ApoB-48 and apoB-100 differentially influence the expression of type-III hyperlipoproteinemia in APOE*2 mice

Myron E. Hinsdale,[1,2] Patrick M. Sullivan,[3] Hafid Mezdour,[4] and Nobuyo Maeda

Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7525

Abstract **Apolipoprotein E (apoE) is essential for the clearance of plasma chylomicron and VLDL remnants. The human *APOE* locus is polymorphic and 5–10% of *APOE*2* homozygotes exhibit type-III hyperlipoproteinemia (THL), while the remaining homozygotes have less than normal plasma cholesterol. In contrast, mice expressing *APOE*2* in place of the mouse *Apoe* (*Apoe*$^{2/2}$ mice) are markedly hyperlipoproteinemic, suggesting a species difference in lipid metabolism (e.g., editing of apolipoprotein B) enhances THL development. Since apoB-100 has an LDLR binding site absent in apoB-48, we hypothesized that the *Apoe*$^{2/2}$ THL phenotype would improve if all *Apoe*$^{2/2}$ VLDL contained apoB-100. To test this, we crossed *Apoe*$^{2/2}$ mice with mice lacking the editing enzyme for apoB (*Apobec*$^{-/-}$). Consistent with an increase in remnant clearance, *Apoe*$^{2/2}$.*Apobec*$^{-/-}$ mice have a significant reduction in IDL/LDL cholesterol (IDL/LDL-C) compared with *Apoe*$^{2/2}$ mice. However, *Apoe*$^{2/2}$.*Apobec*$^{-/-}$ mice have twice as much VLDL triglyceride as *Apoe*$^{2/2}$ mice. In vitro tests show the apoB-100-containing VLDL are poorer substrates for lipoprotein lipase than apoB-48-containing VLDL. Thus, despite a lowering in IDL/LDL-C, substituting apoB-48 lipoproteins with apoB-100 lipoproteins did not improve the THL phenotype in the *Apoe*$^{2/2}$.*Apobec*$^{-/-}$ mice, because apoB-48 and apoB-100 differentially influence the catabolism of lipoproteins.**—Hinsdale, M. E., P. M. Sullivan, H. Mezdour, and N. Maeda. **ApoB-48 and apoB-100 differentially influence the expression of type-III hyperlipoproteinemia in APOE*2 mice. *J. Lipid Res.* 2002.** 43: **1520–1528.**

Supplementary key words lipoproteins • very low density lipoprotein • metabolism • lipoprotein remnants

Apolipoprotein E (apoE) is essential for the clearance of chylomicron and VLDL remnants from the plasma. Variant apoE proteins in humans are known to cause primary dysbetalipoproteinemia [or type-III hyperlipoproteinemia (THL)], which is characterized by the accumulation of chylomicron and VLDL remnants in the plasma and a high incidence of coronary artery and peripheral vessel atherosclerosis (1). The most common form of THL is associated with homozygosity for the APOE*2 allele, whose product, apoE–2, has <2% normal binding to the low density lipoprotein receptor (LDLR) in vitro. However, the majority of APOE*2 homozygotes typically have normal to below normal plasma levels of cholesterol and triglycerides (TG) and only 5–10% of the homozygotes develop THL. Thus, reductions in lipoprotein clearance due to hormonal, dietary, and genetic changes resulting in reduced LDLR function or capacity are thought to trigger the THL (2). In support of this hypothesis, some of these THL patients have other disorders such as hyperuricemia, glucose intolerance, obesity, and hypothyroidism (3, 4). Nevertheless, the basic mechanism why only 5–10% of APOE*2 homozygotes develop THL while the remaining homozygotes have below normal cholesterol levels remains unexplained.

To approach this fundamental question, we previously generated mice (*Apoe*$^{2/2}$ mice) which solely express the APOE*2 allele under the control of the endogenous mouse *Apoe* promoter by using a gene-targeted replacement strategy (5). Surprisingly, all the *Apoe*$^{2/2}$ mice, regardless of age and gender, exhibit many characteristics of THL, while mice similarly made to express only the APOE*3 allele (*Apoe*$^{3/3}$ mice) have a normal plasma lipid

Manuscript received 1 March 2002 and in revised form 21 May 2002.
DOI 10.1194/jlr.M200103-JLR200

Abbreviations: *Apobec,* apolipoprotein B editing complex-1 gene; *Apoe*$^{2/2}$ mice, mice with apolipoprotein E allele *APOE*2* replaced for mouse *Apoe* gene; FC, free cholesterol; FPLC, fast protein liquid chromatography; LDLR, low density lipoprotein receptor; LpL, lipoprotein lipase; PL, phospholipids; TC, total cholesterol; TG, triglycerides; THL, type-III hyperlipoproteinemia.

[1] To whom correspondence should be sent.
e-mail: myron-hinsdale@omrf.ouhsc.edu

Current addresses: [2]Oklahoma Medical Research Foundation, Cardiovascular Biology Division, Oklahoma City, OK 73104; [3]Duke University, Department of Medicine (Neurology division), Durham, NC 27710; [4]Institute Pasteur de Lille, Laboratoire de Genetique Experimentale, 1, rue du Prof. Calmette, 59019 Lille, France.

profile (6). Dissecting the basis for the complete penetrance of THL phenotype in *Apoe*$^{2/2}$ mice would likely contribute to a better understanding of the mechanism of THL in humans. For example, decreased binding of the apoE-2 in the *Apoe*$^{2/2}$ mice to the LDLR as compared with *Apoe*$^{3/3}$ mice is likely one component of the THL phenotype because a modest increase in expression of the LDLR can eliminate the hyperlipoproteinemia in the *Apoe*$^{2/2}$ mice (7).

At present, it is unknown what influence, if any, the different structural protein components of the remnant lipoprotein particles have in THL. In humans, each chylomicron contains a single apoB-48, and each VLDL contains a single apoB-100. The transcripts for the apoB-48 are made by editing of nascent *APOB* transcripts, which introduces a translational stop codon at 48% of the full length coding sequence, as compared with the unedited transcripts which generate apoB-100 (8, 9). Unlike apoB-100, apoB-48 lacks LDLR binding domains, and consequently apoB-48-containing lipoproteins are totally dependent on apoE for clearance. Mice differ from humans, and produce apoB-48-containing particles from both the liver and intestine, and approximately 20–30% of apoB-containing particles in fasting mouse plasma contain apoB-48 (10, 11). The levels of apoB-48 containing particles produced by the liver, in the fed state, are speculated to be higher since feeding increases *Apob* mRNA editing (11). Since apoB-48 is dependent on apoE for clearance, the higher amount of apoB-48-containing lipoproteins in mice likely contributes to the THL phenotype in the *Apoe*$^{2/2}$ mice.

In this paper, we hypothesized that the hyperlipidemic phenotype of the *Apoe*$^{2/2}$ mice would be improved if all chylomicrons and VLDL in the *Apoe*$^{2/2}$ mice had apoB-100 only and were cleared independently of apoE. Therefore, we made *Apoe*$^{2/2}$ mice that are deficient in apoB-100 mRNA editing, thus producing only apoB-100 containing VLDL and chylomicrons.

