N_2, suspended in 8 ml 2 mM EDTA, 1 mM dithiothreitol, pH 7.5, and sonicated at 4°C at a power setting of 4 for four 10-min bursts using a sonicator (model 185; Branson Sonic Power Co., Danbury, CT) fitted with a microtip. The resulting emulsion was centrifuged 15 min at 1,000 rpm to remove Ti. The supernatant was injected into 20 ml of $d > 1.063$ fraction of rabbit plasma and incubated for 16 h at 37°C, with the addition of 2 mM EDTA, 2 mM diethylparanitrophenyl phosphate (an LCAT inhibitor) and 10 U/ml each of penicillin and streptomycin. The mixture was then dialyzed against normal saline, 1% each penicillin and streptomycin, for 24 h at 4°C. After dialysis, it was infused intravenously into a rabbit, over 5 min. The rabbit was exsanguinated after 1 h and [^{3}H]cholesteryl ether HDL was isolated from the $d > 1.063$ fraction of the rabbit plasma. The labeled HDL was extensively dialyzed against normal saline, 1% each penicillin and streptomycin, and passed through a sterile 0.45 μm filter. The resulting HDL preparations had specific activities of 4,200 cpm/μg CE (linoleyl) and 13,700 cpm/μg CE (oleyl). The latter preparation contained 97.5% of the label as cholesteryl ether, 0.5% as free cholesterol, 1.4% as triglyceride and 0.6% as phospholipid. Both [^{3}H]cholesteryl ether HDL preparations showed a similar delay in clearance from plasma during CETP inhibition and the data obtained with the two preparations have therefore been combined.

Preparation of monoclonal antibodies. MAbs to CETP have been described previously (4). CETP-specific and control MAbs were purified from mouse ascites fluid by protein A-Sepharose chromatography. Control MAb was made to a fusion protein of apo-B produced by bacteria; this control MAb did not react with native apo-B. Because of an initial febrile response to MAb injection in our rabbits, the protein A-Sepharose column was pre-washed with ethanol to remove pyrogen, according to the manufacturer's suggestion. MAbs were filtered through a 0.42-μm filter and stored under sterile conditions. These two modifications prevented any further febrile responses.

[1a,2a(n)-^{3}H]Cholesteryl linoleyl and oleyl ethers, Protosol, Biofluor, and Econofluor were obtained from New England Nuclear Research Products (Boston, MA). All enzymes were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Ketamine HCl and xylozine were obtained from Henry Schein Co., Port Washington, NY.

Procedures

Rabbit studies. New Zealand White male rabbits, 2.5–3.5 kg, were studied in pairs (experimental and control), matched for plasma cholesterol and triglyceride. Free access to food and water was allowed throughout the course of the study. During an initial pilot study, rabbits (n = 8) were infused with MAb (0.83 mg/kg) at time 0 hours only. This resulted in modest inhibition of transfer activity during the following 24–48 h (Fig. 2, *dashed line*). Subsequently, our protocol was modified to include a second infusion of MAb at 6 h (0.42 mg/kg). On the morning of study, rabbits were bled for baseline studies, then, at time 0 hours, infused over 5 min with MAb to CETP (inhibited animals), or irrelevant IgG or saline (control animals). Rabbits studied for cholesteryl ether tissue uptake received [^{3}H]cholesteryl ether HDL immediately after MAb infusion. Eight experimental and eight control rabbits were studied using this modified protocol. To reduce the effects of stress, these rabbits were accommodated to handling and restraint for several days before study and were kept in their usual surroundings during the course of the study. Blood volume removed over a 24-h period was kept to < 20% of total blood volume to minimize hemorrhagic lipemia.

Sampling of tissue radioactivity. At the time of sacrifice (24 or 48 h), rabbits were heparinized with 5,000 U per kg i.v., given a sublethal injection of xylozine (5 mg/kg) and ketamine (35 mg/kg) and exsanguinated by open cardiac puncture. Perfusion with normal saline instilled through the left ventricle was carried out to a total volume of 2 liters at which time the perfusate was invariably clear. Puncture of the inferior vena cava was performed at the start of perfusion to aid in clearing of the liver. Organs were placed on ice and weighed. Samples of skin, muscle, and abdominal fat were taken and the percentage of total body weight of each calculated from published tables (11–13). The small intestinal lumen was washed with saline until clear.

Protocols for tissue and RBC digestion were obtained from New England Nuclear. 200-mg samples of each organ in duplicate were digested overnight at 55°C with 1.5 ml Protosol. Decolorization with 100 μl 30% H_2O_2 was carried out at 55°C for 30 min. Samples were counted in 10 ml Econofluor after a 60-min equilibration period. Total counts in each organ were derived from the weight of the organ and the average counts in the digested samples. 250-μl aliquots of RBCs were digested in 1 ml of a Protosol/ethanol mixture (1:2, vol/vol). Samples were incubated at 60°C for 60 min. Decolorization with 500 μl 30% H_2O_2 was carried out at 60°C for 30 min. Samples were counted following the stepwise addition of 15 ml Biofluor and 0.5 ml of 0.5 N HCl. There was a 30-min equilibration period before counting. Organ and RBC samples were counted for 5 min with an open channel in a scintillation counter (LS 5801; Beckman Instruments, Inc., Fullerton, CA) using an Automatic Quench Correction program. 50-μl aliquots of plasma were counted in Hydrofluor. The total injected radioactivity was derived from the 5-min plasma and RBC counts. Blood volume was assumed to be 6% of body weight. 200-μl aliquots of each lipoprotein fraction were counted and the total counts in each circulating lipoprotein fraction derived from the plasma volume and volume plasma spun.

