# Hepatic secretion of small lipoprotein particles in *apobec-1*$^{-/-}$ mice is regulated by the LDL receptor

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**Abstract** **Recent studies have examined the role of the LDL receptor (LDLR) in regulating murine hepatic lipoprotein production and apolipoprotein B (apoB) secretion, with divergent conclusions from in vivo versus in vitro approaches. We have re-examined this question, both in vivo and in vitro, using *apobec-1*$^{-/-}$ mice to model the pattern of human hepatic apoB-100 secretion. Hepatic triglyceride production in vivo (using Triton WR-1339) was unchanged in wild-type (WT) C57BL/6, *apobec-1*$^{-/-}$, *ldlr*$^{-/-}$, and *[apobec-1*$^{-/-}$, *ldlr*$^{-/-}$*]* mice, while apoB-100 production (using [$^{35}$S]methionine incorporation) was increased >2-fold in *[apobec-1*$^{-/-}$, *ldlr*$^{-/-}$*]* mice. Although >90% of newly synthesized apoB floated within the d < 1.006 fraction of serum from all genotypes, fast-performance liquid chromatography separation revealed that nascent triglyceride-rich particles from *[apobec-1*$^{-/-}$, *ldlr*$^{-/-}$*]* mice, but not WT, *apobec-1*$^{-/-}$, or *ldlr*$^{-/-}$ mice, distributed into smaller (intermediate and LDL-sized) particles. Studies in isolated hepatocytes from these different genotypes confirmed secretion of smaller particles exclusively from *[apobec-1*$^{-/-}$, *ldlr*$^{-/-}$*]* mice, and pulse-chase analysis demonstrated increased secretion of apoB-100 with virtual elimination of posttranslational degradation. These results directly support the suggestion that the LDLR regulates hepatic apoB-100 production and modulates secretion of small, triglyceride-rich particles, both in vivo and in vitro.**—Nassir, F., Y. Xie, B. W. Patterson, J. Luo, and N. O. Davidson. **Hepatic secretion of small lipoprotein particles in *apobec-1*$^{-/-}$ mice is regulated by the LDL receptor.** ***J. Lipid Res.*** 45: **1649–1659.**

**Supplementary key words** VLDL secretion • apolipoprotein B-100 • lipoprotein biogenesis • atherosclerosis

Apolipoprotein B-100 (apoB-100) is an essential surface component of hepatic VLDL assembly and serves as a ligand for LDL receptor (LDLR)-mediated lipoprotein clearance (1–4). Elevated concentrations of apoB-100-containing lipoproteins are a key risk factor for atherosclerosis, an observation that has focused considerable attention on the mechanisms involved in the coordinated regulation of apoB-100 production and lipoprotein biogenesis, particularly in regard to hepatic lipoprotein secretion.

Studies using primary hepatocytes have demonstrated that hepatic apoB production is regulated principally through degradation of the newly synthesized protein, a process that occurs at both the co- and posttranslational levels and that involves proteasomal as well as nonproteasomal pathways (5). During initiation of hepatic VLDL assembly, newly synthesized apoB-100 is initially complexed with small amounts of neutral lipid that are transferred through the actions of the microsomal triglyceride transfer protein (MTTP) (6–8). This so-called "first step" in VLDL assembly involves a physical interaction between MTTP and the elongating apoB peptide, as well as the facilitated transfer of lipid, which, in turn, permits optimal folding of nascent apoB-100 (9). In situations of limiting lipid availability or of MTTP deficiency, this initial lipidation step of apoB-100 fails, favoring apoB-100 degradation by the proteasomal pathway (10, 11). When the lipid availability is not limiting, the products of the first step in VLDL assembly acquire additional neutral lipid and following terminal modifications, eventually progress to form mature VLDL particles (12).

In addition to the classical lipidation-dependent, proteasomal pathway of apoB degradation, several studies have demonstrated a role for the LDLR in regulating hepatic apoB production (13–21). These studies have demonstrated that the LDLR regulates hepatic lipoprotein production, a function distinct from the well-recognized consequences of LDLR deficiency that lead to defective clearance of plasma LDL in familial hypercholesterolemia

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(FH) (22). More specifically, earlier studies from Williams, Brocia, and Fisher (21) suggested a modulatory role for the LDLR in the reuptake and net secretion of newly secreted apoB-containing lipoproteins from HepG2 cells. More recently, these findings were extended by Attie and colleagues (14, 20), who demonstrated both increased production and reduced degradation of apoB from hepatocytes prepared from *ldlr*$^{-/-}$ mice and proposed that the LDLR binds to apoB within the secretory pathway, during early lipidation. Although some investigators have confirmed these findings using isolated hepatocytes (15–17), others have demonstrated no change in VLDL apoB production in vivo, using apoB-100-only mice in an *ldlr*$^{-/-}$ background (23). This uncertainty prompted a reexamination of the underlying hypothesis.

In the current study, we have examined apoB and VLDL production both in vivo and in vitro in mice lacking either the *ldlr* and/or *apobec-1* gene(s). *Apobec-1* deletion eliminates hepatic apoB-48 production, resulting in mice that secrete only apoB-100-containing lipoproteins. Accordingly, *apobec-1*-null mice represent a representative model for human apoB secretion (24). Our findings confirm the role of the LDLR in regulating apoB secretion and further demonstrate direct secretion of small, apoB-100-containing lipoprotein particles. These data suggest a novel mechanism by which the LDLR regulates production of atherogenic lipoprotein particles.

## MATERIALS AND METHODS

### Animals

Male C57BL/6 wild-type (WT) and *ldlr*$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). *Apobec-1*$^{-/-}$ mice were previously generated (24) in a mixed 129/sv background and backcrossed more than twelve generations onto a C57BL/6 background. Mice deficient in both the *ldlr* and *apobec-1* genes *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]* (25) were also backcrossed more than twelve generations onto a C57BL/6 background. Male mice aged 12–18 weeks were used for all the studies and were maintained on a 12 h light–dark cycle in a full-barrier facility and fed ad libitum a chow diet (Picolab Rodent Diet 20; fat content, 4.5% w/w, comprising 11.9% calories).

### In vivo studies of hepatic lipid secretion

Hepatic triglyceride secretion rates were determined in vivo as previously described (19). Mice were fasted for 4 h and then injected via tail vein with 500 mg/kg Triton WR-1339 (Tyloxapol, Sigma Chemical Co.). Blood samples were drawn before injection and every 30 min for 2 h for triglyceride determination. The distribution of lipids was also determined by fast-performance liquid chromatography (FPLC) on two Superose® 6 columns connected in series (Pharmacia Biotech, Inc.). Equal volumes of pooled serum (three animals/group) at 0 h or 2 h after Triton injection were fractionated, and 800 μl fractions were collected. Triglyceride, phospholipids, and cholesterol values were determined enzymatically (Wako Chemicals USA, Inc.). Lipid composition of both nascent (2 h after Triton) and circulating particles (0 h post Triton) was also determined as detailed (19). The composition of nascent particles was calculated by subtracting the values for circulating lipoproteins as described (19).