EXPERIMENTAL PROCEDURES

Mice

Mice used in this study were generated from crosses of APOE*2 replacement mice with *Apobec-1* knock out mice (*Apobec*$^{-/-}$) kindly provided by Dr. Edward Rubin, Lawrence Berkley National Laboratory (12). Double homozygous *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice and controls (*Apoe*$^{2/2}$) used in experiments were littermates generated from crosses of compound heterozygous mice (*Apoe*$^{2/+}$·*Apobec*$^{+/-}$) with *Apoe*$^{2/2}$ mice followed by intercrossing *Apoe*$^{2/2}$·*Apobec*$^{+/-}$ mice. They had mixed genetic background between C57BL/6 and 129. The mice were maintained on PROLAB ISOPRO RMH 3000 diet. All procedures were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. Mice were genotyped using PCR. Reaction conditions were 94°C 1 min, followed by 45 cycles of 94°C 20 s, 60°C 30 s, 72°C 30 s, with a final 5 min at 72°C.

*APOE*2 locus primers and products.* PGKpolyA (5′GCAGCCTC TGTTCCACATACACT3′) and exon 4 (5′TTGATTCTCCTGG GCCACTG3′) primers produced an ~350 bp fragment diagnostic for the *APOE*2* locus while intron 2 (5′GCAAGAGGTGA TGGTACTCG3′) and intron 3 (5′GTCTCGGCTCTGAACTAC ATAG3′) primers amplified the wild-type locus giving an ~600 bp product. These primers were used in a multiplex reaction.

Apobec$^{-/-}$ *locus primers and products.* PGKpolyA (5′GCAGCCTC TGTTCCACATACACT3′) and intron 6 (5′TTCCCAGTAGCA ACAACCACAGA3′) primers amplified the *Apobec*$^{-/-}$ locus giving an ~260 bp fragment, and the exon 6 (5′TGAGCCGACACCC CTATGTAACTCT3′) and intron 6 primers amplified the wild-type locus giving an ~350 bp fragment.

Plasma lipoprotein analysis

After a 5 h fast, the mice were anesthetized with avertin, and blood was collected from the retroorbital sinus into microcentrifuge tubes containing 0.007 TIU aprotinin (Sigma), 0.19 mg EDTA, and 50 μg gentamicin per 200 μl of blood. Plasma total cholesterol (TC) was determined using a diagnostic kit (Wako Chemicals Inc.) as per kit instructions. For TG measurements, a Sigma diagnostic kit was used as per kit instructions. HDL cholesterol (HDL-C) was determined as previously described (13). ApoE was measured using an ELISA with antibodies specific for human apoE as described (14). For gel filtration analysis with fast protein liquid chromatography (FPLC), 100 μl of pooled plasma from the indicated mice was fractionated using a Superose 6 HR1/30 column (Pharmacia Biotech Inc.). For each 0.5 ml fraction collected, TC, TG, and apoE concentrations were determined.

Agarose gels, SDS-PAGE, and Western blot analyses

Fasted plasma lipoproteins were fractionated by ultracentrifugation using a Beckmann TLA 120.2 rotor at 70,000 rpm for 8 h at 4°C in a Beckman Optima TLX for each fraction. Fractions collected were d = 1.006, 1.02, 1.04, 1.06, 1.08, 1.10, and 1.21 g/ml. Fractions were dialyzed in 50 mM Tris, 1 mM EDTA, and 10 mM NaCl at 4°C, and plasma equivalents were loaded on precast 4–15% acrylamide gels (BioRad) or precast 1% agarose gels (Helena Laboratories). Molecular weight standards (BioRad) were used to determine size of detected proteins. Stained gels were scanned and analyzed with NIH Image software version 1.62. Western blots were probed with a rabbit polyclonal (1:10,000) anti-mouse apoB antibody (a kind gift from Dr. Harshini DeSilva) followed by a conjugated goat anti-rabbit antibody (1:40,000) (Calbiochem). Chemiluminescence (Amersham Pharmacia Biotech) was used for detection of all secondary antibodies. Hybridization buffer was 1% nonfat dry milk in PBS.

Lipoprotein lipase assay

The ability of VLDL to act as a substrate for lipoprotein lipase (LpL) was measured according to van Dijik et al. (15) with some modifications. Plasma VLDL from individual animals was isolated by ultracentrifugation at d < 1.006 g/ml and dialyzed. Samples were diluted with reaction buffer to 100 μl aliquots containing 2, 4, 8, and 16 μg of VLDL-TG. These were incubated at 37°C for 10 min. Reaction buffer was 0.1 M Tris pH 8.5 plus 1.0% fatty acid free BSA. One hundred and fifty milliunits of bovine milk LpL (Sigma-Aldrich, diluted to 15 U/ml with 0.1 Tris, 20% glycerol v/v, pH 8.5) was added and the samples were incubated for another 10 min. The reactions were terminated by adding 50 μl of stop solution (50 mM KH_2PO_4, 0.1% v/v Triton X-100, ph 6.9) and placed on ice. Free fatty acids (FFA) were measured using the non-esterified free fatty acid kit (NEFA C, Wako Chemicals). Duplicate samples, without LpL, were incubated to determine background FFA release. The FFA concentration for each sample was plotted followed by curve fitting to a rectangular hyperbola using Dataraid software (written by Dr. Jolyon Jesty at the State University of New York at Stony Brook). For 8.0 μg of TG substrate, released FFA per min per unit of enzyme was cal-

culated and then analyzed byStudent's *t*-test (JMP Statistical software version 4.0.3, SAS Institute Inc.).

VLDL compositional analysis

The VLDL used in the lipase assay was analyzed for phospholipid (PL), free cholesterol (FC), TC, and TG using commercial kits from Wako Chemicals (PL, FC, and TC) and Sigma-Aldrich (TG) as per kit instructions. Total protein was measured using the Bradford assay (Pierce). Cholesterol ester (CE) mass was estimated by multiplying the difference between FC and TC by a factor of 1.67 (16). Percentages of TG, FC, PL, CE, and protein in the total mass were then calculated. Two-tailed Student's *t*-test and linear correlation were used to analyze statistical significance.

Triglyceride secretion assay

The liver secretion of TG was assayed as described (17). Briefly, a 10% solution of Triton WR-1339 (Tyloxapol, Sigma-Aldrich) in 0.9% saline was injected into the tail vein at a dose of 0.7 mg/g body weight (total volume injected was ~200 μl). Approximately 75 μl of blood was collected at time 0 (i.e., before Tyloxapol injection), 30 min. (i.e., after tyloxapol injection), 60 min, 120 min, and 180 min using non-heparinized capillary tubes and was immediately added to microcentrifuge tubes containing aprotinin (Sigma-Aldrich), EDTA, and gentamicin. Plasma TG concentrations (mg/dl) were then measured and normalized for liver weight. The μmols TG secreted were then estimated for average total body plasma volume (4.5% of body weight) as previously reported in the mouse (18). These TG values (μmols/g liver weight) were then plotted against time for each mouse, and a linear curve fit was performed. The slopes from the curve fit equations were used to calculate the μmols of TG secreted per gram of liver per hour and analyzed by *t*-test.