Analytical methods. Free and total cholesterol were measured enzymatically (14–16) and cholesteryl ester mass calculated from the difference (mean recovery 72.4±3.0%, i.e., 72.4% of the plasma CE mass was recovered in the isolated lipoproteins). Phospholipid mass was determined enzymatically according to the method of Takayama (17) (mean recovery 81.4±3.0%). Triglyceride enzymatic reagent kits were obtained from Sigma Chemical Co., St. Louis, MO (mean recovery 79.5±2.9%). Cholesterol, TG and PL assays were checked using normal and hyperlipemic human plasma obtained from the Centers for Disease Control. Results were within 13 to 27% of the standard for cholesterol assays, 4 to 34% for triglyceride assays, and 18 to 28% for phospholipid assays. Protein concentration in VLDL was determined according to the method of Lowry (18). Protein concentration in LDL and HDL was determined using the Bradford reagent system. Lipoprotein mass data were adjusted for small changes in hematocrit occurring at the 24- and 48-h timepoints.

Measurement of LCAT and cholesteryl ester transfer activity. LCAT activity was measured from the decrease in free cholesterol mass following plasma incubation at 37°C for 0, 0.5, 1, 1.5, and 2 h. CETP activity in rabbit plasma was assayed as described previously (19).

Statistical methods. Due to the large number of measurements in this study, it was important to control for type I error. Therefore, it was decided a priori to carry out only a limited number of statistical comparisons, based on the original hypotheses and also the changes in lipoprotein composition implied by these changes (see Introduction and Tables II and III). In addition, because we were interested primarily in absolute changes in lipoprotein mass regardless of their time-course, it was decided to define the response of each animal to CETP

inhibition as the difference between the zero time value and the mean of all postinhibition values. Comparisons between the two groups (inhibited and control) were made by unpaired *t* tests.

Three additional comparisons were made related to turnover of [^{3}H]cholesteryl ether. Initial slopes of clearance of radiolabel from HDL and plasma, and the FCR of plasma were calculated, and statistical comparison between groups made by unpaired *t* test. Radioactivity data in plasma and in HDL were fitted by a sum of two exponentials ($Ae^{-\alpha t} + Be^{-\beta t}$), representing the response of a two-compartment system (one intravascular and one extravascular). Two parameters were derived from the two-exponential model: the initial (fractional) slope [$(A\alpha + B\beta)/A + B$] and the (irreversible) fractional clearance rate [$A + B/(A/\alpha + B/\beta)$]. Turnover studies are usually done while the animals are in a steady state (i.e., constant levels of plasma and HDL CE in this case). However, by making the reasonable assumption that the fractional clearance and turnover rates remain constant even as CE levels change in response to CETP inhibition, it is possible to derive these fractional rates from total radioactivity data.

All results are expressed as means±SEM.

Results

Three neutralizing monoclonal antibodies to human cholesteryl ester transfer protein were obtained by immunizing mice with a purified preparation of CETP, and have been previously described (4). To evaluate their activity in the rabbit, each MAb was incubated with rabbit plasma at 37°C in increasing concentration (Fig. 1). There was a 62–85% maximum inhibition of the cholesteryl ester transfer activity. Of the three MAbs, TP1 (formerly 2H4 [4]) was most efficient at inhibiting transfer activity in the rabbit and was thus chosen for in vivo study. Preincubation of MAb TP1 with plasma did not significantly enhance its ability to inhibit rabbit CETP (Fig. 1, *dashed line*). Thus, MAb TP1 appeared suitable for in vivo inhibition of CETP in the rabbit.

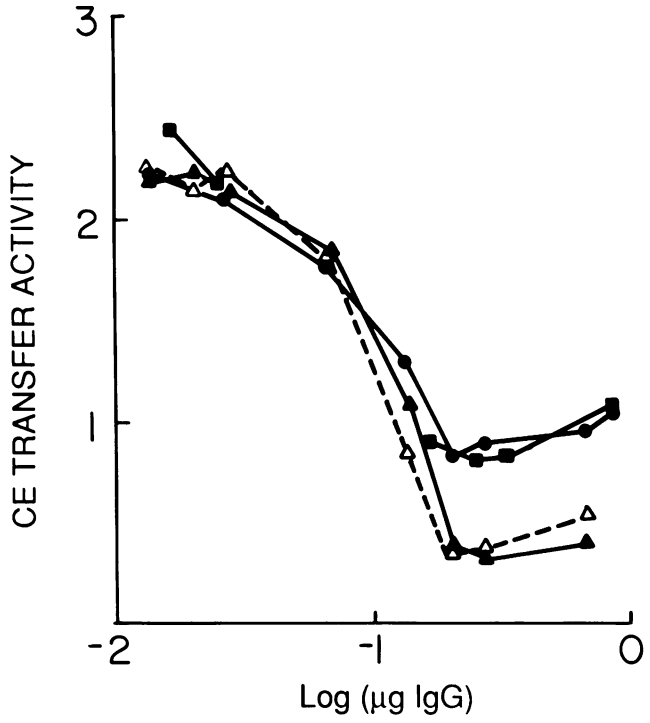

Figure 1. Immunotitration of CE transfer activity in rabbit plasma by three monoclonal antibodies to human CETP. Rabbit plasma (10 μl) was incubated with varying concentrations of each of the TP1, TP2, and TP3 MAbs for 2 h at 37°C in 60 μl of Tris-saline-EDTA, pH 7.5. Isotopic CE transfer activity was then assayed for 2 h at 37°C. Y-axis represents CE transfer activity in cpm $\times 10^{-3}$. ▲, TP1; ●, TP2; ■, TP3; △, TP1 without 2 h preincubation.

