Absence of Hyperlipidemia in LDL Receptor–Deficient Mice Having Apolipoprotein B100 Without the Putative Receptor-Binding Sequences

Lance A. Johnson, Michael K. Altenburg, Rosemary L. Walzem, Lori T. Scanga, Nobuyo Maeda

Objective—To examine the effects of apoB100 structure, specifically a mutation in the LDLr binding region, on the production of LDL and development of atherosclerosis in vivo.

Methods and Results—Ldlr<sup>−/−</sup> Apobec1<sup>−/−</sup> mice lacking the LDLR and apoB editing enzyme accumulated LDL in plasma and developed severe atherosclerosis when they had wild-type apoB100. In marked contrast, in Ldlr<sup>−/−</sup> Apobec1<sup>−/−</sup> mice carrying the Apob100-β mutation, in the 2 putative LDLR-binding domains of apoB prevented both LDL accumulation and atherosclerosis. Intestinal absorption of lipids and triglyceride secretion from the liver were not affected. However, the VLDL particles with apoB100-β were larger in volume by about 70%, and carried approximately four times as much apoE per particle. ApoB100-β synthesis rate in the primary hepatocytes was normal, but its intracellular degradation was enhanced. Additionally, mutant apoB100 VLDL cleared from the circulation more quickly in vivo through apoE-LRP–mediated mechanism than VLDL with wild-type apoB100. In contrast, uptake of the 2 VLDL by macrophages were not different.

Conclusion—While conformational change to apoB100 during conversion of VLDL to LDL exposes LDLR binding domains and facilitates LDLR-mediated lipoprotein clearance, it may also inhibit LRP-mediated VLDL uptake and contribute to LDL accumulation in familial hypercholesterolemia. (Arterioscler Thromb Vas Biol. 2008;28:1745-1752)

Key Words: lipoprotein clearance • atherosclerosis • apolipoprotein B100 • familial hypercholesterolemia • animal models

A poloprotein (apo) B is an essential component of VLDL, LDL, and chylomicrons. ApoB normally exists in 2 forms, apoB100 and apoB48; both are the products of the same gene. ApoB100 comprises 4536 amino acids, synthesized in the liver, and secreted into the circulation as a structural component of VLDL. ApoB48 is 48% of full-length apoB, and is formed as a result of posttranslational editing of ApoB mRNA by the apoB editing complex (apoBEC), which changes Gln at codon 2153 to a stop codon. ApoB48 is synthesized in the small intestine and is required for the packaging of lipids into chylomicrons. Whereas human liver makes exclusively apoB100, a large proportion of message in the mouse liver is edited and consequently mice produce both apoB48 and apoB100 from the liver.

In addition to maintaining the structural integrity of lipoprotein particles, apoB100 also functions as a ligand for the LDLR and is therefore a primary determinant of circulating LDL cholesterol levels. The LDLR-binding domain of apoB100 has not been fully defined; however, biochemical, immunochemical, and genetic evidence suggests that it is a region of net positive change located in the carboxyl-terminal portion of apoB100. Two sequences, residues 3147 to 3157 and 3359 to 3367, are enriched in basic amino acid residues and have been proposed as putative LDLR-binding domains in both species. The sequence at 3359 to 3367 is highly conserved among mammalian species and is also similar to the LDLR-binding site of apoE. Also, Boren et al showed that the removal of positive charges from residues 3359 to 3367 by site-directed mutagenesis renders the LDL containing the modified apoB defective in LDLR binding.

To define the regions of apoB that bind the LDLR, we previously introduced mutations into the mouse Apob gene. The apoB100-β protein is the same length as apoB100 but contains 2 peptide sequences for human β-globin in place of the residues 3147 to 3157 and 3359 to 3367. The modification also drastically reduced the net positive charges and amphipathic helices of the 2 domains. We expected that the mice producing apoB100-β would model defective apolipoprotein B100 in humans by accumulating binding-defective LDL in plasma. However, we found that the apoB100-β<sub>B100-β</sub> mice have slightly, but not significantly lower than normal, total plasma cholesterol and HDL cholesterol, and the amount of...
plasma LDL was not different from that in wild-type mice. One explanation is that these 2 regions are not essential for apoB100 binding to the LDLR in vivo. The interpretation, however, is complicated because mice normally have very little apoB100-containing LDL particles in circulation. In addition, the production of apoB48 from the liver and the efficient clearance of apoB48-containing remnants mediated by apoE make the metabolism of apoB100 difficult to study in vivo in mice.

The present study examined the effect of apoB100-containing LDL by introducing the mutation onto a background of Ldlr−/−/Apobec1−/− double mutants; a model of human familial hypercholesterolemia with severe atherosclerosis. Apobec1−/− mice that lack the mRNA editing enzyme produce only apoB100, whereas Ldlr−/− mice that lack LDLR accumulate LDL cholesterol in plasma. Surprisingly, when these mice also carry the apoB100−/− mutation, they are completely protected from hypercholesterolemia and atherosclerosis that normally occurs in Ldlr−/−/Apobec1−/− mice.

