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patient-oriented research

Rapid turnover of apolipoprotein C-III-containing triglyceride-rich lipoproteins contributing to the formation of LDL subfractions

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Abstract The atherogenicity theory for triglyceride-rich lipoproteins (TRLs; VLDL + intermediate density lipoprotein) generally cites the action of apolipoprotein C-III (apoC-III), a component of some TRLs, to retard their metabolism in plasma. We studied the kinetics of multiple TRL and LDL subfractions according to the content of apoC-III and apoE in 11 hypertriglyceridemic and normolipidemic persons. The liver secretes mainly two types of apoB lipoproteins: TRL with apoC-III and LDL without apoC-III. Approximately 45% of TRLs with apoC-III are secreted together with apoE. Contrary to expectation, TRLs with apoC-III but not apoE have fast catabolism, losing some or all of their apoC-III and becoming LDL. In contrast, apoE directs TRL flux toward rapid clearance, limiting LDL formation. Direct clearance of TRL with apoC-III is suppressed among particles also containing apoE. TRLs without apoC-III or apoE are a minor, slow-metabolizing precursor of LDL with little direct removal. Increased VLDL apoC-III levels are correlated with increased VLDL production rather than with slow particle turnover. Finally, hypertriglyceridemic subjects have significantly greater production of apoC-III-containing VLDL and global prolongation in residence time of all particle types. ApoE may be the key determinant of the metabolic fate of atherogenic apoC-III-containing TRLs in plasma, channeling them toward removal from the circulation and reducing the formation of LDLs, both those with apoC-III and the main type without apoC-III.—Zheng, C., C. Khoo, K. Ikewaki, and F. M. Sacks. **Rapid turnover of apolipoprotein C-III-containing triglyceride-rich lipoproteins contributing to the formation of LDL subfractions.** ***J. Lipid Res.*** **2007.** 48: **1190–1203.**

Supplementary key words kinetics • stable isotopes • apolipoprotein B-100 • apolipoprotein E • low density lipoprotein

Epidemiological studies demonstrate that apolipoprotein C-III (apoC-III) and the apoB lipoproteins that have apoC-III as a component independently predict coronary heart disease (1–3). ApoC-III is present on ∼40–80% of triglyceride-rich lipoproteins (TRLs) and ∼5–10% of LDLs in plasma (4–6). The mechanisms by which apoC-III causes hypertriglyceridemia and atherosclerosis are incompletely understood.

Experiments in vitro show that apoC-III can inhibit lipoprotein lipase (7, 8) and hepatic lipase (9) and retard the clearance of VLDL by interfering with the binding of apoB-100 (10) or apoE to hepatic receptors (11, 12). Direct evidence supporting a role of high apoC-III level in abnormal TRL metabolism has come from transgenic animal studies. Overexpression of apoC-III in mice causes hypertriglyceridemia (13–17), whereas apoC-III deficiency protects against it (18, 19). In these studies, impaired particle clearance via LDL receptors (14–16), reduced binding affinity to cell surface proteoglycans (15, 17), inhibition of lipolysis (17, 19), and overproduction of VLDL triglyceride (14, 16) have all been implicated as mechanisms for the hypertriglyceridemic effect of apoC-III. In humans, there is also evidence for apoC-III affecting TRL metabolism. Patients with combined deficiency of apoC-III and apoA-I experience rapid VLDL clearance (20). In a kinetic study, the plasma concentrations and secretion rates of VLDL apoC-III were correlated with those of VLDL triglyceride (21). In another kinetic study in a similar group of humans, VLDL apoC-III concentrations and secretion rates were correlated with VLDL triglyceride and VLDL apoB residence times but not with secretion rates (22). Thus, although in vitro experiments, animal models, and human kinetic studies all provide evidence that apoC-III adversely affects the metabolism of TRL, the studies do not form a consensus about which mechanisms are dominant.

The observation that high levels of apoC-III cause hypertriglyceridemia by retarding VLDL catabolism has

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led to a kinetic theory for the atherogenicity of TRL: inhibition by apoC-III of TRL catabolism increases the residence times for TRLs and their atherogenic remnants. This view follows the long-established kinetic theory for LDL atherogenicity: reduced LDL receptor activity causes long residence times, high concentrations, and prolonged exposure of vascular wall cells to circulating LDLs. However, there is no direct evidence that apoC-III-containing TRLs, the particles that strongly predict coronary heart disease, have long residence times in plasma.

In contrast to the actions of apoC-III, apoE assists in the clearance of apoB lipoproteins by binding to cell surface receptors (23, 24) and proteoglycans (25). Overexpression of apoE in mice corrects the hypertriglyceridemia produced by the overexpression of apoC-III (16). Humans who are deficient in apoE, or who are homozygous for a defective isoform of apoE, have retarded metabolism of TRL and abnormal TRL composition (26, 27). Thus, apoC-III and apoE may be viewed functionally as antagonists in apoB lipoprotein metabolism.

The objective of the current study is to understand in as much depth as feasible apoB lipoprotein metabolism, focusing on the types of particles that are likely candidates for kinetic distinctness and that are likely relevant to remnant lipoprotein metabolism and atherogenic dyslipidemia. A central aim is to learn about apoC-III-containing apoB lipoproteins. Because most actions on lipoprotein metabolism of apoC-III and apoE are contrary, if not directly antagonistic, the concomitant presence of apoC-III and apoE on the same TRL particle presents an obstacle to the understanding of the normal role of either apolipoprotein, as we found in a previous kinetic study (28). Thus, we consider it essential to distinguish the effects of apoC-III and apoE on the metabolism of apoB lipoproteins, and we prepared from plasma distinct apoB lipoprotein types with either apoC-III or apoE, both of these proteins, or neither of them. This approach gains clinical relevance from two lines of evidence. First, the apoB concentration of TRLs that have apoC-III is an exceptionally strong risk factor for cardiovascular disease, eclipsing the effect of plasma triglyceride (3). Second, we recently demonstrated that apoC-III by itself and as a component of TRL and LDL is directly involved in atherogenesis by activating adhesion molecules in monocytes and endothelial cells and by activating proinflammatory nuclear factor-κB (29–31). For these reasons, we aim for better understanding of the mechanisms that produce or sustain high concentrations of apoB lipoproteins with apoC-III.

METHODS

Subjects

Five hypertriglyceridemic and six normolipidemic participants were recruited into the study. All were nondiabetic and had normal serum chemistry profiles (**Table 1**). Exclusion criteria included secondary hyperlipidemia, (APO)E2/E2, E4/E4, or E2/E4 genotype, and the use of medications that affect lipid metabolism. The study was approved by the Human Subjects Committees at the Harvard School of Public Health and Brigham and Women's Hospital. All participants gave informed consent.

TABLE 1. Baseline characteristics of the participants

Characteristic	Normolipidemic Subjects (n = 6)	Hypertriglyceridemic Subjects (n = 5)
Age (years)	58 ± 11	52 ± 4
Gender (female/male)	3/3	2/3
Body mass index (kg/m^2)	28 ± 6	28 ± 4
Plasma lipids and apolipoproteins (mg/dl)		
Total apoB	76 ± 20	84 ± 19
Total triglyceride	113 ± 53	262 ± 54[a]
Total cholesterol	168 ± 24	175 ± 33
VLDL cholesterol	8 ± 3	20 ± 5[b]
Intermediate density lipoprotein cholesterol	10 ± 4	18 ± 5[b]
LDL cholesterol	94 ± 17	104 ± 21
LDL_1	24 ± 4	18 ± 3
LDL_2	32 ± 10	26 ± 6
LDL_3	38 ± 12	60 ± 14[b]
HDL cholesterol	57 ± 18	33 ± 5[b]
VLDL apoB	3.0 ± 0.7	5.8 ± 2.0[b]
VLDL apoE	1.3 ± 0.7	2.2 ± 0.8
VLDL apoC-III	2.1 ± 0.8	7.9 ± 4[b]

apoB, apolipoprotein B. Values are means ± SD. Concentrations were measured in the samples after an overnight fast. LDL_1, density of 1.025–1.032 g/ml; LDL_2, density of 1.032–1.038 g/ml; LDL_3, density of 1.038–1.050 g/ml.

[a] $P < 0.01$ between the two groups.

[b] $P < 0.05$ between the two groups.

Controlled dietary intake

There was a 3-week controlled dietary period before the kinetic study. The entire diet was provided to the participants as outpatients, and they were asked not to consume alcoholic beverages or any other source of caloric intake. Dietary energy levels were adjusted to reports of hunger or satiety and to trends in body weight that were measured every other day. The diet was prepared at the metabolic kitchen of the General Clinical Research Center at Brigham and Women's Hospital (Boston, MA). The diet consists of 37% energy from fat (8% from saturated fat, 24% from monounsaturated fat, and 5% from polyunsaturated fat), 48% from carbohydrate, and 15% from protein.