The mean level of inhibition of CE transfer activity achieved in rabbits after intravenous infusion of MAb is shown in Fig. 2. After a single MAb infusion at time 0 h, there was an early peak of 70% inhibition with a fairly rapid fall to 47% by 24 h (Fig. 2, *upper dashed line*). To achieve a more pronounced inhibition of activity, a second injection of antibody was given at 6 h. This resulted in a more sustained inhibition over the first 24 h, with a decrease to 44% by 48 h (Fig. 2, *upper solid line*). Control rabbits showed a small but consistent degree of inhibition possibly reflecting stress or blood loss, with accompanying loss of CETP. This nonspecific inhibition of CE transfer activity was less pronounced after modification of the protocol to reduce stress and blood loss (*lower solid line*). There was no difference in degree of inhibition in controls receiving nonspecific monoclonal IgG vs. saline infusion, hence the data have been combined. The compositional data that follow are presented for inhibited and control animals (n = 8 pairs) treated by the modified protocol (i.e., two MAb injections plus minimization of stress and blood withdrawal).

The mean changes from baseline values for HDL CE, and TG after injection of MAb are shown in Fig. 3, *A* and *B*, respectively. Most notable was the pronounced rise in HDL CE in the inhibited animals, which continued throughout the time course of the study, and reached levels that were almost twice the initial values (~ 20 mg/dl). By contrast, there was little change in HDL CE in the control animals. There was a pronounced fall in HDL TG in the TP1-treated animals (Fig. 3 *B*). The control animals also showed a transient, less marked fall in HDL TG, which tended to recover by 24 h.

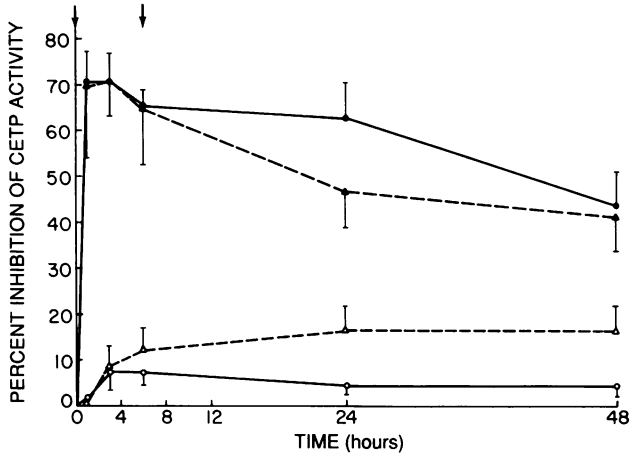

Figure 2. Inhibition of CE transfer activity in rabbit plasma after TP1 MAb infusion. Rabbits were initially infused at 0 hours with 0.75 mg/kg TP1 (▲, n = 8) or nonspecific monoclonal IgG (0.75 mg/kg) or saline (△, n = 7, data combined). Subsequently, the protocol was modified to achieve more sustained inhibition. Inhibited animals (n = 8) received 0.83 mg/kg at 0 hours with a boosting infusion of 0.42 mg/kg at 6 h (●). Control rabbits (n = 8) also received two infusions of IgG (0.83 and 0.42 mg/kg) or saline (○). Aliquots of plasma (50 μl of a 1:10 dilution) were incubated in duplicate at 37°C for 18 h in a 250-μl volume of Tris-saline-EDTA, pH 7.5 to determine CE transfer activity. The percent inhibition (y-axis) was calculated as [1 − (activity after inhibition/initial activity) × 100%]. Results are means±SEM.

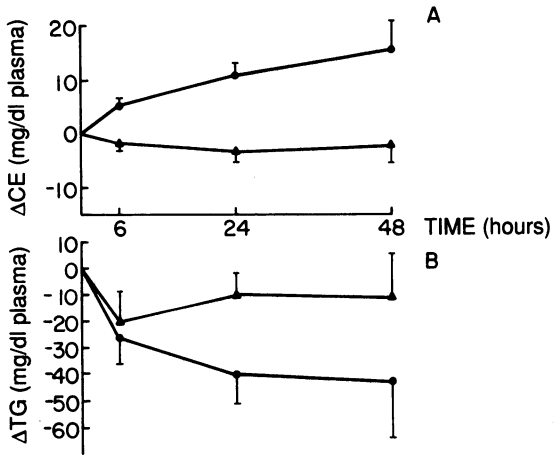

Figure 3. Mean change from baseline of HDL CE (*A*) and TG (*B*). HDL was assayed in duplicate for CE and TG concentration in TP1 (n = 8) and control (n = 8) animals. Results, expressed in mg/dl plasma, are means±SEM of the change from the 0 hour value for each animal. The mean initial HDL CE and TG values are shown in Table I. ●, TP1 injected animals; ▲, control animals.

The full results of lipid and protein analysis of plasma, VLDL, LDL, and HDL in inhibited and control rabbits are presented in Table I. In addition to the changes in HDL lipids enumerated above, there was a rise in plasma CE level in the inhibited group, which largely reflected the rise in HDL CE. There were no other striking differences between the inhibited and control groups. Although there was a moderate increase in all VLDL constituents at 24 h, this was similar in control and inhibited animals, and thus represents a nonspecific response. Similarly, there were nonspecific increases in LDL constituents in both control and inhibited animals. It is notable that the dominant neutral lipid in LDL and HDL was TG, not CE, in control animals. Previous workers have also found that HDL and LDL are markedly enriched in TG in nonfasted rabbits (20, 21). Separation of IDL from the LDL subfraction did not alter the high LDL TG.