RESULTS

Plasma lipids

Plasma lipid and lipoprotein profiles in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice were significantly different from those in the *Apoe*$^{2/2}$ mice. Both females and males had significantly increased plasma TG (~60% and 100%, respectively) (**Table 1**). In addition, the females had an ~18% decrease in TC ($P = 0.03$, student's *t*-test) while the males had an ~60% increase in HDL-C concentrations ($P = 0.01$, student's *t*-test) (Table 1). FPLC fractionation of the plasma followed by lipid analysis of each fraction showed the increase in TG of the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice was localized to the VLDL fractions and the decrease in TC in the females was localized to the LDL fractions (**Fig. 1**, left panel). A slight decrease in LDL-C was also seen in males (Fig. 1, right panel).

ApoE concentration and distribution

Plasma apoE-2 concentrations were similar between the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ and *Apoe*$^{2/2}$ mice (Table 1). However, apoE-2 distribution in FPLC fractions was very different in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice having a reduction of ~50–60%, particularly in the IDL/LDL fractions (Fig. 1). This apoE-2 distribution parallels the cholesterol reduction as seen in the same FPLC fractions. These findings suggest the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice have markedly less apoE bound to lipoproteins (~50% and ~30% less in females and males, respectively). In the males, but not in the females, apoE-2 was reduced slightly in the HDL fractions. In both male and female *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice, the amount of apoE-2 associated with the VLDL fraction was similar to that in the *Apoe*$^{2/2}$ mice despite the larger difference in VLDL-TG.

Characteristics of lipoproteins fractionated by ultracentrifugation

To further investigate the apolipoprotein content of plasma lipoproteins in the females, plasma from 10 mice of each genotype was pooled and fractionated by ultracentrifugation. Plasma equivalents of these fractions were separated by SDS polyacrylamide gel electrophoresis (**Fig. 2A, B**). The *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ plasma had less apoE-2 in the 1.02–1.04 fractions (Fig. 2A, right), as compared with the *Apoe*$^{2/2}$ plasma where apoE-2 was more evenly present in fractions 1.006–1.04 (Fig. 2A, left). ApoA-I was primarily associated with fractions 1.08–1.21 g/ml in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice as compared with the *Apoe*$^{2/2}$ mice that had a wider distribution of apoA-I, including the 1.04 and 1.06 fractions. Gels with reduced sample volume show (Fig. 2B) about a 1:2 ratio of apoB-100:B48 in the *Apoe*$^{2/2}$ VLDL while no apoB-48 was detectable in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ VLDL. We estimated, by scanning densitometry of the coomassie stained SDS-PAGE gel, the total amount of apoB (apoB-48 plus apoB-100) in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ plasma VLDL was twice that in the *Apoe*$^{2/2}$ plasma VLDL. This increase in VLDL apoB was also seen in males (data not shown). In addition, the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ plasma had ~18% more apoE in the 1.006 fraction but ~26% less in the 1.02 fraction than the same fractions from *Apoe*$^{2/2}$ plasma. Therefore, in *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ plasma, VLDL are doubled, and the distribution of apoE and apoA-I is restricted to the VLDL fractions and HDL fractions, respectively.

When these ultracentrifugation fractions were separated on agarose gels and stained with fat red 7B, we found increased staining in the 1.006 fraction and less in the 1.02 and 1.04 fractions in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice as compared with the *Apoe*$^{2/2}$ mice (Fig. 2C, top). These findings confirm the FPLC distribution of high VLDL-TG levels in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ plasma. Immunoblots of the

TABLE 1. Plasma lipids and apoE concentration

Gender	Genotype	TC	TG	HDL-C	ApoE
		mg/dl			
Females	*Apoe*$^{2/2}$ (n = 14)	347 ± 71	165 ± 65	49 ± 10	33 ± 8
	Apoe$^{2/2}$·*Apobec*$^{-/-}$ (n = 11)	285 ± 39[a]	263 ± 48[b]	53 ± 14	36 ± 8
Males	*Apoe*$^{2/2}$ (n = 6)	290 ± 40	159 ± 44	48 ± 22	28 ± 9
	Apoe$^{2/2}$·*Apobec*$^{-/-}$ (n = 8)	281 ± 47	325 ± 56[c]	77 ± 13[d]	34 ± 7

Data are means ± SD in mg/dl in plasma from mice fed normal chow diet with 5.0% fat. TC, total cholesterol; TG, triglyceride; HDL-C, HDL cholesterol.

[a] $P = 0.03$, compared to *Apoe*$^{2/2}$ females.
[b] $P < 0.001$, compared to *Apoe*$^{2/2}$ females.
[c] $P < 0.001$, compared to *Apoe*$^{2/2}$ males.
[d] $P = 0.01$, compared to *Apoe*$^{2/2}$ males.

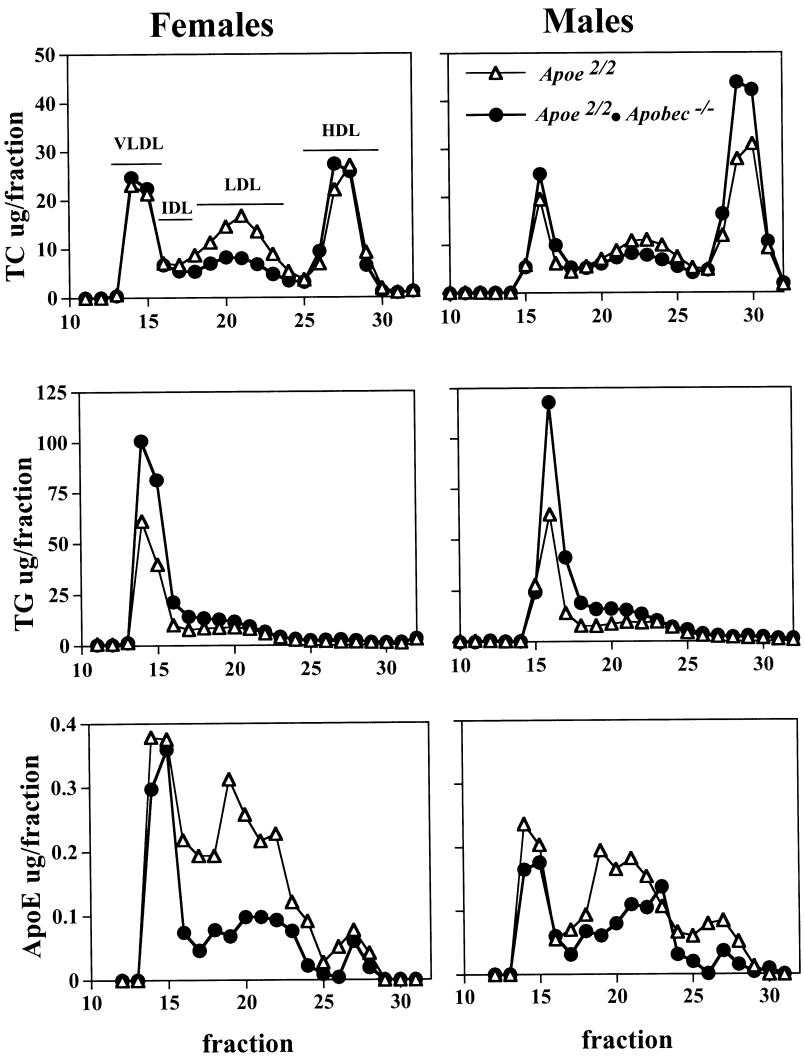

Fig. 1. Fast protein liquid chromatography (FPLC) fractionation of plasma. Total cholesterol (TC), triglyceride (TG), and apoE-2 were measured in each fraction. Fractions containing VLDL, IDL, LDL, and HDL are indicated.

