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Research Article

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Hyperalphalipoproteinemia in Human Lecithin Cholesterol Acyltransferase Transgenic Rabbits

In Vivo Apolipoprotein A-I Catabolism Is Delayed in a Gene Dose-dependent Manner

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Abstract

Lecithin cholesterol acyltransferase (LCAT) is an enzyme involved in the intravascular metabolism of high density lipoproteins (HDLs). Overexpression of human LCAT (hLCAT) in transgenic rabbits leads to gene dose-dependent increases of total and HDL cholesterol concentrations. To elucidate the mechanisms responsible for this effect, ^{131}I-HDL apoA-I kinetics were assessed in age- and sex-matched groups of rabbits ($n = 3$ each) with high, low, or no hLCAT expression. Mean total and HDL cholesterol concentrations (mg/dl), respectively, were 162±18 and 121±12 for high expressors (HE), 55±6 and 55±10 for low expressors (LE), and 29±2 and 28±4 for controls. Fast protein liquid chromatography analysis of plasma revealed that the HDL of both HE and LE were cholesteryl ester and phospholipid enriched, as compared with controls, with the greatest differences noted between HE and controls. These compositional changes resulted in an incremental shift in apparent HDL particle size which correlated directly with the level of hLCAT expression, such that HE had the largest HDL particles and controls the smallest. In vivo kinetic experiments demonstrated that the fractional catabolic rate (FCR, d^{-1}) of apoA-I was slowest in HE (0.328±0.03) followed by LE (0.408±0.01) and, lastly, by controls (0.528±0.04). ApoA-I FCR was inversely associated with HDL cholesterol level ($r = -0.851$, $P < 0.01$) and hLCAT activity ($r = -0.816$, $P < 0.01$). These data indicate that fractional catabolic rate is the predominant mechanism by which hLCAT overexpression differentially modulates HDL concentrations in this animal model. We hypothesize that LCAT-induced changes in HDL composition and size ultimately reduce apoA-I catabolism by altering apoA-I conformation and/or HDL particle regeneration. (*J. Clin. Invest.* 1996. 97:1844–1851.) Key words: high density lipoproteins • lipoproteins • apolipoprotein A-I • metabolism • cholesterol

Introduction

Glomset was the first to propose lecithin cholesterol acyltransferase (LCAT)[1] as a pivotal enzyme involved in the intravascular metabolism of high density lipoproteins (HDLs) (1). LCAT is synthesized primarily by hepatocytes and catalyzes the reaction whereby a fatty acid is transferred from the *sn*-2 position of phosphatidylcholine to the 3-hydroxyl group of cholesterol, yielding cholesteryl esters and lysolecithin (1, 2). In plasma, LCAT preferentially associates with HDL (α-LCAT) but may also act on low density lipoproteins (β-LCAT) (3). The esterification of free cholesterol by LCAT is essential for the HDL particle maturation process by providing cholesteryl esters for the hydrophobic core, thus, converting discoidal particles into spherical HDL (4, 5). Cholesteryl esters generated by LCAT may be transported directly to the liver or, alternatively, may be transferred to apolipoprotein (apo) B-containing particles by the action of cholesteryl ester transfer protein (CETP) (6) and, ultimately, removed by hepatic receptor-mediated pathways (7–9). Hence, LCAT has been suggested to play an important role in reverse cholesterol transport by creating a concentration gradient for the efflux of free cholesterol from peripheral cells to HDL particles (10).

Apolipoprotein A-I, the principal protein component of HDL, is the most potent physiological activator of LCAT (11, 12). The strong inverse association which has consistently been demonstrated between plasma apoA-I concentrations and the risk of coronary heart disease (CHD) has generated much interest in elucidating the precise mechanisms which modulate such concentrations in vivo. In general, plasma apoA-I concentrations are subject to regulation at the levels of a) synthesis, b) catabolism, and c) lipoprotein particle remodeling. Kinetic studies in both human (13–17) and nonhuman (18, 19) primates have established that plasma apoA-I concentrations are primarily determined by the rate of apoA-I catabolism, rather than by production. However, the processes responsible for defining the life span of apoA-I-containing particles are not completely understood.

Data from our laboratory (20) and others (14, 16, 18, 19, 21–23) suggest that HDL particle size may affect the metabolism of apoA-I, with smaller particles having a shorter mean life span than larger ones. LCAT has been shown to be a sig-

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1. *Abbreviations used in this paper:* CE, cholesteryl ester; CER, cholesterol esterification rate; CETP, cholesterol ester transfer protein; CHD, coronary heart disease; CLD, classic LCAT deficiency; FC, free cholesterol; FCR, fractional catabolic rate; FED, fish eye disease; FPLC, fast protein liquid chromatography; HE, high expressors; HL, hepatic lipase; IDL, intermediate density lipoprotein; LCAT, lecithin cholesterol acyltransferase; LE, low expressors; PL, phospholipid; PR, production rate; TC, total cholesterol; TG, triglyceride.

nificant determinant of HDL particle size, as evidenced by individuals with mutations in the LCAT gene. Patients with either total or partial deficiency of plasma LCAT activity present with two clinically distinct syndromes: classic LCAT deficiency (CLD) and fish eye disease (FED), respectively (24). Characterization of the lipoprotein particles isolated from the plasma of patients with LCAT deficiency reveals the presence of abnormal, small spherical, as well as aggregated discoidal, HDL particles, establishing the importance of LCAT for the proper synthesis and maturation of HDL (25, 26). These defective particles are rapidly catabolized (27, 28), resulting in marked hypoalphalipoproteinemia (HDL $<$ 15 mg/dl) (24).

Recently, we have described the generation of transgenic mice (29) and rabbits (30) which express human LCAT to varying degrees. Control New Zealand White rabbits have low total cholesterol levels (31), high cholesteryl ester transfer protein activity (32), low hepatic lipase (HL) activity (33), and lack an analogue of human apoA-II (31, 34), providing a unique model in which to assess the interaction between enzymes affecting HDL metabolism. The overexpression of human LCAT in these animals leads to alterations in the concentration, composition, and size of HDL. To elucidate the mechanisms responsible for these changes, we selected age- and sex-matched rabbits which expressed high, low, or no level of human LCAT. Our data indicate that human LCAT overexpression differentially modulates HDL concentrations in this animal model by altering apoA-I catabolism in a gene dose-dependent manner, such that high expressors have the largest HDL particles with the longest plasma half-life.