Tracer infusion

Participants were admitted to the General Clinical Research Center of Brigham and Women's Hospital in the evening before study. After an overnight fast, they received a priming dose of 4.2 μmol/kg [D_3]L-leucine (Tracer Technologies, Cambridge, MA), followed by a constant infusion of [D_3]L-leucine at 4.8 μmol/kg/h for 15 h. A bolus injection of [D_5]L-phenylalanine (1.2 mg/kg) was also administered at the same time. Blood samples were collected at baseline, every 20 min in the first 2 h after the infusion, and hourly thereafter. For the first 4 h, participants were restricted to noncaloric drinks. After this, they were given a standardized lunch and supper that had no fat, leucine or phenylalanine and that contained 60% of the calories required for maintenance, to avoid discomfort and the abnormal metabolic effects of a prolonged fast.

Sequential immunoaffinity chromatography

Blood was collected into EDTA coated vacuum tubes and protease inhibitors were added. Plasma was separated immediately after sampling by centrifugation at 2,500 rpm for 20 min, divided

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into aliquots in polypropylene vials, immediately sealed under nitrogen gas, and kept frozen at −80°C until analysis. Separation of lipoproteins by apoE and apoC-III content was carried out with affinity-purified polyclonal antibodies against apoE and apoC-III (Academy Biomedical, Houston, TX) coupled to Sepharose 4B resin, as described previously (5, 6). Plasma was first incubated with anti-apoE immunoaffinity resin, and the unbound fraction (E−) was collected by gravity flow. The bound fraction (E+) was eluted by incubation with 3 M NaSCN and was immediately desalted and dialyzed. Both bound and unbound fractions from the anti-apoE column were then incubated with anti-apoC-III immunoaffinity resin. This sequential column procedure separated plasma into four immunofractions: those with both apoE and apoC-III (E+CIII+), those with apoE but not apoC-III (E+CIII−), those with apoC-III but not apoE (E−CIII+), and those without apoE or apoC-III (E−CIII−). The efficiency of the procedure, defined as the percentage of ligand (apoE or apoC-III) that is removed from plasma by the resin, is 98% for apoC-III and 97% for apoE. In addition, reincubation of eluted E+ and CIII+ fractions on the respective resin resulted in complete rebinding and no recovery of cholesterol or apoB in the unbound fractions, suggesting that any possible dissociation of apoE or apoC-III from apoB lipoproteins occurring during NaSCN elution, desalting, or concentrating procedures did not convert E+ or CIII+ particles to E− or CIII−. We also evaluated the effect of freezing and storage. Column efficiency for the anti-apoE and anti-apoC-III columns was 98% and 97% for fresh samples and 99% and 98% for frozen samples. Moreover, the percentages and plasma concentrations of apoB lipoproteins in the four immunofractions were similarly unaffected. Therefore, freezing and storage of plasma under these conditions do not affect the subsequent immunoaffinity columns.

Ultracentrifugation

The four immunofractions described above were then ultracentrifuged separately at 25,000 rpm on a Ti 25 rotor in an L8-70M instrument (Beckman, Brea, CA) to isolate light VLDL (Svedberg units of flotation, 60–400), dense VLDL (Svedberg units, 20–60), intermediate density lipoprotein (IDL; 1.006–1.025 g/ml), LDL_1 (1.025–1.032), LDL_2 (1.032–1.038), and LDL_3 (1.038–1.050) using a modification of the methods of Lindgren, Jensen, and Hatch (32). A density of 1.050 g/ml was selected as the cutoff point for LDL to avoid contamination by lipoprotein [a], which is concentrated at densities between 1.050 and 1.080 g/ml (33).

Determination of lipids and apolipoproteins

Triglyceride and cholesterol concentrations were determined enzymatically on a Cobas MIRA Plus Autoanalyser (Roche, Nutley, NJ). ApoE, apoC-III, apoB, apoC-I, and apoC-II concentrations were determined by sandwich ELISA procedures using affinity-purified antibodies (Academy Biomedical). Intra-assay coefficients of variation for lipid and apolipoprotein measurements were between 2% and 6%, and interassay coefficients of variation were between 4% and 8%.

Measurement of tracer enrichment and pool size

After ultracentrifugation, apoB was precipitated from the lipoproteins with isopropanol, and the precipitate was free of apolipoproteins other than apoB, as determined by high-sensitivity SDS-PAGE with silver staining. Norleucine internal standard was added, and the mixture was converted to volatile heptafluorobutyric acid derivatives as described previously (28). Tracer enrichment was measured on a 5890 gas chromatograph/5988A mass spectrometer (Hewlett-Packard, Palo Alto, CA) using negative chemical ionization and selective ion monitoring. ApoB mass was measured by comparing the ratio between the area under the leucine curve and the area under the norleucine curve with a standard curve of various leucine/norleucine ratios (34, 35). Plasma total apoB concentration was measured by ELISA and applied equally among the individual lipoprotein fractions to correct their apoB masses for loss. Plasma volume (liters) was assumed to be 4.4% of body weight (kg).

Model development and kinetic analysis

Tracer enrichment and apoB were measured in 24 apoB lipoprotein fractions (four immunofractions further separated into six density fractions) for each sample (**Fig. 1**). A multiple-compartment model was used to find the best fit to the observed data using SAAM II software (SAAM Institute, Seattle, WA). **Figure 2** shows the diagram of the model. A plasma amino acid-forcing function is followed by a hepatic intracellular delay compartment, accounting for the time required for the synthesis and secretion of apoB-100 into plasma. The model is developed by designating every physically separated lipoprotein fraction to a single compartment. Enrichment curves for light VLDL show a slow-turnover tail and thus require an additional slow-turnover compartment. Compartments 11–17 correspond to apoB lipoproteins without apoE and apoC-III (E−CIII−). Among them, compartments 11 and 12 are fast- and slow-turnover components of light VLDL, compartment 13 is for dense VLDL, compartment 14 is for IDL, and compartments 15–17 are for LDL_1, LDL_2, and LDL_3, respectively. Similarly, compartments 21–27 correspond to E−CIII+ apoB lipoproteins, compartments 31–34 correspond to E+CIII−, and compartments 41–47 correspond to E+CIII+. ApoB masses in three fractions (E+CIII− LDL_1, LDL_2, and LDL_3) are too low to measure; thus, these three fractions are not included.

The model allows direct secretion and direct removal of apoB into and out of every lipoprotein fraction. The model allows stepwise delipidation among apoB lipoproteins of the same apoE and apoC-III composition (e.g., E+CIII+ VLDL → E+CIII+ IDL → E+CIII+ LDL). Lipolysis pathways for IDL directly to LDL_2 and LDL_3, bypassing LDL_1, are also tested, and found to be required (i.e., having a nonzero parameter value in the best-fit model) only for the E−CIII− fraction. Another important feature of our model is selective conversion pathways among apoB lipoproteins of different apoE and apoC-III composition (e.g., E+CIII+ light VLDL → E+CIII− dense VLDL). When plasma apoB lipoproteins undergo lipolysis, their apoE and apoC-III content becomes lower (4), indicating a loss of apoE and apoC-III. When a TRL particle completely loses its apoC-III or apoE content, its phenotype, based on the presence of apoE and apoC-III, changes accordingly. On the other hand, when apoB lipoproteins free of apoC-III or apoE interact with HDL or another apoB lipoprotein, they may gain apoC-III or apoE and also change their phenotype. When evaluating potential conversion pathways between fractions with different apoE and apoC-III composition, we first compare the enrichment curves of the originating and destination compartments to eliminate pathways that strongly prohibit a precursor-product relationship. Next, pathways that are compatible with the tracer enrichment curves are added to the existing model one by one and are fitted to the data. Pathways for which rate constants are zero or negligible are eliminated. Average particle size and triglyceride content are also taken into consideration when selecting a pathway. After the model structure was established by the mean tracer and tracee data of all 11 subjects, each participant's data were fitted individually to obtain the parameter values. Tracer and tracee data for both $[D_5]$L-phenylalanine bolus and $[D_3]$L-leucine primed,