Methods
Please see supplemental methods (available online at http://atvb.ahajournals.org) for details on plasma lipid, lipoprotein, tissue weight, triglyceride secretion, lipolysis, uptake of Dil and 125I-labeled lipoproteins, LRP inhibition by adenoRAP, gene expression, and apoB synthesis, secretion, and degradation.

Animals and Diets
The apoB100−/− allele codes for “VHLTVPVEKSAVT” and “KEFT-PPVQAAYAYQ” instead of “LSVKAQYYKNND” and “GTSRLMRRKRLK” of the wild-type apoB100 allele at residues 3143 to 3154 and 3356 to 3366, respectively. ΔLdlr−/− mice (B6:129S7-Ldlrtm1Her/J) were obtained from the Jackson Laboratory. Apobec1−/− mice were obtained from Dr Eddy Rubin at the Lawrence Berkeley National Laboratory. Three strains of mutants were crossed to generate mice that are heterozygous for the Apob locus and doubly homozygous for the Apobec1 and the Ldlr loci. These mice were then crossed, and ΔLdlr−/−/Apobec1−/− mice with Apob genotypes of 100/100 (wild type), 100/100−/− (heterozygous), and 100−/−/100−/− (homozygous) were generated for experiments. Their genetic backgrounds were complex mixes between C57BL/6J, 129/SvEv, and 129/Ola. Animals were maintained on normal chow (NC; 4.5% fat, 0.022% cholesterol; ProLab Isopro 3000; Agway Inc), or were fed a high-fat Western-type diet (HFW; 21% fat, 0.2% cholesterol; TD 88137; Harlan Teklad). Mice in all experiments were age-matched within 3 weeks. All procedures for the handling of mice were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Biochemical Analyses and Atherosclerosis Evaluation
Mice were fasted 4 hours before analysis. Liver and fecal lipids were extracted with chloroform/methanol. Plasma lipids, lipoprotein distribution, and triglyceride secretion rate, were determined as described. Lipoprotein particle diameters were determined by dynamic light scattering analysis using a Microtrac 250. Peritoneal macrophages and hepatocytes were isolated as described. The VLDL (d <1.006 g/mL) and LDL (d=1.06 to 1.10 g/mL) fraction was isolated from pooled plasma by ultracentrifugation and labeled with 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiI C18; Molecular Probes Inc) or with 125I (Iodine-125 Radionuclide, Perkin Elmer) for clearance assays. Fibroblasts were kindly provided by Dr J. Herz at the University of Texas Southwestern Medical Center. Cellular lipids were extracted with isopropanol and measured with a microscope fluorometer. Gene expression in the liver was analyzed by real-time polymerase chain reaction (PCR), and quantification of atherosclerosis was carried out as described.

Data Analysis
Values are reported as mean±SE in mg/dl. Plasma samples were collected from mice after a 4-hr fast. The numbers in parentheses are the No. of animals. F indicates females; M, males; TC, total cholesterol; TG, triglycerides; FC, free cholesterol; PL, phospholipids; n.d., not determined. Effects of Apob genotypes are highly significant in all categories (P<0.0001).

Table. Plasma Lipid Levels in Ldlr−/−/Apobec1−/− Mice With Wild-Type ApoB100 or ApoB100−/− Mutation

| Apob Genotype | Normal Chow Diet | | | | High-Fat Western Diet |
|---------------|-----------------|-----------------|-----------------|-----------------|
|               | TC (mg/dl) | TG (mg/dl) | FC (mg/dl) | PL (mg/dl) |
| F 100/100     | 380±31 | 78±6 | 1005±35 | 253±37 | 627±23 |
| 100/100−/−   | 196±7  | 36±3 | 476±86  | 124±56  | 143±36  |
| 100−/−/100−/− | 93±6  | 23±3 | 292±24  | 60±5   | 84±14   |
| M 100/100     | 478±23  | 99±7  | 1161±26 | 599±22 | n.d.    |
| 100/100−/−   | 256±10 | 60±9  | n.d.    | n.d.   | n.d.    |
| 100−/−/100−/− | 126±7 | 39±4 | 329±20 | 149±29 | n.d.    |

Data are mean±SE in mg/dl. Plasma samples were collected from mice after a 4-hr fast. The numbers in parentheses are the No. of animals. F indicates females; M, males; TC, total cholesterol; TG, triglycerides; FC, free cholesterol; PL, phospholipids; n.d., not determined. Effects of Apob genotypes are highly significant in all categories (P<0.0001).

Results
ApoB100−/− Causes Marked Reduction of LDL in Ldlr−/−/Apobec1−/− Mice
Ldlr−/−/Apobec1−/− mice with wild-type apoB100 had high levels of plasma cholesterol and triglycerides on NC, and further increased plasma lipids on a HFW diet (Table). In contrast, both plasma cholesterol levels in the Ldlr−/−/Apobec1−/− mice that are heterozygous and homozygous for the apoB100−/− mutation were reduced in an allele dose-dependent manner. The protective effect of the apoB100−/− mutation compared to controls was retained when mice were fed a HFW diet, although plasma cholesterol levels increased about 3-fold in all mice. Plasma levels of triglyceride, free cholesterol, and phospholipids in mice with apoB100−/− were also significantly lower than those with apoB100 mice.