Methods

Animals. The production of the human LCAT transgenic rabbits used in these experiments has been described elsewhere (30). Briefly, fertilized eggs from New Zealand White rabbits were microinjected with a 6.2-kb genomic fragment consisting of the entire LCAT gene, including 0.85 and 1.134 kb of the 5′ and 3′-flanking regions, respectively. Integration of the human LCAT gene in newborn rabbits was determined by Southern blot analysis. Three experimental groups, comprised of three animals each, were defined on the basis of total and HDL cholesterol concentrations, as well as by LCAT activity, and were, thus, designated as high expressors (HE), low expressors (LE), and controls. It has previously been demonstrated that the number of integrated copies of the LCAT transgene correlates highly with plasma human LCAT concentrations, as well as with total and HDL cholesterol levels in transgenic animals (29). Both the high and low expressors were generated from a single founder which integrated the human LCAT gene into more than one site. All of the rabbits used in these studies were age-matched males of equivalent body weight, with values (kilograms) of 3.44 ± 0.02, 3.28 ± 0.24, and 3.03 ± 0.08 for HE, LE, and controls, respectively. Animals were maintained on a standard rabbit chow diet (NIH-09) and had free access to water for the duration of the studies. The research protocol was approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute.

Plasma lipids and apolipoprotein A-I. To facilitate blood sampling for the metabolic studies, a central venous catheter was placed via the right external jugular vein. Samples were collected from the central vein catheter at each designated time point and were added to tubes containing tripotassium EDTA. Plasma was isolated by centrifugation at 2500 rpm for 30 minutes at 4°C. Total cholesterol (TC) and triglycerides (TG) (Sigma, St. Louis, MO) and free cholesterol (FC) and phospholipids (PL) (Wako Chemicals USA, Inc., Richmond, VA) in both whole plasma and FPLC fractions were measured with a Hitachi 911 Autoanalyzer (Hitachi USA, Indianapolis, IN) using enzymic reagents. Plasma HDL cholesterol was determined after dextran sulfate-Mg^{2+} precipitation of very low density and low density lipoproteins (35). Cholesteryl ester (CE) values were calculated by subtracting free cholesterol from total cholesterol concentrations. Plasma apoA-I concentrations were determined by a competitive ELISA assay, utilizing a monoclonal antiserum directed against purified rabbit apoA-I.

Human LCAT mass and activity. Human LCAT mass was quantified by radioimmunoassay. This previously reported method (36) uses a polyclonal antibody generated against human LCAT and radiolabeled, purified human LCAT standards. α-LCAT activity was determined, in duplicate, as previously described (37). Cholesterol esterification rate (CER) was assessed in duplicate plasma samples by determining the rate of esterification of [^{14}C]cholesterol, as has been described elsewhere in detail (38).

Gel filtration chromatography. 200 μl of rabbit plasma were applied to a fast protein liquid chromatography (FPLC) system consisting of two Superose 6 columns connected in series (Pharmacia Biotech, Inc., Piscataway, NJ). Lipoproteins were eluted at 0.3 ml/min with phosphate-buffered saline, containing 1 mM EDTA and 0.02% (wt/wt) sodium azide (39). After the initial 10 ml were eluted, the next 30 ml were collected in 0.5-ml fractions. The distribution of ^{131}I-apoA-I among the lipoproteins was assessed by quantitating the radioactivity in each FPLC fraction on a Packard Cobra gamma counter (Packard Instrument Co., Downers Grove, IL). Total, free, and esterified cholesterol concentrations, as well as phospholipids, were determined for each fraction.

In vivo metabolic studies. Rabbit apoA-I was purified (40) and then radiolabeled with ^{131}I (Dupont/NEN, Boston, MA), using a modification of the iodine monochloride method previously described (41, 42). ^{131}I-apoA-I was reassociated with autologous rabbit plasma for 30 min at 39°C. Unbound iodine was removed by extensive dialysis against 0.1% (vol/vol) PBS, 0.01% (wt/vol) EDTA. Four milliliters of the dialyzed, autologous plasma from each rabbit were then subjected to density gradient ultracentrifugation for 22 h at 39,000 rpm, 10°C, according to the method of Terpstra and Pels (43), for the isolation of ^{131}I-HDL–apoA-I. Each isolated radiolabeled HDL fraction was further dialyzed against PBS/EDTA to remove excess potassium bromide. Each preparation was filter-sterilized (0.22 μm Millex-GV filters, Millipore, Bedford, MA) before injection.

While there is evidence in the literature which suggests that exchange-labeled HDL is cleared more rapidly from the plasma than is whole-labeled HDL (22, 44), the present study employed similar methodology as that described by Schaefer et al. (13) where exchange-labeled HDL, which were generated in vivo by incubating radiolabeled apoA-I with autologous plasma, were not cleared at a faster rate than were whole-labeled HDL. These data are corroborated by the results of Vega et al. (45) which provide further evidence that isolated radioiodinated apoA-I has similar kinetic behavior to apoA-I radiolabeled as part of intact HDL. Additionally, Ikewaki et al. (46) have shown that endogenous labeling of apoA-I with a stable isotope ($^{13}C_6$-phenylalanine) generated kinetic parameters which were highly comparable with those obtained by exchange-labeling.

25 μCi of radiolabeled, autologous HDL preparation were infused into the marginal ear vein of each rabbit. Blood samples were collected at 5 min and at 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, and 168 h after injection. Plasma was isolated by centrifugation at 2500 rpm, 4°C for 30 min. 500 μl of each sample were analyzed for radioactivity on a Packard Cobra gamma counter. Residence time (RT) was determined from the area under the plasma radioactivity decay curve, using a multiexponential computer curve-fitting program (SAAM31) (47). Fractional catabolic rate (FCR) was calculated as the reciprocal of the RT. ApoA-I pool size was derived from the formula: [plasma volume (dl) × plasma apoA-I concentration (mg/dl)]/body weight (kg). Plasma volume was estimated as 3.28% of body weight (48). Production rate (PR) was calculated as the product of FCR and pool size.

Table I. LCAT Concentration and Activity in the Plasma of Control and hLCAT Transgenic Rabbits

Level of expression	hLCAT mass	α-LCAT activity	CER
	μg/ml	*nmol/ml per h*	*nmol/ml per h*
High	28±3*‡	2463±61‖	111±11¶
Low	7±2*§	643±32‖	101±7**
Control‡‡	< 0.2‡§	103±6‖	43±10¶**

Values are mean±SEM. Mean values with a common superscript are significantly different from one another. LCAT, lecithin cholesterol acyltransferase; CER, cholesterol esterification rate. *$P < 0.025$; ‡ $P < 0.001$; § $P < 0.05$; ‖ $P < 0.000$; ¶ $P < 0.01$; ** $P < 0.01$. ‡‡The immunoassay detected < 0.2 μg/ml of human LCAT in all 3 nontransgenic rabbits.