Tests for statistical significance of several changes in lipoprotein mass constituents hypothesized a priori (see Introduction) are presented in Table II. Because we made no prior assumption regarding the time course of lipoprotein compositional changes, and because we found no uniform time of peak change in lipoprotein composition, we elected to average all postinjection values (i.e., 6-, 24-, and 48-h values) for statistical analysis. The result is designated "mean postinjection response" in Table II. A statistical comparison of the mean change from initial to postinjection response within each group is also presented.

HDL CE rose significantly comparing the inhibited and control groups ($P < 0.001$), and also the postinjection to the initial value in the inhibited group ($P < 0.01$). HDL TG fell in the inhibited group ($P < 0.05$) but the mean change was not significantly different from that of the control group. Plasma CE rose in the inhibited group ($P < 0.05$), but the difference between the groups did not quite reach significance ($P = 0.10$). Additional hypotheses tested but not found to be statistically significant included changes in VLDL CE and TG, LDL CE and TG, and HDL PL and protein.

The percentage composition of each lipoprotein fraction is presented in Table III. The percent CE in VLDL decreased in the inhibited animals, while it tended to rise in the control group. Primarily as a result of these changes, the CE/TG ratio fell in the inhibited group and rose in the controls. At 24 and 48 h, the CE/TG ratio is significantly different ($P < 0.01$) between the two groups. Although there were some changes in LDL composition, none were significantly different, comparing control and inhibited animals.

Paralleling the changes in HDL mass outlined previously, the percent CE in HDL in the inhibited group rose to twice its starting value, while there was a reciprocal decrease in HDL percent TG. By contrast, there were no changes in HDL CE or TG in the control group. Additionally, there was a fourfold increase in the HDL CE/TG ratio in the inhibited animals, not seen in controls, while the lipid/protein ratio did not change significantly. Thus, the lipid compositional changes of HDL in CETP-inhibited animals largely constituted a CE for TG substitution. As determined by scanning SDS gels, HDL apoprotein composition showed no change with CETP inhibition and was predominantly apo A-I (85% in controls, 87% in inhibited animals).

As in human studies (5), the rate of cholesterol esterification in rabbit plasma was unaffected by CETP inhibition (data not shown), indicating that the increase in HDL CE was probably due to a decrease in CE catabolism. The effect of CETP inhibition on clearance of [^{3}H]cholesteryl ether HDL was therefore studied in five pairs of rabbits receiving double injections of MAb. The results for a representative pair of animals (TP1 injected and control) are shown in Fig. 4 *A*. There was a marked delay in clearance of radioactivity from HDL in the inhibited rabbit. Although the control animal showed lower radioactivity at the initial timepoint and recovery of radioactivity during isolation of the lipoproteins was 56.2%, these were not consistent findings in all pairs of rabbits. For the same pair of rabbits, CETP inhibition also produced a delay in the clearance of radioactivity from plasma (Fig. 4 *B*). This suggests that CETP activity regulates the catabolism of HDL CE and perhaps thereby affects the clearance from whole plasma.

Table IV shows the mean rate of removal of [^{3}H]-cholesteryl ether from HDL and plasma for five pairs of animals studied. These values are the means of the initial slopes of each animal's decay curve using a two-exponential model (see Methods). There was a significant delay in clearance of radioactivity from both HDL and plasma ($P < 0.05$ and $P < 0.01$, respectively) when the inhibited group was compared with the control group. Similar clearance data are not shown for VLDL and LDL, because, in general, radioactivity recovered in these lipoproteins was < 10% of the injected dose and there were no consistent differences between the TP1 injected and control groups. At this level of radioactivity, the 2.5% of injected radiolabel not found in cholesteryl ether becomes significant (see Methods).

Since the initial slope reflects both redistribution of radiolabel into a noncirculating pool and irreversible removal from the plasma pool, the plasma FCR was calculated from the area under the plasma decay curve. For the TP1-treated animals, the mean plasma FCR was 2.73±0.25 pools/d, while for the control group, it was 3.80±0.37 pools/d ($P < 0.05$ by unpaired t test).

The distribution of [^{3}H]cholesteryl ether radioactivity in tissues is shown for inhibited and control animals in Table V.

*Table I. Plasma and Lipoprotein Lipid and Protein Concentrations in TP1-treated and Control Rabbits**