ultracentrifugation fractions using a polyclonal apoB antibody showed an ~60% decrease in apoB containing lipoproteins in the 1.06 and their absence in 1.08 fractions (Fig. 2C, bottom). Again, these findings were consistent with the FPLC analyses that showed low IDL/LDL-C levels in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice. Phospholipid levels measurements of the ultracentrifugation fractions showed an ~25% increased in VLDL-PL and an ~50–70% decreased in LDL-PL (fractions 1.04–1.06) in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ compared with those in the *Apoe*$^{2/2}$ (Fig. 2D). In summary, the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice have reduced IDL/LDL particles and increased VLDL particles as compared with the *Apoe*$^{2/2}$ mice.

Triglyceride secretion

An increase in TG particle secretion could increase steady state levels of plasma TG in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice. To test this possibility, we injected the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ and *Apoe*$^{2/2}$ mice with Triton WR-1339 and measured the accumulation of plasma TG over time. As shown in **Fig. 3**, there was no significant difference in TG secretion rates between the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$and *Apoe*$^{2/2}$ mice. Therefore, the difference in plasma TG is not due to differences in secretion rates of VLDL.

VLDL composition

The proportional masses of CE, FC, PL, TG, and protein in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ VLDL (n = 5) and the *Apoe*$^{2/2}$ VLDL (n = 5) (**Fig. 4**) showed that the *Apoe*$^{2/2}$· *Apobec*$^{-/-}$ VLDL, as compared with *Apoe*$^{2/2}$, have significantly high TG (47.6 ± 1.6% vs. 37.4 ± 2.8%, $P = 0.01$) and low esterified cholesterol (18.0 ± 1.4% vs. 25.3 ± 2.4%, $P = 0.03$). The percent of core lipids (TG plus CE mass) were, however, about equal. On an individual animal basis, CE content and TG content were inversely correlated each other ($r = -0.99$). Protein content was not different (10.6 ± 0.3% vs. 10.3 ± 0.1%, $P = 0.4$). Because VLDL-CE in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice is 30% lower than that in the *Apoe*$^{2/2}$ mice (Fig. 4), the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ VLDL could be up to 30% larger in size, or 30% more in number, or a combination of both. However, since VLDL apoB levels are higher in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice, as judged from PAGE gels (Fig. 2), the actual number of VLDL particles in these mice is likely increased. Thus, these data suggest

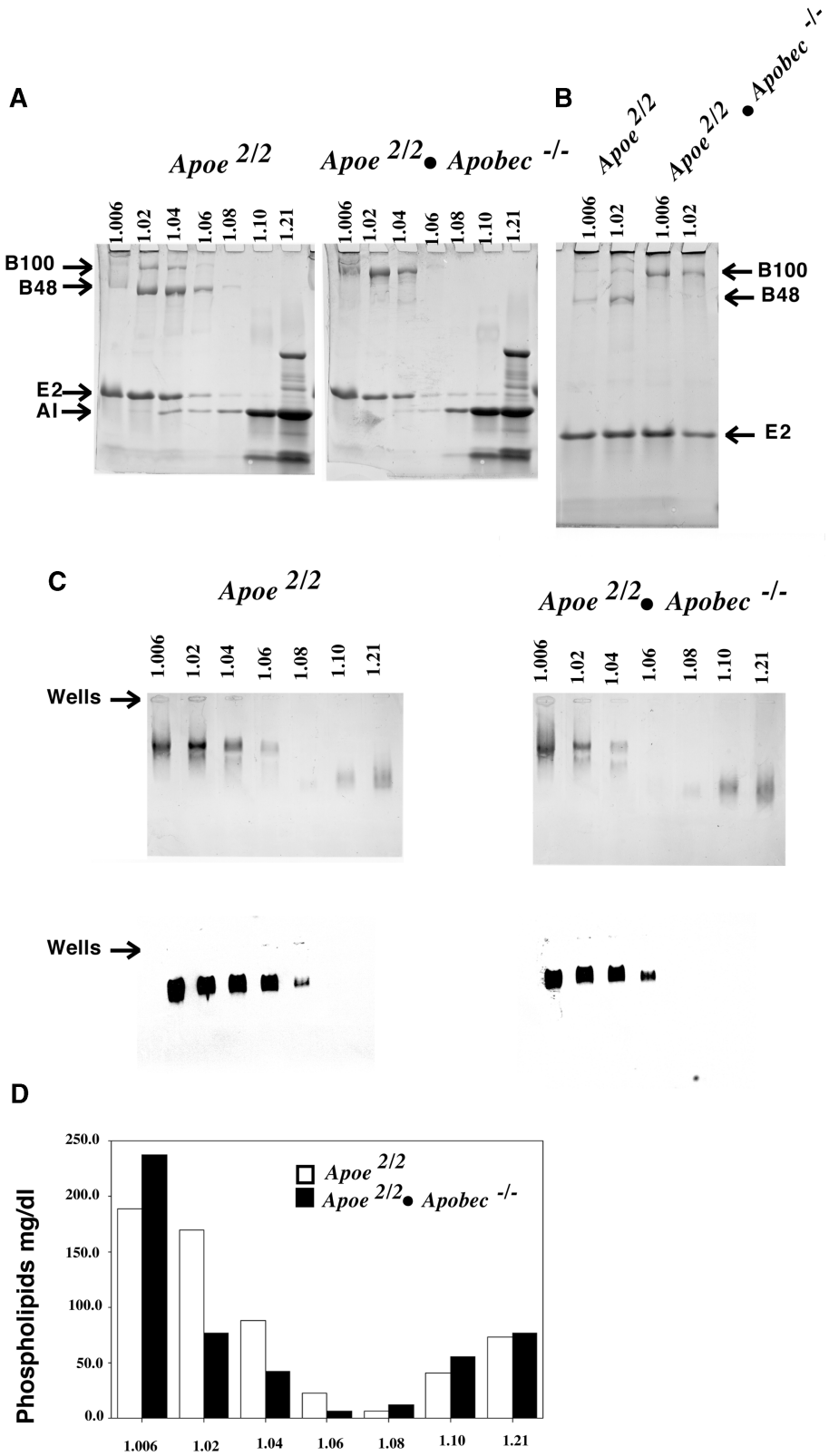

Fig. 2. Analyses of ultracentrifugation fractions of plasma from *Apoe*$^{2/2}$ and *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ female mice. A: SDS polyacrylamide gel (4–15%) analysis using fractions from the *Apoe*$^{2/2}$ mice (left) and fractions from *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice (right). ApoB-100 (B100), apoB-48 (B48), apoE-2 (E2), and apoA-I (A1) are indicated. B: *Apoe*$^{2/2}$and *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ fractions 1.006 and 1.02 loaded with half the amounts of gels in A. C (top): Agarose gel electrophoresis of fractions from *Apoe*$^{2/2}$ (left) and *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ (right) and stained with fat red 7B. (bottom) Western blots of same fractions with an anti-apoB antibody are shown. D: Phospholipid levels of each fraction are shown.