Statistical analysis. Data were assessed for significance with Student's nonpaired, two-tailed *t* test, and correlation coefficients were determined by the method of Pearson. Multiple regression analysis was performed using SPSS for Windows (release 5.0; SPSS Inc., Chicago, IL). In all cases, statistical significance was set at $P < 0.05$. Data presented in the text, tables, and figures represent mean±SEM.

Results

Human LCAT mass and activity. The data for hLCAT mass and activity for HE, LE, and control rabbits are provided in Table I. As expected, plasma hLCAT concentrations were markedly elevated in HE as compared with both LE and controls. The difference between LE and controls was also significant. For all three control rabbits, less than 0.2 μg/ml of LCAT were detected, indicating that our immunoassay was specific for the human enzyme. The correlation between plasma hLCAT mass and α-LCAT activity was positive and highly significant ($r = 0.955$, $P < 0.01$). Accordingly, similar differences were noted between the groups when the values for α-LCAT activity were compared. HE had four times the level of α-LCAT activity than did LE, with LE having ~ 6.5 times the activity of controls. These ratios were almost identical to those of the hLCAT mass data.

Interestingly, the CER was lower in the LCAT transgenic rabbits relative to the LCAT activity and was not significantly different between HE and LE. Potential explanations for this finding include: (*a*) differences in substrate specificities between the two assays, (*b*) coactivators or inhibitors in rabbit plasma which may modify the esterification rate in vivo, and (*c*) reduced activation of human LCAT by rabbit, versus human, apoA-I. However, in spite of this observation, the mean CER of both HE and LE was significantly increased as compared with controls, and CER was significantly correlated with apoA-I FCR.

Plasma lipids and lipoprotein composition. Table II summarizes the mean plasma lipid data of control and hLCAT transgenic rabbits. HE exhibited significant increases in plasma total (295%), esterified (312%), and HDL (220%) cholesterol concentrations relative to LE. As expected, the differences in these parameters between HE and controls were

Table II. Plasma Lipids of Control and hLCAT Transgenic Rabbits

Level of expression	TC	FC	CE	HDL-C	PL	TG
High	162±18*‡	35±7‖§	128±12‡¶	121±12§‡	156±27‖	46±7
Low	55±6*§	14±3‖	41±3‡*	55±10§‖	100±15	45±7
Control	29±2‡§	9±1§	20±2¶*	28±4‡‖	74±1‖	52±9

Values are mean±SEM (mg/dl). Mean values with a common superscript are significantly different from one another. *$P < 0.005$; ‡ $P < 0.002$; § $P < 0.025$; ‖ $P < 0.05$; ¶ $P < 0.001$.

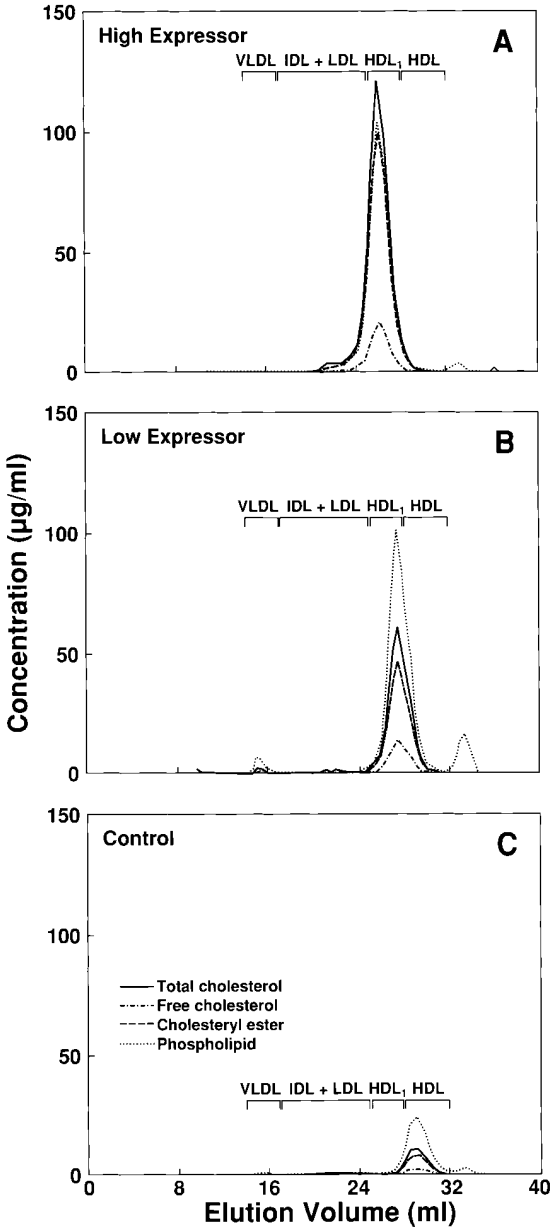

Figure 1. Compositional analysis of gel filtration chromatography fractions. *A*, *B*, and *C* illustrate the concentrations of total cholesterol, free cholesterol, cholesteryl ester, and phospholipid in the FPLC fractions of representative HE, LE, and control rabbits, respectively. The HDL particles of both HE and LE had a relative enrichment of total cholesterol, cholesteryl ester, and phospholipid, as compared with controls, with HE having the most significant increases.

even more dramatic, with levels which were 559, 640, and 432% greater in HE, respectively, as compared with those of age- and sex-matched controls. Although only the HE had a notable difference between total and HDL cholesterol levels, SDS-PAGE analysis of the lipoprotein fractions isolated by gel filtration chromatography, followed by immunoblotting, has shown that this was not due to increased LDL apoB (data not shown). Rather, this difference was likely due to the fact that HE have an apoE-rich HDL_1 which was precipitated by the HDL method used in this study.

Plasma FC content was significantly greater in HE relative to both LE and controls. Plasma total, esterified, and HDL cholesterol concentrations were all significantly elevated in LE relative to controls. However, despite a more than 1.5-fold increase in LE, the difference in FC between LE and controls was not significant. A similar trend was noted in plasma phospholipids, with HE having the highest levels, followed by LE and controls. Plasma triglyceride concentrations were not significantly different when the three groups were compared.

In Fig. 1 FPLC lipoprotein profiles from representative HE (*A*), LE (*B*), and control (*C*) rabbits are given. FPLC analysis revealed that HDL was the predominant lipoprotein class in each group, independent of the degree of LCAT expression. However, distinct alterations in particle concentrations and composition were observed among the groups. In agreement with the plasma lipid determinations, the FPLC profile of HE was characterized by the presence of a large, CE- and PL-enriched HDL, analogous to HDL_1, the levels of which were markedly elevated relative to LE and controls. Compared with controls, an accumulation of CE and PL was also noted in the HDL of LE, but to a far lesser extent than that observed in HE. Moreover, the elution profile of LE indicated that the apparent HDL particle size of these animals was intermediate to those of HE and controls.