### In vivo studies of hepatic apoB secretion

Mice were fasted for 4 h and injected intraperitoneally with 500 μCi of [$^{35}$S]protein-labeling mix (NEN Life Science Products, Inc.); 30 min later, the mice were injected intravenously with Triton WR-1339 500 mg/kg (body weight). Blood samples were drawn before injection (0 h) and 2 h after Triton injection. Serum was separated by centrifugation at 4°C, and lipoprotein fractions ($d < 1.063$ g/ml) were isolated by ultracentrifugation at 100,000 rpm for 4 h at 10°C using a Beckman MLA-130 rotor in a table-top ultracentrifuge (Beckman Instruments). Aliquots of $d < 1.063$ lipoproteins (representing equal volumes of starting plasma) or aliquots of total serum were separated through 5–15% SDS-PAGE to determine apoB or albumin secretion, respectively. Albumin secretion was independently quantitated by immunoprecipitation from aliquots of whole serum. ApoB secretion and albumin secretion were quantitated by phosphorImager using the ImageQuant software (SI, Molecular Dynamics, Sunnyvale, CA). ApoB secretion was calculated by subtracting the 0 h (before Triton injection) values from the 2 h (post Triton injection) values and normalized to albumin secretion. In other experiments, newly synthesized apoB distribution was determined in both the $d < 1.006$ and d 1.006–1.063 fractions, 2 h after Triton injection.

### In vitro studies of hepatic apoB production

Primary mouse hepatocytes were isolated by liver perfusion as described in (20), with minor modifications. Hepatocytes were collected by centrifugation, washed three times in hepatocyte wash medium, and cultured in collagen-coated T75 flasks (Falcon) in attachment medium (hepatocyte wash medium with 5% FBS, 100 mg/ml streptomycin, and 100 U/ml penicillin) overnight. Cells were preincubated for 1 h in methionine- and cysteine-free DMEM supplemented with 200 μM oleic acid (Sigma) (without serum), pulse labeled for 10 min in the same medium containing 250 μCi/ml [$^{35}$S]protein-labeling mix, and chased in complete medium containing 3 mM cysteine and 10 mM methionine as indicated in the figure legends. Where indicated, 10 mg/ml heparin (Sigma) was present in the medium during the pulse-chase study. Following the pulse-chase, media were collected and a cocktail of protease inhibitors (Roche Diagnostics, Indianapolis, IN) was added. The cells were washed three times with ice-cold PBS and subsequently lysed in cold lysis buffer (M-Per, Pierce Chemicals, Chicago, IL, containing 0.1% SDS) along with protease inhibitors. Cell lysates and media were clarified by centrifugation at 10,000 rpm at 4°C for 5 min to remove cellular debris, and immunoprecipitation was conducted as described previously (26, 27).

### Modeling apoB secretion in vitro

Multicompartmental modeling was performed using the SAAM II program (SAAM Institute, University of Washington, Seattle, WA) with minor modifications to the model of Attie and colleagues (20) (Fig. 6A). The [$^{35}$S]methionine tracer was applied to compartment 1 for a 10 min period. Compartments 2 through 6 represent proteins in various stages of synthesis. Full-length mature protein (compartment 7) may be degraded by a "slow" process (compartment 10), or passed to a delay, (compartment 8, e.g., transit through the secretory pathway). Protein emerging from this delay (compartment 9) can either be secreted into the medium (compartment 11) or degraded without being secreted.

The experimental data (arbitrary units) were normalized to cell protein. The intracellular protein data were assigned to the sum of the amounts in compartments 7–10, whereas the values for protein secreted into the medium were assigned to compartment 11. The rate constants of the model k(i,j) (the fraction of compartment i converted to compartment j per minute) were

optimized to provide a minimum sum of squares difference between the model-projected solution and the observed data.

The parameter of principal interest determined from this model was the fraction of full-length protein (compartment 7) that was secreted as calculated by the expression:

$$\text{fraction secreted} = \frac{k(8,7)}{k(8,7)+k(10,7)} \times \frac{k(11,9)}{k(11,9)+k(0,9)} \quad \textit{(Eq. 1)}$$

The first term of this expression defines the fraction of compartment 7 passed to compartments 8 and 9, and the second term defines the fraction of compartment 9 that was secreted.

### In vitro examination of hepatic lipoprotein size distribution

Lipoprotein particle size distribution was determined on the $d < 1.006$ g/ml fraction prepared from media of hepatocytes incubated in serum-free media for 4 h. The $d < 1.006$ fraction was isolated by ultracentrifugation at 100,000 rpm for 4 h at 10°C using a Beckman MLA-130 rotor in a table-top ultracentrifuge (Beckman Instruments). The $d < 1.006$ fractions were concentrated using a 10K NMWL centrifugal filter and subjected to negative staining and electron microscopy. Negatively stained particles were viewed in a Zeiss 902 electron microscope.

### Statistical analysis

The data are shown as mean ± SEM. Differences between groups were evaluated using Student's *t*-test or ANOVA (Bartlett's test for homogeneity of variance and Bonferroni's multiple comparisons test). Differences were considered significant when $P < 0.05$.

## RESULTS

### Hepatic triglyceride and apoB secretion in vivo

Fasting serum triglyceride concentrations were similar in WT, *apobec-1*$^{-/-}$, and *ldlr*$^{-/-}$ mice (45 ± 4, 44 ± 2, and 55 ± 9 mg/dl, n = 10 per group) but ~2-fold higher in *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]* mice (99 ± 8 mg/dl, $P < 0.0001$). Serum cholesterol levels were elevated in both *ldlr*$^{-/-}$ and *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]* mice (300 ± 20 mg/dl and 488 ± 13 mg/dl), respectively, findings similar to previously published results (23, 25). Hepatic triglyceride secretion rates were determined following Triton injection, revealing comparable values among all genotypes (**Fig. 1A**). By contrast, apoB-100 secretion was 1.7-fold higher in *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]* compared with *apobec-1*$^{-/-}$ mice (Fig. 1B, C). ApoB-100 secretion was also higher (~1.5-fold) in *ldlr*$^{-/-}$ compared with WT mice (Fig. 1B, C), findings consistent with the previously postulated role of the LDLR in regulating apoB secretion (20). In addition, apoB-48 secretion was increased in *ldlr*$^{-/-}$ compared with WT mice, as previously noted by Attie and colleagues (20) in isolated hepatocytes. Albumin secretion, an internal control for hepatic protein production, was comparable in all genotypes (Fig. 1B). Taken together, these results demonstrate comparable in vivo hepatic triglyceride secretion rates regardless of LDLR status, coupled with increased in vivo production of apoB in the *ldlr*$^{-/-}$ background.

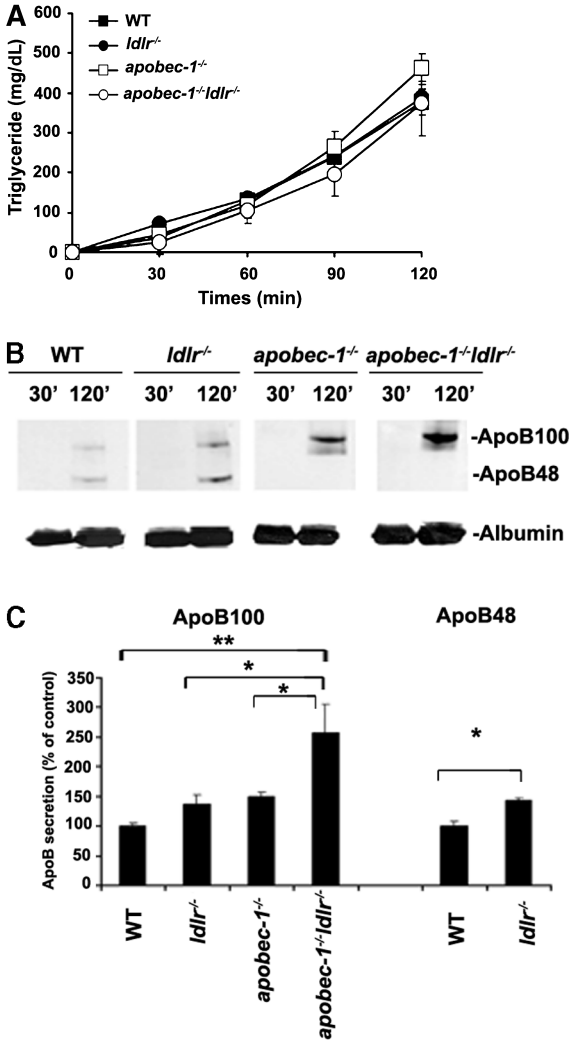
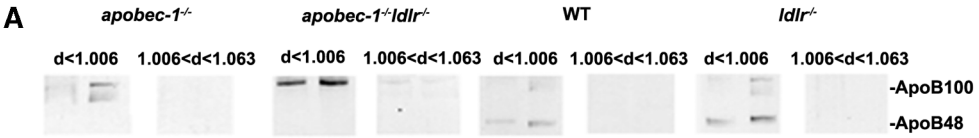