	Time	FC	CE	PL	TG	Protein
	h					
Plasma (*mg/dl±SEM*)						
TP1	0	10.2±2.3	45.1±7.8	94.1±8.0	181.5±33.1	
	6	12.7±2.8	46.7±8.2	81.8±6.5	170.8±20.7	
	24	24.1±5.4	65.4±12.7	124.2±14.5	257.4±76.4	
	48	19.3±4.0	58.0±15.0	103.0±14.6	138.2±21.2	
Control	0	14.0±2.7	47.1±8.3	96.9±8.9	148.8±17.7	
	6	13.0±2.4	45.9±8.1	88.0±9.4	115.6±31.5	
	24	21.9±3.1	50.9±10.4	123.1±15.1	175.6±47.0	
	48	23.6±3.9	45.1±5.2	121.7±12.8	126.5±26.9	
VLDL (*mg/dl±SEM*)						
TP1	0	4.9±2.0	5.2±1.4	9.3±2.9	77.7±17.8	11.3±2.7
	6	4.2±1.5	2.5±0.7	7.3±2.2	68.6±18.0	8.3±2.1
	24	10.2±4.1	4.8±1.6	15.2±4.9	115.4±38.5	15.3±4.1
	48	3.7±1.3	3.0±1.2	9.5±3.8	64.2±21.1	10.8±2.7
Control	0	3.6±0.9	4.9±1.3	6.8±1.5	57.8±10.3	8.2±2.3
	6	2.6±1.1	3.9±1.6	6.7±2.2	51.6±18.8	7.8±3.1
	24	6.3±2.4	7.9±2.4	10.9±3.1	85.2±34.0	15.0±4.4
	48	2.4±1.2	4.1±1.8	4.3±1.0	28.4±4.2	7.5±2.6
LDL (*mg/dl±SEM*)						
TP1	0	1.5±1.2	3.0±1.5	3.7±1.0	17.7±3.1	2.6±0.8
	6	2.0±1.0	2.9±1.1	3.8±0.6	11.0±1.1	2.4±0.4
	24	4.3±1.9	5.9±2.4	7.9±1.8	15.2±2.1	5.2±1.1
	48	3.7±0.7	5.6±2.3	8.3±1.4	14.7±1.0	5.3±0.9
Control	0	1.6±0.7	4.5±2.8	4.7±1.4	11.8±2.6	6.0±2.4
	6	2.2±0.8	3.7±2.0	5.2±1.3	10.4±1.0	3.2±1.1
	24	3.8±1.4	8.0±4.6	7.4±1.4	12.6±1.0	5.0±0.8
	48	4.1±1.7	5.8±1.2	10.0±2.9	18.0±5.6	5.8±1.3
HDL (*mg/dl±SEM*)						
TP1	0	7.6±3.6	18.2±6.9	57.9±5.5	70.2±12.5	68.7±13.1
	6	9.4±4.5	23.6±6.5	55.7±6.0	44.3±5.7	51.6±8.2
	24	10.7±5.3	29.1±7.9	58.9±7.0	29.8±4.8	57.6±11.4
	48	7.5±0.8	35.0±15.8	69.9±12.8	31.9±5.3	62.2±8.3
Control	0	7.4±2.5	23.3±4.8	65.6±6.0	59.4±9.1	86.7±12.0
	6	8.2±3.0	21.9±4.3	62.0±4.8	39.7±3.4	76.9±9.7
	24	9.0±3.3	18.9±5.2	65.4±4.6	49.2±7.8	79.6±11.6
	48	8.4±3.5	21.3±3.9	76.8±12.6	56.0±8.7	84.0±13.3

* Plasma, VLDL, LDL, and HDL at each timepoint from CETP-inhibited (n = 8) and control (n = 8) rabbits were assayed for PL, TG, free and total cholesterol. Cholesteryl ester was calculated from the difference between free and total cholesterol. Lipoproteins were also assayed for protein. Results are means in mg/dl plasma±SEM.

Also shown is a group of rabbits not included in the earlier analysis, which was sacrificed at 6 h. We hypothesized that inhibition of CETP would lead to less uptake of HDL CE in the liver, since CETP is thought to promote transfer of HDL CE to VLDL, and VLDL are cleared predominantly in the liver. There was a trend toward decreased liver uptake of labeled cholesteryl ether in the inhibited animals, especially at 24 h after injection of labeled HDL (13% less uptake). However, the differences in liver uptake, comparing inhibited and control animals were not statistically significant. In a further analysis of the data, the percent uptake of radiolabel by liver after normalization to 100% recovery in each animal was compared. In paired comparisons, there was a decrease in uptake of radiolabel by liver in 7 of 10 inhibited animals, no change in one, and a rise in two (P = 0.07 by paired t test). Lending further weight to this trend, there was a strong negative correlation (r = −0.87) in inhibited animals between liver uptake of [^{3}H]cholesteryl ether and the level of HDL CE following inhibition of CETP (i.e., the higher HDL CE after CETP inhibition, the fewer counts recovered in the liver). Together with the delay in clearance of [^{3}H]cholesteryl ether from HDL and plasma shown above, these data suggest that CETP enhances the clearance of HDL CE by the liver, probably by transfer into VLDL and subsequent catabolism of VLDL by the liver.

The remainder of the organs sampled showed only slight differences in uptake of [^{3}H]cholesteryl ether between the two groups, with the exception of the kidneys. At all timepoints, there was more radiolabel in the kidneys of the inhibited animals than those of the controls with nearly a twofold difference

*Table II. Tests of Statistical Significance of Changes in Lipoprotein Composition**

		Initial value	Mean postinjection response	*P* Value: Initial vs. mean postinjection response	Mean change	*P* Value: Mean change, inhibited vs. control group
			mg/dl±SEM			
Plasma	CE_I	45.1±7.8	56.8±10.1	<0.05	11.7±4.3	=0.10
	CE_C	47.1±8.3	49.0±8.8	NS	1.9±3.7	
VLDL	CE_I	5.2±1.4	3.5±1.0	NS	−1.8±0.9	NS
	CE_C	4.9±1.3	5.0±1.5	NS	0.2±1.6	
	TG_I	77.7±17.8	87.0±24.8	NS	9.3±14.8	NS
	TG_C	57.8±10.3	54.4±17.1	NS	−3.3±9.6	
LDL	CE_I	3.0±1.5	5.1±1.7	NS	2.1±1.2	NS
	CE_C	4.5±2.8	6.6±3.2	NS	2.1±0.4	
	TG_I	17.7±3.1	13.7±1.0	NS	−4.0±3.4	NS
	TG_C	11.8±2.6	13.0±1.4	NS	1.2±2.3	
HDL	CE_I	18.2±6.9	27.9±8.1	<0.01	9.7±2.3	<0.001
	CE_C	23.3±4.8	20.9±4.6	NS	−2.4±1.7	
	TG_I	70.2±12.5	36.9±3.2	<0.05	−33.3±13.1	NS
	TG_C	59.4±9.1	46.0±4.9	NS	−13.4±7.8	
	PL_I	57.9±5.5	59.7±7.1	NS	1.8±2.5	NS
	PL_C	65.6±6.0	66.3±5.6	NS	0.7±2.4	
	$PROT_I$	68.7±13.1	54.6±9.3	NS	−14.1±6.8	NS
	$PROT_C$	86.7±12.0	78.2±10.3	NS	−8.4±5.8	