When plasma lipoproteins from male mice on normal chow diet were analyzed by fast protein liquid (FPLC), more than 60% of the plasma cholesterol was in the LDL fraction in apoB100−/−/Apobec1−/− mice with wild-type apoB100. In contrast, a striking absence of LDL-cholesterol was noted in the plasma of Ldlr−/−/Apobec1−/− mice homozygous for apoB100−/− (Figure 1A). Mice with one copy of apoB100−/−...
had approximately half the amount of LDL as those with wild-type apoB100. All mice had very low levels of VLDL cholesterol, and there was no difference in the amount of HDL. The possession of apoB100-β resulted in a similar reduction of triglycerides in the LDL fraction (Figure 1B). SDS gel electrophoresis of lipoprotein fractions from plasma of mice fed a HFW diet showed that the distribution of apoB100-β among various classes of lipoproteins was similar to that of normal apoB100 with the highest concentration in the LDL range (1.02 g/mL to 1.06 g/mL) density fractions. Samples equivalent to 5 μL of apoB100 plasma and 15 μL of apoB100-β plasma were loaded. M, weight markers. D, Tissue weight normalized to body weight, (E) liver lipid contents, and (F) fecal lipids. Number of animals is in each bar. Error represents SEM. *P<0.005, **P<0.001.

Figure 1. Plasma lipoprotein distribution by FPLC (A) cholesterol, (B) triglycerides. C, SDS-PAGE of apoB100, apoB-100β, and apoE in VLDL to LDL (1.006 to 1.06 g/mL) density fractions. Samples equivalent to 5 μL of apoB100 plasma and 15 μL of apoB100-β plasma were loaded. M, weight markers. D, Tissue weight normalized to body weight, (E) liver lipid contents, and (F) fecal lipids. Number of animals is in each bar. Error represents SEM. *P<0.005, **P<0.001.

Ldlr−/−Apobec1−/− Mice With apoB100-β Secrete Larger VLDL

A possible source of the disparity in plasma LDL levels is a difference in hepatic VLDL production rates. To estimate the secretion rate of triglyceride-rich lipoprotein (TRL) particles from the liver, we injected Triton WR1339 (Tyloxapol) intravenously into mice to inhibit lipolysis and uptake of TRLs, and measured plasma triglycerides (Figure 2A). Although the basal triglyceride levels differ in the 2 groups of mice, triglyceride secretion rates were nearly identical at 280 to 300 μg/mL/h regardless of whether they have apoB100 or apoB100-β.

We next analyzed VLDL particle size at 2 hours post-Tyloxapol injection. The size of particles in the <1.006 g/mL density fraction was significantly different between the 2 groups of Ldlr−/−Apobec1−/− mice; the mean±SD diameter of apoB100-β VLDL particles were larger (55.0±15 nm) than those with wild-type apoB100 (45.6±14 nm). Based on the difference in the diameter, we estimate that the average apoB100-β VLDL has approximately 46% more surface area and 76% greater volume than that of the normal apoB100 VLDL. Assuming that the triglyceride content of a particle is relative to its volume, this implies that the number of VLDL particles secreted from the Ldlr−/−Apobec1−/− liver with apoB100-β is approximately 60% that from the liver with wild-type apoB100.

To examine the production and degradation of apoB100 proteins, we conducted a pulse-chase experiment with radio-labeled methionine in the primary hepatocytes isolated from the Ldlr−/−Apobec1−/− mice with apoB100 and with apoB100-β (Figure 2B). Immunoprecipitable apoB protein in the apoB100-β cells after the 30-minute pulse was not significantly different from that in apoB100 cells, suggesting...
apoB100 did not differ from that of 125I-VLDL with apoB100 in the five organs measured (Figure 3B).

To assess the conversion of VLDL to smaller particles in vivo, plasma samples were isolated from mice 2 hours after 125I-VLDL injection, pooled, and separated into VLDL, IDL, and LDL fractions using ultracentrifugation. Proportions of counts in the density fractions containing each class of lipoproteins were similar between mice received 125I-VLDL with apoB100 and those with apoB100-β (Figure 3C). Furthermore, the apoB100-β VLDL particles incubated with postheparin plasma released FFA at rates of 12±1 nmol FFA/min compared to apoB100 VLDL at 11±1 nmol FFA/min (Figure 3D), suggesting that the lack of putative LDLR binding domain sequences of apoB does not affect the lipolysis of VLDL in Ldlr-/- Apobec1-/- apoB100-β mice.

Taken together, these experiments suggest that the absence of hyperlipidemia in LDLR-deficient mice having apoB100 without the putative receptor-binding sequences is likely because their VLDL particles are quickly cleared from the circulation before they become small, cholesterol-enriched LDL particles.