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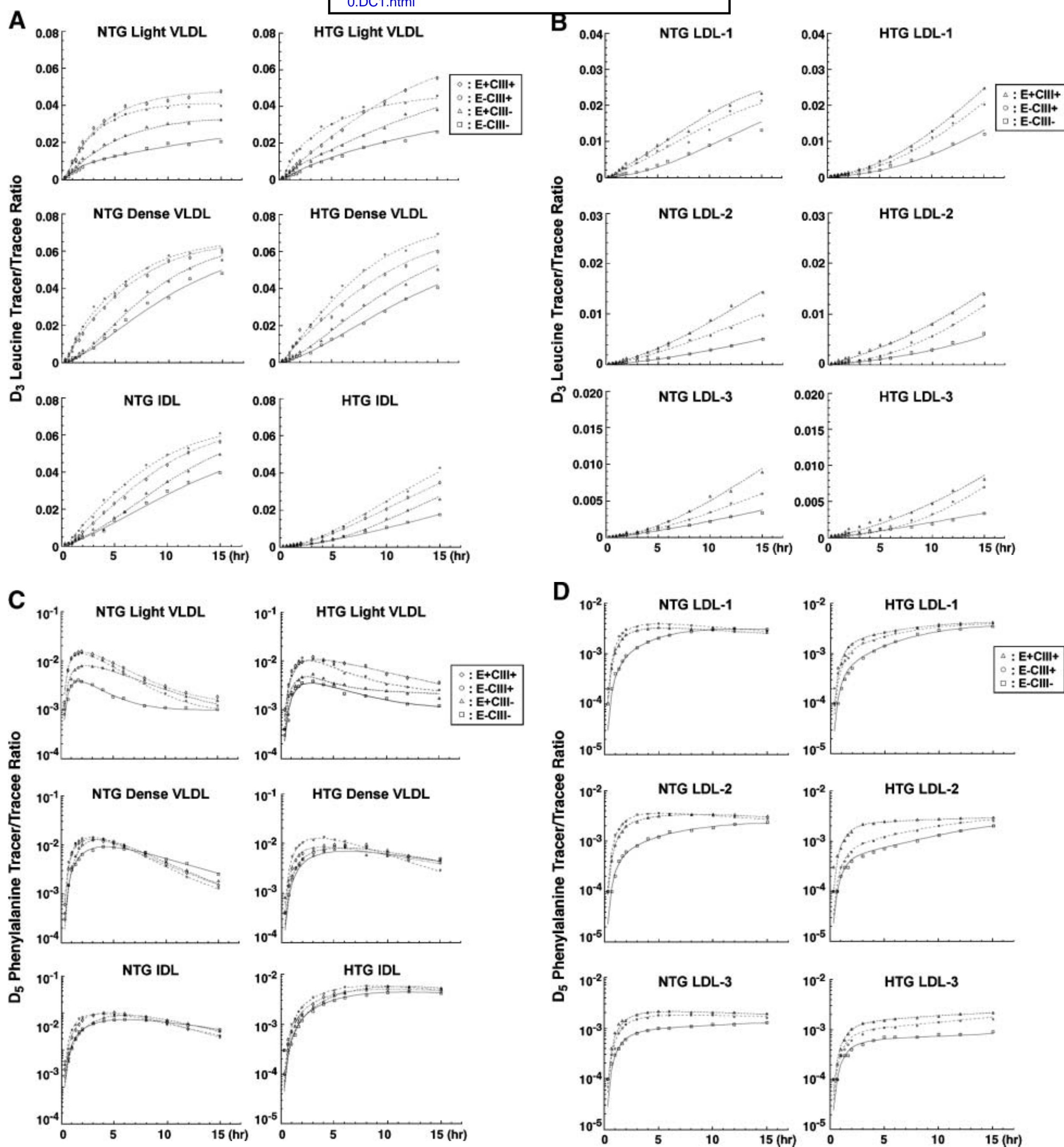

Fig. 1. Tracer-tracee ratios of D_3-leucine (A, B) and D_5-phenylalanine (C, D) in VLDL, intermediate density lipoprotein (IDL), and LDL subfractions in normolipidemic (NTG; n = 6) and hypertriglyceridemic (HTG; n = 5) participants. E+CIII+, apolipoprotein B (apoB) lipoproteins with both apoE and apoC-III; E−CIII+, particles with apoC-III but not apoE; E+CIII−, particles with apoE but not apoC-III (concentrations of E+CIII− LDL were too low to measure, and they were not modeled); E−CIII−, particles without apoE or apoC-III. Data points represent average leucine and phenylalanine tracer-tracee ratios in each group. Lines represent model-derived curves fitted to the data. Phenylalanine data are presented on a logarithmic scale. See Methods for details. ApoB mass and model predicted pool size data are available in the supplementary data.

continuous infusions were combined in a parallel multiple-compartment model and were solved simultaneously by making the rate constants equal for leucine and phenylalanine experiments. Thus, for every study participant, a single set of rate constants was produced. This model is able to generate excellent fits to tracer and tracee data for both leucine and phenylalanine. Fitting of apoB mass data is also excellent, and the results are shown in the supplementary data. The coefficients of variation for most parameter estimates were <30% and those for the major pathways were <15%.

Statistical analysis

Data were analyzed using the SAS software (SAS Institute, Cary, NC) and are presented as means ± SD. An unpaired *t*-test was used for between-group comparisons and a paired *t*-test was

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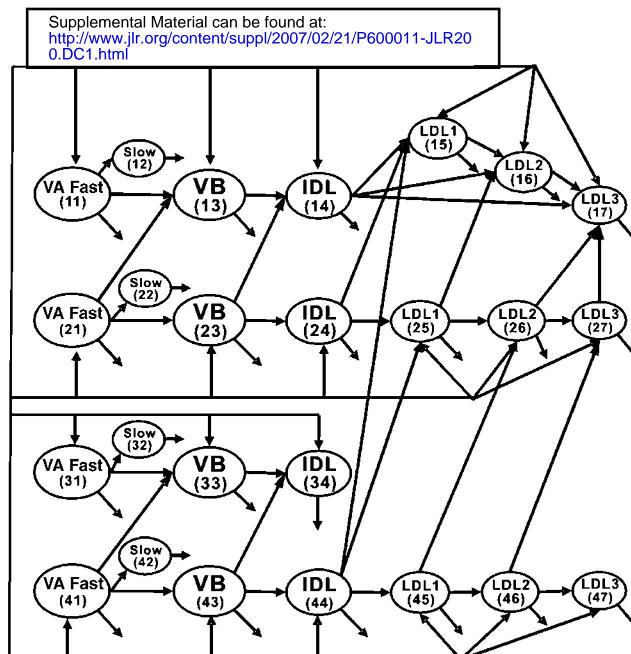

Fig. 2. Multicompartmental model. Plasma tracer-forcing function supplies a delay compartment responsible for the production and secretion of apoB-100. Compartments 11–47 represent plasma pools of apoB-100. VA, light VLDL; VB, dense VLDL; LDL_1, density of 1.025–1.032 g/ml; LDL_2, density of 1.032–1.038 g/ml; LDL_3, density of 1.038–1.050 g/ml. See Methods for details.

used for within-person comparisons, unless specified otherwise. $P \leqslant 0.05$ was considered statistically significant. Spearman correlation analysis and regression analysis were also conducted to study the associations between apoE, apoC-III, hypertriglyceridemia, and VLDL metabolism.

RESULTS

Demographic information and baseline measurements of lipoproteins for the participants are summarized in Table 1. Hypertriglyceridemic and normolipidemic participants have similar age, gender, and body mass index. The majority of them are overweight but not obese. Concentrations of plasma total apoB, total cholesterol, and LDL cholesterol are similar in the two groups. The hypertriglyceridemic participants have significantly higher concentrations of plasma total triglyceride, VLDL apoB, VLDL cholesterol, and VLDL apoC-III, higher LDL_3 cholesterol, and lower HDL cholesterol.

ApoC-III is secreted on ~90% of VLDL and 15% of LDL

Figure 3 shows secretion rates of apoB-100 for each particle type. (The numerical data and data for LDL subfractions are available online.) The vast majority of VLDL particles are secreted as apoC-III-containing (90% in both hypertriglyceridemic and normolipidemic subjects). VLDL without apoE or apoC-III constitutes <10% of total VLDL secretion. In IDL, the percentage secreted as E−CIII− increases substantially to 36% in normolipidemic subjects and 43% in hypertriglyceridemic subjects, although the majority are still secreted as apoC-III-containing. The secretion pattern is reversed in LDL, most secreted without apoC-III.

ApoE is secreted with apoC-III in TRL

Approximately 40% of TRLs that are secreted with apoC-III also contain apoE (Fig. 3). In contrast, nearly all of the apoB lipoproteins secreted with apoE have apoC-III. Less than 2% of TRLs are secreted with apoE but not apoC-III in both groups. Similar to apoC-III, secretion of apoE-containing apoB lipoproteins (E+CIII− and E+CIII+) is concentrated within TRLs (41% of total VLDL secretion, 24–28% of IDL). Less than 8% of LDL is secreted with apoE.