Isotopic distribution of apoA-I. The distribution of radiolabeled apoA-I in the 24 h postinjection plasma samples of HE, LE, and controls was assessed by FPLC gel filtration chromatography, as depicted in Fig. 2. In accord with the FPLC lipoprotein profiles, these data revealed an incremental shift in apparent HDL particle size which correlated directly with the level of LCAT expression, such that HE had the largest HDL particles and controls the smallest. As expected, radiolabeled apoA-I was only associated with HDL in vivo.

Metabolic parameters of HDL apoA-I. The kinetic parameters of HDL apoA-I are shown in Table III. The mean plasma apoA-I pool size was significantly increased in HE as compared with both LE and controls, with the difference between the latter two groups also achieving statistical significance. As illustrated in Fig. 3, these differences in circulating apoA-I levels were directly attributable to distinctive rates of HDL apoA-I catabolism between the three groups, such that the decline in plasma radioactivity over time of ^{131}I-HDL apoA-I was slowest in HE, followed by LE, and, lastly, by controls. This translated into a mean apoA-I FCR for HE which was 20% lower than that for LE and 38% lower than that for controls. The 23% reduction in apoA-I FCR for LE relative to controls was also significant. The preceding corresponded with

Table III. Metabolic Parameters of HDL apoA-I in Control and hLCAT Transgenic Rabbits

Level of expression	ApoA-I Pool Size	ApoA-I PR	ApoA-I FCR
	mg/kg	*mg·kg·d*$^{-1}$	*pools/d*
High	113±12*‡	10.8±1.4	0.328±0.028‡
Low	67±3*§	8.4±0.8	0.408±0.014‖
Control	46±2‡§	8.1±0.6	0.528±0.036‡‖

Values are mean±SEM. Mean values with a common superscript are significantly different from one another. *$P < 0.02$; ‡§$P < 0.01$; ‖$P < 0.04$.

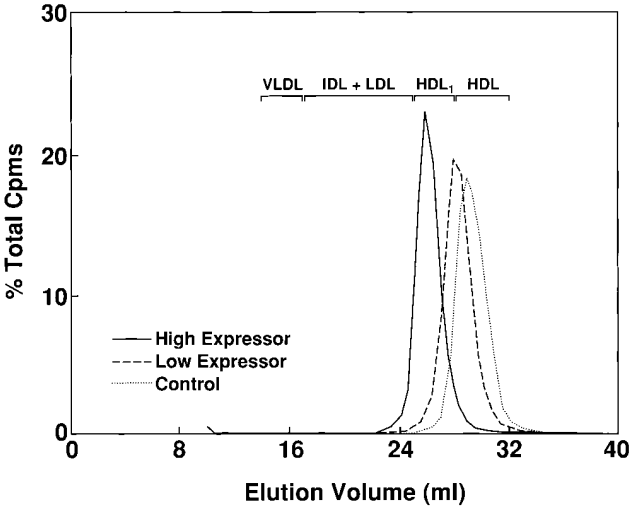

Figure 2. Distribution of radiolabeled apoA-I in the 24-h postinjection plasma samples of representative HE, LE, and control rabbits, as assessed by gel filtration chromatography. Note the incremental shift in apparent HDL particle size which correlates directly with the level of LCAT expression.

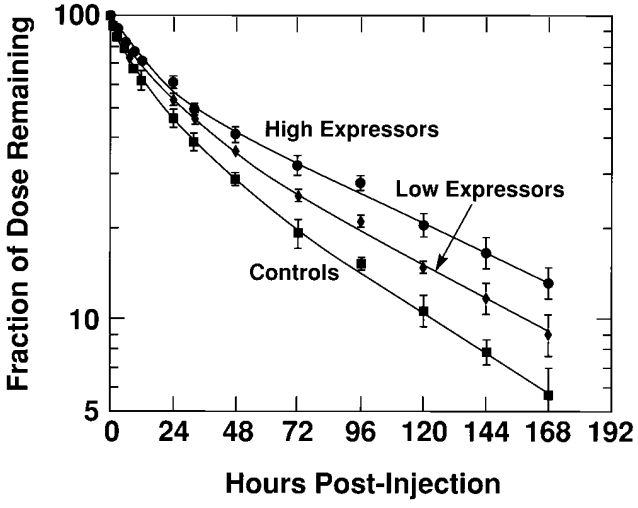

Figure 3. In vivo metabolism of apoA-I. Purified rabbit apoA-I was radiolabeled, and HDL isolated, as described in Methods. The radioactivity decay curves for HE (○), LE (◇), and controls (□), $n = 3$ per group (mean±SEM), are illustrated using a two-log scale as the ordinate. The plasma clearance of apoA-I was slowest in HE, followed by LE and, lastly, by controls, suggesting a gene dose-dependent reduction in apoA-I FCR.

apoA-I residence times of 3.1 d for HE, 2.5 d for LE, and 1.9 d for controls. Although the apoA-I PR was slightly elevated in HE, no significant differences were noted between the groups.

Further evidence that fractional catabolic rate was the predominant mechanism by which hLCAT overexpression differentially modulated HDL apoA-I concentrations in these animals is provided in Fig. 4. Plasma obtained 1, 24, and 48 h after injection of radioiodinated HDL apoA-I were analyzed by FPLC gel filtration chromatography. When the data in panels *A*, *B*, and *C* are compared, it is clear that the decline in HDL-associated radioactivity over time is slowest in HE (*A*) and fastest in controls (*C*), with LE having an intermediate rate of decay (*B*). Moreover, all of the radioactivity remains associated with HDL and is not redistributed.