**Fig. 1.** Hepatic triglyceride secretion rates in vivo. A: Mice were fasted for 4 h and injected intravenously with Triton WR-1339, and triglyceride accumulation was measured every 30 min for 2 h. Results are mean ± SEM (n = 15 per group). B, C: Hepatic apolipoprotein B (apoB) secretion determined in vivo. Mice were fasted for 4 h and injected intraperitoneally with 500 μCi of [$^{35}$S]protein-labeling mix and 30 min later injected with Triton WR-1339. Equal aliquots from serum or from lipoprotein fractions ($d < 1.063$ g/ml) isolated 30 min or 2 h after Triton injection were subjected to SDS-PAGE analysis to determine albumin and apoB secretion, respectively. B: Representative gels for apoB and albumin secretion. C: ApoB secretion was quantitated, normalized to albumin secretion, and expressed as percentage of apoB secretion in wild type (WT). Results are mean ± SEM (n = 8 per group). *$P < 0.05$; **$P < 0.005$.

### Distribution of apoB and lipids in nascent lipoprotein particles in vivo

In view of the known stoichiometry of apoB and core lipid, i.e., one apoB molecule per lipoprotein particle, the findings above led us to ask whether LDLR deficiency alters the profile of newly secreted hepatic lipoprotein particles in order to accommodate the increased secretion of apoB. As examined using ultracentrifugation, the distribution of newly synthesized apoB revealed that >90% ap-




**B**

**Circulating d<1.006 particles**

| | Triglyceride (%) | Cholesterol (%) | Phospholipid (%) |
|---|---|---|---|
| *apobec-1*$^{-/-}$ | 62.1 ± 8.0 | 12.0 ± 2.0 | 26.2 ± 5.6 |
| *apobec-1*$^{-/-}$*ldlr*$^{-/-}$ | 59.6 ± 5.5 | 17.9 ±2.4 | 22.5 ± 2.4 |
| WT | 58.5 ± 8.1 | 23.4 ± 5.5 | 18.1 ± 2.5 |
| *ldlr*$^{-/-}$ | 54.6 ± 1.3 | 24.2 ± 2.5 | 21.2 ± 2.2 |

**C**

**Nascent d<1.006 particles**

| | Triglyceride (%) | Cholesterol (%) | Phospholipid (%) |
|---|---|---|---|
| *apobec-1*$^{-/-}$ | 78.9 ± 2.0 | 8.7 ± 1.7 | 12.1 ± 0.1 |
| *apobec-1*$^{-/-}$*ldlr*$^{-/-}$ | 79.8 ± 1.5 | 9.9 ± 1.1 | 10.1 ± 0.4 |
| WT | 70.8 ± 2.0 | 11.0 ± 0.1 | 14.3 ± 2.0 |
| *ldlr*$^{-/-}$ | 73.3 ± 1.9 | 11.8 ± 1.3 | 14.9 ± 0.8 |

**Fig. 2.** Distribution of newly synthesized apoB in d < 1.006 g/ml and 1.006 < d < 1.063 g/ml fractions. Mice were injected intraperitoneally with 500 μCi of [$^{35}$S]protein-labeling mix and 500 mg/kg Triton WR-1339. Lipoprotein fractions at d < 1.006 and 1.006 < d < 1.063 g/ml were isolated from equal aliquots of serum 2 h after Triton injection. A: Lipoprotein fractions from the 2 h time point were subjected to SDS-PAGE analysis to determine the distribution of de novo-synthesized apoB. Each panel is a representative gel from two different mice. B: Lipid composition (triglyceride, total cholesterol and phospholipid) of d < 1.006 fraction was determined at 0 h (circulating particles). C: Lipid composition of d < 1.006 2 h after Triton injection (nascent particles). Data are expressed as percentage of total lipids, and composition of nascent particles at 2 h was corrected for by subtracting the circulating values at the 0 h time point. Data are mean ± SEM of six different experiments.

peared in the d < 1.006 fraction in all genotypes, with less than 10% isolated in the IDL/LDL (1.006–1.063) fraction (**Fig. 2A**). These findings suggest that newly synthesized lipoproteins in all genotypes float in the VLDL density range. In addition, the lipid composition of the newly synthesized particles revealed a similar distribution of triglyceride, cholesterol, and phospholipid in all genotypes, with the majority of lipid represented by triglyceride (Fig. 2C). Circulating lipoprotein particle composition was also similar between the different genotypes but with relatively less enrichment with triglyceride than nascent particles (Fig. 2B). These data suggest that newly synthesized, triglyceride-rich lipoprotein particles from all four genotypes float within the d < 1.006 g/ml VLDL density range.

To address the question of lipoprotein size distribution, we undertook FPLC fractionation of serum triglyceride before and 2 h after Triton injection. These experiments revealed the presence of smaller particles in *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]* mice (**Fig. 3**) in a distribution extending from small VLDL to the IDL size range. By contrast, serum from *apobec-1*$^{-/-}$, *ldlr*$^{-/-}$, and WT mice contained only VLDL-sized particles (Fig. 3, right panel). Fractions from the FPLC profiles of animals administered [$^{35}$S]methionine were analyzed by SDS-PAGE to determine the distribution of newly synthesized apoB-100 (Fig. 3E), the findings demonstrating a shift from VLDL (fractions 3–5) in *apobec-1*$^{-/-}$ mice to IDL/LDL (fractions 11–14) in *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]* mice. These results imply the possibility of