**Role of ApoE-LRP–Mediated Clearance**

To examine the specific roles of the LRP on apoB100-β particle uptake, we measured uptake of DiI labeled VLDL and LDL in mouse fibroblasts deficient in either LDLR (LDLR-/-LRP+/+), in LRP (LDLR+/+LRP-/-), or in both LDLR and LRP (LDLR-/-LRP-/-, negative control cells). Two hours after DiI-labeled apoB100-LDL was added to the medium, the uptake into LDLR-/-LRP+/- cells was significantly higher than in LDLR+/+LRP-/- negative control cells (Figure 4A). The uptake of apoB100-LDL by the LDLR+/+LRP-/- cells was also higher than in negative control cells. Uptake of apoB100-β LDL was not increased by the expression of either LDLR or LRP. The opposite pattern of uptake was observed in studies with VLDL. In 30 minutes, DiI-labeled apoB100-β-VLDL was efficiently taken up by cells expressing LDLR, and particularly LRP, whereas no such increase over the uptake by negative control cells was found in cells given DiI-labeled-apoB100 VLDL (Figure 4B).

We next blocked hepatic LRP function using Ad-RAP to determine the role of the LRP in the clearance of apoB100-β VLDL in vivo. While basal cholesterol and triglyceride levels in plasma are significantly lower in Ldlr-/- Apobec1-/- mice with apoB100-β than those with normal apoB100, the levels 5 days after Ad-RAP injection were not different between the 2 groups of mice. FPLC analyses showed a similar accumulation of cholesterol in the VLDL fraction after 5 days in both groups (supplemental Figure I). These data imply that apoB100-β VLDL is removed by receptors inhibited by RAP, such as LRP. We also examined the contribution of HSPG binding by incubating LDLR+/+LRP-/- cells at 4°C with DiI-VLDL. The amount of VLDL released from the surface by heparinase after 2 hours was not significantly different (supplemental Figure II), suggesting that apoB100-β does not affect the binding ability of VLDL to proteoglycans.

To gain further insight into the apparent enhancement of lipoprotein clearance in Ldlr-/- Apobec1-/- mice with apoB100-β, we analyzed the expression of the Apob, Apoe,
and Lrp1 genes in the liver by real-time PCR. Although there was no Apob genotype effect on the mRNA levels for Apob and Lrp1, liver expression of Apoe was approximately twice as high in mice with apoB100-1/H9252 as in mice with wild-type apoB100 (supplemental Figure III). These data, combined with the higher apoE:apoB protein ratio, suggest that the increased production of apoE protein may be contributing to the accelerated clearance of apoB100-1/H9252 containing VLDL-remnants and the resistance to hyperlipidemia in the apoB100-1/H9252 mice even in the absence of LDLR.

**Ldlr-/-Apobec1-/-** Mice With ApoB100-β Are Protected From Development of Atherosclerosis

High levels of LDL are a well-documented risk factor for atherosclerosis. Ldlr-/- Apobec-/- female mice with apoB100 had a significant size of lesions (34±6×10³µm²), even when they were on NC diet and were as young as 4 to 5 months old (Figure 5A). In marked contrast, the apoB100-β mutation demonstrated a significant atheroprotective effect. Although 3 of 5 Ldlr-/- Apobec-/- mice heterozygous for apoB100-β had visible plaques (16±7×10³µm²), there were absolutely no plaques seen in mice homozygous for the apoB100-β mutation. The overall effect of the Apob genotype on plaque development was P<0.002 by ANOVA. Feeding a HFW diet for 2 months accelerated the plaque development in the Ldlr-/- Apobec-/- mice with apoB100 (mean lesions size 54±8×10³µm²; n=5). In contrast, there were virtually no lesions present in apoB100-β mice but only very small foam cell aggregations (1.7±0.8×10³µm², P<0.002).