* Nine a priori hypotheses were tested for statistical significance (see Introduction). For each hypothesis, results are given for inhibited (I, $n = 8$) and control (C, $n = 8$) animals. Results are means of 0 hour values (Initial value); means of 6-, 24-, and 48-h values for each animal (Mean postinjection value); and means of the change from Initial value to Mean postinjection value for each animal (Mean change); all expressed in mg/dl plasma±SEM. *P* values were calculated by unpaired *t* tests.

at 24 h. As noted above, the total counts recovered was somewhat variable, but without clear differences between control and inhibited animals at each timepoint. However, the recovery for both control and inhibited rabbits was less at 6 h than at 24 and 48 h, raising the possibility of distribution to a pool not measured. Of interest in this regard, Hussain et al. have established active uptake of CE from chylomicron remnants by rabbit bone marrow cells (22).

Discussion

The recent development of monoclonal antibodies to the cholesteryl ester transfer protein has made possible the study in vivo of the effect of CETP inhibition on lipoprotein composition. Although several earlier studies have suggested a significant role for CETP in HDL CE catabolism, its importance has never been directly addressed. The present study has demonstrated that CETP inhibition causes a pronounced increase in HDL CE, accompanied by a decrease in HDL TG. The increase in HDL CE was due to a delay in catabolism, as suggested by the decrease in fractional catabolic rate of [^{3}H]-cholesteryl ether in the inhibited animals. These changes occurred even though the CETP inhibition was only partial (from 70 to 45%, Fig. 2), largely due to the use of a heterologous MAb (Fig. 1). The data show that CETP is of major quantitative importance in the catabolism of HDL CE in the rabbit.

A role of CETP in determining HDL CE mass has been suggested previously by injection of CETP into rats (7–9) and by human studies (6, 23–25). Japanese patients with familial hyperalphalipoproteinemia develop large, CE-rich HDL due to the ongoing activity of LCAT without transfer of CE to apo-B containing lipoproteins. A lack of CETP has been postulated as the cause of their high HDL and low LDL levels (23, 24). However, Groener et al., studying an Italian family with familial hyperalphalipoproteinemia, showed these patients to have normal cholesteryl ester transfer activity (25). High levels of HDL interfere with the assay of CETP activity in plasma. Thus, it is unclear whether a deficiency of CETP activity is secondary to, or the cause of, hyperalphalipoproteinemia. Cross-sectional studies of human lipoprotein composition have suggested that the well known inverse correlation between VLDL TG and HDL CE levels could be due to increased CETP-mediated transfer of CE from HDL to VLDL (6). Recently, Yen et al. have shown a high correlation between VLDL TG and the rate of transfer of CE into VLDL from HDL in incubated plasma, suggesting that the mass of TG-rich acceptor lipoproteins influences the rate of CE transfer and thereby HDL CE mass (5). Also, accelerated transfer of CE from HDL to apo B-containing lipoproteins has been found in hypertriglyceridemic patients with dysbetalipoproteinemia, perhaps accounting for the low HDL CE in this condition (19). However, these findings were different from those reported by others (26).

In our study, the increase in HDL CE mass was accompanied by a reciprocal fall in HDL TG (Fig. 3), confirming that CETP mediates exchange of HDL CE with VLDL TG (27). The molar ratio of the change in mass of HDL CE and TG (inhibited minus control, Fig. 3) was ~ 1:1.3, approximating an equimolar hetero-exchange process (27). Consistent with the suggestion of a hetero-exchange of VLDL TG with HDL

*Table III. Percentage Lipoprotein Composition**

	Time	FC	CE	PL	TG	CE/TG	Lipid/Protein
	h			*%±SEM*			
VLDL							
TP1	0	4.0±0.8	5.2±0.9	8.7±1.5	82.1±2.1	0.06±0.01	9.6±1.4
	6	4.3±0.8	2.9±0.6	7.9±1.6	84.8±2.4	0.04±0.01	10.7±1.5
	24	5.7±0.8	3.4±0.5	10.6±1.5	80.3±2.0	0.04±0.01	8.8±0.9
	48	4.2±1.2	3.7±1.4	10.7±1.5	81.4±2.3	0.05±0.02	10.5±4.4
Control	0	4.7±0.5	6.3±1.0	9.9±1.9	79.2±3.0	0.08±0.01	10.9±1.9
	6	3.2±0.8	4.7±1.1	12.1±2.7	80.0±2.7	0.06±0.02	9.1±1.2
	24	4.9±0.7	7.8±1.0	14.4±2.6	72.9±2.9	0.11±0.02	6.5±1.1
	48	4.9±1.8	8.7±2.6	11.8±2.6	74.6±4.2	0.12±0.04	4.9±0.7
LDL							
TP1	0	4.3±2.4	10.3±4.1	14.9±2.7	70.5±6.6	0.19±0.08	11.0±1.6
	6	8.2±2.8	12.3±4.3	19.8±2.1	59.7±5.8	0.27±0.11	8.6±0.9
	24	9.5±2.8	13.2±3.9	24.0±2.5	53.2±6.1	0.33±0.11	6.3±0.5
	48	12.0±1.9	14.6±5.2	25.2±2.3	48.2±5.0	0.35±0.14	6.4±0.4
Control	0	6.4±2.0	14.5±3.4	23.1±4.2	79.2±3.0	0.34±0.13	5.7±1.0
	6	8.8±2.6	13.1±2.8	23.8±3.4	54.3±4.8	0.30±0.11	6.8±0.5
	24	9.4±2.8	17.9±5.0	24.3±1.4	48.4±6.0	0.26±0.04	6.1±0.4
	48	10.2±2.3	16.6±2.1	26.1±2.0	47.1±3.0	0.37±0.06	6.3±0.6
HDL							
TP1	0	4.8±1.9	10.9±3.2	39.5±3.6	44.7±4.9	0.31±0.12	2.8±0.7
	6	6.2±2.2	16.8±3.1	42.7±2.5	34.2±3.3	0.57±0.17	3.1±0.9
	24	7.3±2.5	21.3±3.6	46.7±2.4	24.7±4.1	1.19±0.43	2.0±0.5
	48	6.0±1.4	20.7±5.5	49.2±2.2	24.1±4.2	1.16±0.48	2.0±0.5
Control	0	4.6±1.5	15.3±3.2	42.1±2.1	38.0±4.8	0.54±0.17	2.2±0.4
	6	5.9±2.1	16.2±2.7	46.7±1.4	31.2±3.6	0.63±0.17	1.9±0.3
	24	6.0±2.1	13.5±3.7	45.8±2.6	34.7±5.4	0.66±0.34	2.1±0.4
	48	4.8±1.7	13.6±2.9	46.4±4.1	35.2±5.4	0.45±0.12	2.1±0.4