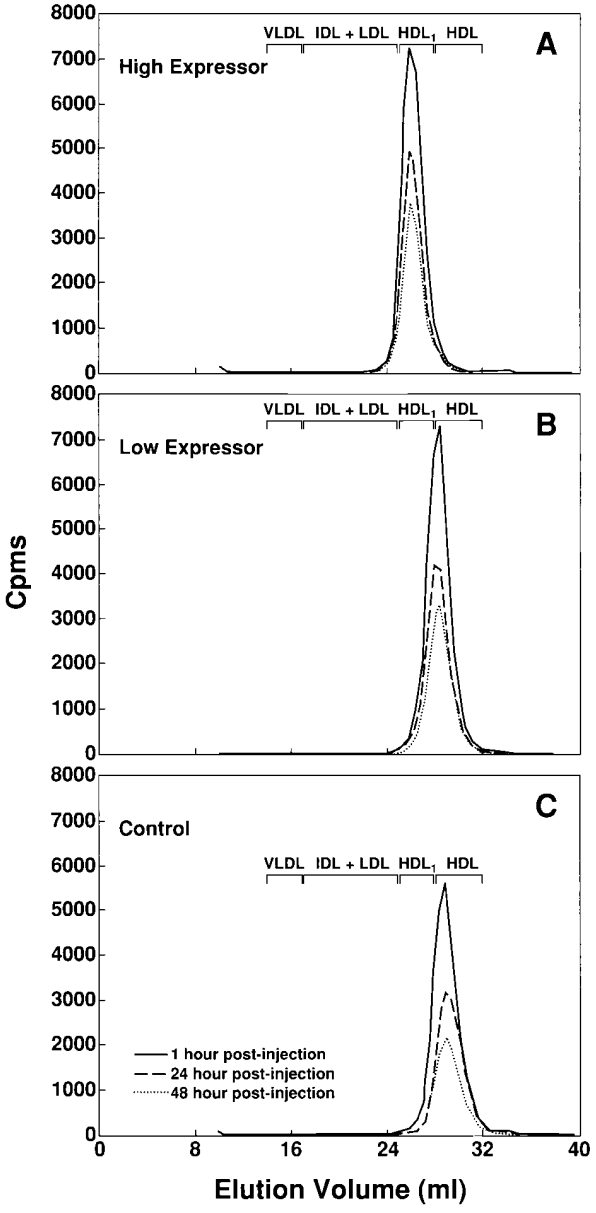

Figure 4. FPLC elution profiles of plasma obtained 1, 24, and 48 h after injection of radiolabeled HDL apoA-I. (*A–C*) demonstrate that the decline in HDL-associated radioactivity over time is slowest in HE and fastest in controls, with LE having an intermediate rate of decay.

Relationships between apoA-I metabolic parameters and hLCAT mass and activity. The results of linear regression analyses are shown in Table IV. Plasma HDL-C concentrations were positively associated with apoA-I PR and hLCAT mass, α-LCAT activity, and CER in these animals, whereas a strong negative correlation was noted between HDL-C and apoA-I FCR. LCAT activity and CER were also negatively associated with apoA-I clearance rate. Although a strong trend was noted, the relationship between apoA-I FCR and hLCAT mass did not reach statistical significance, most likely due to the small sample size ($n = 6$). With the exception of HDL-C, LCAT activity was the sole parameter with which apoA-I PR was significantly correlated, although trends were noted with hLCAT mass and CER.

Multiple stepwise regression analysis was also performed in order to further define those variables which were most closely linked to the changes in HDL-C levels and apoA-I FCR. Using HDL-C as the dependent variable and apoA-I FCR, apoA-I PR, CER, and LCAT activity as the independent variables, forward analysis yielded an r^2 of 0.915 for LCAT activity, with this being the only variable required in the model to explain the variability in HDL-C concentrations. With respect to apoA-I FCR, both forward and backward analyses revealed that LCAT activity was the primary determinant of apoA-I FCR, when CER, LCAT activity, and LCAT mass were entered into the model. We realize that these analyses are limited by the fact that we do not have CETP and HL data for these animals. However, data from littermates of these rabbits demonstrate that CETP and HL activities are not significantly different between LCAT transgenics and controls (data not shown), suggesting that these two variables are not likely to be as significant in the determination of HDL-C levels in these rabbits as was LCAT activity.

Discussion

Plasma HDL cholesterol concentrations are regulated by a complex series of metabolic processes involving synthesis, catabolism, and lipoprotein particle remodeling. Several studies (13–19) have demonstrated that variation in HDL fractional catabolic rate is the primary predictor of HDL cholesterol levels, with HDL particle size correlating inversely with the rate of clearance (14, 16, 18–23). Critical to the proper functioning of the HDL metabolic cascade are a number of different enzymes, including hepatic and lipoprotein lipases, CETP, and LCAT. LCAT is the key enzyme involved in the esterification of intravascular free cholesterol. The esterification of free cholesterol by LCAT is essential for HDL particle maturation, converting discoidal particles into spherical HDL only when

Table IV. Correlation Coefficient Analysis

Parameter	HDL-C	ApoA-I FCR	ApoA-I PR
HDL-C	—	−0.851‡	0.694¶
hLCAT mass**	0.969§	−0.634	0.731
LCAT activity	0.956*	−0.816§	0.668¶
CER	0.769‖	−0.823§	0.524

*$P < 0.001$; ‡$P < 0.005$; §$P < 0.01$; ‖$P < 0.03$; ¶$P < 0.05$. **$n = 6$.

sufficient cholesteryl esters are generated for the hydrophobic core (4, 5). Thus, LCAT is a significant determinant of HDL particle size. With these concepts in mind, the present studies were designed to elucidate the mechanisms responsible for the changes in HDL cholesterol concentrations observed in rabbits overexpressing human LCAT.

The obligatory role of LCAT in the regulation of HDL cholesterol levels is clearly evidenced by individuals with LCAT gene defects, who have marked hypoalphalipoproteinemia characterized by the presence of abnormal, small spherical and aggregated, discoidal HDL particles. Detailed kinetic studies of patients with CLD and FED reveal hypercatabolism of both apoA-I and apoA-II, with LpA-I:A-II particles cleared at an even faster rate than LpA-I particles (27). Since LpA-I has been purported to be a specific antiatherogenic particle within HDL, this may, in part, explain why LCAT deficiency is not associated with an increased risk of premature atherosclerosis, despite the very low plasma levels of HDL and apoA-I (24). Although such patients have provided insights into the involvement of LCAT in HDL metabolism, reverse cholesterol transport, and atherogenesis, presently, the precise physiologic roles of this enzyme remain elusive.

The studies reported here further establish the importance of LCAT in the modulation of HDL cholesterol levels. We observed that the overexpression of human LCAT in the transgenic rabbit model resulted in significant elevations of plasma total and HDL cholesterol concentrations, the extent of which correlated directly with LCAT gene expression. Hence, high expressors had the greatest HDL cholesterol concentrations, while low expressors had levels intermediate to those of high expressors and controls. In order to investigate the metabolic mechanism(s) responsible for this gene dose-dependent hyperalphalipoproteinemia, we assessed ^{131}I-HDL apoA-I kinetics in these animals using autologous HDL. These in vivo experiments revealed that the fractional catabolic rate of apoA-I was slowest in high expressors, followed by low expressors, and, lastly, by controls, with no significant differences noted in apoA-I production rates. Moreover, apoA-I FCR was inversely associated with HDL cholesterol level and LCAT activity, indicating that fractional catabolic rate was the predominant mechanism by which LCAT overexpression differentially modulated HDL concentrations in this animal model. While these data are consistent with the concept that plasma HDL concentrations are primarily determined by the rate of apoA-I catabolism, rather than by production (13–19), they provide new information regarding the role of LCAT in the regulation of HDL metabolism by demonstrating that alterations in catabolism may be related to gene dosage.