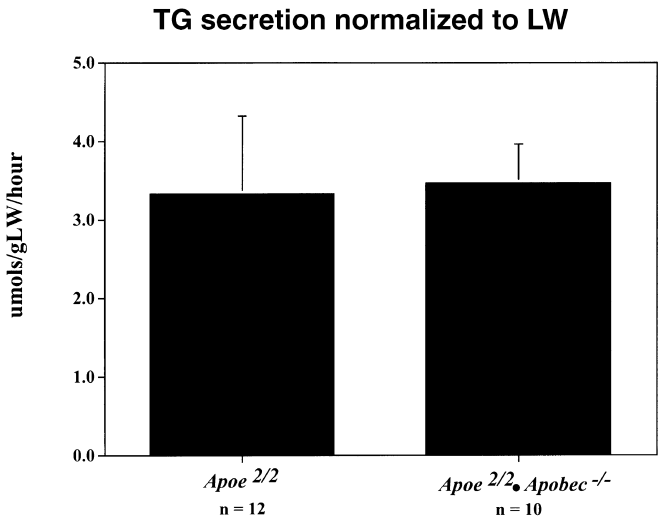

Fig. 3. TG secretion rate in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ and *Apoe*$^{2/2}$ mice. Mice were injected intravenously with Triton WR-1339 at 0.7 mg/g body weight. Plasma TG was measured at time 0 (i.e., before Tyloxapol injection), 30 min (i.e., after tyloxapol injection), 60 min, 120 min, and 180 min and normalized for plasma volume and liver weight. Micromoles of TG secreted per h per g liver weight were calculated using the slope of the linear curve fit lines.

that the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice have twice as much TG-rich VLDL as compared with the *Apoe*$^{2/2}$ mice whose plasma VLDL are more enriched with CE.

LpL assay

We examined whether the increased steady state levels of VLDL-TG in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice was caused by a reduced lipolysis of VLDL-TG by LpL as compared with *Apoe*$^{2/2}$ mice. Free fatty acid released from VLDL-TG by LpL was 2.8 ± 0.5 nmol FFA/min/U in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice (n = 5) as compared with 4.5 ± 0.8 nmol FFA/ml/U in the *Apoe*$^{2/2}$ mice (n = 4, **Fig. 5**). This 62% difference was significant ($P < 0.01$). There is a significant correlation between the VLDL-FFA release (Fig. 5) and the PL percentage of VLDL particle mass (Fig. 4) ($r = 0.69$, $P = 0.04$). This implies that the VLDL with a lower percentage of PL release less FFA under our assay conditions. We also find that the mass percentage of PL per VLDL particle is lowest in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice ($P < 0.0001$). This difference in individual VLDL-PL is probably a result of the increased VLDL-TG and not a cause of the decreased LpL lipolysis. Taken together with the fact that the TG secretion rates in the two groups of mice are the same, a significant part of the increase in steady state levels of plasma VLDL-TG in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice results from a reduction in lipolysis of VLDL-TG.

DISCUSSION

Apoe$^{2/2}$ mice expressing human apoE-2 isoform in place of mouse apoE exhibit exaggerated THL compared with the majority of humans homozygous for APOE*2 who have relatively low plasma cholesterol levels. The predominant apoB containing lipoprotein accumulating in the *Apoe*$^{2/2}$ mice contains apoB-48 (5). In this paper, we examined whether or not this exaggerated THL phenotype is because mice produce apoB-48 containing VLDL particles from the liver. This is in contrast to human liver VLDL that are entirely apoB-100 containing lipoproteins. We hypothesized that if the *Apoe*$^{2/2}$ mice had only apoB-100 containing lipoprotein particles, their hyperlipoproteinemic phenotype would be improved due to increased clearance of the human-like VLDL remnants. Various other studies support this hypothesis. For example, apoB-48-containing particles are the primary circulating apoB-containing lipoprotein in apoE deficient mice (19). Changing the apoB in *Apoe*$^{-/-}$ mice to totally apoB-100 by crossing them to *Apobec*$^{-/-}$ mice or with apoB-100 only mice have shown to decrease their total plasma cholesterol by about 55% (20) or 20% (21), respectively. These decreases were in both VLDL-C and LDL-C, and resulted from increased clearance of lipoprotein particles most likely via the apoB-100 binding to LDLR (20, 21). Thus, it can be argued that the plasma lipid profile in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice should improve. However, the overall THL phenotype did not improve in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice when they produced only apoB-100-containing VLDL and chylomicrons.

The exclusive presence of apoB-100 on lipoprotein particles in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice did alter the metabolism of the lipoprotein particles. Similar to the majority of human *APOE*2* homozygotes without THL, female *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice had a small but significant reduction in IDL/LDL. The reduction in males was however not significant. It should be noted there is a sex predilection for low LDL in female *APOE*2* homozygotes that also have familial hypercholesterolemia (22). In the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice, there are two possible explanations for the reduction of cholesterol in the IDL/LDL range particles. It could directly result from increased clearance of apoB-100 lipoproteins through binding of apoB-100 to the LDLR. Furthermore, LDL clearance may be enhanced by the poor competition of apoE-2-containing remnants with apoB-100 containing LDL for the LDLR (23) or by the up regulation of the LDLR in THL (24). Alternatively, the reduced IDL/LDL-C could be from decreased conversion of VLDL particles to IDL/LDL particles. ApoB-100 associated with VLDL is not in a conformation that binds well to the LDLR. For apoB-100 to become an effective ligand, conversion of VLDL to IDL/LDL mainly through lipolysis is required to expose LDLR binding sites in apoB-100. Although there were no significant increases in either VLDL-C or apoE in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice compared with the *Apoe*$^{2/2}$ mice, there was a 2-fold increase in TG-rich apoB-100 VLDL. This suggests that there is decreased conversion of VLDL to IDL/LDL and/or increased VLDL secretion.