* Percent lipid composition of each lipoprotein fraction was calculated for CETP-inhibited (n = 8) and control (n = 8) animals (e.g., 100*FC/(FC + CE + PL + TG), all in mg/dl plasma). The CE to TG ratio and the total lipid to protein ratio are also presented. Results are mean percent±SEM. HDL protein concentration in one TP1 treated animal was excluded due to extreme variation from the mean.

CE, there was a significant decrease in the VLDL CE to TG ratio in the inhibited animals, compared with controls. However, the absolute mass of VLDL CE was low and not significantly changed by CETP inhibition, and < 10% of [^{3}H]-cholesteryl ether radioactivity was recovered in VLDL in both sets of animals. These results could be explained by extremely rapid transfer through the VLDL fraction, i.e., transfer of CE from HDL to VLDL with high turnover of VLDL. Large VLDL are the preferred acceptors of CETP-mediated transfer (28–30), and are in large part rapidly removed from plasma (31, 32). Similarly, transfer could have occurred into chylomicrons in these nonfasting rabbits.

Although the rabbit is an adequate species in which to study the effects of CETP on lipoprotein metabolism, various limitations were imposed by this model. An unexpected finding in this study was the TG-rich nature of these rabbits' HDL and LDL. Although the plasma TG levels were comparable to values found in the literature for nonfasted rabbits (20, 21, 33, 34), a major portion of this was in the HDL. A similar phenomenon has been observed in nonfasted rabbits by others, but not to this degree (20, 21). Presumably, this reflects in part the high level of transfer activity in this species, since with CETP inhibition, the HDL composition came to resemble that of nonfasting rabbits. Stress-mediated hypertriglyceridemia may also have contributed to this TG enrichment. Despite measures to minimize stress, plasma TG increased an average of 54 mg/dl during the accommodation period, just before study, and showed a further increase during the course of the study. Even though total plasma TG and cholesterol values were matched in each pair of rabbits, there were substantial variations in cholesterol and TG distributions among the lipoproteins, reflecting variability between rabbits. This is reflected in large SEMs for lipoprotein composition and differences in absolute levels (not significant) at 0 hours in control versus experimental animals (Tables I and III). This interanimal variability might have contributed to an insensitivity of this study to subtler changes in lipoprotein composition (e.g., in LDL). An additional concern was that binding of IgG to CETP while the CETP was bound to lipoprotein could have altered lipoprotein metabolism (e.g., by promoting clearance in the spleen). However, the molar ratio of CETP to HDL is of the order of 1:1,000, so that even if all HDL particles containing CETP were cleared by the spleen, this would have a miniscule effect on lipoprotein composition.

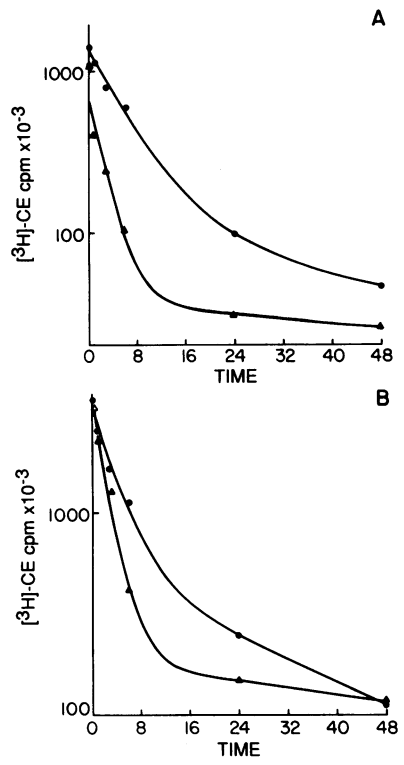

Figure 4. Radioactivity remaining in HDL (*A*) and plasma (*B*) after injection of [^{3}H]-cholesteryl ether HDL, shown for a representative pair of animals. CETP-inhibited and control rabbits were injected with ~ 3.5×10^6 cpm [^{3}H]cholesteryl ether HDL after the initial infusion of MAb (t = 0). Aliquots of plasma (50 μl) or HDL (200 μl) were counted in duplicate. The results are given as the total counts per minute remaining in plasma or HDL assuming a blood volume of 6% of body weight. Computer generated curves were drawn using a two exponential model of decay (see Methods). ●, TP1 injected animal; ▲, control animal.