It has consistently been demonstrated that HDL particle size can influence the metabolism of apoA-I (14, 16, 18–23). In fact, one study has reported that as much as 70% of the variability in apoA-I FCR was due to variability in estimates of HDL size or density (16). Accordingly, the differences in apoA-I FCR observed in our study could be explained by LCAT-induced compositional changes in HDL which, in turn, resulted in alterations in HDL particle size. Fast protein liquid chromatography analysis of plasma revealed that the HDL of both high and low expressors were cholesteryl ester and phospholipid enriched, relative to controls, resulting in an incremental shift in apparent HDL particle size which correlated directly with the level of LCAT expression, such that high expressors had the largest HDL particles and controls the smallest. These changes in particle size were inversely associated with the rate of HDL catabolism, with the large HDL of high expressors having the longest life span and the small HDL of controls the shortest.

We hypothesize that the relationship between increased particle size and decreased apoA-I FCR is not one of cause and effect, but rather that the alterations in catabolism may be secondary to changes in apoA-I conformation. Both in vivo and in vitro work support this concept. Horowitz et al. (22) have clearly demonstrated that HDL composition and size can affect the metabolic fate of apoA-I. Their data suggest that increased renal clearance of apoA-I occurs when HDL are cholesterol-depleted and relatively triglyceride enriched, as were our control HDL, due to the dissociation of apoA-I from HDL. The in vitro experiments of Liang et al. (49) have further shown that the dissociation of apoA-I from HDL is a concentration-dependent phenomenon, such that increasing the level of HDL decreases both the reduction in HDL particle size and the dissociation of apoA-I. Additional support for our hypothesis also comes from the fact that the affinity of apolipoproteins for lipoprotein particles has been shown to inversely correlate with their metabolic clearance (50, 51).

Several factors, in addition to LCAT, have been shown to promote changes in HDL particle size. For example, CETP and HL activities (52) have been reported to reduce the size of HDL, whereas lipoprotein lipase activity (53) can increase it. An alternative hypothesis to that of the role of apoA-I conformation in catabolism involves HDL particle regeneration. As previously noted, the rabbit has an inherently low level of HL activity. This relative HL deficiency in concert with LCAT overexpression may have created a metabolic schema in which large, cholesteryl ester rich HDL particles were ineffective substrates for HL, preventing a diminution in HDL size and, ultimately, the regeneration of nascent HDL which are more rapidly catabolized than are mature HDL.

Previous studies have suggested that LCAT activity may also be sensitive to the size of HDL particles, with small HDL particles the preferred substrate of this enzyme (54). Normal rabbit plasma is characterized by the presence of small HDL which would presumably be excellent substrates for the LCAT reaction. However, consistent with our results, a recent study with reconstituted LpA-I (55) demonstrated that increasing cholesterol content corresponded with increases in both HDL particle size and LCAT activity. Based on their observations, these investigators concluded that HDL particle size does not modulate LCAT activity, but rather that changes in lipoprotein surface charge can mediate LCAT activity. Such changes could also affect the binding of apoA-I to the lipoprotein particle surface, resulting in alterations of apoA-I metabolism such as those observed in our study.

The final issue which must be considered concerns the implications of our findings with respect to understanding the involvement of LCAT in atherosclerosis susceptibility. In view of the strong inverse relationship which is known to exist between HDL cholesterol levels and the risk of premature coronary heart disease, it would not be unreasonable to propose that the overexpression of LCAT could be of potential benefit. However, as noted earlier in the discussion, LCAT deficiency is not associated with an increased risk of premature CHD. Whether or not high levels of LCAT would actually prove protective remains to be determined. Although one study has shown that LCAT levels were significantly greater in women

as compared with men (56), the role of this enzyme as a predictor of atherosclerosis susceptibility is yet to be established.

In summary, our data indicate that human LCAT overexpression differentially modulates HDL concentrations in this animal model by reducing apoA-I catabolism in a gene dose-dependent manner. The observed variation in apoA-I FCR could be explained by LCAT-induced changes in HDL composition and size which, in turn, ultimately resulted in altered HDL particle catabolism. These findings provide new insights into the complex metabolic processes involved in the regulation of HDL concentrations.

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References

1. Glomset, J.A., E.T. Janssen, R. Kennedy, and J. Dobbins. 1966. Role of plasma lecithin:cholesterol acyltransferase in the metabolism of high density lipoproteins. *J. Lipid Res.* 7:638–648.

2. Glomset, J.A. 1968. The plasma lecithin:cholesterol acyltransferase reaction. *J. Lipid Res.* 9:155–167.

3. Albers, J.J., C.H. Chen, and J.L. Adolphson. 1981. Lecithin:cholesterol acyltransferase (LCAT) mass; its relationship to LCAT activity and cholesterol esterification rate. *J. Lipid Res.* 22:1206–1213.

4. Castro, G.R., and C.J. Fielding. 1988. Early incorporation of cell-derived cholesterol into pre-beta-migrating high-density lipoprotein. *Biochemistry.* 27: 25–29.

5. Fielding, P.E., M. Kawano, A.L. Catapano, A. Zoppo, S. Marcovina, and C.J. Fielding. 1994. Unique epitope of apolipoprotein A-I expressed in pre-beta-1 high density lipoprotein and its role in the catalyzed efflux of cellular cholesterol. *Biochemistry.* 33:6981–6985.

6. Tall, A.R. 1993. Plasma cholesteryl ester transfer protein. *J. Lipid Res.* 34:1255–1274.

7. Goldstein, J.L., M.S. Brown, R.G. Anderson, D.W. Russell, and W.J. Schneider. 1985. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol.* 1:1–39.

8. Herz, J., U. Hamann, S. Rogne, O. Myklebost, H. Gausepohl, and K.K. Stanley. 1988. Surface location and high affinity for calcium of a 500-kd liver membrane protein closely related to the LDL-receptor suggest a physiological role as lipoprotein receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:4119–4127.

9. Strickland, D.K., J.D. Ashcom, S. Williams, W.H. Burgess, M. Migliorini, and W.S. Argraves. 1990. Sequence identity between the alpha 2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor. *J. Biol. Chem.* 265:17401–17404.

10. Fielding, C.J. 1984. The origin and properties of free cholesterol potential gradients in plasma and their relation to atherogenesis. *J. Lipid Res.* 25: 1624–1628.