ApoC-III-containing TRLs and LDLs have fast turnover rates

ApoC-III-containing apoB lipoproteins have faster rates of appearance of tracer than their counterparts without apoC-III (Fig. 1). With the injection of the bolus phenylalanine tracer, the peak tracer-tracee ratio is higher and the rate of disappearance is faster for apoC-III-containing lipoproteins. These patterns are true for all 11 participants. Accordingly, apoC-III-containing VLDL, IDL, and LDL have fast fractional catabolic rates (FCRs): 40–150% higher than those without apoC-III (**Fig. 4**). FCRs of E+CIII−, E−CIII+, and E+CIII+ TRL and LDL, although all significantly higher than those of E−CIII−, are generally not statistically different from each other. However, as shown below, the presence of apoE and apoC-III each appears to affect flux through specific catabolic pathways.

TRLs without apoC-III or apoE are almost exclusively lipolyzed into LDL

In both hypertriglyceridemic and normolipidemic subjects, a substantial proportion of E−CIII− VLDL and IDL

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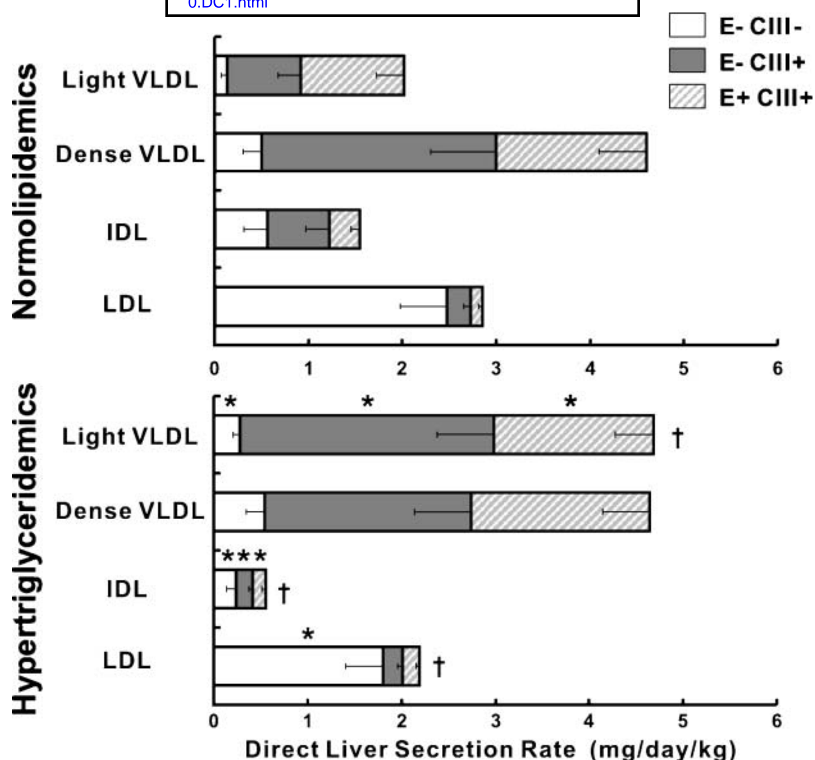

Fig. 3. Liver secretion rates of apoB lipoproteins. Data represent model-predicted secretion rates (mg/day/kg body weight; means ± SD) of light VLDL, dense VLDL, IDL, and LDL according to their apoC-III and apoE composition. Average total apoB secretion rates in normotriglyceridemic and hypertriglyceridemic participants are 11 and 12 mg/day/kg, respectively ($P = 0.77$). Secretion of E+CIII− constitutes <2% of total apoB lipoproteins in both groups and thus is not plotted. * $P < 0.05$ between hypertriglyceridemic and normolipidemic groups for corresponding fractions; † $P < 0.05$ in total secretion rates of light VLDL, IDL, or LDL between hypertriglyceridemic and normolipidemic groups. See supplementary data for exact values and LDL subfractions.

is formed from apoC-III-containing TRLs by loss of apoC-III from E−CIII+ associated with the lipolytic conversion of larger to smaller particles. VLDLs that do not contain apoE or apoC-III are poor candidates for direct removal and are almost exclusively lipolyzed to IDL. Subsequently, >90% of E−CIII− IDL is hydrolyzed to LDL, about half going to LDL_1 and the rest going directly to LDL_2 and LDL_3.

TRLs with apoC-III but not apoE are the major contributors to plasma LDL formation

Approximately 70% of E−CIII+ TRLs are hydrolyzed to LDL, either E−CIII+ LDL (20%) or E−CIII− LDL (50%); the remaining 30% are removed from the circulation as E−CIII+ TRLs (Fig. 5). Thus, lipolytic conversion from E−CIII+ TRLs to E−CIII− LDLs is a major pathway responsible for LDL formation in plasma. Indeed, E−CIII+ TRLs are precursors for more than half (55%) of total plasma E−CIII− LDLs among hypertriglyceridemic subjects, generating twice as much LDL as direct liver secretion (27%) (**Fig. 6**; detailed data are available in the supplementary data). In addition, the majority of E−CIII+ LDLs are also eventually converted to E−CIII− LDLs. In all, E−CIII+ TRLs and LDLs provide 45–61% of the total production of E−CIII− LDLs.

TRLs with apoE but not apoC-III are rapidly removed from the circulation

E+CIII− TRLs constitute <2% of liver secretion of apoB lipoproteins. Instead, the majority of these particles are products of E+CIII+ TRLs after the loss of apoC-III during lipolysis (Fig. 5). E+CIII− VLDLs are ∼10 times more likely to undergo direct removal than conversion to IDLs. The rate constants for removal are higher in E+CIII− than for any other type of TRL. We could not detect any measurable amount of LDL particles with apoE but not apoC-III, presumably because of the fast removal of E+CIII− IDL.

Metabolism of TRLs with both apoE and apoC-III is a function of both apolipoproteins

Ninety percent of E+CIII+ TRLs are cleared from the circulation before reaching LDL size (Fig. 5). Most are removed as E+CIII+ VLDL or IDL, and some are converted to E+CIII− before leaving the circulation. Only 10% of E+CIII+ TRLs are converted into LDLs. The contribution of E+CIII+ TRLs to LDL formation is very low, producing only 5–8% of total plasma LDL compared with 45–61% for E−CIII+ TRLs ($P < 0.01$) (Fig. 6). This disparity between these two TRL particle types as precursors for LDL contrasts with their similar secretion rates

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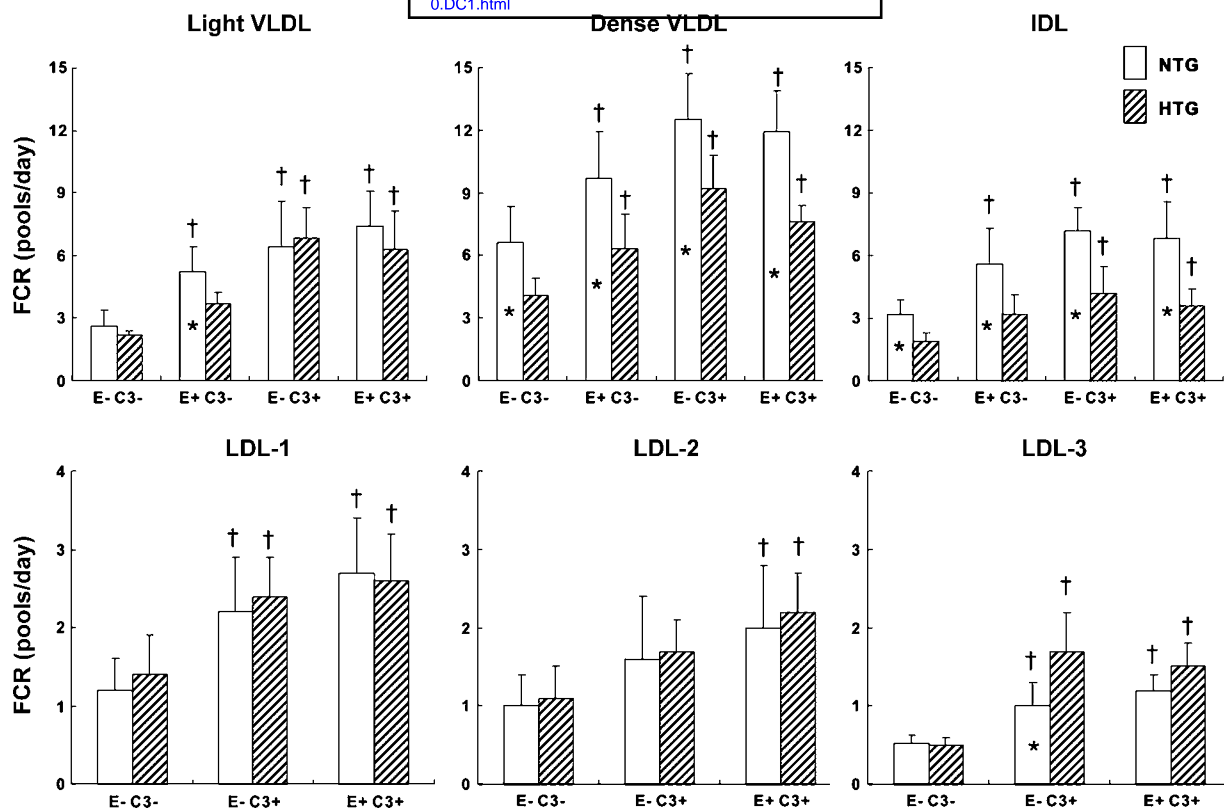

Fig. 4. Fractional catabolic rates (FCRs) of apoB lipoproteins according to contents of apoE and apoC-III. * $P < 0.05$ between hypertriglyceridemic subjects (HTG; n = 5) and normolipidemic controls (NTG; n = 6); † $P < 0.05$ compared with the corresponding E−CIII− fraction in each density fraction. Data are means ± SD.