We found that the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ VLDL particles are poorer substrates for LpL than the *Apoe*$^{2/2}$VLDL, showing a reduced rate (62%) of free fatty acid release in vitro. This appears to be a major reason for the TG increase because there is no difference in TG secretion rates between

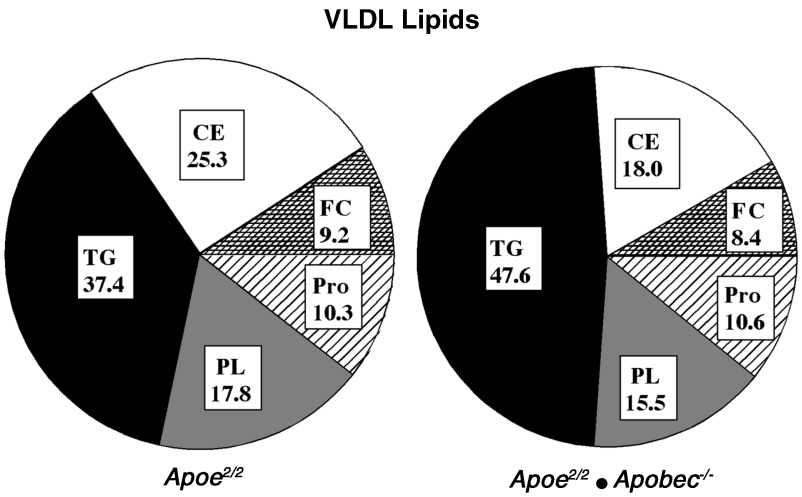

Fig. 4. Compositional analysis of VLDL from *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ and *Apoe*$^{2/2}$ mice. Percentage of cholesterol ester (CE), free cholesterol (FC), protein (Pro), phospholipid (PL), and TG in total lipoprotein particle mass are shown.

the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ and *Apoe*$^{2/2}$ mice. Increases in apoE plasma levels can have a dose dependent inhibition on LpL activity (25–28). However, we found the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice have similar amounts of plasma and VLDL apoE-2 as compared with the *Apoe*$^{2/2}$ mice, which excludes the levels of apoE-2 as a cause for decrease in VLDL lipolysis. We note, however, that a possibility remains that the reduced clearance of VLDL through receptor-mediated pathways and/or HSPG-mediated endocytosis could also be contributing to the increased VLDL and VLDL TG in the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice. To further characterize the catabolism of TG in vivo, the response of *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ and *Apoe*$^{2/2}$ mice to oral fat challenge was performed and revealed no difference between the mice (data not shown). This was a surprise, but may be partly explained by the recent finding that the *Apobec1*$^{-/-}$ mice have reduced fat absorption as compared with wild-type mice (29).

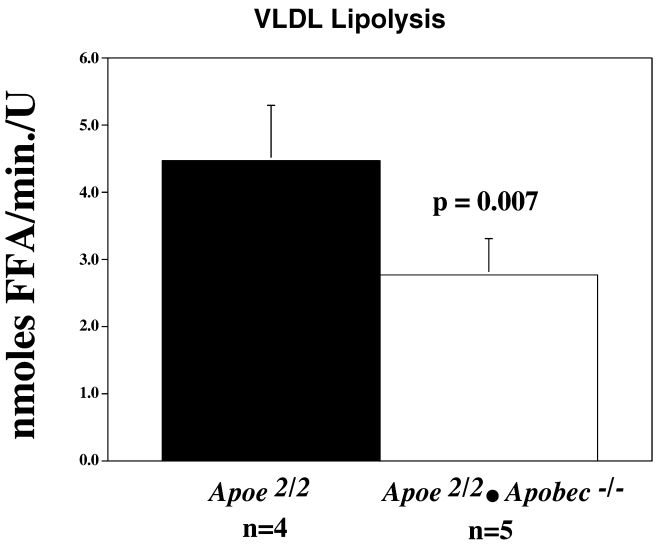

Fig. 5. In vitro lipolysis of VLDL-TG from *Apoe*$^{2/2}$ and *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ female mice. The data was curve fitted and the nmoles of free fatty acids released from 8.0 μg of VLDL TG is shown.

Similar degrees of increase in VLDL-TG have been seen in other animal models where apoB-100 was exclusively present on lipoproteins, although their mechanism has not been explored. These models include *Apoe*$^{-/-}$ *Apobec*$^{-/-}$ mice (20), *LDLR*$^{-/-}$ *Apobec*$^{-/-}$ mice (20), and apoB-100 only mice with or without a functional apoE (21). Thus, our results of apoB-100 containing lipoproteins not being as efficient a substrate for LpL as compared with apoB-48 lipoproteins can also explain the increased VLDL-TG in these other apoB-100 exclusive mice. In fact, absence of apoE and decreased VLDL conversion are likely the reason for the accumulation of the less atherogenic large VLDL in the *Apoe*$^{-/-}$ apoB-100 only mice verses the more atherogenic small LDL that accumulate in the *LDLR*$^{-/-}$ apoB-100 only mice (30, 31). The decreased lipolysis could be due to a decreased efficiency of interaction of apoB-100 VLDL with LpL as compared with apoB-48 VLDL. These findings suggest a possibility that apoB-100-containing particles are more resistant to lipolysis by LpL, possibly due to the presence of the C-terminal half of the apoB-100 protein that is missing in apoB-48. If true, lipoproteins containing truncated apoB could be used to "map" this LpL inhibition segment in apoB-100. For example, an increase in apoB lipoprotein clearance was first seen in humans with truncated apoB protein (apoB-75 and apoB-89) containing lipoproteins (32–35). Furthermore, mice producing apoB-70 (36) or apoB-81 (37) in place of apoB-100 have below normal TG levels. In mice with truncated apoB-81 and apoB-83, a decrease in production of apoB lipoproteins and an increase in truncated apoB lipoprotein clearance have been reported (38, 39). This increased clearance of the truncated apoB lipoproteins is due to a loss of the C-terminus of apoB, which possibly inhibits VLDL binding to the LDLR (40). VLDL lipolysis in these apoB-truncated mice has not been measured. In vitro assays have shown that apoE inhibits LpL and some of this inhibition is associated with the LDLR binding region of apoE (41). This region is highly homologous to the LDLR binding site B of apoB-100 (40). Therefore, site B and other C-terminal areas may be re-

sponsible for the inhibitory activity of apoB-100 on LpL hydrolysis of VLDL.

In summary, we have shown that the presence of an additional LDLR binding site on apoB containing lipoproteins does not improve the type-III hyperlipoproteinemia seen in *Apoe*$^{2/2}$ mice. The *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ mice had slightly improved LDL-C levels but much worsened plasma VLDL-TG levels. These changes result from the *Apoe*$^{2/2}$·*Apobec*$^{-/-}$ apoB-100 VLDL being a less suitable substrate for LpL as compared with the predominately apoB-48 VLDL in *Apoe*$^{2/2}$ mice. We speculate that the C-terminus of apoB-100 has an additional and important role in controlling the interaction of apoB lipoproteins with LpL just as it does in apoB-100 interactions with the LDLR (40, 42). This difference in LpL catabolism may explain the common observation that chylomicrons (apoB-48 lipoproteins) can out-compete VLDL (apoB-100 lipoproteins) for conversion to remnant lipoproteins (43). Why the majority of human *APOE*2* homozygotes with defective apoE-2 have better than normal plasma lipid and lipoprotein profiles remains to be explained.