In inhibited rabbits, total plasma CE rose significantly, and clearance of labeled CE from plasma was delayed by CETP inhibition. The fact that whole plasma, as well as HDL [^{3}H]-cholesteryl ether turnover, was delayed implies that CETP-mediated catabolism of HDL CE is a rate-limiting step for removal of CE from plasma. One interpretation of these data is that CETP, by affecting the movement of CE out of HDL, regulates the rate of transfer of CE from plasma into tissue. The tissue fate of [^{3}H]cholesteryl ether strongly suggested decreased uptake by liver in inhibited rabbits. This could be due to the influence of CETP on transfer of CE from HDL to VLDL, or perhaps a more direct effect of CETP on uptake of HDL CE by liver (35).

The effect of CETP inhibition on the clearance of CE from plasma suggests that CETP may play a regulatory role in the process of reverse cholesterol transport. This pathway of cholesterol metabolism postulates the centripetal movement of cholesterol from peripheral tissues to HDL, esterification by LCAT, and transfer of the CE to TG-rich lipoproteins with ultimate uptake by the liver. CETP could regulate this process, either by an effect on the rate of cholesterol esterification by LCAT, or on the delivery of CE to tissues. Recently, CETP has been shown to have no effect on LCAT activity in incubated human plasma (5). We found a similar lack of effect of CETP inhibition on cholesterol esterification in rabbit plasma. However, our data suggest that in the rabbit, CETP regulates the rate of transfer of CE from plasma into tissues, probably by mediating the transfer of CE from HDL to TG-rich lipoproteins, with subsequent rapid clearance by the liver.

Table IV. Initial Rate of Removal of [^{3}H]CE (pools/d±SEM)*

HDL	TP1	4.1±0.6	$P < 0.05$
	Control	6.8±1.0	
Plasma	TP1	5.2±0.4	$P < 0.01$
	Control	7.8±0.5	

* Radioactivity remaining in HDL and in plasma after injection of [^{3}H]cholesteryl ether HDL was determined. For each animal, the slope of the initial portion of a computer generated decay curve was calculated using a two-exponential model of decay. Results are means for five pairs of animals, in pools/d±SEM. Data from the same six pairs of animals are presented in this table and Table V, sacrificed at 24 and 48 h. However, one set of lipoproteins was lost during ultracentrifugation (animals sacrificed at 24 h). P values were calculated using an unpaired t test.

*Table V. Tissue Uptake of Radioactivity after [^{3}H]Cholesteryl Ether HDL Injection**

	Time after injection of radiolabel (%±SEM)		
	6 h (n = 4 pairs)	24 h (n = 3 pairs)	48 h (n = 3 pairs)
$Liver_I$	29.66±4.06	54.78±15.83	63.72±2.78
$Liver_C$	30.86±5.14	67.85±8.40	71.18±9.46
$Kidneys_I$	0.59±0.16	0.66±0.05	0.62±0.08
$Kidneys_C$	0.48±0.16	0.36±0.02	0.38±0.02
$Heart_I$	0.10±0.03	0.14±0.01	0.11±0.01
$Heart_C$	0.09±0.02	0.08±0.01	0.10±0.02
$Lungs_I$	0.32±0.10	1.23±0.47	0.85±0.15
$Lungs_C$	0.40±0.12	0.85±0.22	0.51±0.10
$Spleen_I$	0.06±0.01	0.17±0.03	0.14±0.02
$Spleen_C$	0.10±0.02	0.21±0.07	0.20±0.02
$Adrenals_I$	0.17±0.04	0.63±0.01	0.62±0.03
$Adrenals_C$	0.15±0.04	0.70±0.11	0.56±0.18
$Small\ intestine_I$	0.74±0.06	1.79±0.19	1.92±0.45
$Small\ intestine_C$	0.58±0.09	2.23±0.91	1.31±0.08
$Testes_I$	0.16±0.02	0.46±0.06	0.33±0.04
$Testes_C$	0.12±0.02	0.30±0.09	0.26±0.10
$Skin_I$	3.34±0.54	6.04±0.84	4.61±0.19
$Skin_C$	2.29±0.65	3.24±0.32	4.84±0.98
$Skeletal\ muscle_I$	3.92±1.57	15.98±1.06	12.30±3.49
$Skeletal\ muscle_C$	5.08±1.92	14.16±4.60	11.05±1.35
$Adipose\ tissue_I$	1.23±0.16	1.78±0.42	1.70±0.23
$Adipose\ tissue_C$	1.62±0.63	1.58±0.54	1.43±0.42
$Erythrocytes_I$	3.09±1.52	1.08±0.18	0.63±0.14
$Erythrocytes_C$	2.88±1.78	0.72±0.49	0.34±0.04
$Plasma_I$	31.02±3.11	9.07±2.48	3.21±0.44
$Plasma_C$	22.94±2.90	4.60±1.26	2.68±0.36
$Recovery_I$	75.20±4.82	107.37±7.87	90.76±4.83
$Recovery_C$	68.42±9.74	98.82±1.18	94.81±8.36

* TP1 treated and control rabbits were injected with [^{3}H]cholesteryl ether HDL and tissue radioactivity sampled at 6, 24, or 48 h. 200-mg aliquots of each tissue, and 250 μl of erythrocytes were digested in duplicate and counted. 50-μl aliquots of plasma were counted in duplicate. For each tissue, total organ counts were calculated based on the weight of the organ, and are expressed as percentage of total radioactivity recovered in plasma and erythrocytes 5 min after injection of labeled HDL. For each tissue, results are given for TP1 treated animals (subscript I) and control animals (subscript C) in mean percent±SEM.

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