(i.e., 3.0 mg/kg/day for E+CIII+ vs. 3.9 mg/kg/day for E−CIII+ among normolipidemic subjects; $P = 0.48$) (Fig. 3). Rate constants for direct removal for E+CIII+ TRLs are intermediate between E−CIII+ and E+CIII− (i.e., faster than E−CIII+ but slower than E+CIII−) (Fig. 5). These findings strongly implicate apoE in redirecting TRL catabolism toward the direct removal as well as apoC-III in inhibiting this pathway.

LDL subfraction kinetics

LDL without apoE or apoC-III constitutes >90% of LDL in the circulation (Fig. 5). They are either products of TRL lipolysis in plasma or are directly secreted from the liver. Figure 6 summarizes the contribution of each pathway to LDL production. Overall, significantly more LDLs come from TRL lipolysis than from direct liver secretion (~3:1 in hypertriglyceridemic subjects and 2:1 in normolipidemic subjects; both $P < 0.01$). Among E−CIII− LDLs formed by lipolysis, E−CIII+ TRLs contribute by far the most (75% and 63% in hypertriglyceridemic and normolipidemic subjects, respectively), followed by E−CIII− TRLs (19% and 25%) and E+CIII+ TRLs (5% and 12%).

ApoC-III-containing LDL subfractions demonstrate different metabolic patterns than LDL that does not have apoC-III. First, both E−CIII+ and E+CIII+ LDL subfractions have higher FCRs than their E−CIII− counterparts (Fig. 4). Second, >80% of E−CIII+ LDL_1, LDL_2, and LDL_3 lose apoC-III during circulation in plasma to form E−CIII− LDL_3, similar to the metabolic fate of E−CIII+ TRLs (Fig. 5). Finally, among both hypertriglyceridemic and control subjects, >50% of E+CIII+ LDL_1 and LDL_2 are removed from the circulation before being lipolyzed into E−CIII+ LDL_2 or LDL_3, presumably as a result of the positive effect of apoE on LDL receptor-mediated clearance. In contrast, only 30–40% of E−CIII− LDL_1 and LDL_2 are removed from the circulation before reaching LDL_3.

VLDL apoC-III and apoE levels are determined mainly by increased VLDL secretion, not by retarded catabolism

Concentrations of VLDL apoC-III and apoE are strongly and positively associated with VLDL triglyceride ($r = 0.98$, $P < 0.001$ for apoC-III and $r = 0.88$, $P = 0.002$ for apoE). Concentrations of apoC-III and apoE found in VLDL are strongly associated with the secretion rates of VLDL apoB but not with the FCR (**Fig. 7**, far left and middle left panels). Among VLDL particles that contain apoC-III with or without apoE, the number of apoC-III molecules per particle is not associated with altered FCRs (Fig. 7, upper middle right panel) or rate constants for lipolysis or clearance (data not shown). Among VLDLs that contain both apoC-III and apoE, the molar ratio of apoE to apoB is significantly correlated with their FCRs ($r = 0.70$, $P = 0.03$) (Fig. 7, lower middle right panel). Overall, apoE/apoB ratios in apoE-containing VLDLs are positively associated with rate constants for direct removal ($r = 0.68$, $P = 0.03$ in E+CIII+ VLDL).

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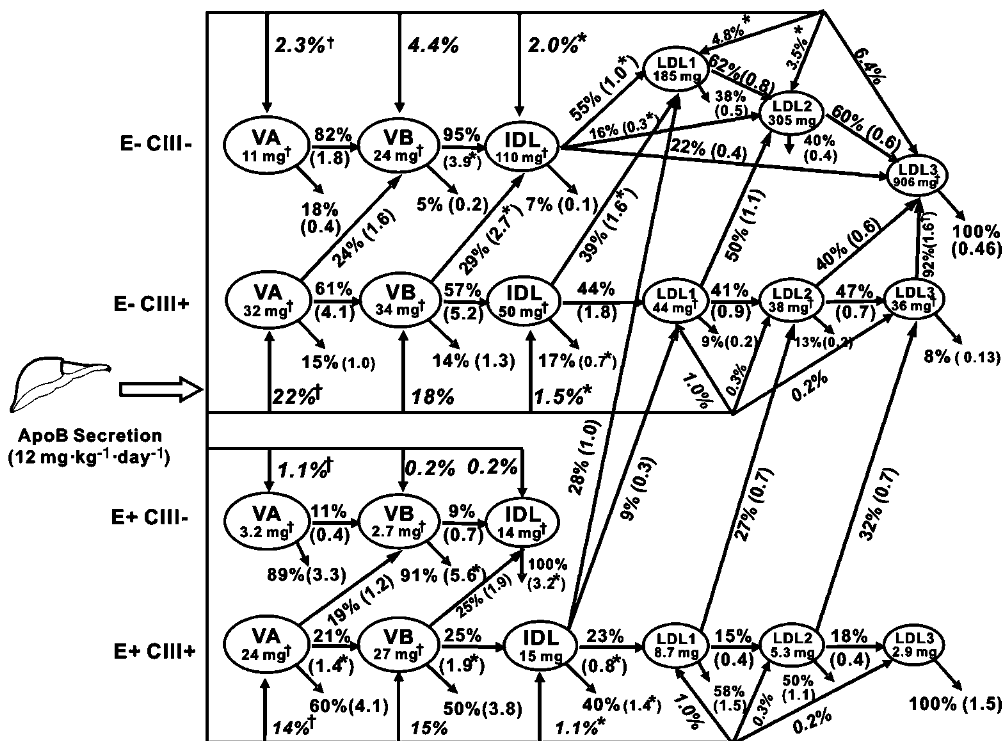

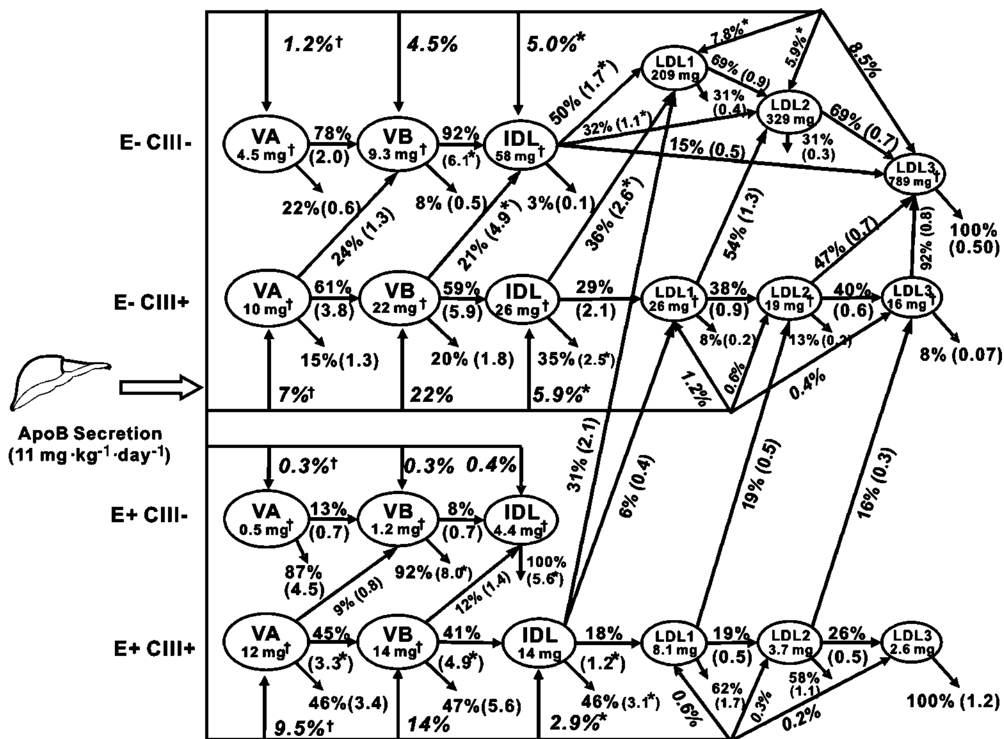

Fig. 5. Flux of apoB-100 in hypertriglyceridemic (A) and normolipidemic (B) participants. Oval boxes represent apoB lipoprotein fractions separated by apoC-III and apoE content and by density; numbers inside indicate pool sizes (mg). Arrows out of the liver represent direct liver secretion; percentages next to the arrows indicate the percentage of total liver secretion into each fraction. Arrows out of lipoprotein compartments represent conversion and direct removal; percentages above the arrows indicate the relative proportion of flux out of each compartment, and numbers in parentheses indicate the rate constant for each pathway (pools/day). VA, light VLDL; VB, dense VLDL. Data are means ± SD. † The parameter is significantly higher in hypertriglyceridemic than in normolipidemic subjects; * the parameter is significantly lower in hypertriglyceridemic subjects ($P < 0.05$).