The authors thank Dr. John Parks (Wake Forest University School of Medicine, Deptartment of Pathology, Winston-Salem, NC) for helpful comments on this manuscript, and Ben Roberts for excellent technical assistance. We also thank Dr. Pierre Neuenschwander (University of Texas Health Science Center at Tyler, Biomedical Research) for help with the curve fitting of the VLDL lipolysis assay data. This research was supported by Grants RR00111 (M.E.H.), and HL42630 (N.M.) from the National Institutes of Health.

REFERENCES

1. Utermann, G., M. Hees, and A. Steinmetz. 1977. Polymorphism of apolipoprotein E and occurrence of dysbetalipoproteinaemia in man. *Nature.* **269:** 604–607.
2. Mahley, R. W., and S. C. Rall, Jr. 1995. Type III hyperlipoproteinemia (Dysbetalipoproteinemia): The Role of Apolipoprotein E in Normal and Abnormal Metabolism. *In* The Molecular and Metabolic Bases of Inherited Disease. 7th edition. Volume 2. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle. McGraw-Hill, Inc., New York, New York. 1953–1980.
3. Mahley, R. W., Y. Huang, and S. C. Rall, Jr. 1999. Pathogenesis of type III hyperlipoproteinemia (dysbetalipoproteinemia). Questions, quandaries, and paradoxes. *J. Lipid Res.* **40:** 1933–1949.
4. Davignon, J., J. S. Cohn, L. Mabile, and L. Bernier. 1999. Apolipoprotein E and atherosclerosis: insight from animal and human studies. *Clin. Chim. Acta.* **286:** 115–143.
5. Sullivan, P. M., H. Mezdour, S. H. Quarfordt, and N. Maeda. 1998. Type III hyperlipoproteinemia and spontaneous atherosclerosis in mice resulting from gene replacement of mouse Apoe with human Apoe*2. *J. Clin. Invest.* **102:** 130–135.
6. Knouff, C., M. E. Hinsdale, H. Mezdour, M. K. Altenburg, M. Watanabe, S. H. Quarfordt, P. M. Sullivan, and N. Maeda. 1999. Apo E structure determines VLDL clearance and atherosclerosis risk in mice. *J. Clin. Invest.* **103:** 1579–1586.
7. Knouff, C., S. Malloy, J. Wilder, M.K. Altenburg, and N. Maeda. 2000. Doubling expression of the LDL receptor by truncation of the 3′ UTR sequence ameliorates type III hyperlipoproteinemia in mice expressing the Human ApoE2 isoform. *J. Biol. Chem.* 276: 3856–3862
8. Powell, L. M., S. C. Wallis, R. J. Pease, Y. H. Edwards, T. J. Knott, and J. Scott. 1987. A novel form of tissue-specific RNA processing produces apolipoprotein- B48 in intestine. *Cell.* **50:** 831–840.
9. Chen, S. H., G. Habib, C. Y. Yang, Z. W. Gu, B. R. Lee, S. A. Weng, S. R. Silberman, S. J. Cai, J. P. Deslypere, M. Rosseneu, A. M. Gotto Jr., W. H. Li, and L. Chan. 1987. Apolipoprotein B-48 is the product of a messenger RNA with an organ-specific in-frame stop codon. *Science.* **238:** 363–366.
10. Oka, K., K. Kobayashi, M. Sullivan, J. Martinez, B. B. Teng, K. Ishimura-Oka, and L. Chan. 1997. Tissue-specific inhibition of apolipoprotein B mRNA editing in the liver by adenovirus-mediated transfer of a dominant negative mutant APOBEC-1 leads to increased low density lipoprotein in mice. *J. Biol. Chem.* **272:** 1456–1460.
11. Higuchi, K., K. Kitagawa, K. Kogishi, and T. Takeda. 1992. Developmental and age-related changes in apolipoprotein B mRNA editing in mice. *J. Lipid Res.* **33:** 1753–1764.
12. Morrison, J. R., C. Paszty, M. E. Stevens, S. D. Hughes, T. Forte, J. Scott, and E. M. Rubin. 1996. Apolipoprotein B RNA editing enzyme-deficient mice are viable despite alterations in lipoprotein metabolism. *Proc. Natl. Acad. Sci. USA.* **93:** 7154–7159.
13. Warnick, G. R., J. Benderson, and J. J. Albers. 1982. Dextran sulfate-Mg2+ precipitation procedure for quantitation of high- density-lipoprotein cholesterol. *Clin. Chem.* **28:** 1379–1388.
14. Sullivan, P. M., H. Mezdour, Y. Aratani, C. Knouff, J. Najib, R. L. Reddick, S. H. Quarfordt, and N. Maeda. 1997. Targeted replacement of the mouse apolipoprotein E gene with the common human APOE3 allele enhances diet-induced hypercholesterolemia and atherosclerosis. *J. Biol. Chem.* **272:** 17972–17980.
15. van Dijk, K. W., B. J. van Vlijmen, H. B. van't Hof, A. van der Zee, S. Santamarina-Fojo, T. J. van Berkel, L. M. Havekes, and M. H. Hofker. 1999. In LDL receptor-deficient mice, catabolism of remnant lipoproteins requires a high level of apoE but is inhibited by excess apoE. *J. Lipid Res.* **40:** 336–344.
16. Maugeais, C., U. J. Tietge, K. Tsukamoto, J. M. Glick, and D. J. Rader. 2000. Hepatic apolipoprotein E expression promotes very low density lipoprotein-apolipoprotein B production in vivo in mice. *J. Lipid Res.* **41:** 1673–1679.
17. Li, X., F. Catalina, S. M. Grundy, and S. Patel. 1996. Method to measure apolipoprotein B-48 and B-100 secretion rates in an individual mouse: evidence for a very rapid turnover of VLDL and preferential removal of B-48- relative to B-100-containing lipoproteins. *J. Lipid Res.* **37:** 210–220.
18. Wish L., J. Furth, and R. H. Storey. 1950. Direct determination of plasma, cell, and organ-blood volumes in normal and hypervolemic mice. *Proc. Soc. Exp. Biol. Med.* **74:** 644–648.
19. Zhang, S. H., R. L. Reddick, J. A. Piedrahita, and N. Maeda. 1992. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science.* **258:** 468–471.
20. Nakamuta, M., S. Taniguchi, B. Y. Ishida, K. Kobayashi, and L. Chan. 1998. Phenotype interaction of apobec-1 and CETP, LDLR, and apoE gene expression in mice: role of apoB mRNA editing in lipoprotein phenotype expression. *Arterioscler. Thromb. Vasc. Biol.* **18:** 747–755.
21. Farese, R. V., Jr., M. M. Veniant, C. M. Cham, L. M. Flynn, V. Pierotti, J. F. Loring, M. Traber, S. Ruland, R. S. Stokowski, D. Huszar, and S. G. Young. 1996. Phenotypic analysis of mice expressing exclusively apolipoprotein B48 or apolipoprotein B100. *Proc. Natl. Acad. Sci. USA.* **93:** 6393–6398.
22. Ferrieres, J., C. F. Sing, M. Roy, J. Davignon, and S. Lussier-Cacan. 1994. Apolipoprotein E polymorphism and heterozygous familial hypercholesterolemia. Sex-specific effects. *Arterioscler. Thromb.* **14:** 1553–1560.
23. Woollett, L. A., Y. Osono, J. Herz, and J. M. Dietschy. 1995. Apolipoprotein E competitively inhibits receptor-dependent low density lipoprotein uptake by the liver but has no effect on cholesterol absorption or synthesis in the mouse. *Proc. Natl. Acad. Sci. USA.* **92:** 12500–12504.
24. Davignon, J., R. E. Gregg, and C. F. Sing. 1988. Apolipoprotein E polymorphism and atherosclerosis. *Arteriosclerosis.* **8:** 1–21.
25. Jong, M. C., V. E. Dahlmans, M. H. Hofker, and L. M. Havekes. 1997. Nascent very-low-density lipoprotein triacylglycerol hydrolysis by lipoprotein lipase is inhibited by apolipoprotein E in a dose-dependent manner. *Biochem. J.* **328:** 745–750.
26. Huang, Y., X. Q. Liu, S. C. Rall, Jr., and R. W. Mahley. 1998. Apolipoprotein E2 reduces the low density lipoprotein level in transgenic mice by impairing lipoprotein lipase-mediated lipolysis of triglyceride-rich lipoproteins. *J. Biol. Chem.* **273:** 17483–17490.
27. Huang, Y., X. Q. Liu, S. C. Rall, Jr., J. M. Taylor, A. von Eckardstein, G. Assmann, and R. W. Mahley. 1998. Overexpression and accumulation of apolipoprotein E as a cause of hypertriglyceridemia. *J. Biol. Chem.* **273:** 26388–26393.