11. Fielding, C.J., V.G. Shore, and P.E. Fielding. 1972. A protein cofactor of lecithin:cholesterol acyltransferase. *Biochem. Biophys. Res. Commun.* 46:1493–1498.

12. Jonas, A. 1991. Lecithin-cholesterol acyltransferase in the metabolism of high-density lipoproteins. *Biochim. Biophys. Acta.* 1084:205–220.

13. Schaefer, E.J., L.A. Zech, L.L. Jenkins, T.J. Bronzert, E.A. Rubalcaba, R.T. Lindgren, R.L. Aamodt, and H.B. Brewer, Jr. 1982. Human apolipoprotein A-I and A-II metabolism. *J. Lipid Res.* 23:850–862.

14. Brinton, E.A., S. Eisenberg, and J.L. Breslow. 1989. Elevated high density lipoprotein cholesterol levels correlate with decreased apolipoprotein A-I and A-II fractional catabolic rate in women. *J. Clin. Invest.* 84:262–269.

15. Brinton, E.A., S. Eisenberg, and J.L. Breslow. 1990. A low-fat diet decreases high density lipoprotein (HDL) cholesterol levels by decreasing HDL apolipoprotein transport rates. *J. Clin. Invest.* 85:144–151.

16. Brinton, E.A., S. Eisenberg, and J.L. Breslow. 1994. Human HDL cholesterol levels are determined by apoA-I fractional catabolic rate, which correlates inversely with estimates of HDL particle size: effects of gender, hepatic and lipoprotein lipases, triglyceride and insulin levels, and body fat distribution. *Arterioscler. Thromb.* 14:707–720.

17. Ikewaki, K., L.A. Zech, M. Kindt, H.B. Brewer, Jr., and D.J. Rader. 1995. Apolipoprotein A-II production is a major factor regulating the distribution of apolipoprotein A-I among HDL subclasses LpA-I and LpA-I:A-II in normolipidemic humans. *Arterioscler. Thromb. Vasc. Biol.* 15:306–312.

18. Melchior, G.W., C.K. Castle, T.J. Vidmar, H.G. Polites, and K.R. Marotti. 1990. ApoA-I metabolism in cynomolgus monkeys: male-female differences. *Biochim. Biophys. Acta.* 1043:97–105.

19. Pape, M.E., E.F. Rehberg, K.R. Marotti, and G.W. Melchior. 1991. Molecular cloning, sequence, and expression of cynomolgus monkey cholesteryl ester transfer protein. Inverse correlation between hepatic cholesteryl ester transfer protein mRNA levels and plasma high density lipoprotein levels. *Arterioscler. Thromb.* 11:1759–1771.

20. Rader, D.J., G. Castro, L.A. Zech, J.C. Fruchart, and H.B. Brewer, Jr. 1991. In vivo metabolism of apolipoprotein A-I on high density lipoprotein particles LpA-I and LpA-I,A-II. *J. Lipid. Res.* 32:1849–1859.

21. Brinton, E.A., S. Eisenberg, and J.L. Breslow. 1991. Increased apoA-I and apoA-II fractional catabolic rate in patients with low high density lipoprotein-cholesterol levels with or without hypertriglyceridemia. *J. Clin. Invest.* 87: 536–544.

22. Horowitz, B.S., I.J. Goldberg, J. Merab, T. Vanni, R. Ramakrishnan, and H.N. Ginsberg. 1991. Increased plasma and renal clearance of an exchangeable pool of apolipoprotein A-I in subjects with low levels of high density lipoprotein cholesterol. *J. Clin. Invest.* 91:1743–1752.

23. Melchior, G.W., C.K. Castle, R.W. Murray, W.L. Blake, D.M. Dinh, and K.R. Marotti. 1994. Apolipoprotein A-I metabolism in cholesteryl ester transfer protein transgenic mice: insights into the mechanisms responsible for low plasma high density lipoprotein levels. *J. Biol. Chem.* 269:8044–8051.

24. Glomset, J.A., G. Assmann, E. Gjone, and K.R. Norum. 1995. Lecithin: cholesterol acyltransferase deficiency and fish eye disease. *In* The Metabolic and Molecular Bases of Inherited Disease. C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle, editors. McGraw-Hill, Inc., New York. 1933–1952.

25. Forte, T., K.R. Norum, J.A. Glomset, and A.V. Nichols. 1971. Plasma lipoproteins in familial lecithin:cholesterol acyltransferase deficiency: structure of low and high density lipoproteins as revealed by electron microscopy. *J. Clin. Invest.* 50:1141–1148.

26. Forte, T.M., and L.A. Carlson. 1984. Electron microscopic structure of serum lipoproteins from patients with fish eye disease. *Arteriosclerosis.* 4:130–137.

27. Rader, D.J., K. Ikewaki, N. Duverger, H. Schmidt, H. Pritchard, J. Frolich, M. Clerc, M.-F. Dumon, T. Fairwell, L. Zech, S. Santamarina-Fojo, and H.B. Brewer, Jr. 1994. Markedly accelerated catabolism of apolipoprotein A-II (apoA-II) and high density lipoproteins containing apoA-II in classic lecithin: cholesterol acyltransferase deficiency and fish-eye disease. *J. Clin. Invest.* 93: 321–330.

28. Gylling, H., and T.A. Miettinen. 1992. Noncholesterol sterols, absorption and synthesis of cholesterol and apolipoprotein A-I kinetics in a Finnish lecithin-cholesterol acyltransferase deficient family. *Atherosclerosis.* 95:25–33.

29. Vaisman, B.L., H.-G. Klein, M. Rouis, A.M. Berard, M.R. Kindt, G.D. Talley, S.M. Meyn, R.F. Hoyt, Jr., S.M. Marcovina, J.J. Albers, J.M. Hoeg, H.B. Brewer, Jr., and S. Santamarina-Fojo. 1995. Overexpression of human lecithin cholesterol acyltransferase leads to hyper-alphalipoproteinemia in transgenic mice. *J. Biol. Chem.* 270:12269–12275.

30. Hoeg, J.M., B.L. Vaisman, S.J. Demosky, Jr., S.M. Meyn, G.D. Talley, R.F. Hoyt, Jr., S. Feldman, A.M. Bérard, N. Sakai, D. Wood, M.E. Brousseau, S. Marcovina, H.B. Brewer, Jr., and S. Santamarina-Fojo. 1996. Lecithin: cholesterol acyltransferase overexpression generates hyperalpha-lipoproteinemia and a nonatherogenic lipoprotein pattern in transgenic rabbits. *J. Biol. Chem.* 271:4396–4402.