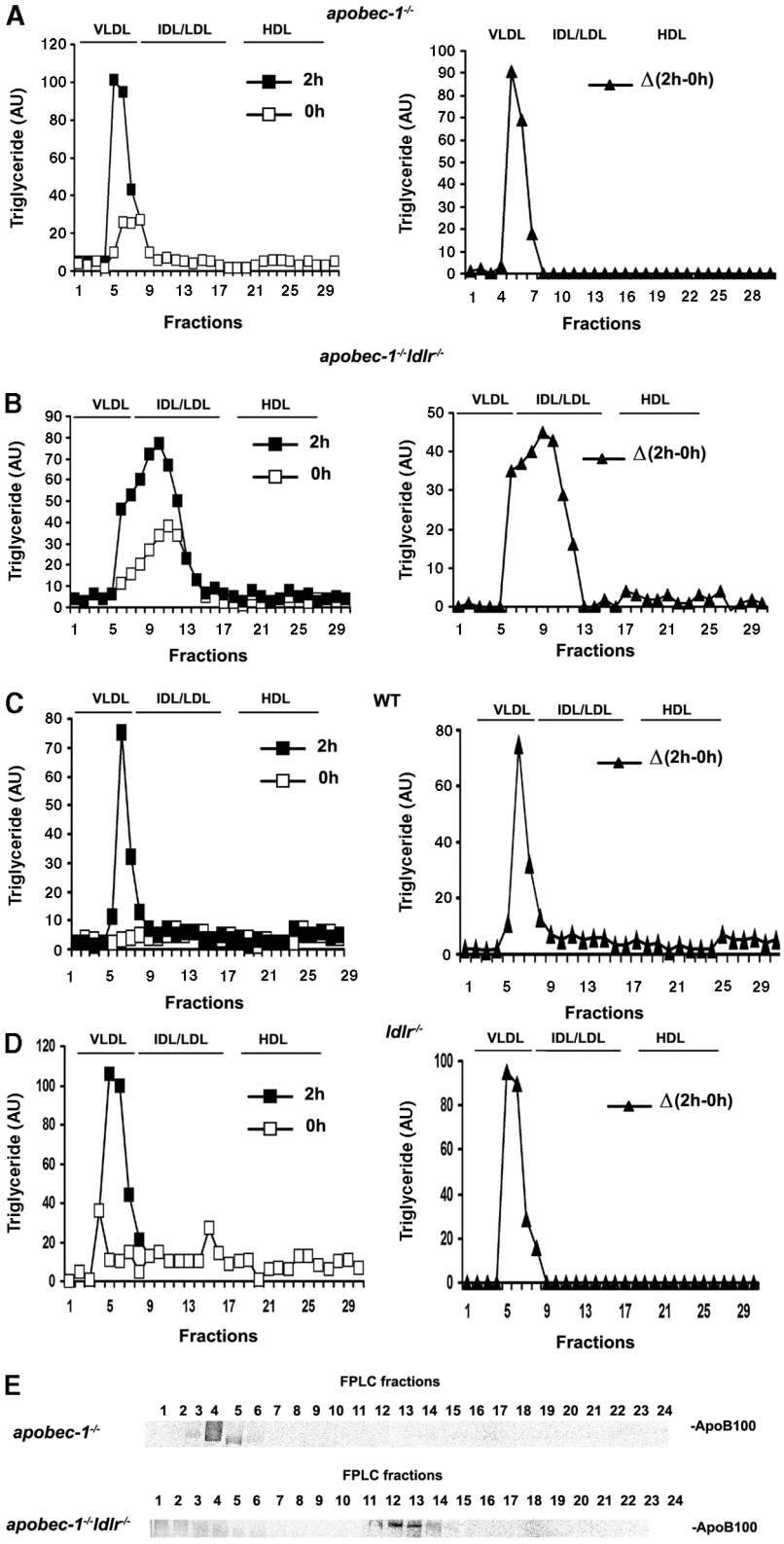



**Fig. 3.** Fast-performance liquid chromatography (FPLC) distribution of circulating and nascent triglyceride-rich lipoprotein particles. Triglyceride distribution was determined in serum pooled from three mice (0 h or 2 h after Triton injection) and fractionated by FPLC. Left panels: distribution of triglyceride in lipoprotein fractions at 0 h and 2 h after Triton injection. Right panels: distribution of triglyceride accumulated during the 2 h after Triton injection (Δ 2 h–0 h). The genotypes are presented as follows: A: *apobec-1*$^{-/-}$; B: *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]*; C: WT; D: *ldlr*$^{-/-}$. E: Mice were injected intraperitoneally with 500 μCi of [$^{35}$S]protein-labeling mix and 500 mg/kg Triton WR-1339. The animals were exsanguinated 2 h later, and the distribution of newly synthesized apoB-100 in lipoprotein fractions was determined by SDS-PAGE analysis of the indicated fractions from FPLC. AU, area units.

direct hepatic secretion of apoB-100 in association with small, triglyceride-rich, IDL-sized particles when LDLR deficiency is imposed on an apoB-100-only background. To resolve this possibility directly, further studies were undertaken in vitro.

### Secretion of small lipoprotein particles from primary hepatocytes in *[apobec-1$^{-/-}$, ldlr$^{-/-}$]* mice

To directly determine the size of lipoprotein particles secreted from isolated hepatocytes, lipoproteins of ($d < 1.006$) were prepared from hepatocyte media and subjected to negative-stain electron microscopy. The results demonstrate comparable distribution of VLDL-sized particles (40–80 nm) from WT, *ldlr*$^{-/-}$, and *apobec-1*$^{-/-}$ hepatocytes (**Fig. 4A**, **B**). By contrast, ~80% of lipoproteins secreted from *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]* hepatocytes were in the IDL/LDL-sized particle (<40 nm diameter) (Fig. 4A, B). These in vitro finding are consistent with the FPLC results above and confirm the shift in hepatic lipoprotein secretion in *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]* mice. Taken together, the data strongly imply that the LDLR directly modulates secretion of small triglyceride-rich particles in the apoB-100-only background.

### ApoB secretion from murine primary hepatocytes

To pursue the mechanisms underlying the alterations in hepatic apoB-100 and VLDL secretion from *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]* mice, we undertook in vitro studies of apoB production using primary hepatocytes. As determined from inspection of representative autoradiographs, >90% of newly synthesized apoB-100 was recovered at the end of the chase using hepatocytes from *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]* mice (**Fig. 5B**), contrasted with only ~33% recovery from *apobec-1*$^{-/-}$ mice (Fig. 5A). Similar findings were encountered in *ldlr*$^{-/-}$ mice (~85% of apoB-100 recovered) compared with ~31% in WT hepatocytes (Fig. 5C). These data are summarized also in Fig. 5E. The recovery of apoB-48 was also higher in *ldlr*$^{-/-}$ compared with WT mice (Fig. 5C, F). Albumin recovery was comparable in all genotypes (~100%) (Fig. 5A–D).

Additional studies were undertaken to further delineate the role of cell surface LDLR expression versus an effect mediated via interactions occurring within an intracellular compartment. These studies were conducted with isolated hepatocytes in which pulse-chase studies were performed with or without heparin in the chase medium (20). The findings of these studies (**Table 1**) demonstrate that heparin partially rescues the degradation of apoB-100 and apoB-48 in WT hepatocytes and of apoB-100 in *apobec-1*$^{-/-}$ hepatocytes, implying a role for cell surface interactions with the LDLR in regulating the secretion and recovery of newly synthesized apoB. Nevertheless, inclusion of heparin fails to restore apoB-100 secretion and recovery to the levels found in *ldlr*$^{-/-}$ and *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]* mice (Table 1), confirming the findings of Attie and colleagues (20), that intracellular interactions are also relevant.

Multicompartmental modeling (**Fig. 6**) of apoB-100 fit the prediction that over 90% of newly synthesized apoB-100 was destined for secretion in *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]* and *ldlr*$^{-/-}$ hepatocytes (Fig. 6C, E). Using the same assumptions, only 30% of newly synthesized apoB-100 was destined for secretion in WT and *apobec-1*$^{-/-}$ hepatocytes (Fig. 6B, D). Consistent with previous findings from multicompartmental modeling, the secretion of apoB-48 was ~2-fold higher in *ldlr*$^{-/-}$ compared with WT hepatocytes (~50% vs. 20%) (Fig. 6F, G).