28. Huang, Y., Z. S. Ji, W. J. Brecht, S. C. Rall, Jr., J. M. Taylor, and R. W. Mahley. 1999. Overexpression of apolipoprotein E3 in transgenic rabbits causes combined hyperlipidemia by stimulating hepatic VLDL production and impairing VLDL lipolysis. *Arterioscler. Thromb. Vasc. Biol.* **19:** 2952–2959.
29. Kendrick, J. S., L. Chan, and J. A. Higgins. 2001. Superior role of apolipoprotein B48 over apolipoprotein B100 in chylomicron assembly and fat absorption: an investigation of apobec-1 knock-out and wild-type mice. *Biochem. J.* **356:** 821–827.
30. Veniant, M. M., S. Withycombe, and S. G. Young. 2001. Lipoprotein size and atherosclerosis susceptibility in Apoe(−/−) and Ldlr(−/−) mice. *Arterioscler. Thromb. Vasc. Biol.* **21:** 1567–1570.
31. Veniant, M. M., M. A. Sullivan, S. K. Kim, P. Ambroziak, A. Chu, M. D. Wilson, M. K. Hellerstein, L. L. Rudel, R. L. Walzem, and S. G. Young. 2000. Defining the atherogenicity of large and small lipoproteins containing apolipoprotein B100. *J. Clin. Invest.* **106:** 1501–1510.
32. Krul, E. S., M. Kinoshita, P. Talmud, S. E. Humphries, S. Turner, A. C. Goldberg, K. Cook, E. Boerwinkle, and G. Schonfeld. 1989. Two distinct truncated apolipoprotein B species in a kindred with hypobetalipoproteinemia. *Arteriosclerosis.* **9:** 856–868.
33. Parhofer, K. G., P. H. Barrett, D. M. Bier, and G. Schonfeld. 1992. Lipoproteins containing the truncated apolipoprotein, Apo B-89, are cleared from human plasma more rapidly than Apo B-100-containing lipoproteins in vivo. *J. Clin. Invest.* **89:** 1931–1937.
34. Parhofer, K. G., A. Daugherty, M. Kinoshita, and G. Schonfeld. 1990. Enhanced clearance from plasma of low density lipoproteins containing a truncated apolipoprotein, apoB-89. *J. Lipid Res.* **31:** 2001–2007.
35. Krul, E. S., K. G. Parhofer, P. H. Barrett, R. D. Wagner, and G. Schonfeld. 1992. ApoB-75, a truncation of apolipoprotein B associated with familial hypobetalipoproteinemia: genetic and kinetic studies. *J. Lipid Res.* **33:** 1037–1050.
36. Homanics, G. E., T. J. Smith, S. H. Zhang, D. Lee, S. G. Young, and N. Maeda. 1993. Targeted modification of the apolipoprotein B gene results in hypobetalipoproteinemia and developmental abnormalities in mice. *Proc. Natl. Acad. Sci. USA.* **90:** 2389–2393.
37. Toth, L. R., T. J. Smith, C. Jones, H. V. de Silva, O. Smithies, and N. Maeda. 1996. Two distinct apolipoprotein B alleles in mice generated by a single 'in- out' targeting. *Gene.* **178:** 161–168.
38. Kim, E., C. M. Cham, M. M. Veniant, P. Ambroziak, and S. G. Young. 1998. Dual mechanisms for the low plasma levels of truncated apolipoprotein B proteins in familial hypobetalipoproteinemia. Analysis of a new mouse model with a nonsense mutation in the Apob gene. *J. Clin. Invest.* **101:** 1468–1477.
39. Srivastava, R. A., L. Toth, N. Srivastava, M. E. Hinsdale, N. Maeda, A. B. Cefalu, M. Averna, and G. Schonfeld. 1999. Regulation of the apolipoprotein B in heterozygous hypobetalipoproteinemic knock-out mice expressing truncated apoB, B81. Low production and enhanced clearance of apoB cause low levels of apoB. *Mol. Cell. Biochem.* **202:** 37–46.
40. Boren, J., I. Lee, W. Zhu, K. Arnold, S. Taylor, and T. L. Innerarity. 1998. Identification of the low density lipoprotein receptor-binding site in apolipoprotein B100 and the modulation of its binding activity by the carboxyl terminus in familial defective apo-B100. *J. Clin. Invest.* **101:** 1084–1093.
41. McConathy, W. J., and C. S. Wang. 1989. Inhibition of lipoprotein lipase by the receptor-binding domain of apolipoprotein E. *FEBS Lett.* **251:** 250–252.
42. Boren, J., U. Ekstrom, B. Agren, P. Nilsson-Ehle, and T. L. Innerarity. 2001. The molecular mechanism for the genetic disorder familial defective apolipoprotein B100. *J. Biol. Chem.* **276:** 9214–9218.
43. Chappell, D. A., and J. D. Medh. 1998. Receptor-mediated mechanisms of lipoprotein remnant catabolism. *Prog. Lipid Res.* **37:** 393–422.