To examine whether direct VLDL scavenging by macrophages rather than LDL accumulation is responsible for the dramatic differences in atherosclerosis, we isolated peritoneal macrophages from Ldlr-/- mice and incubated with equal amounts of Dil-labeled VLDL in the medium. Uptake of the Dil-VLDL with apoB100 by the macrophages was a little more enhanced compared to that with apoB100-β, but the differences were not statistically significant (Figure 5B). A
To investigate the mechanisms for the uptake of apoB48- and apoB100-containing lipoproteins by the LDLR and by the LRP, Veniant et al previously characterized plasma lipoproteins in the \textit{Ldlr}\textsuperscript{-/-} mice homozygous for an “apoB48-only” allele or homozygous for an “apoB100-only” allele.\textsuperscript{24} The authors concluded that the LDLR plays a significant role in the clearance of both apoB100- and apoB48-containing lipoproteins, and that the LRP is important for apoB48- containing lipoproteins but has little if any capacity to remove apoB100-containing lipoproteins from the plasma. The “apoB100-only” \textit{Ldlr}\textsuperscript{-/-} mice are phenotypically identical to the \textit{Ldlr}\textsuperscript{-/-}\textit{Apobec1}\textsuperscript{-/-} mice with wild-type apoB100 we used in the current study. Interestingly, the plasma lipids and lipoprotein distribution in \textit{Ldlr}\textsuperscript{-/-}\textit{Apobec1}\textsuperscript{-/-} mice with mutant apoB100-\textbeta are very similar to “apoB48-only” \textit{Ldlr}\textsuperscript{-/-} mice, despite that apoB100-\textbeta retains the full length of the apoB protein. Both strains of mice have no substantial accumulation of LDL particles, suggesting that apoB100-\textbeta remnants, like apoB48-only remnants, are cleared via LRP in the absence of LDLR and that the length of apoB protein does not influence this process. However, whereas Veniant et al showed that LRP inhibition with RAP of “apoB48-only” \textit{Ldlr}\textsuperscript{-/-} mice leads to higher VLDL levels than in “apoB100-only” \textit{Ldlr}\textsuperscript{-/-} mice,\textsuperscript{23} VLDL accumulation in the \textit{Ldlr}\textsuperscript{-/-}\textit{Apobec1}\textsuperscript{-/-} mice with apoB100 and those with apoB100-\textbeta were similar after LRP inhibition, suggesting that additional mechanisms may present in the protective effects noted in the apoB100-\textbeta-producing mice.

The \textit{Ldlr}\textsuperscript{-/-}\textit{Apobec1}\textsuperscript{-/-} mice with apoB100-\textbeta do not accumulate substantial LDL particles in plasma even when fed a HFW diet. This lack of LDL accumulation occurs despite the inability of apoB100-\textbeta-containing LDL to be cleared in vitro. Importantly, the livers of \textit{Ldlr}\textsuperscript{-/-}\textit{Apobec1}\textsuperscript{-/-} mice with apoB100-\textbeta appear to produce a smaller number of larger VLDL particles than the livers of the \textit{Ldlr}\textsuperscript{-/-}\textit{Apobec1}\textsuperscript{-/-} mice with apoB100, despite equal expression of the Apob gene and protein synthesis. However, the reduction of LDL cholesterol in the \textit{Ldlr}\textsuperscript{-/-}\textit{Apobec1}\textsuperscript{-/-} mice with apoB100-\textbeta is more than the reduction of apoB secreted. This is in contrast to the report by Crooke et al that \textit{Ldlr}\textsuperscript{-/-} mice treated with an apoB antisense oligonucleotides had a reduction of apoB mRNA by 74% but still had 48% levels of LDL-cholesterol compared to the pretreatment levels.\textsuperscript{24} It has long been recognized that the larger VLDL particles are removed faster and less likely converted to LDL than smaller VLDL, and a larger surface area of apoB100-\textbeta VLDL may allow more apoE to associate with the particle and facilitate LRP mediated uptake.\textsuperscript{25,26} The \textit{Ldlr}\textsuperscript{-/-}\textit{Apobec1}\textsuperscript{-/-} mice with apoB100-\textbeta have plasma lipoproteins containing 4-fold higher apoE protein per particle, and 2-fold higher \textit{Apoe} gene expression in the liver than mice with apoB100. All together, these changes favor the enhanced clearance of apoB100-\textbeta containing particles via the LRP.

We also observed an enhanced degradation of apoB100-\textbeta in primary hepatocytes from the \textit{Ldlr}\textsuperscript{-/-}\textit{Apobec1}\textsuperscript{-/-} mice with apoB100-\textbeta in culture. Whether the accelerated degradation of apoB100-\textbeta results from its abnormal protein folding or is the consequence of enhanced turnover has yet to be determined. Although a limited apoB protein available for lipoprotein assembly could account for the larger size of VLDL, a

\begin{figure}[h]
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\caption{A, Atherosclerotic plaque sizes at the aortic roots of 4-month-old female \textit{Ldlr}\textsuperscript{-/-}\textit{Apobec1}\textsuperscript{-/-} mice. Number of animals is in parentheses. B, Macrophage VLDL uptake. \textit{Ldlr}\textsuperscript{-/-}\textit{Apobec1}\textsuperscript{-/-} macrophages were incubated with Dil-labeled VLDL with apoB100 or with apoB100-\textbeta. Cellular florescence is expressed as Arbitrary Units (AU) per cell gram of cell protein.}
\end{figure}

similar result was obtained in the macrophages isolated from wild-type mice (data not shown), indicating that the scavenging by macrophages of the VLDL is not affected by the apoB100-\textbeta mutation.

Taken together our data demonstrate that, even in the absence of LDLR, a mutation in the putative receptor binding domains of apoB prevents LDL accumulation, and dramatically reduces atherosclerosis.