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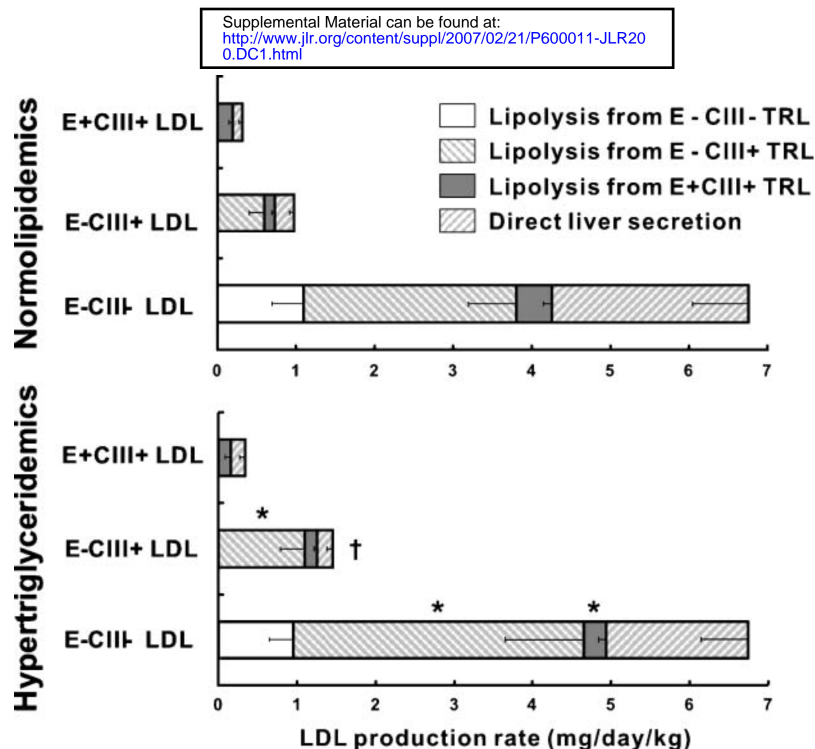

Fig. 6. Contribution of lipolysis pathways and direct liver secretion to LDL formation. Data represent production rates (mg/day/kg; means ± SD) of different types of LDL particles from direct liver secretion and various lipolytic conversion pathways. * $P < 0.05$ in corresponding fractions between hypertriglyceridemic and normolipidemic groups; † $P < 0.05$ in total E−CIII+ LDL production rates between hypertriglyceridemic and normolipidemic groups. See supplementary Table II for exact numerical data. TRL, triglyceride-rich lipoprotein.

ApoC-III-containing TRLs are enriched in apoC-II and apoC-I

Consistent with our earlier observations (5), there is a trend toward a higher content of triglyceride and cholesterol per particle among apoC-III-containing TRLs and LDLs compared with those without apoC-III (**Fig. 8**). However, the most striking finding is that apoB lipoproteins with apoC-III contain significantly more apoC-II and apoC-I molecules per particle than those without apoC-III. Within every density fraction, E+CIII+ apoB lipoproteins

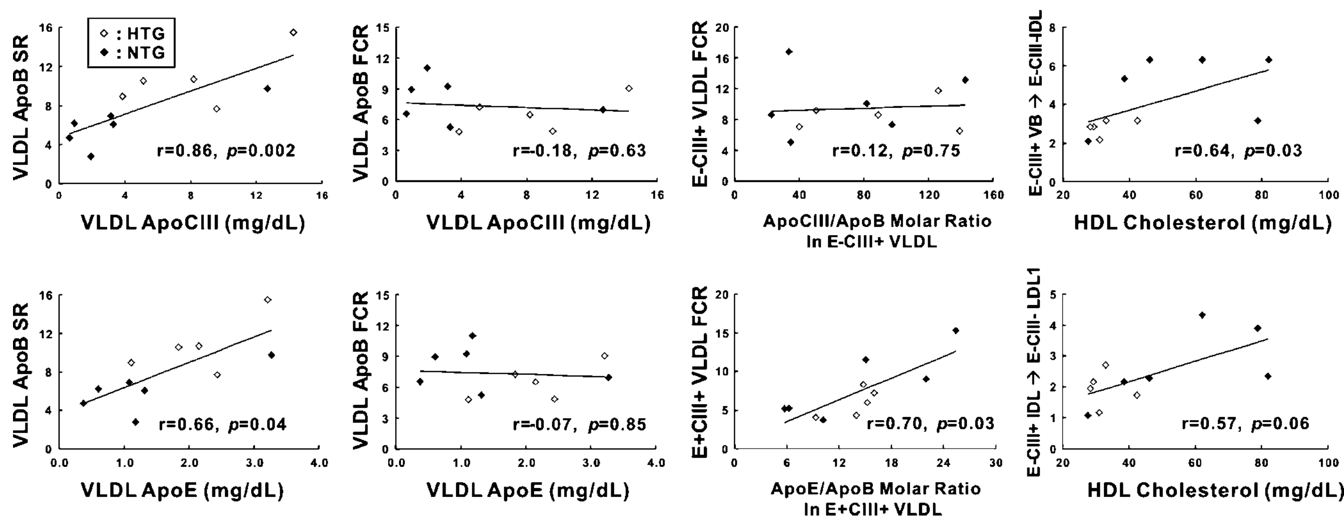

Fig. 7. Relationship between VLDL apoC-III, VLDL apoE, HDL cholesterol, and kinetic parameters. Linear regression lines are shown for each relationship in all 11 subjects [open diamonds, hypertriglyceridemic subjects (HTG); closed diamonds, normolipidemic subjects (NTG)]. Spearman correlation coefficients (r) are shown together with P values. SR, direct liver secretion rates (mg/day/kg); FCRs are measured in pools/day. Molar ratios are calculated using concentrations of VLDL apoC-III, apoE, and apoB in the stated fractions and their respective molecular weights. The y axes in the far right panels indicate rate constants for lipolytic conversion pathways between E−CIII+ dense VLDL (VB) and E−CIII− IDL (top) and between E−CIII+ IDL and E−CIII− LDL_1 (bottom).

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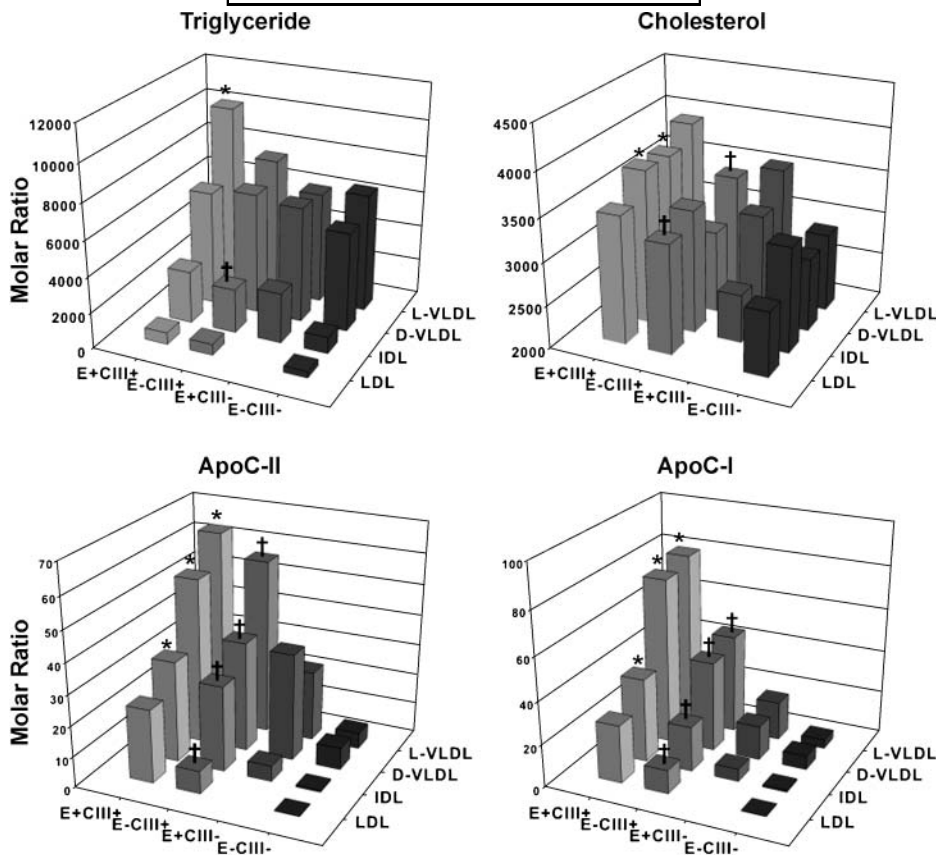