31. Chapman, M.J. 1980. Animal lipoproteins: chemistry, structure, and comparative aspects. *J. Lipid Res.* 21:789–853.

32. Ha, Y.C., and P.J. Barter. 1982. Differences in plasma cholesteryl ester transfer activity in sixteen vertebrate species. *Comp. Biochem. Physiol.* [B] 71: 265–269.

33. Clay, M.A., G.J. Hopkins, C.P. Ehnholm, and P.J. Barter. 1989. The rabbit as an animal model of hepatic lipase deficiency. *Biochim. Biophys. Acta.* 1002:173–181.

34. Borresen, A.L., T.J. Kindt, and K. Berg. 1978. Partial purification and characterization of the inherited Hl 1 and R 67 antigens of rabbit serum high density lipoprotein. *J. Immunogenet.* 5:71–86.

35. Warnick, G.R., J. Benderson, and J.J. Albers. 1982. Dextran sulfate-Mg^{2+} precipitation procedure for quantitation of high density lipoprotein cholesterol. *Clin. Chem.* 28:1379–1388.

36. Albers, J.J., J.L. Adolphson, and C.-H. Chen. 1981. Radioimmunoassay of human plasma lecithin-cholesterol acyltransferase. *J. Clin. Invest.* 67:141–148.

37. Klein, H.-G., P. Lohse, N. Duverger, J.J. Albers, D.J. Rader, L.A. Zech, S. Santamarina-Fojo, and H.B. Brewer, Jr. 1993. Two different allelic mutations in the lecithin:cholesterol acyltransferase (LCAT) gene resulting in classic LCAT deficiency: LCAT (tyr83→stop) and LCAT (tyr156→asn). *J. Lipid Res.* 34:49–58.

38. Stokke, K.T., and K.R. Norum. 1971. Determination of lecithin:cholesterol acyltransfer in human blood plasma. *Scand. J. Clin. Lab. Invest.* 27:21–27.

39. Jiao, S., T.G. Cole, R.T. Kitchens, B. Pfleger, and G. Schonfeld. 1990. Genetic heterogeneity of lipoproteins in inbred strains of mice: analysis by gel-permeation chromatography. *Metabolism.* 39:155–160.

40. Brewer, H.B., Jr., R. Ronan, M. Meng, and C. Bishop. 1986. Isolation and characterization of apolipoproteins A-I, A-II, and A-IV. *In* Methods in En-

zymology. J.P. Segrest and J. Albers, editors. Academic Press, Orlando. 223–246.

41. Gregg, R.E., L.A. Zech, E.J. Schaefer, and H.B. Brewer, Jr. 1984. Apolipoprotein E metabolism in normolipoproteinemic human subjects. *J. Lipid Res.* 25:1167–1176.

42. Ikewaki, K., D.J. Rader, T. Sakamoto, M. Nishiwaki, N. Wakimoto, J.R. Schaefer, T. Ishikawa, T. Fairwell, L.A. Zech, H. Nakamura, M. Nagano, and H.B. Brewer, Jr. 1993. Delayed catabolism of high density lipoprotein apolipoproteins A-I and A-II in human cholesteryl ester transfer protein deficiency. *J. Clin. Invest.* 92:1650–1658.

43. Terpstra, A.H.M., and A.E. Pels. 1988. Isolation of plasma lipoproteins by a combination of differential and density gradient ultracentrifugation. *Fresenius Z Anal. Chem*. 330:149–151.

44. Shepherd, J., C.J. Packard, A.M. Gotto, and D.O. Taunton. 1978. A comparison of two methods to investigate the metabolism of human apolipoproteins A-I and A-II. *J. Lipid Res.* 19:656–661.

45. Vega, G.L., H. Gylling, A.V. Nichols, and S.M. Grundy. 1991. Evaluation of a method for study of kinetics of autologous apolipoprotein A-I. *J. Lipid Res.* 32:867–875.

46. Ikewaki, K., D.J. Rader, J.R. Schaefer, T. Fairwell, L.A. Zech, and H.B. Brewer, Jr. 1993. Evaluation of apoA-I kinetics in humans using simultaneous endogenous stable isotope and exogenous radiotracer methods. *J. Lipid Res.* 34:2207–2215.

47. Berman, M., and M. Weiss. 1978. SAAM Manual. National Institutes of Health, Bethesda, MD. DHEW Publication No. (NIH) 78:180.

48. Ross, A.C., and D.B. Zilversmit. 1977. Chylomicron remnant cholesteryl esters as the major constituent of very low density lipoproteins in plasma of cholesterol-fed rabbits. *J. Lipid Res.* 18:169–181.

49. Liang, H.-Q., K.-A. Rye, and P.J. Barter. 1994. Dissociation of lipid-free apolipoprotein A-I from high density lipoproteins. *J. Lipid Res.* 35:1187–1199.

50. Garber, D.W., Y.V. Venkatachalapathi, K.B. Gupta, J. Ibdah, M.C. Phillips, J.B. Hazelrig, J.P. Segrest, and G.M. Anantharamaiah. 1992. Turnover of synthetic class A amphipathic peptide analogues of exchangeable apolipoproteins in rats. Correlation with physical properties. *Arterioscler. Thromb.* 12: 886–894.

51. Westerlund, J.A., and K.H. Weisgraber. 1993. Discrete carboxyl-terminal segments of apolipoprotein E mediate lipoprotein association and protein oligomerization. *J. Biol. Chem.* 268:15745–15750.

52. Hopkins, G.J., and P.J. Barter. 1986. Role of triglyceride-rich lipoproteins and hepatic lipase in determining the particle size and composition of high density lipoproteins. *J. Lipid Res.* 27:1265–1277.

53. Patsch, J.R., A.M. Gotto, Jr., T. Olivecrona, and S. Eisenberg. 1978. Formation of high density lipoprotein$_2$-like particle during lipolysis of very low density lipoproteins in vitro. *Proc. Natl. Acad. Sci. USA*. 75:4519–4523.

54. Barter, P.J., G.J. Hopkins, and L. Gorjatschko. 1985. Lipoprotein substrates for plasma cholesterol esterification. Influence of particle size and composition of the high density lipoprotein subfraction 3. *Atherosclerosis.* 58:97–107.

55. Sparks, D.L., G.M. Anantharamaiah, J.P. Segrest, and M.C. Phillips. 1995. Effect of the cholesterol content of reconstituted LpA-I on lecithin:cholesterol acyltransferase activity. *J. Biol. Chem*. 270:5151–5157.

56. Albers, J.J., R.O. Bergelin, J.L. Adolphson, and P.W. Wahl. 1982. Population-based reference values for lecithin-cholesterol acyltransferase (LCAT). *Atherosclerosis.* 43:369–379.