**Discussion**

LDL is generated in the circulation from VLDL produced by the liver after lipolysis and exchange of surface apolipoproteins. During this conversion, conformational changes occur in its structural protein, apoB100, allowing for the exposure of domain(s) that interact with LDLR.\textsuperscript{15–17} Exposure of the receptor-binding domain and subsequent binding of apoB100 to the LDLR is the major pathway for the clearance of LDL cholesterol by the liver, as illustrated by the marked accumulation of LDL in plasma of patients and in animals lacking LDLR.\textsuperscript{18–21} Particles that lack full-length apoB, such as apoB48-containing chylomicron remnants, can acquire apoE which mediates efficient clearance of these particles by the LDLR, LRP, and other receptors which may act in concert with proteoglycans.\textsuperscript{22}

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question remains as to whether apoB100-β fails to form subsets of VLDL particles that are predestined to form LDL particles. Studies have demonstrated that a substantial amount of newly synthesized apoB protein is degraded rather than secreted, and that its interaction with LDLR channels apoB toward presecretory degradation. Reuptake of newly synthesized lipoproteins by LDLR can also attenuate VLDL secretion, and both apoE and apoB are important for this process. Loss of these regulations results in an increased secretion of apoB proteins and smaller, underlipidated VLDL particles in humans and mice that lack functional LDLR.

The metabolism of lipoproteins with apoB100-β mutation is consistent with other observations. For example, truncations of apoB on the C-terminal side of amino acid 3500 result in more efficient clearance of VLDL. Individuals heterozygous for a R3480P mutation in apoB exhibit hypobetalipoproteinemia because of a reduced conversion of VLDL to LDL, despite that this mutation caused reduced binding of LDL to the LDLR. Similarly, milder than expected hyperlipidemia in individuals with familial defective apolipoprotein B-100 attributable to mutations at R3500 has been attributed to an enhanced removal of apoE-containing VLDL and decreased production of LDL. Individuals heterozygous for a R3480P mutation in apoB exhibit hypobetalipoproteinemia because of a reduced conversion of VLDL to LDL, despite that this mutation caused reduced binding of LDL to the LDLR.

In conclusion, we have demonstrated that the mutation in the LDLR binding domains of apoB100 dramatically protects mice from both hypercholesterolemia and atherosclerosis that develop in the absence of LDLR. Our observations raise an intriguing possibility that an interference of the exposure of the putative LDLR-binding domains to the lipoprotein surface may indeed enhance remnant clearance through apoE-mediated mechanisms. This may be applicable as a potential therapeutic approach for preventing LDL accumulation in patients with familial hypercholesterolemia.

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Disclosures
None.

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Absence of Hyperlipidemia in LDL Receptor-deficient Mice having Apolipoprotein B100 without the Putative Receptor-Binding Sequences

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Detailed Methods

Lipid/lipoprotein analyses. Plasma was isolated from animals that had been fasted for 4 hr. Animals were anesthetized with Avertin (2,2,2-tribromoethanol), and blood was collected from the retro-orbital sinus into tubes containing EDTA (final concentration of 8 mM), and stored on ice. Plasma was removed after centrifugation at 8,000g for 10 min at 4 °C, and the plasma concentrations of total cholesterol (kit cat#439-17501; Wako Chemicals) and triglyceride (kit cat#2150-101; Stanbio Laboratory) were determined by enzymatic colorimetric methods. Liver and fecal cholesterol and triglyceride concentrations were determined using the same kits as in the plasma after extraction with chloroform/methanol as previously described by Folch et al.¹

Lipoproteins in pooled plasma samples (100 µL) were fractionated by fast protein liquid chromatography (FPLC) using a Superose 6 HR10/30 column (Pharmacia Biotech Inc., Piscataway, New Jersey, USA) at a flow rate of 0.4 ml/min. Cholesterol and triglyceride concentrations were measured in each fraction (0.5 ml) by the method described above. For apolipoprotein analysis, 1 ml of pooled plasma was separated by sequential density
ultracentrifugation into seven fractions ranging in densities from <1.006 g/ml to >1.21 g/ml, using the procedure described by de Silva et al. Lipoprotein fractions were dialyzed against 10 mM Tris, pH 7.4, 1 mM EDTA, 150 mM NaCl.

To determine relative amounts of apoB100, apoB-100β, and apoE, VLDL (1.006 – 1.02 g/ml), IDL (1.02 – 1.06 g/ml) and LDL (1.06 – 1.10 g/ml) density fractions of the plasma from \( Ldlr^{-/-} Apobec1^{-/-} \) mice with wild type apoB100 and with apoB100-β were subjected to electrophoresis in a 3-20% denaturing SDS-polyacrylamide gel. Plasma was pooled from 4 mo old male mice (3 animals per group) fed a HFW for 2 months, and fasted for 4 hours.

**Tissue weight analysis.** Female mice of each genotype were fasted for 4h prior to analysis. Visceral fat refers to combined weight of the two gonadal fat pads and two perirenal fat pads. Subcutaneous fat refers to combined weight of left and right inguinal fat pads. All animals were 5-8 month old females on a high fat diet for 2 months prior to analysis.