## DISCUSSION

The current studies add further support to the hypothesis advanced by Attie and colleagues (20) that the LDLR plays a direct role in regulating murine hepatic apoB secretion by modulating the rate of export of nascent apoB-containing lipoprotein particles from the endoplasmic reticulum (14). These studies extend earlier findings in HepG2 cells, utilizing physiological manipulation of cholesterol content and LDLR function, demonstrating that blocking antibodies to the LDLR increased apoB-100 secretion, whereas lovastatin treatment (which would be anticipated to increase LDLR expression) resulted in decreased apoB-100 secretion (21). In contrast to the increased secretion of apoB-100, hepatic triglyceride secretion and (as evidenced by ultracentrifugal analysis) VLDL triglyceride secretion rates appear comparable regardless of LDLR genotype. We propose a resolution to this apparent paradox by demonstrating that this combination of unchanged triglyceride transport coupled with increased secretion of apoB-100 in *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]* mice is accommodated by secretion of greater numbers of smaller particles.

There has been controversy surrounding the role of the LDLR in regulating murine hepatic apoB production and lipoprotein secretion. This may, in part, reflect differences in the approaches used to examine hepatic VLDL secretion, with findings from isolated hepatocytes demonstrating increased secretion of apoB (20) whereas in vivo studies using Triton WR-1339 have shown either a small increase (19) or no change (23) in VLDL triglyceride and apoB production in the *ldlr*$^{-/-}$ background. An additional layer of complexity is that the age, sex, and genetic background of mice used to examine these parameters may be critical, with reported studies including both young and older mice of both genders, and with inbred C57BL/6 as well as mixed genetic backgrounds (15, 19, 23). Although no attempt was made to systematically evaluate the relative importance of each of these variables, our approach was to examine hepatic apoB and triglyceride production rates, both in vivo and in vitro using inbred C57BL/6 male mice within the ages of 12 and 18 weeks. The results support the central conclusions of Attie and colleagues (20), who used isolated hepatocytes to demonstrate increased secretion of apoB. Our findings also confirm certain elements of the report of Millar et al. (23), specifically, in relation to the absence of change in hepatic triglyceride production in vivo with the LDLR genotype. However, Millar et al. concluded that the LDLR failed to alter hepatic VLDL

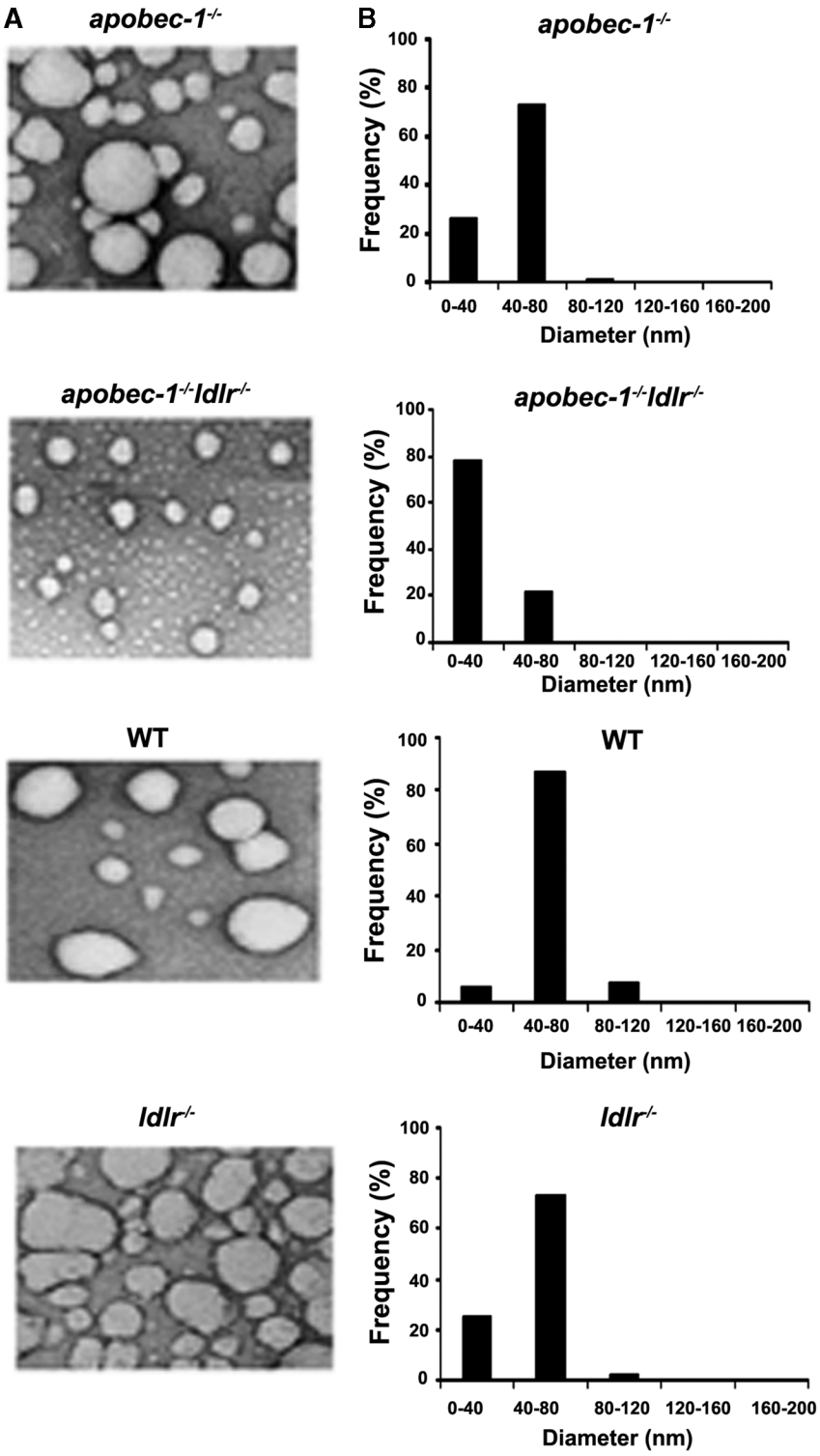
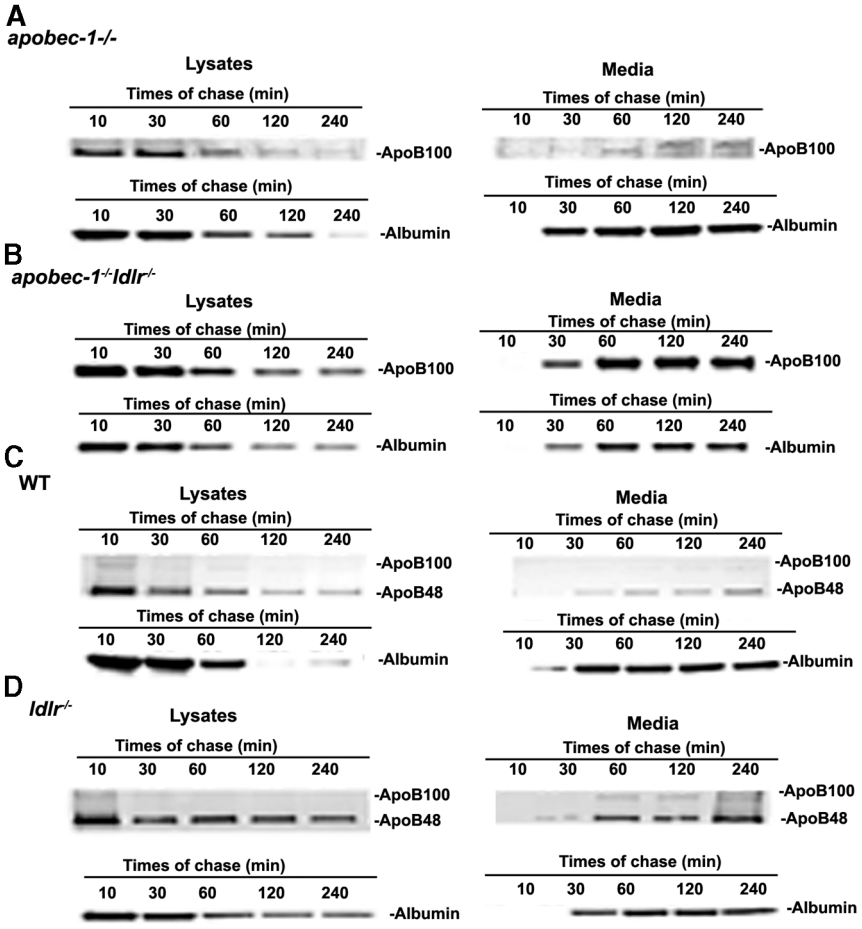



**Fig. 4.** Electron micrographs of newly secreted lipoproteins from primary murine hepatocytes. The size of secreted hepatic lipoproteins was determined on media from hepatocytes incubated for 4 h. Lipoprotein fractions ($d < 1.006$ g/ml) were isolated from media and were subjected to negative staining and electron microscopy. A: Representative electron micrographs from *apobec-1*$^{-/-}$, *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]*, WT, and *ldlr*$^{-/-}$ mice, respectively. B: Calculated frequencies of the size of lipoprotein particles. Two hundred particles were sized for each genotype. The data shown are representative examples from three individual experiments, each using hepatocytes pooled from three animals.




E

| ApoB100 | | | |
|---|---|---|---|
| | Secretion (%) | Degradation (%) | Recovery (%) |
| *apobec-1*$^{-/-}$ | 27 ± 7 | 63 ± 5 | 33 ± 9 |
| *apobec-1*$^{-/-}$*ldlr*$^{-/-}$ | 79 ± 16$^{***}$ | 23 ± 9$^{***}$ | 93 ± 13$^{***}$ |
| WT | 21 ± 2 | 66 ± 4 | 31 ± 5 |
| *ldlr*$^{-/-}$ | 85 ± 3$^{***}$ | 27 ± 7$^{***}$ | 85 ± 17$^{***}$ |

F

| | ApoB48 | | |
|---|---|---|---|
| | Secretion (%) | Degradation (%) | Recovery (%) |
| WT | 24 ± 9 | 63 ± 13 | 34 ± 11 |
| *ldlr*$^{-/-}$ | 52 ± 4$^{*}$ | 23 ± 7$^{*}$ | 85 ± 15$^{*}$ |

**Fig. 5.** Hepatic apoB synthesis and secretion from murine primary hepatocytes. Hepatocytes were isolated and subjected to pulse-chase analysis as described in Materials and Methods. Lysates and media were collected at the indicated times; apoB and albumin were quantitatively immunoprecipitated. The immunoprecipitates were quantitated by SDS-PAGE and phosphoimager analysis. The genotypes are presented as follows: A: *apobec-1*$^{-/-}$; B: *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]*; C: WT; and D: *ldlr*$^{-/-}$. ApoB-100 secretion (E) and apoB-48 secretion (F) were quantitated and expressed as percentage of newly synthesized proteins. Data are means ± SEM from three individual experiments, each using hepatocytes pooled from two to four animals. $^{*}P < 0.05$, $^{***}P < 0.001$.

TABLE 1. Secretion of apoB from murine primary hepatocytes in the presence of heparin

| | Secretion | | Recovery | |
|---|---|---|---|---|
| | − Heparin | + Heparin | − Heparin | + Heparin |
| | % | | | |
| apoB-100 | | | | |
| *apobec-1*$^{-/-}$ | 25 ± 7 | 47 ± 9* | 41 ± 10 | 64 ± 9* |
| *apobec-1*$^{-/-}$, *ldlr*$^{-/-}$ | 69 ± 13 | 77 ± 7 | 91 ± 5 | 88 ± 10 |
| WT | 22 ± 5 | 42 ± 6* | 49 ± 13 | 66 ± 7* |
| *ldlr*$^{-/-}$ | 75 ± 7 | 82 ± 9 | 89 ± 12 | 96 ± 5 |
| apoB-48 | | | | |
| WT | 34 ± 4 | 71 ± 11* | 49 ± 14 | 85 ± 10* |
| *ldlr*$^{-/-}$ | 59 ± 4 | 67 ± 9 | 95 ± 5 | 87 ± 7 |

Hepatocytes were isolated and subjected to pulse-chase analysis as described in Materials and Methods. Cells were preincubated, pulse-labeled for 30 min, and chased in the presence (+) or absence (−) of 10 mg/ml heparin. Lysates and media were collected at 0 h and 4 h of chase; apoB was immunoprecipitated and quantitated by phosphorImager. ApoB-100 secretion and apoB-48 secretion were quantitated and expressed as percentage of newly synthesized proteins. Data are expressed as percent newly synthesized apoB after the 30 min pulse and are shown as mean ± SEM from two individual experiments

ApoB, apolipoprotein B; WT, wild type.

* $P < 0.05$.

and IDL/LDL apoB production in *ldlr*$^{-/-}$ versus *ldlr*$^{+/-}$ mice (23), a discrepancy we cannot readily explain, other than to invoke an effect of heterozygous LDLR deletion in female mice. Our current findings, taken together with earlier studies in HepG2 cells (21) and those of Attie et al. (20), certainly support a role for the LDLR in physiological regulation of hepatic apoB-100 secretion.

A major finding emerging from the current studies is that hepatocytes from *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]* mice secrete small, triglyceride-rich particles directly. This finding supports the suggestion that an intracellular ligand–receptor interaction between apoB-100 and the LDLR modulates hepatic apoB secretion, results consistent with some, but not all, studies in human subjects with FH (28, 29). Of particular interest however, Fisher, Zech, and Stacpoole (29) demonstrated that FH heterozygotes exhibit increased production of smaller (intermediate and LDL-sized) lipoprotein particles, findings consistent with the predictions emerging from the current and other recent data (17) in murine hepatocytes. To our knowledge, studies of lipoprotein particle secretion from isolated hepatocytes have not been reported in patients with FH, necessitating an alternative approach to this question. *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]* mice provide a surrogate model of human hepatic lipoprotein metabolism in FH and offer one such approach. The *apobec-1*$^{-/-}$ background overcomes intrinsic differences in the affinity of apoB-100 and apoB-48 for the LDLR and surmounts potential differences in the regulation of hepatic secretion of apoB-100 versus apoB-48. This is particularly relevant, because murine hepatocytes secrete predominantly apoB-48-containing particles. ApoB-48 lacks the LDLR binding domain, yet apoB-48-containing lipoproteins bind the LDLR at high affinity by virtue of the presence of apoE. Interestingly, although apoE-null mice have reduced hepatic VLDL secretion (30), Teusink and colleagues (19) demonstrated that the effects of apoE on hepatic VLDL secretion were independent of LDLR status, suggesting that other receptors may be involved in the regulation of hepatic apoB-48 production.

In relation to the current study, we anticipated that the phenotype favoring small lipoprotein particle secretion in *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]* mice would be greatly abrogated in *ldlr*$^{-/-}$ mice in an *apobec-1*-sufficient background, where greater numbers of apoB-48-containing particles are produced (20). However, an inevitable question is how to explain the hypertriglyceridemia in *[apobec-1*$^{-/-}$*, ldlr*$^{-/-}$*]* mice, particularly inasmuch as triglyceride secretion is unchanged. We concur with the suggestion of Rader and colleagues (23), that defective triglyceride catabolism may be involved, although we have no direct evidence for this speculation. Additionally, higher serum triglyceride levels have been previously demonstrated in apoB-100-only mice (25), a finding that has been speculated by Young and colleagues (31, 32) to be caused by incomplete triglyceride hydrolysis.

Studies in HepG2 cells (21) and in isolated murine hepatocytes (20) demonstrated that the LDLR regulates secretion of apoB-containing lipoproteins, through actions mediated both intracellularly and at the cell surface, via reuptake. These (14, 20) and more recent findings (17) suggest that binding of the LDLR to apoB within the secretory pathway leads to presecretory degradation and consequently elimination of small, presumably underlipidated, lipoprotein particles. Fusion of a nascent apoB-100-containing triglyceride-rich particle with the LDLR is thought to occur within the endoplasmic reticulum, an interaction that has been suggested to represent a quality control mechanism to limit direct production of atherogenic lipoprotein particles (14, 20). Once the nascent particle acquires sufficient lipid, its affinity for the LDLR deceases, and it is then released and continues through the secretory pathway, where it matures into a larger, VLDL-sized particle (14, 20). Additional support for this general model of lipoprotein assembly has been recently provided through studies using conditional deletion of the MTTP, another dominant restriction point in intestinal and hepatic lipoprotein secretion, whose mechanism of action is intimately connected with the lipidation and processing of apoB (5, 9, 12). Findings emerging from these recent studies demonstrated that conditional ("floxed") MTTP-deletor mice in an *ldlr*$^{-/-}$ background secreted apoB-100-containing lipoproteins that were isolated in the LDL and HDL size range, in contrast to the floxed deletor mice in an LDLR-sufficient background, where essentially no apoB-100 was secreted (17). These results are in accord with the current findings demonstrating that elimination of LDLR expression leads to secretion of small, dense, triglyceride-rich lipoprotein particles. Together, the findings strongly suggest that the LDLR plays an important role in the surveillance process in lipoprotein assembly, specifically in regard to events surrounding the earliest phases of lipoprotein biogenesis.

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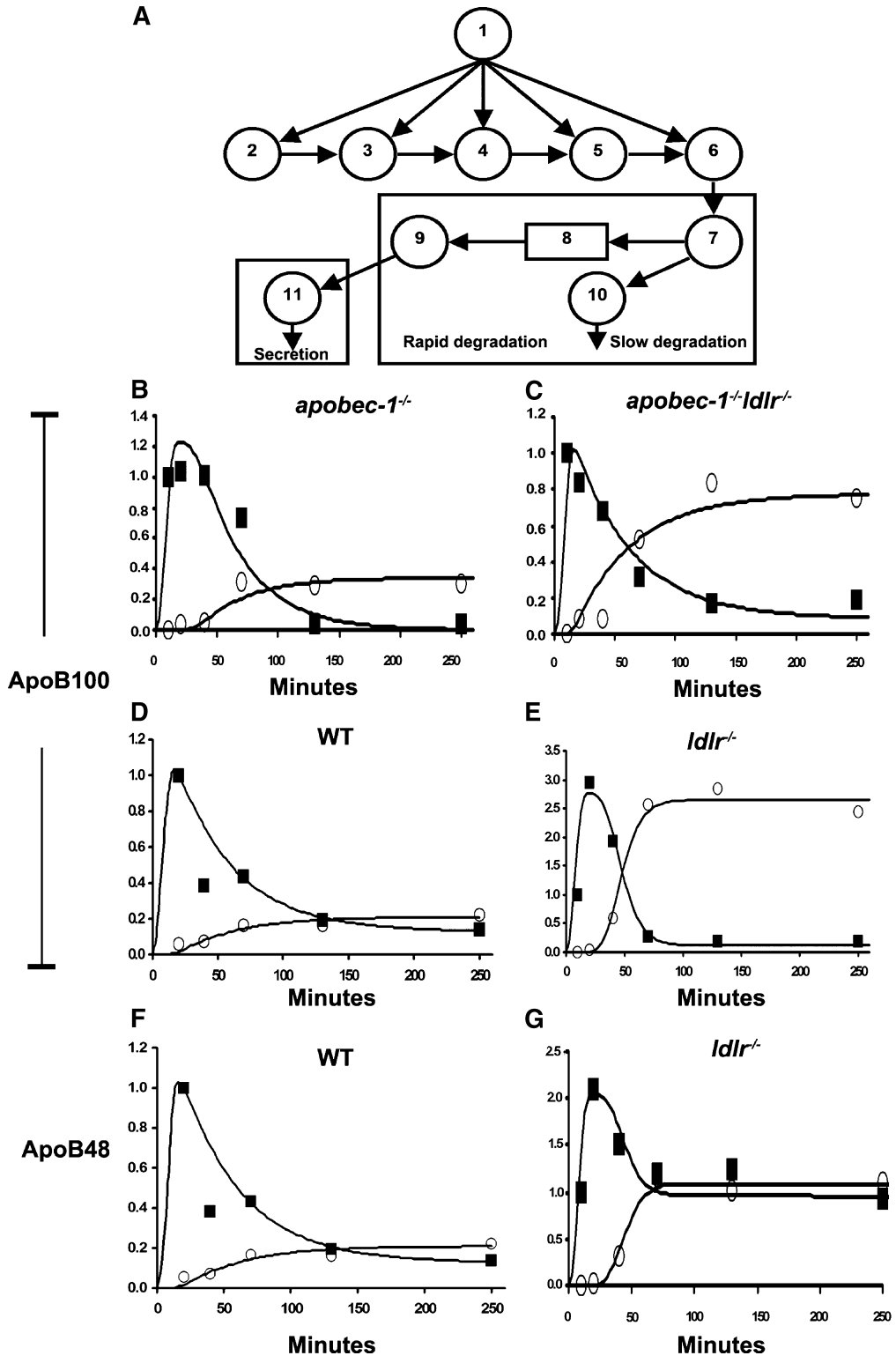


**Fig. 6.** Multicompartmental modeling of apoB secretion and degradation from murine hepatocytes of different genotypes. The assumptions used are summarized in Materials and Methods. [$^{35}$S]methionine was applied to compartment 1 for 10 min. A: Multicompartmental model for apoB secretion and intracellular degradation. Compartments 7–10 represent fully synthesized, immunoprecipitable intracellular apoB. Secreted apoB is represented in compartment 11. ApoB can be "lost" from compartment 9 (rapid degradation) or compartment 10 (slow degradation). ApoB-100 secretion is modeled from the various genotypes as follows: B: *apobec-1*$^{-/-}$; C: *[apobec-1*$^{-/-}$, *ldlr*$^{-/-}$*]*; D: WT; and E: *ldlr*$^{-/-}$. ApoB-48 secretion is modeled as follows: F: WT; and G: *ldlr*$^{-/-}$. Open square, secreted protein; filled square, intracellular protein.

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## REFERENCES


1. Attie, A. D., R. C. Pittman, and D. Steinberg. 1982. Hepatic catabolism of low density lipoprotein: mechanisms and metabolic consequences. *Hepatology.* **2:** 269–281.
2. Brown, M. S., and J. L. Goldstein. 1986. A receptor-mediated pathway for cholesterol homeostasis. *Science.* **232:** 34–47.
3. Goldstein, J. L., M. S. Brown, R. G. Anderson, D. W. Russell, and W. J. Schneider. 1985. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol.* **1:** 1–39.
4. Pittman, R. C., T. E. Carew, A. D. Attie, J. L. Witztum, Y. Watanabe, and D. Steinberg. 1982. Receptor-dependent and receptor-independent degradation of low density lipoprotein in normal rabbits and in receptor-deficient mutant rabbits. *J. Biol. Chem.* **257:** 7994–8000.
5. Fisher, E. A., and H. N. Ginsberg. 2002. Complexity in the secretory pathway: the assembly and secretion of apolipoprotein B-containing lipoproteins. *J. Biol. Chem.* **277:** 17377–17380.
6. Benoist, F., and T. Grand-Perret. 1997. Co-translational degradation of apolipoprotein B100 by the proteasome is prevented by microsomal triglyceride transfer protein. Synchronized translation studies on HepG2 cells treated with an inhibitor of microsomal triglyceride transfer protein. *J. Biol. Chem.* **272:** 20435–20442.
7. Berriot-Varoqueaux, N., L. P. Aggerbeck, M. Samson-Bouma, and J. R. Wetterau. 2000. The role of the microsomal triglygeride transfer protein in abetalipoproteinemia. *Annu. Rev. Nutr.* **20:** 663–697.
8. Yao, Z., K. Tran, and R. S. McLeod. 1997. Intracellular degradation of newly synthesized apolipoprotein B. *J. Lipid Res.* **38:** 1937–1953.
9. Shelness, G. S., and J. A. Sellers. 2001. Very-low-density lipoprotein assembly and secretion. *Curr. Opin. Lipidol.* **12:** 151–157.
10. Ellgaard, L., M. Molinari, and A. Helenius. 1999. Setting the standards: quality control in the secretory pathway. *Science.* **286:** 1882–1888.
11. Yeung, S. J., S. H. Chen, and L. Chan. 1996. Ubiquitin-proteasome pathway mediates intracellular degradation of apolipoprotein B. *Biochemistry.* **35:** 13843–13848.
12. Wang, S., R. S. McLeod, D. A. Gordon, and Z. Yao. 1996. The microsomal triglyceride transfer protein facilitates assembly and secretion of apolipoprotein B-containing lipoproteins and decreases cotranslational degradation of apolipoprotein B in transfected COS-7 cells. *J. Biol. Chem.* **271:** 14124–14133.
13. Barrett, P. H., and G. F. Watts. 2002. Shifting the LDL-receptor paradigm in familial hypercholesterolemia: novel insights from recent kinetic studies of apolipoprotein B-100 metabolism. *Atheroscler.* **3 (Suppl.):** 1–4.
14. Gillian-Daniel, D. L., P. W. Bates, A. Tebon, and A. D. Attie. 2002. Endoplasmic reticulum localization of the low density lipoprotein receptor mediates presecretory degradation of apolipoprotein B. *Proc. Natl. Acad. Sci. USA.* **99:** 4337–4342.
15. Horton, J. D., H. Shimano, R. L. Hamilton, M. S. Brown, and J. L. Goldstein. 1999. Disruption of LDL receptor gene in transgenic SREBP-1a mice unmasks hyperlipidemia resulting from production of lipid-rich VLDL. *J. Clin. Invest.* **103:** 1067–1076.
16. Jiang, X. C., S. Qin, C. Qiao, K. Kawano, M. Lin, A. Skold, X. Xiao, and A. R. Tall. 2001. Apolipoprotein B secretion and atherosclerosis are decreased in mice with phospholipid-transfer protein deficiency. *Nat. Med.* **7:** 847–852.
17. Larsson, S. L., J. Skogsberg, and J. Bjorkegren. 2003. The low density lipoprotein receptor prevents secretion of dense apoB-100-containing lipoproteins from the liver. *J. Biol. Chem.* **279:** 831–836.
18. Liao, W., T. Y. Hui, S. G. Young, and R. A. Davis. 2003. Blocking microsomal triglyceride transfer protein interferes with apoB secretion without causing retention or stress in the ER. *J. Lipid Res.* **44:** 978–985.
19. Teusink, B., A. R. Mensenkamp, H. van der Boom, F. Kuipers, K. W. van Dijk, and L. M. Havekes. 2001. Stimulation of the in vivo production of very low density lipoproteins by apolipoprotein E is independent of the presence of the low density lipoprotein receptor. *J. Biol. Chem.* **276:** 40693–40697.
20. Twisk, J., D. L. Gillian-Daniel, A. Tebon, L. Wang, P. H. R. Barrett, and A. D. Attie. 2000. The role of the LDL receptor in apolipoprotein B secretion. *J. Clin. Invest.* **105:** 521–532.
21. Williams, K. J., R. W. Brocia, and E. A. Fisher. 1990. The unstirred water layer as a site of control of apolipoprotein B secretion. *J. Biol. Chem.* **265:** 16741–16744.
22. Goldstein, J. L., and M. S. Brown. 1989. Familial hypercholesterolemia. *In* The Metabolic Basis of Inherited Disease. 6th edition. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw Hill Information Services, New York. 1215–1250.
23. Millar, J. S., C. Maugeais, I. V. Fuki, and D. J. Rader. 2002. Normal production rate of apolipoprotein B in LDL receptor-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **22:** 989–994.
24. Hirano, K., S. G. Young, R. V. Farese, Jr., J. Ng, E. Sande, C. Warburton, L. M. Powell-Braxton, and N. O. Davidson. 1996. Targeted disruption of the mouse apobec-1 gene abolishes apolipoprotein B mRNA editing and eliminates apolipoprotein B-48. *J. Biol. Chem.* **271:** 9887–9890.
25. Powell-Braxton, L., M. Veniant, R. D. Latvala, K. I. Hirano, W. B. Won, J. Ross, N. Dybdal, C. H. Zlot, S. G. Young, and N. O. Davidson. 1998. A mouse model of human familial hypercholesterolemia: markedly elevated low density lipoprotein cholesterol levels and severe atherosclerosis on a low-fat chow diet. *Nat. Med.* **4:** 934–938.
26. Nassir, F., D. K. Bonen, and N. O. Davidson. 1998. Apolipoprotein(a) synthesis and secretion from hepatoma cells is coupled to triglyceride synthesis and secretion. *J. Biol. Chem.* **273:** 17793–17800.
27. Nassir, F., Y. Xie, and N. O. Davidson. 2003. Apolipoprotein[a] secretion from hepatoma cells is regulated in a size-dependent manner by alterations in disulfide bond formation. *J. Lipid Res.* **44:** 816–827.
28. Cummings, M. H., G. F. Watts, M. Umpleby, T. R. Hennessy, J. R. Quiney, and P. H. Sonksen. 1995. Increased hepatic secretion of very-low-density-lipoprotein apolipoprotein B-100 in heterozygous familial hypercholesterolaemia: a stable isotope study. *Atherosclerosis.* **113:** 79–89.
29. Fisher, W. R., L. A. Zech, and P. W. Stacpoole. 1994. ApoB metabolism in familial hypercholesterolemia. Inconsistencies with the LDL receptor paradigm. *Arterioscler. Thromb.* **14:** 501–510.
30. Kuipers, F., M. C. Jong, Y. Lin, M. Eck, R. Havinga, V. Bloks, H. J. Verkade, M. H. Hofker, H. Moshage, T. J. Berkel, R. J. Vonk, and L. M. Havekes. 1997. Impaired secretion of very low density lipoprotein-triglycerides by apolipoprotein E-deficient mouse hepatocytes. *J. Clin. Invest.* **100:** 2915–2922.
31. 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.
32. Veniant, M. M., V. Pierotti, D. Newland, C. M. Cham, D. A. Sanan, R. L. Walzem, and S. G. Young. 1997. Susceptibility to atherosclerosis in mice expressing exclusively apolipoprotein B48 or apolipoprotein B100. *J. Clin. Invest.* **100:** 180–188.