Fig. 8. Contents of triglyceride, cholesterol, apoC-II, and apoC-I in light (L-) and dense (D-) VLDL, IDL, and LDL types defined by the presence of apoC-III and apoE. Molar ratios of lipids and apolipoproteins per apoB in each particle type are calculated based on their concentrations and those of apoB-100 in the samples taken before the infusion (i.e., time 0). Data shown are averages from all 11 participants. * $P < 0.05$ between corresponding E+CIII+ and E+CIII− fractions; † $P < 0.05$ between corresponding E−CIII+ and E−CIII− fractions.

have the highest content of apoC-II and apoC-I, followed closely by E−CIII+ and then E+CIII−, whereas E−CIII− fractions always have the lowest content. In addition, within each type of particle (E−CIII−, E+CIII−, etc.), apoC-II and apoC-I contents are highest in VLDL, progressively diminishing as particle density increases.

Slow TRL metabolism and overproduction of apoC-III-containing light VLDLs are two hallmarks of hypertriglyceridemia

Hypertriglyceridemic subjects have significantly slower FCRs than normolipidemic subjects in all dense VLDL and IDL fractions, regardless of their apoE and apoC-III composition (Fig. 4). This suggests that slow TRL FCR in hypertriglyceridemia involves factors other than apoC-III and apoE. Hypertriglyceridemic subjects also have lower rate constants for some conversion pathways from E−CIII+ TRLs to E−CIII− IDLs and LDLs (Fig. 5), resulting in retention of these apoC-III-containing TRLs in plasma and higher flux to E−CIII+ LDLs. This may be attributable to low levels of HDLs among hypertriglyceridemic subjects, because HDLs are significantly correlated with rate constants for these conversion pathways (Spearman correlation coefficient: $r = 0.64$, $P = 0.03$ between HDL cholesterol levels and rate constants for the E−CIII+ dense VLDL → E−CIII− IDL pathway; $r = 0.57$, $P = 0.06$ for the E−CIII+ IDL → E−CIII− LDL_1 pathway) (Fig. 7, far right panels). Another major metabolic perturbation in hypertriglyceridemia is the overproduction of light VLDL (Fig. 3). Although secretion rates for total apoB lipoproteins are similar in both groups, there is a major difference in the secretion pattern. Secretion of light VLDL E−CIII+ constitutes 22% of total apoB secretion in hypertriglyceridemic subjects, more than three times as high as in normolipidemic subjects ($P < 0.01$). Together with increased secretion of E−CIII+ light VLDL, hypertriglyceridemic subjects have a >2-fold increase in the secretion of total light VLDL ($P < 0.05$). Overall, the combination of these factors, slow metabolism of the majority of TRLs and overproduction of apoC-III-containing light VLDLs, results in significantly increased plasma levels of apoC-III-containing TRLs and LDLs found among hypertriglyceridemic patients.

DISCUSSION

This study reveals substantial heterogeneity in TRL and LDL metabolism according to whether they have apoC-III

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or apoE. ApoC-III-containing particles dominate VLDL metabolism in both hypertriglyceridemic and normolipidemic subjects, whereas those with neither apoC-III nor apoE dominate LDL metabolism. ApoB lipoproteins containing apoC-III have more rapid tracer enrichment, higher peak tracer-tracee ratios, and faster disappearance than particles without apoC-III in both hypertriglyceridemic and normolipidemic participants. These patterns of the tracer enrichment curves indicate that possession of apoC-III is a marker for a short residence time in plasma for VLDL, IDL, or LDL. Multiple-compartment modeling further showed that the presence of apoC-III is associated with relative suppression of direct removal of these lipoproteins from the circulation and enhancement of their conversion to smaller and denser particles. ApoE, in contrast, apparently modulates the direction of flux in favor of direct removal. TRLs that do not have apoC-III or apoE, constituting a minor portion of TRL secretion (<20%), are mostly stepwise converted to smaller particles down to LDLs, and there is little direct removal from the circulation before that end point. LDLs thus arise from two sources, plasma VLDLs and IDLs that have apoC-III but not apoE and direct hepatic secretion.

In general, these observations are consistent with some known actions of apoC-III and apoE. First, considering E−CIII+ VLDL, the fraction with the highest secretion rate, conversion to IDL and LDL dominates direct removal by 4:1 or 5:1. This could be attributable to the inhibitory action of apoC-III on particle clearance by way of apoB-100 interacting with the LDL receptor or other hepatic receptors (10). Second, considering E+CIII+ VLDL metabolism, the rate constants for particle clearance are higher and the rate constants for conversion to IDL and LDL are lower than those of E−CIII+ VLDL, reflecting the role of apoE in enhancing receptor-mediated clearance (23, 24). Correlation analysis also suggests that VLDL apoE content, the molar ratio of apoE to apoB, is positively related to rate constants for the direct removal of VLDL. Consistent with these observations, apoC-III transgenic animal studies show that retarded tissue uptake of VLDL or chylomicron remnants in these animals can be corrected by adding exogenous apoE or by coexpressing apoE transgene (15, 16). Conversely, the absence of apoE could explain the preference for lipolysis over direct removal observed among E−CIII− and also E−CIII+ particles.

A major unresolved question raised by this study is why the presence of apoC-III is not associated with a retarded metabolism of apoB lipoproteins. We are confident that this is true for several reasons. First, rapid turnover for apoC-III-containing TRLs and LDLs is clear from the tracer enrichment curves and is not a result of any particular features of the kinetic model. Second, all 11 participants had the general pattern of tracer enrichments shown for the group, particularly the characteristics of fast tracer appearance and disappearance for apoC-III-containing particles. Third, fast turnover of apoC-III-containing particles relative to those without apoC-III occurred for all density classes. Fourth, the apoC-III-containing particles were extracted completely from plasma by immunoaffinity chromatography before ultracentrifugation, ensuring that the native apoC-III-containing particles are segregated before further procedures that could affect their properties. Finally, we are finding the same pattern of tracer enrichment in the same participants studied a second time under different nutritional conditions and in another group of 12 participants (6 normolipidemic and 6 hypertriglyceridemic) studied on a different diet (unpublished observations).

This result contrasts with those from apoC-III transgenic animal studies and provokes several thoughts unanticipated at the outset of this study. First, impaired lipolysis has been proposed as the main defect behind apoC-III-induced hypertriglyceridemia, because in vitro experiments suggest that apoC-III may be a direct noncompetitive inhibitor for lipoprotein lipase (8). However, it has been called into question whether this is the case in vivo, as mice overexpressing human apoC-III do not have reduced lipoprotein lipase activity (14, 16, 17) and TRLs isolated from these apoC-III transgenic mice do not retard lipase action in vitro (14, 16). In support of this view, our study finds no significant correlation between the number of molecules of apoC-III per particle and the rate constants for lipolytic conversion among apoC-III-containing VLDLs. Alternatively, it has been reported that VLDLs from apoC-III transgenic mice may have reduced binding to cell surface heparin sulfate proteoglycan as another, indirect, mechanism for retarded lipolysis in these animals (15, 17). However, reduced binding to heparan sulfate proteoglycan of VLDL apoC-III could also operate at the hepatocyte surface and be a mechanism for decreased receptor-mediated uptake and reduced clearance, as found in our study. In another study, in vitro, apoC-III content of apoB lipoproteins in humans was associated with increased binding affinity to vascular proteoglycan biglycan (36). Thus, these divergent findings from distinct model systems do not provide a conclusion regarding whether VLDL metabolism is affected by apoC-III interacting with vascular proteoglycan. Evidence for a possible species difference in how apoC-III affects VLDL metabolism comes from lipoprotein composition analysis. VLDLs separated from apoC-III transgenic mice have significantly lower contents of both apoE (14–16) and apoC-II (14, 16) per particle than those from wild-type mice. However, apoC-III-containing TRLs and LDLs in humans in this and previous studies (28) have high contents of these apolipoproteins. Thus, the displacement of other apolipoproteins by apoC-III overexpression in mice contrasts directly with the apparent coexistence of considerable amounts of apoC-III, apoC-II, and apoE on TRLs in humans. In addition, the species difference may be related to the lack of cholesteryl ester transfer protein and the low levels of apoB lipoproteins relative to the amount of apoC-III expressed in the mouse models.

Nonetheless, the kinetic hypothesis for the link between apoC-III and coronary heart disease, requiring that apoC-III-rich remnant lipoproteins be slowly metabolized in plasma, needs revision, because these lipoproteins are metabolized as fast or faster than their apoC-III-free counterparts and only some can be defined as remnants. The

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strong correlation between apoC-III and risk of coronary heart disease among population studies (1–3) may be attributable to apoC-III's direct involvement in atherogenesis. We recently reported that apoC-III alone or as a component of VLDL and LDL stimulates the adhesion of monocytes to vascular endothelial cells (29–31). ApoC-III activates β1-integrin in human monocytic cells and vascular cell adhesion molecule-1 and intercellular cell adhesion molecule-1 in endothelial cells. Upstream pathways include the activation of protein kinase C, RhoA, and nuclear factor-κB, all of which could have proinflammatory effects on cells in developing atheromatous lesions. Furthermore, apoC-III may also increase the binding of apoB lipoproteins to vascular proteoglycan biglycans (36), an action that would increase the retention of apoB lipoproteins in the arterial wall. Thus, the abundance of apoC-III on TRLs may be a crucial factor in explaining their atherogenicity. In addition to the content of apoC-III, their high potential for LDL formation further renders the E−CIII+ apoB lipoproteins possibly the most atherogenic of lipoproteins.

This study demonstrates a global reduction in FCR for TRLs in hypertriglyceridemia, regardless of particle apoC-III and apoE composition. Previous studies have found slow VLDL turnover among hypertriglyceridemic subjects (37, 38). This has at times been attributed to the action of a high apoC-III concentration in hypertriglyceridemic subjects (22). However, we also find that hypertriglyceridemia status is independently associated with reduced TRL FCR, indicating that factors other than apoC-III or apoE retard TRL catabolism in hypertriglyceridemia. Our results also show that hypertriglyceridemic subjects have a three-fold greater secretion rate of light VLDL particles containing apoC-III without apoE than normolipidemic subjects. VLDL apoC-III levels are strongly correlated with both VLDL secretion rates and VLDL triglyceride concentrations but have no relationship with VLDL FCR. These observations are compatible with the concept that apoC-III may serve as a regulator for hepatic triglyceride production. Overproduction of VLDL triglyceride but not apoB has been reported in some apoC-III transgenic animal models (14, 16). In humans, production of VLDL apoC-III correlates strongly with the secretion of VLDL triglyceride (21), consistent with the findings of our study. It is possible that apoC-III could stimulate the secretion of triglyceride-rich VLDL at the cellular level, and the exact mechanisms warrant further investigation.

This study finds no evidence for the acquisition in plasma of apoC-III or apoE by TRLs that do not already contain them when they enter the systemic circulation. This is surprising, because a considerable proportion of plasma apoC-III and apoE is found on HDLs, and studies have suggested that apoC-III and apoE can exchange between VLDL and HDL (39, 40). This may reflect limitations of the design of this study. First, transfer of apoC-III or apoE from HDL or other TRLs to nascent TRLs at the hepatocyte surface is compatible with our data and would be seen by our system as direct hepatic secretion. Second, our system also does not recognize the transfers or exchanges of apoC-III or apoE that undoubtedly occur among particles that already contain these apolipoproteins, because they would not cause a phenotype change under immunoaffinity chromatography. Third, because the current study was conducted in the fasting state, it does not exclude the possibility that deviation from the observed pattern could occur under other physiological conditions. In fact, during alimentary lipemia, it has been reported that apoE is able to redistribute from HDL to VLDL (40). Fourth, the results could be specific to the nutritional condition of this study: a high-monounsaturated-fat diet. A high-carbohydrate diet reduces the conversion of VLDL to IDL (41), a process that this study links to the content of both apoE and apoC-III.

Studies of apoC-III and apoE kinetics have not been able to resolve to what extent they are freely exchangeable among apoB lipoproteins, and between apoB lipoproteins and HDL, with some finding evidence of separate exchangeable and nonexchangeable pools in VLDL or HDL and limited transfer (42, 43) and others favoring quick exchange to equilibrium producing a single homogeneous pool (39, 44). Because apoC-III-containing VLDLs have on average ~50 copies of apoC-III per particle, the coexistence in the circulation of these apoC-III-laden particles with those free of any apoC-III, and the coexistence of apoE-containing VLDLs with VLDLs free of apoE, are compatible only with conditional, and not free, exchange of apoC-III and apoE between VLDL and HDL. Our finding that VLDL particles not having apoC-III or apoE, the smallest type of VLDL (5, 6), do not acquire apoE during circulation is supported by in vitro experiments showing that apoE does not adhere readily to small apoB lipoproteins (45). However, this does not explain why VLDL E−CIII+, which are very large particles, do not acquire apoE. Perhaps apoE does not attach to VLDLs that are undergoing rapid lipolysis, the principal metabolic pathway for VLDL E−CIII+.

ApoC-III especially, but also apoE, detaches from apoB lipoproteins during lipolysis, and they are likely taken up by HDL. The magnitude of these lipolytic conversion pathways among apoB lipoproteins may depend on the availability of HDLs as acceptors for apoC-III and apoE. Our results demonstrate significant correlations between HDL cholesterol and rate constants of conversion pathways from E−CIII+ TRLs to E−CIII− LDLs. This may explain why hypertriglyceridemic subjects in our study, having low HDL concentrations, accumulate atherogenic E−CIII+ apoB lipoproteins as a result of the impaired ability to convert these particles to E−CIII−. Finally, the movement of apoE and apoC-III from TRLs to HDLs may be a way that apoB lipoprotein metabolism affects HDL metabolism, because apoC-III and apoE are shown to interact with hepatic lipase, ABCA1, scavenger receptor class B type I, and other regulators of HDL metabolism (24, 46).

In conclusion, by uniting concepts of apoB lipoprotein metabolism related to particle size and apoC-III and apoE content, a new structure for lipoprotein physiology is revealed. A lipoprotein system that has low potential for atherogenesis and optimal provision of lipids for nutrition would secrete triglycerides on VLDLs that have both apoC-

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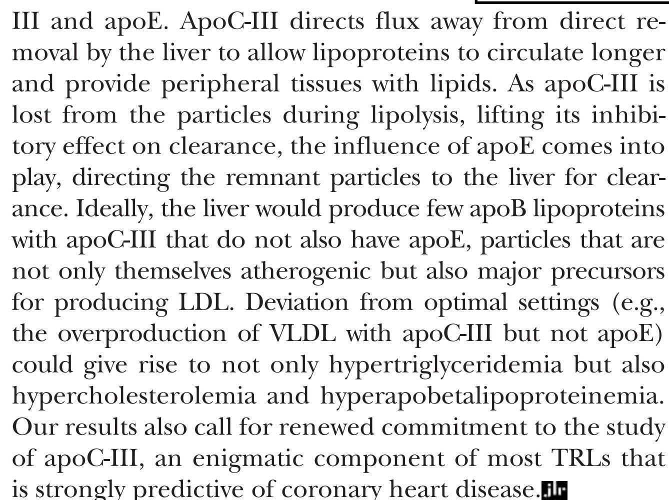
III and apoE. ApoC-III directs flux away from direct removal by the liver to allow lipoproteins to circulate longer and provide peripheral tissues with lipids. As apoC-III is lost from the particles during lipolysis, lifting its inhibitory effect on clearance, the influence of apoE comes into play, directing the remnant particles to the liver for clearance. Ideally, the liver would produce few apoB lipoproteins with apoC-III that do not also have apoE, particles that are not only themselves atherogenic but also major precursors for producing LDL. Deviation from optimal settings (e.g., the overproduction of VLDL with apoC-III but not apoE) could give rise to not only hypertriglyceridemia but also hypercholesterolemia and hyperapobetalipoproteinemia. Our results also call for renewed commitment to the study of apoC-III, an enigmatic component of most TRLs that is strongly predictive of coronary heart disease.

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