**Triglyceride secretion rate** Female mice of each genotype were fasted for 4h prior to injection of Tyloxapol (Triton WR-1339; Sigma Chemical Co., St. Louis, Missouri, USA) via tail vein at a dose of 0.7 mg/g body weight. Plasma was collected for triglyceride measurement at time points of 0, 5, 15, 30, 60, and 120 minutes after injection.

**Particle size.** Plasma samples from \( Ldlr^{-/-} Apobec1^{-/-} \) mice with apoB100 or apoB100-β at 2 hr after Tyloxapol injection were pooled (n = 3 in each group) and the d < 1.006 g/ml fraction was prepared by ultracentrifugation. Lipoprotein particle diameters were determined by dynamic light scattering analysis with a Microtrac Series 150 Ultrafine particle analyzer fitted with a
flexible conduit-sheathed probe tip (UPA-150; Microtrac, Clearwater, Florida, USA). Raw particle-size distributions were converted to population percentiles, which were used to calculate the median particle diameter for each decile of lipoprotein size distribution.

**LRP inhibition by adenoRAP.** Recombinant adenovirus containing the cDNA for rat receptor-associated protein (Ad-RAP) was kindly provided by Dr. Joachim Herz at The Southwestern Medical Center at Texas and prepared in 293 cells as described. Four 8 week old male mice of each genotype were injected via tail vein with 200 µl of Ad-RAP diluted in PBS, at a concentration of 1.5 x 10^9 PFU. Plasma lipoprotein analyses were carried out 5 days after Ad-RAP injection.

**Gene expression.** Gene expression in the liver was analyzed by real time PCR. Liver pieces of approximately 100 mg were dissected from the median lobe and placed in RNA-later solution (Ambion) for 24 hr prior to analysis. mRNA was purified using an automated nucleic acid workstation ABI 6700 and real-time PCR was performed in an ABI PRISM 7700 Sequence Detector (Applied Biosystems). mRNA levels were normalized to β-actin expression. Primer sequences used were:

**Apoe:** Forward 5’-TCCAGGAAGAGCTGCAGAG-3’,  
Reverse 5’-AGTTCCGTATAGTGTCCCTC-3’,  
Probe 5’-Fam CCCAAGTCACACAAGAACTGACGGCA Tamra-3’.

**Apob:** Forward 5’-CTTCTCTTCATATAAGGCGTG-3’,  
Reverse 5’-CATTGTTAGACAAACAGACGCT-3’,  
Probe 5’-Fam TGCAAGACCTTCCCGGGCA Tamra-3’. 
\[\text{Lrp1:} \quad \text{Forward 5'}-\text{CCCTGACAAGCCTACCAACT-3'}, \]
\[\text{Reverse 5'}-\text{TCCGTGCTGGCCAGGGAAT-3'}, \]
\[\text{Probe 5'}-\text{Fam ACCCAGTGTATGCCACGCTCTACATG Tamra-3'}. \]

\[\beta\text{-actin:} \quad \text{Forward 5'}-\text{CTGCCTGACGGCCAGGTC-3'}, \]
\[\text{Reverse 5'}-\text{CAAGAAGGAAGGCTGGAAAAGA-3'}, \]
\[\text{Probe 5'}-\text{Fam CACTATTGGCAACGAGCGTTCCG Tamra-3'}. \]

**Lipolysis.** Plasma lipolysis assay was adapted from a technique previously described. Basal blood samples were collected retro-orbitally from \(Ldlr^{+/-}Apobec1^{+/-}\) mice homozygous for apoB100 or apoB100-\(\beta\). Mice were then injected subcutaneously with 100 Units/kg body weight of heparin. After 10 minutes, a second blood sample was collected to serve as a source of lipases. 50 µl of pre-heparin plasma was then incubated at 37°C with 5 µl of post-heparin plasma, or 5 µl PBS as a control. FFA release was measured at 15 minute intervals after the addition of post-heparin plasma. Lipolysis rate is estimated from the slope of the linear regression of FFA (nmol/min).

**ApoB synthesis, secretion and degradation in primary mouse hepatocytes** Primary hepatocytes were isolated from \(Ldlr^{+/-}Apobec1^{+/-}\) mice homozygous for apoB100 or apoB100-\(\beta\) as described. The yield of hepatocytes ranged from \(3 \times 10^6\) to \(6 \times 10^6\) cells/g of liver, and viability was greater than 80%. The cells were plated onto 60-mm mouse collagen IV-coated dishes (Falcon) at a density of \(1.2 \times 10^6\) viable cells/dish and cultured for 16h in low glucose DMEM.
containing 1% bovine serum albumin. Cells were incubated in serum-free media for 4 hours prior to a 30 minute-pulse with 0.5 ml medium containing \( ^{35}\text{S} \) methionine (100 µCi/ml, Amersham). Cells were then chased for 1 and 4 hours with fresh medium containing an excess of cold methionine.\(^6\)-\(^8\) Cell-associated apoB and apoB in the medium were immunoprecipitated using a Goat anti-human apoB antibody (Calbiochem), separated by SDS-PAGE, and visualized by a Fla-3000 phosphoimager (FujiFilm).

**Di-I labeling and uptake by fibroblasts in culture.** The VLDL and LDL fraction were isolated from pooled plasma by ultracentrifugation at d<1.006 g/ml and labeled with 1,1\(^\prime\)-dioctadecyl-3,3,3\(^\prime\),3\(^\prime\)-tetramethylindocarbocyanine perchlorate (DiI C\(_{18}\); Molecular Probes Inc.), as described.\(^7\) Equal amount of total DiI-labeled protein was added in 1 ml serum-free, phenol red-free medium to mouse fibroblasts plated at 5x10\(^4\) cells in 6-well plates. After incubation at 37° C for 30 min or 2 hr, cellular lipid was extracted with isopropanol and measured with a microscope fluorometer as described.\(^9\)

**Uptake of DiI-VLDL by the peritoneal macrophage.** Macrophages were obtained from the peritoneal cavity of the \( Ldlr^{-/-} \) mice 4 days after intra-peritoneal injection of 1 ml of 4% (w/v) thioglycolate. The cells obtained were washed with DMEM, spun at 1000 g for 5 minutes and plated in 12-well plates at a density of 6 x 10\(^5\) cells/well in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Cells were washed 2 hours later to remove non-sticking cells, and cultured at least 24 hours in DMEM 10% FBS prior to
experiments. Macrophage uptake of DiI-VLDL with apoB100 or with apoB100-β was measured as described above.

**125-I VLDL Clearance, Conversion and Tissue Uptake in vivo.** VLDL was isolated from \(Ldlr^{-/-}\) \(\text{Apobec1}^{-/-}\) mice homozygous for apoB100 or apoB100-β by ultracentrifugation and labeled with \(^{125}\text{I}\) as previously described and injected to \(Ldlr^{-/-}\) mice via the tail vein for the *in vivo* clearance assay. Plasma was collected at 0, 2, 5, 15, 30, 60 and 120 minutes after injection and radioactivity remaining in plasma counted on a Wallac 1470 Wizard Gamma Counter (EG&G Wallac, Turku, Finland). A dose of \(5 \times 10^5\) counts, diluted in 200 µl of PBS, was administered to each mouse, and at least 3 mice were used for each VLDL. After 2 hours, plasma was isolated from mice injected in the clearance assay and lipoprotein fractions were obtained by sequential ultracentrifugation. Counts were measured in VLDL (1.006 – 1.02 g/ml), IDL (1.02 – 1.06 g/ml) and LDL (1.06 – 1.10 g/ml) density fractions and expressed as a percentage of the count registered by injected dose VLDL.

In a separate experiment, tissues were isolated from each mouse at 20 min after \(^{125}\text{I}\)-VLDL injection and radioactivity was counted.

**Atherosclerosis.** Mice were fed a HFW diet for three months before euthanization with a lethal dose of 2,2,2-tribromoethanol. The heart and the vascular tree were perfused at physiological pressure with 4% phosphate buffered paraformaldehyde, pH 7.4. Morphometric analysis of plaque size at the aortic root was made on four segments using methods previously described.
Supplemental Figure I

Figure I. **VDL and LDL cholesterol distribution after RAP mediated blocking of LRP.**

Total plasma cholesterol (A) and triglyceride (B) at 0 and 5 days after injection of adRAP. White bars and black bars indicate Ldlr\(^{-/-}\) Apobec\(^{-/-}\) mice with wild type apoB100 and those with apoB100-\(\beta\), respectively. The distribution of plasma VLDL and LDL cholesterol in Ldlr\(^{-/-}\) Apobec\(^{-/-}\) mice with wild type apoB100 (C) and in those with apoB100-\(\beta\) (D). Blood samples were collected from mice after a 4 hr fast prior (filled symbols) and at 5 days after injection of adRAP (open symbols). A total of 50 \(\mu\)l plasma, pooled from groups of 3 male mice, was size-fractionated by FPLC and cholesterol amount in each fraction was measured. The plasma lipids and lipoprotein distributions in the two groups of mice were not different after the blocking of LRP by RAP. ** denotes P<0.005 between mice with apoB100 and mice with apoB100-\(\beta\).
Figure II. **Heparinase releasable VLDL.** Dii-VLDL released by LDLR-/-LRP-/- fibroblasts after treatment with heparinase. Cells were incubated at 4°C for 2 hr with Dii-VLDL. VLDL was isolated from Ldlr-/-Apobec1-/- mice homozygous for apoB100 or apoB100-β.
Supplemental Figure III.

Figure III. **Hepatic Apoe, Apob and Lrp1 gene expression.** Liver tissues were isolated from 6-8 month old mice fed a HFW diet for 1-2 months. mRNA amounts are normalized to β-actin and expressed relative to the mean of B100/B100 mice as 100%. Error represents SEM. The number of animals is shown inside each bar. * P<0.002.
REFERENCES


Book Chapters: