Severe Hypercholesterolemia, Hypertriglyceridemia, and Atherosclerosis in Mice Lacking Both Leptin and the Low Density Lipoprotein Receptor*

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Leptin-deficient mice (ob/ob) are an excellent murine model for obesity, insulin resistance, and diabetes, all of which are components of a multiple risk factor syndrome that, along with hypercholesterolemia, precipitates a potential high risk for atherosclerosis. In the current study, we show an unexpectedly severe hyperlipidemia in ob/ob mice on a background of low density lipoprotein receptor (LDLR) deficiency (−/−). Doubly mutant mice (LDLR−/−; ob/ob) exhibited striking elevations in both total plasma cholesterol (TC) and triglyceride (TG) levels (1715 ± 87 and 1016 ± 172 mg/dl, respectively), at age 3–4 months, resulting in extensive atherosclerotic lesions throughout the aorta by 6 months. Lipoprotein analyses revealed the elevated TC and TG levels to be due to a large increase in an apoB-containing broad-β remnant lipoprotein fraction. While fasting, diet restriction, and low level leptin treatment significantly lowered TG levels, they caused only slight changes in TC levels. Hepatic cholesterol and triglyceride contents as well as mRNA levels of cholesterologenic and lipogenic enzymes suggest that leptin deficiency increased hepatic triglyceride production but did not change cholesterol production in ob/ob mice regardless of their LDLR genotype. These data provide evidence that the hypertriglyceridemia and hypercholesterolemia in the doubly mutant mice are caused by distinct mechanisms and point to the possibility that leptin might have some impact on plasma cholesterol metabolism, possibly through an LDLR-independent pathway. This model will be an excellent tool for future studies on the relationship between impaired fuel metabolism, increased plasma remnant lipoproteins, diabetes, and atherosclerosis.

Dyslipidemia, diabetes, and obesity are among the many risk factors associated with coronary artery disease. Although many human patients present with varying combinations of these risk factors, most animal models produced to date have focused on only one or two and their effects on atherogenesis. Leptin-deficient mice (ob/ob)[1] are an excellent murine model for obesity, insulin resistance, and diabetes, all of which are components of a multiple risk factor syndrome that, along with hypercholesterolemia, precipitates a potential high risk for atherosclerosis (1, 2). These mice also have fatty livers, presumably due to hepatic overproduction of triglycerides, reflecting an imbalanced energy metabolism. However, they show only a modest increase in plasma triglyceride and HDL cholesterol levels (3) and thus do not develop atherosclerosis on a regular chow diet as shown in the current study. It has been reported that ob/ob mice might have an impaired secretion of very low density lipoproteins (VLDL) (4); however, extensive studies on apoB-containing lipoprotein metabolism in these mice have not been undertaken.

Remnant lipoprotein particles are the apolipoprotein B (apoB)-containing lipoproteins that remain in the circulation after hepatic VLDL and intestinal chylomicrons are lipolyzed. They include lipoproteins in the size range of VLDL, intermediate density lipoproteins (IDL), and low density lipoproteins (LDL). Remnant particles can exist in individuals in a normolipidemic state, especially postprandially, but are generally processed by lipoprotein lipase and quickly cleared from plasma. However, the triglyceride-rich lipoproteins that are increased in diabetic patients as well as cholesterol-rich remnants referred to as β-VLDL that are found in patients with type III hyperlipidemia are also remnant lipoproteins and in these cases are known to be atherogenic. Several mouse models of chronically increased remnant lipoproteins have recently been created by targeted disruption of genes that are crucial in lipoprotein metabolism. These include apoE-deficient mice (5, 6), LDL receptor-deficient mice on a high fat diet (7), and LDLR/LDLR-related protein (LRP) double knockout mice (8), confirming the importance of apoE as a ligand and LDLR and LRP as receptors for plasma remnant clearance. Recently, it was reported that disruption of the LDLR gene unmasked severe hyperlipidemia with increased remnant particles in sterol regulatory element-binding protein (SREBP)-1a transgenic

[1] The abbreviations used are: ob/ob, leptin-deficient mice; LDLR, low density lipoprotein receptor; TC, total plasma cholesterol; TG, total plasma triglycerides; LRP, LDLR-related protein; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoprotein; IDL, intermediate density lipoproteins; SREBP, sterol regulatory element-binding protein; TgSREBP-1a;LDLR−/−, SREBP-1a transgenic mice crossed with LDLR(−/−) mice; apo, apolipoprotein; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline.

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mice (TgSREBP-1a;LDLR−/−), providing another model for studying the effects of excessive remnant lipoproteins in plasma (9). In this model, hepatic overexpression of nuclear SREBP-1a, a potent transcription activator for both cholesterol and triglyceride synthesis, caused overproduction of lipids and large VLDL in the liver. Normally, these large particles can be taken up by the LDLR very efficiently, which prevented the development of hyperlipidemia in the TgSREBP-1a mice; however, in the absence of the LDLR, remnant lipoproteins accumulated in plasma, providing support for the importance of the LDLR in remnant clearance. The TgSREBP-1a;LDLR−/− mouse model is different from other hyperlipidemic animal models because hepatic lipid overproduction, rather than simply a lack of lipoprotein clearance, contributes to the pathological mechanism. This observation prompted us to study the metabolic consequences of hepatic triglyceride overproduction as seen in ob/ob mice, on the background of a state of impaired remnant clearance.

We sought to analyze the effects of leptin deficiency on remnant lipoprotein metabolism by producing mice that were deficient in both leptin and the LDLR (LDLR−/−;ob/ob). These doubly mutant mice showed extreme elevations in both plasma cholesterol and triglyceride levels and also had extensive atherosclerotic lesions throughout the aorta. While fasting, diet restriction, and low level leptin treatment of LDLR−/−;ob/ob mice significantly lowered TG levels, they caused only slight changes in total plasma cholesterol (TC) levels. This model supports the idea that a relationship exists between energy imbalance/hepatic overproduction of triglycerides and plasma remnant accumulation during impaired lipoprotein clearance.

EXPERIMENTAL PROCEDURES

Animals—LDLR(−/−) mice (10) were back-crossed onto a C57Bl/6 background to the 10th generation. ob/+ mice on a C57Bl/6 background were purchased from Jackson Laboratories and were also maintained in our colonies. To obtain leptin deficient (ob/ob) on a background of LDLR deficiency, ob/+ mice were crossed with LDLR(−/−) mice, and the F1 progeny of these matings (LDLR+/−;ob/+) were then crossed to obtain mice that had either zero, one, or both normal LDLR alleles and were leptin-deficient (LDLR−/−;ob/ob, LDLR+/−;ob/ob, and LDLR+/+;ob/ob, respectively) as well as control LDLR(−/−), LDLR(+/−), and wild type mice. Male and female mice were caged separately, and data was combined for all experiments, since no gender difference was noted. Mice were maintained and cared for according to the regulations of the Tokyo University Animal Care Committee. Mice were kept in microisolator cages with 12-h light/dark cycles and were fed *ad libitum* except during food restriction procedures. All mice were fed a normal rodent chow diet containing 0.075% cholesterol (MF; Oriental Yeast Co., Ltd. (Osaka, Japan)). A cholesterol-free diet was also purchased from Oriental Yeast Co. and fed to mice *ad libitum* for the cholesterol-free diet studies. For caloric restriction, mice were given 1.6 g of normal chow diet per day, and control age-matched mice were fed *ad libitum*.

Plasma Analyses—Blood collections were performed via optical venous plexus puncture with EDTA-treated tubes. Collections were performed between 10 and 12 a.m. on animals fed *ad libitum*. Samples were preserved with EDTA and NaN$_3$. Plasma cholesterol, triglyceride, and HDL cholesterol levels were measured with colorimetric assays by Determiner TC555, TG555, and HDL-C (Kyowa Medex, Co., Ltd. Tokyo, Japan), respectively. Glucose was measured by a standard enzymatic method. Insulin levels were tested with an enzyme-linked immunosorbent assay kit by LABIS (Japan) for mouse insulin. For analysis of lipoprotein distribution, pooled plasma samples from three mice per group were subjected to high performance liquid chromatography (HPLC) (SRL, Japan). Estimation of HDL levels in some samples were obtained from HPLC plots by calculation of the area under the curve. Agarose gel electrophoresis for lipoproteins was performed per standard protocol on gels purchased from Ciba/Corning.

Determination of Liver Cholesterol and Triglyceride Content—Livers were immediately collected and snap frozen in liquid nitrogen. A 50-mg piece of liver was homogenized in PBS. Folch's reagent (CHCl$_3$/MeOH, 2:1) (0.75 ml) was added to the homogenate. The nonaqueous phase was collected, and 30 μl of 200 mg/ml Triton X-100 in CHCl$_3$ was added. Samples were dried and used for cholesterol and triglyceride analysis as

TABLE I
Blood TC and TG levels in 12–16-week-old mice

Mice were maintained on a chow diet (0.075% cholesterol). TC and TG (mg/dl) of 3–4-month-old mice were measured as described under "Experimental Procedures." Values are given as the mean ± S.E.

Genotype	n	Cholesterol	Triglyceride
		mg/dl	
Wild type	4	81 ± 16	60 ± 12
LDLR(+/−)	3	149 ± 11	100 ± 12
LDLR(−/−)	6	265 ± 40	161 ± 25
LDLR+/+;ob/ob	5	119 ± 25	120 ± 12
LDLR+/−;ob/ob	6	282 ± 29	78 ± 8
LDLR−/−;ob/ob	6	1715 ± 87	1016 ± 172

described above for plasma samples.

RNA Preparation and Northern Blotting—Total RNA was prepared from the snap frozen livers with TRIzol reagent (Life Technologies, Inc.). RNA (12 μg) was electrophoresed through formalin-denatured agarose gels and transferred to Hybond-N membranes (Amersham Pharmacia Biotech). cDNA probes were prepared as described (11, 12). Probes were labeled with [α-³²P]dCTP using Megaprime DNA Labeling System kit (Amersham Pharmacia Biotech). Membranes were hybridized with the radiolabeled probes in Rapid-hyb Buffer (Amersham Pharmacia Biotech) and washed in 0.1× SSC, 0.1% SDS at 65 °C. Membranes were exposed to Eastman Kodak Co. XAR-5 film for 2–5 h at −80 °C.

Lipoprotein Lipase Measurements—Lipoprotein lipase measurements in postheparin plasma (10 min after intravenous injection of 0.1 mg/Kg heparin) were performed using a modification of the standard technique by Muir (21).

Leptin Treatment—Leptin was purchased from Calbiochem and was prepared according to the manufacturer's instructions. Leptin was diluted in PBS for individual mice according to their weight, and 0.3 μg/g body weight was injected in 100-μl doses per day.

*Atherosclerotic Lesion Analyses*—Whole aortae were collected and stained with Sudan IV as described (7, 13). Cross-sections of proximal aorta were prepared and stained with Oil-Red-O by SKK (Japan) per standard techniques as described (14).

Statistical Analyses—All values are stated as mean ± S.E., and differences between groups were evaluated with Student's *t* test.

RESULTS

Plasma Analyses—Blood was collected from wild type, LDLR(−/−), LDLR(+/−), LDLR+/+;ob/ob, LDLR+/−;ob/ob, and LDLR−/−;ob/ob mice that were 3–4 months of age. Plasma was analyzed for TG and TC levels (Table I). LDLR+/+;ob/ob mice had slightly elevated TC levels (119 ± 25 mg/dl), as was previously reported (3). LDLR+/−;ob/ob mice showed a significant elevation in TC levels to 282 ± 29 mg/dl with no increase in TG levels; however, the LDLR−/−;ob/ob mice showed a dramatic increase in TC levels with an average of 1715 ± 87 mg/dl with values ranging from 1430 to 2030 mg/dl. TG levels in these mice were also highly elevated (1016 ± 172 mg/dl).

Since well known phenotypes of ob/ob mice such as obesity, increased plasma glucose, and insulin levels are age-dependent, a time course study of TC and TG levels from additional animals was performed (Fig. 1). At 1 month (upon weaning), the LDLR−/−;ob/ob mice already displayed modest elevations in both TC and TG levels (369 ± 20 mg/dl and 137 ± 26 mg/dl, respectively; *n* = 6). These values increased significantly to 716 ± 48 and 393 ± 97 after mice were placed on a normal diet for 2 weeks. The LDLR−/−;ob/ob mice showed an age-dependent increase in TC and TG levels between 1 and 4 months, with values becoming maximal at 3–4 months, followed by a gradual decrease to 585 ± 44 and 306 ± 36 mg/dl (*n* = 3) by 8 months. LDLR+/−;ob/ob mice also showed a similar pattern of reaching peak TC levels at 3–4 months of age and maintained stable TC levels of ~280 mg/dl thereafter. TG levels in LDLR+/−;ob/ob mice were not different from wild type animals at any time (Fig. 1). HDL levels in all groups of mice were estimated from HPLC results and remained unchanged over the course of the
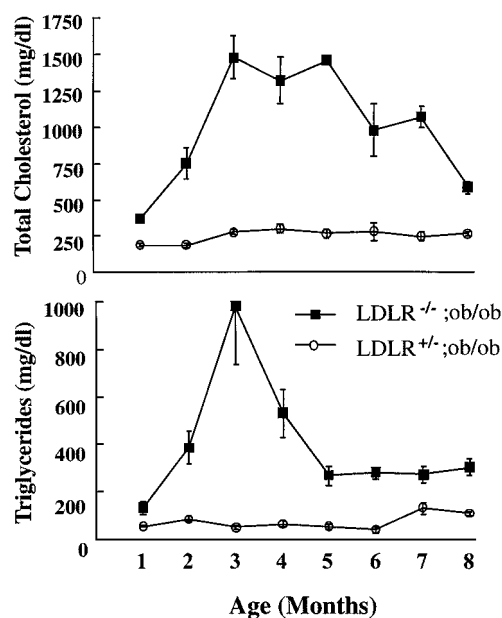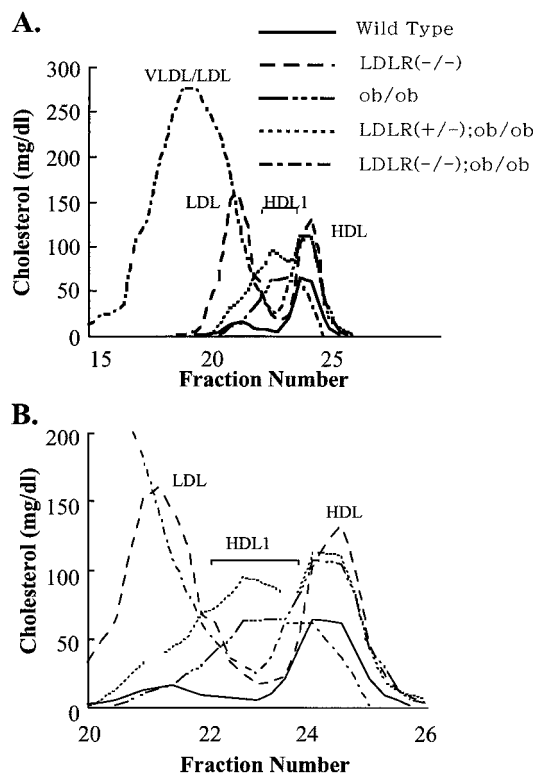
FIG. 1. **Time course study of TG and TC in LDLR−/−;ob/ob and LDLR+/−;ob/ob mice.** Blood was collected from LDLR−/−;ob/ob and LDLR+/−;ob/ob mice monthly, from age 1 month (at weaning) to 8 months and analyzed for TG and TC levels as described under "Experimental Procedures." LDLR−/−;ob/ob mouse values are shown in *closed squares*, and LDLR+/−;ob/ob values are in *open circles*. The *top panel* shows TC values, and the *bottom panel* shows TG levels. *Error bars* represent S.E. $n > 6$ for 1–6-month time points; $n = 3$ for 7- and 8-month data.


FIG. 2. **HPLC analyses of serum samples from LDLR−/−;ob/ob mice.** Blood was collected from 3–4-month old mice, and three pooled mouse serum samples for each group were separated by gel filtration chromatography as described under "Experimental Procedures." *A*, the entire protein profile with VLDL/IDL, LDL, HDL1, and HDL peaks indicated. *B*, a magnification of the HDL1/HDL region of the graph for better clarity. The *key* for genotype of mice is shown in the *inset* of *A*.

study. Wild type mice had HDL levels of 54 mg/dl at 3 months and 47 mg/dl at 8 months. LDLR(−/−) HDLs were slightly elevated to 69 and 65 mg/dl at 3 and 8 months. LDLR+/+;ob/ob mice were 89 and 56 mg/dl, LDLR+/−;ob/ob were 69 and 62 mg/dl, and LDLR−/−;ob/ob had levels of 90 and 94 mg/dl at 3 and 8 months, respectively. Glucose levels in LDLR−/−;ob/ob mice were ~200 mg/dl at 2 months and rose to between 300 and 400 mg/dl from 2 to 7 months. There were no significant differences between LDLR−/−;ob/ob and LDLR+/−;ob/ob mice during the study (data not shown). Insulin levels in LDLR−/−;ob/ob and LDLR+/−;ob/ob mice rose steadily from ~30 to ~300 ng/ml blood between 1 and 7 months (data not shown).

Lipoprotein Profiles—Plasma samples from 12–16-week-old mice were further analyzed for the distribution of lipoproteins (Fig. 2). As expected, wild type mouse plasma contained primarily HDL-sized lipoproteins. LDLR+/+;ob/ob mice showed a unique profile containing mostly HDL with a shoulder on the HDL peak with particles sized between LDL and HDL, as has been recently described (15). This peak most likely contains HDL1 particles and will be referred to as such. LDLR(−/−) mice displayed elevated levels of LDL, similar to previous reports (10). The distribution of lipoproteins in LDLR+/−;ob/ob mice was similar to the LDLR+/+;ob/ob mice except that the HDL1 peak was higher and broader. In contrast, plasma from LDLR−/−;ob/ob mice contained a severely elevated and broadened lipoprotein peak ranging from VLDL/IDL-sized particles to LDL-sized particles, presumably remnant lipoproteins, and retained HDL levels similar to wild type animals.

Further analysis of lipoprotein profiles was performed by lipoprotein-agarose gel electrophoresis (data not shown). As expected, LDLR+/+;ob/ob mouse plasma had a pattern similar to wild type mice, although the α-band was slightly broader, presumably due to the HDL1 particles. Interestingly, LDLR+/−;ob/ob mice had both α and β migrating particles, and LDLR−/−;ob/ob plasma contained a broad β-migrating band similar to that seen from apoE(−/−) lipoproteins.

Whole lipoprotein fractions ($d < 1.21$) were separated from pooled plasma samples of each group ($n = 3$) and were subjected to SDS-polyacrylamide gel electrophoresis to analyze apoproteins (Fig. 3). LDLR(−/−) mice had elevated apoB-100 levels as compared with wild type mice (*lanes 1* and *2*). LDLR+/+;ob/ob mouse plasma contained barely detectable apoBs (*lane 3*); however, LDLR+/−;ob/ob plasma did have apoB-100 and low levels of B-48 (*lane 4*). The amounts of apoBs in these groups were consistent with their LDL peaks in the HPLC. In striking contrast, the LDLR−/−;ob/ob lipoproteins consisted of highly elevated levels of both apoB-100 and apoB-48, with a greater ratio of B-100 to B-48 particles (*lane 5*), indicating that their elevated TC and TG levels may be at least partially due to an increase in particle number. In addition, the LDLR−/−;ob/ob mice had an apparent increase in apoAIV and apoE. This is probably due to the increased remnant lipoproteins. In comparison, apoE(−/−) mouse lipoproteins are enriched in apoAIV, and LDLR(−/−) mice on a high fat diet show increases in apoE (7).

Lipoprotein lipase activities were measured in pooled samples from mice to determine if lipolytic activity was decreased in the LDLR−/−;ob/ob mice. Postheparin lipolytic activity for wild type mice was 0.45 μmol of FFA/ml/min. LDLR(−/−), LDLR+/+;ob/ob, and LDLR−/−;ob/ob all had slightly elevated lipolytic activity (0.61, 0.72, and 0.57 μmol of FFA/ml/min).

Liver Analyses—To determine if the increased plasma TC and TG in the LDLR−/−;ob/ob mice was related to hepatic cholesterologenic and lipogenic enzymes, livers were collected from 6-month-old animals and analyzed for both cholesterol and triglyceride content as well as various enzyme RNA levels. The livers of LDLR(−/−) mice as well as all three ob/ob groups were found to contain slightly, but significantly, higher levels
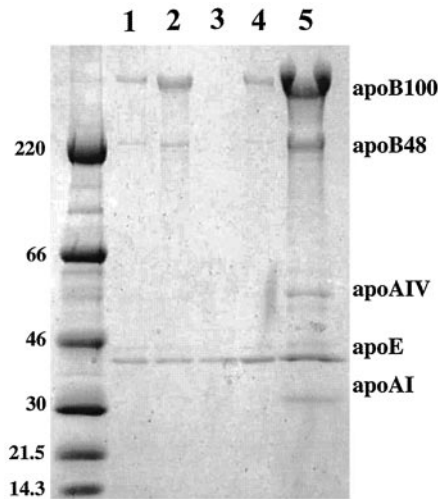
FIG. 3. **SDS-polyacrylamide gel electrophoresis of lipoproteins from LDLR−/−;ob/ob mice.** Lipoproteins were isolated from pooled serum samples of 3–4-month-old mice by ultracentrifugation to a density of 1.21 as described under "Experimental Procedures." Lipoproteins were loaded onto 3–20% SDS-polyacrylamide gels, electrophoresed, and stained with Coomassie Blue. Mouse genotypes are as follows: *lane 1*, wild type; *lane 2*, LDLR(−/−); *lane 3*, LDLR+/+;ob/ob; *lane 4*, LDLR−/−;ob/ob; *lane 5*, LDLR−/−;ob/ob.

TABLE II
Liver cholesterol and triglyceride content

Liver cholesterol and triglyceride content was measured as described under "Experimental Procedures." For all groups, $n = 3$. Values are given as mean $\pm$ S.E. in mg/g of liver.

Genotype	Cholesterol content[a]	Triglyceride content[b]
	mg/g of liver	
Wild type	1.77 ± 0.32	27.0 ± 5.2
LDLR(−/−)	2.91 ± 0.22	24.2 ± 3.3
LDLR+/+;ob/ob	4.13 ± 0.9	244 ± 4.2
LDLR+/−;ob/ob	3.45 ± 0.1	222 ± 40
LDLR−/−;ob/ob	5.01 ± 0.5	278 ± 40

[a] $p < 0.05$ wild type versus all other groups; LDLR(−/−) *versus* LDLR−/−;ob/ob.
[b] $p < 0.005$ wild type and LDLR(−/−) *versus* all ob/ob groups.

of cholesterol than wild type mice (Table II), which is consistent with previous reports (9, 16). LDLR−/−;ob/ob mice contained slightly higher levels of cholesterol than the other two ob/ob groups and the LDLR(−/−) mice ($p < 0.05$) (Table II). Triglyceride levels were ~10 times higher in the three ob/ob groups compared with the wild type and LDLR(−/−) mice, but there was no association between LDLR genotype and liver triglyceride content in the ob/ob mice. These data indicate that leptin deficiency causes a marked increase in liver triglyceride levels but only a small increase in liver cholesterol content.

Northern blot analyses on liver RNAs revealed that cholesterologenic enzymes HMG-CoA synthase (Fig. 4), HMG-CoA reductase and SREBP-2 (data not shown), were unchanged among all five groups. In contrast, lipogenic enzyme RNAs (SREBP-1, fatty acid synthase, ATP citrate lyase, and stearoyl-CoA desaturase-1) were increased in the ob/ob groups compared with wild type and LDLR(−/−); however, there was very little difference in the ob/ob mice between the three LDLR genotypes. ApoAI was slightly decreased in ob/ob mice; however, apoAII, and apoE RNA levels were unchanged in the ob/ob mice compared with controls. A control probe for the ribosomal enzyme 36B4 demonstrates equal loading (Fig. 4).

Special Diets—To determine the possible mechanism by which lipoproteins become elevated in the LDLR−/−;ob/ob mice, initial studies were focused on the possibility that the increased caloric intake of ob/ob mice was overwhelming and surpassed backup lipoprotein clearance systems when the LDLR was not present. To test this hypothesis, LDLR+/−; ob/ob and LDLR−/−;ob/ob mice were fasted for 2 days, after which glucose, TC, and TG levels were analyzed ($n = 3$ in each group). Glucose levels were reduced by 57 and 44% in LDLR+/−;ob/ob and LDLR−/−;ob/ob mice, respectively. Furthermore, TG levels fell by 58 and 53%, respectively, in the fasted mice. In contrast, TC levels increased slightly (although not significantly; $p = 0.12$) in the LDLR−/−;ob/ob mice from 585 ± 44 to 712 ± 47 mg/dl, while TC levels in the LDLR+/−;ob/ob mice were unchanged (265 ± 14 to 244 ± 12 mg/dl). These data are consistent with the faster turnover rate of fatty acids and triglycerides than cholesterol.

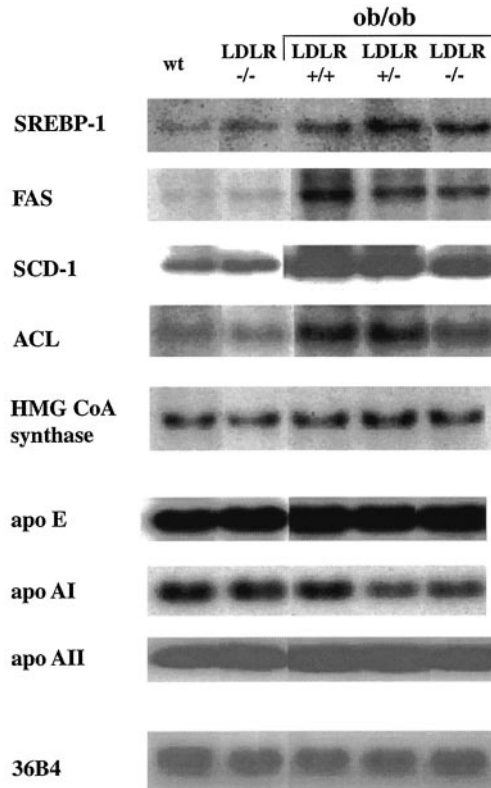Following this short term fasting experiment, a long term caloric restriction diet, in which 4-week-old LDLR−/−;ob/ob

FIG. 4. **Northern blot analysis of total liver RNA from LDLR−/−;ob/ob mice.** Livers were collected from 6-month-old mice, and total RNA was prepared as described under "Experimental Procedures." Total RNA was electrophoresed on formalin-denatured gels, transferred to nylon membranes, and probed with ^{32}P-labeled probes. *Lanes 1–5* contain wild type (*wt*), LDLR(−/−), LDLR+/+;ob/ob, LDLR+/−;ob/ob, and LDLR−/−;ob/ob RNA samples, respectively. Probes used (from *top* to *bottom*) were SREBP-1, fatty acid synthase (*FAS*), stearoyl-CoA desaturase (*SCD-1*), HMG-CoA synthase, ATP citrate lyase (*ACL*), apoE, apoAI, apoAII, and a control probe, 36B4.

mice were given only 50% of what a wild type mouse would consume, was initiated. Both diet-restricted mice ($n = 2$) and mice fed *ad libitum* ($n = 3$) showed a significant increase in TC over the 2-week study, confirming the age-dependent increase in TC; however, this increase was more drastic in the mice fed *ad libitum*, so that after 2 weeks there was a significant difference in TC levels between the two groups (Fig. 5). TG levels increased significantly in the control group but remained unchanged in the diet-restricted group (Fig. 5), a pattern that paralleled changes in glucose levels in the mice (data not shown). There was a slight reduction in HDL levels in both control and diet-restricted LDLR−/−;ob/ob mice (92 ± 35 to 89 ± 28 and 90 ± 35 to 79 ± 32, respectively); however, there were no significant differences, reflecting the smaller contribu-
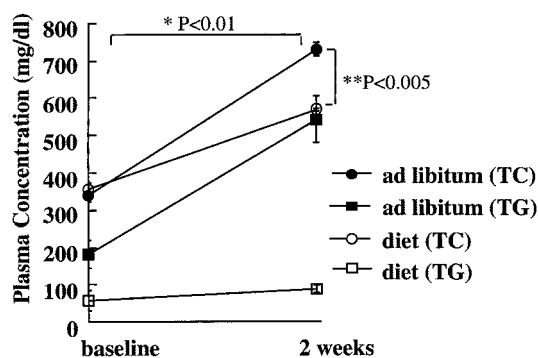
FIG. 5. **TC and TG of LDLR−/−;ob/ob mice placed on caloric restriction.** Four-week-old LDLR−/−;ob/ob mice were placed on a restricted diet (50% of wt calories/day) for 2 weeks ($n = 2$). Age-matched control mice were fed *ad libitum* ($n = 3$). Blood samples were collected before starting the diet and at 2 weeks. Serum samples were analyzed for TC and TG as described under "Experimental Procedures." TC are shown in *circles*, and TG are in *squares*. Diet-restricted mice are represented by *open shapes*, and control mice are represented by *filled shapes*. *Error bars* represent S.E. *, $p < 0.01$ for base-line *versus* 2-week values of all parameters excluding TG of diet-restricted mice. **, $p < 0.005$ for TC levels of diet-restricted and *ad libitum* mice at 2 weeks.

tion of HDL to the hyperlipidemia in these mice. Based on these studies, while the elevated TG levels in the LDLR−/−; ob/ob mice were basically associated with the amount of short term food consumption, the increased caloric intake of these mice, due to their leptin deficiency, cannot account for the elevations in TC levels.

To determine whether the increase in TC levels was derived from the cholesterol that was present in the normal chow diet, these mice were placed on a cholesterol-free diet. After 2 weeks on the cholesterol-free diet, there were no detectable changes in TC (542 ± 20 to 560 ± 102 mg/dl) or TG levels (272 ± 19 to 281 ± 34 mg/dl) of LDLR−/−;ob/ob animals. HDL levels in LDLR−/− mice were unchanged by the cholesterol-free diet (83 ± 12 to 86 ± 13.5 mg/dl). HDL levels in LDLR−/−;ob/ob mice were decreased from 75 ± 5 to 65 ± 13 mg/dl; however, it was not statistically significant. Glucose levels were reduced by 29% in LDLR+/−;ob/ob and LDLR−/−;ob/ob mice.

Leptin Treatment—To determine whether the high plasma TC and TG levels observed in the LDLR−/−;ob/ob were reversible with leptin treatment, 6–8-week-old LDLR(−/−), LDLR+/ −;ob/ob, and LDLR−/−;ob/ob mice were injected with a low dose of leptin (0.3 μg of leptin/g body weight) daily for 10 days. This low dose of leptin has been shown to be suboptimal for weight reduction so that body weight in the treated mice was not significantly changed by this treatment (15). Control mice were injected daily with a similar volume of PBS. The LDLR(−/−) mice did not show any significant changes in TC, TG, or glucose levels (data not shown). Mice in all groups gained weight within the 10-day treatment (predictable from their age), but there were no significant differences due to the leptin treatment (Table III). Insulin levels were typical of ob/ob mice of this age (20–30 ng/ml) throughout the study. Insulin levels in the PBS-treated mice increased during the treatment, while leptin treatment tended to retard this increase in both LDLR−/−;ob/ob and LDLR+/−;ob/ob groups, although there was no significant difference due to a high variability (data not shown). Glucose levels were slightly decreased in the leptin-treated LDLR−/−;ob/ob and LDLR+/−;ob/ob mice (Table III). TG levels were substantially decreased in the leptin-treated LDLR−/−;ob/ob mice, while they tended to be increased in the PBS-treated mice during the course of the study (Table III). In contrast, TC levels in the leptin-treated LDLR−/−;ob/ob mice were only slightly reduced, whereas those of the PBS-treated LDLR−/−;ob/ob increased significantly ($p < 0.05$) during the

course of the study (Table III). HPLC analyses of pooled serum samples from leptin and PBS-injected LDLR−/−;ob/ob mice are shown are Fig. 6. Leptin treatment caused a substantial decrease in the VLDL/IDL fraction compared with little change seen in the PBS-injected mice (data not shown). This noticeable shift from VLDL/IDL size particles to particles in the LDL size range is consistent with the marked reduction in TG and minimal decrease in TC detected from total serum. Interestingly, HDL levels appeared to increase upon leptin injection. The leptin-injected LDLR+/−;ob/ob mice also displayed a lowering in TG levels, which was concomitant with a reduction in the HDL1 peak compared with their base-line profile and with PBS-injected animals (data not shown). These data indicate that leptin treatment in LDLR−/−;ob/ob mice can revert their hypertriglyceridemia but not their hypercholesterolemia, since the remnant cholesterol was presumably converted to LDL cholesterol.

Atherosclerosis—Animals were sacrificed at 6 months of age, and whole aortae and hearts were collected for the analysis of atherosclerotic development. As expected, wild type mice had no detectable lesions in either their whole aortae (Fig. 7, *panels A*) or the proximal aortic sinus (data not shown). LDLR(−/−) mice had slight lesions in the arch region of the whole aorta and fatty streak lesions on the cusps in the aortic sinus (Fig. 7, *panels B*, and Fig. 8*B*). Although the aortae of all three ob/ob groups were coated on the outside extensively with adipose tissue, the LDLR+/+;ob/ob mice had no detectable lesions in either the whole aorta (Fig. 7, *panels C*) or the aortic sinus (Fig. 8*A*). Interestingly, while the LDLR+/−;ob/ob had elevated TC levels, comparable with LDLR(−/−) mice, they had no detectable atherosclerotic lesions in either the whole aorta (Fig. 7, *panels D*) or the proximal aortic sinus (Fig. 8*C*). In contrast, LDLR−/−;ob/ob had extensive lesions throughout the aorta (Fig. 7, *panels E*; Fig. 8*D*). Lesions were smaller in the abdominal and thoracic aortic regions but were most extensive in the aortic arch. These data indicate that LDL cholesterol is a stronger risk factor for atherogenesis than are the combined effects of obesity, diabetes, and insulin resistance in murine models on a normal diet.

DISCUSSION

LDLR−/−;ob/ob Mice Are a Good Model for Hyperlipoproteinemia—There are several available animal models for elevated remnant lipoprotein particles: apoE(−/−) mice, LDLR(−/−) mice on a high fat diet, TgSREBP-1a/LDLR−/−, and LDLR and LRP double knockout mice (5–9). All of these models were shown to have large increases in remnant particles, although the mechanisms, such as lack of receptors and ligands and overproduction of VLDL, are different. Our current animals provide another model of increased remnant lipoproteins with several unique characteristics. One feature of this model is the increase in apoB-100-containing lipoproteins in addition to apoB-48. Mice produce both apoB-100 and apoB-48 in their livers; however, apoB-48 particles are more predominant in the previously mentioned models of hyperlipidemia. Considering the noticeable elevations in apoB-100 in humans with hyperlipidemia, this remnant model could be more relevant to the study of human disease. Currently, the mechanism by which apoB-100 is preferentially accumulated in the plasma of LDLR−/−;ob/ob is unknown. Another characteristic of this model is the sustained levels of HDL cholesterol. While other mouse models show an increase in VLDL/LDL lipoproteins, they are also characterized by low HDL levels. The LDLR−/−; ob/ob and LDLR+/−;ob/ob, in contrast, maintain normal, if not slightly elevated, HDL levels, making them a valuable tool for the study of the distinct role of remnant lipoproteins in atherogenesis without the confounding variable of low HDL.

| Group	Control		Leptin-treated	
	Base line	10 days	Base line	10 days
LDLR+/$-$;ob/ob				
Body weight (g)	21 ± 3	30 ± 2	32.5 ± 6	36 ± 5
Glucose (mg/dl)	194 ± 26	201 ± 31	303 ± 41	215 ± 67
TG (mg/dl)	84 ± 17	193 ± 27^a	128 ± 17	114 ± 42
TC (mg/dl)	235 ± 21	227 ± 6	249 ± 16	190 ± 21
LDLR$-/-$;ob/ob				
Body weight (g)	21 ± 5	29 ± 2	23 ± 7	28 ± 4
Glucose (mg/dl)	295 ± 84	303 ± 35	377 ± 196	196 ± 48
TG (mg/dl)	364 ± 182	459 ± 174	401 ± 246	270 ± 102
TC (mg/dl)	803 ± 36	951 ± 28^a	636 ± 90	593 ± 128

^{a} $p < 0.05$ compared with base line.
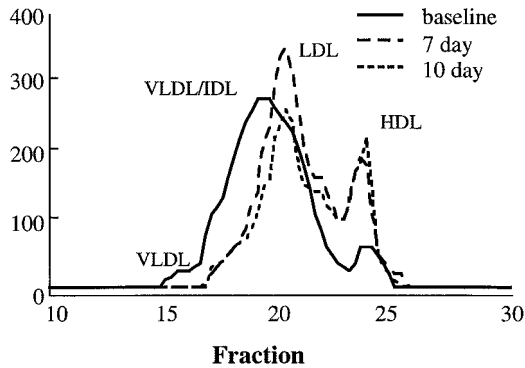
FIG. 6. **HPLC analysis of serum samples from LDLR$-/-$;ob/ob mice treated with leptin.** Blood was collected from LDLR$-/-$;ob/ob mice before and at 7 and 10 days after injections with 3 μg/g body weight leptin. Serum was separated and pooled for HPLC analyses as described under "Experimental Procedures." *Solid lines*, base-line values; *large dashes*, 7-day time point; *small dashes*, 10-day time point (as indicated on the *figure*).
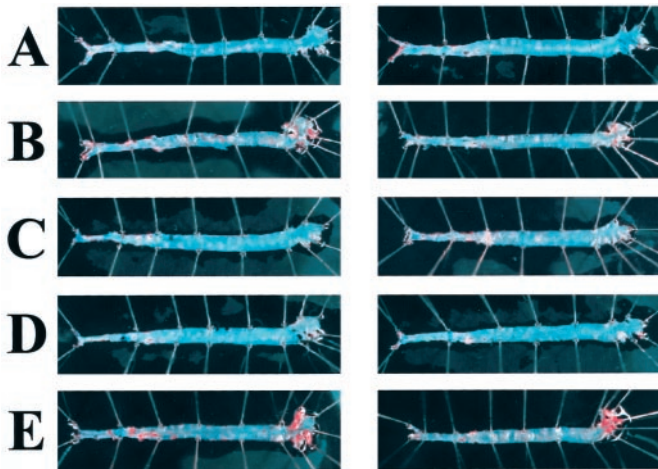

FIG. 7. **En fac aortas from LDLR$-/-$;ob/ob mice.** Mice ($n = 3$ for each group) were sacrificed at 6 months, and whole aortas were collected and stained for lipids as described under "Experimental Procedures." Shown are representative aortas from wild type mice (*panels A*), from LDLR($-/-$) mice (*panels B*), from LDLR+/+;ob/ob mice (*panels C*), from LDLR+/$-$;ob/ob (*panels D*), and from LDLR$-/-$;ob/ob mice (*panels E*).

FIG. 8. **Cross-sections of the proximal aorta from LDLR$-/-$; ob/ob mice.** Hearts collected from 6-month-old mice ($n = 3$ for each group) were fixed in formalin and embedded in OCT, and frozen cross-sections of the proximal aorta were cut as described under "Experimental Procedures." Sections with stained with Oil-Red-O and counterstained with hematoxylin. Sections shown are representative. *A* is from an LDLR+/+;ob/ob mouse; *B* is from an LDLR($-/-$) mouse; *C* is from an LDLR+/$-$;ob/ob; and *D* is from an LDLR$-/-$;ob/ob mouse.

Hypertriglyceridemia and Hypercholesterolemia Are Caused by Distinct Mechanisms—One important conclusion that can be made from this study, is that the hypertriglyceridemia and hypercholesterolemia in the LDLR$-/-$;ob/ob mice are caused by distinct mechanisms. This distinction becomes apparent when hepatic lipid production is contrasted with plasma lipid levels. ob/ob mice sh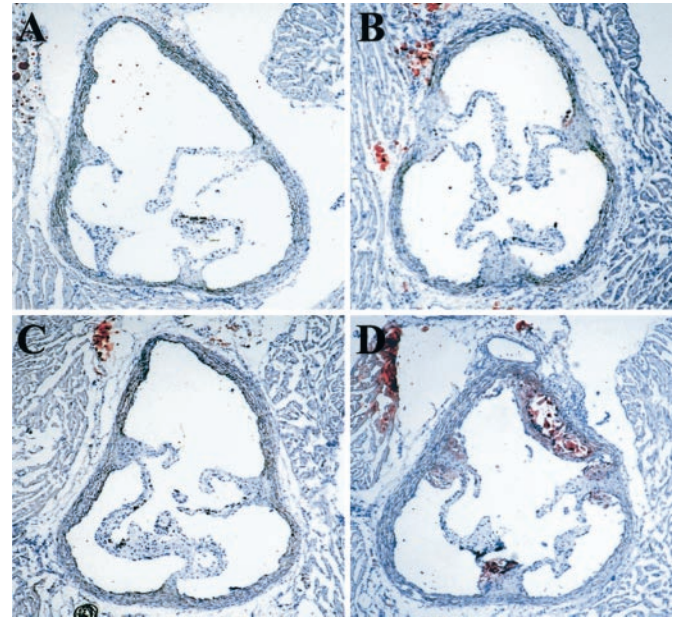ow hepatic overproduction of triglycerides and have fatty livers, presumably due to the activation of nuclear SREBP-1 (see Fig. 4 and Ref. 16), a key transcription factor for lipogenic enzymes (12). Fasting and caloric restriction in mice have both been shown to inhibit hepatic SREBP-1c production (17, 18) and also reverted the hypertriglyceridemia in LDLR$-/-$;ob/ob mice. These data suggest that overconsumption of dietary calories by ob/ob mice and the subsequent increase in hepatic production of triglycerides through activation of SREBP-1 lead to hepatic overproduction and oversecretion of triglyceride-rich VLDL particles. These particles tend to accumulate as remnant lipoproteins due to LDLR deficiency, and this burden of triglycerides might be beyond the capacity of the plasma lipoprotein lipase activity, leading to hypertriglyceridemia. In contrast, hepatic production of cholesterol has been reported to be decreased in ob/ob mice with a slight increase in liver cholesterol content (16). In addition, cholesterol synthesis has been shown to be at normal levels in LDLR($-/-$) mice, because although there is a decreased return of cholesterol through the LDLR, this is compensated for by an increase in LDLR-independent influx (19). Therefore, it is unlikely that the hypercholesterolemia in the LDLR$-/-$;ob/ob

mice is caused by hepatic overproduction of cholesterol. The current data on mRNA levels of cholesterologenic enzymes and liver cholesterol content from the doubly mutant mice also support this interpretation. Taken together with the increased apoB-100 in plasma, suggesting an increase in lipoprotein particle number, it can be speculated that impaired catabolism of remnant particles is the main cause of hypercholesterolemia in these mice. It is interesting to compare the LDLR−/−;ob/ob mice with TgSREBP-1a/LDLR−/− mice (9), which show a similar phenotype, with a dramatic increase in TC and TG levels (1050 mg/dl and 900 mg/dl, respectively). This severe hyperlipidemia in TgSREBP-1a/LDLR−/− mice was primarily due to the plasma appearance of very large VLDL particles that contained mainly apoB-48 and were enriched in both cholesterol and triglycerides. However, in contrast to our LDLR−/−;ob/ob mice, the TgSREBP-1a;LDLR−/− mice had a marked elevation in cholesterol synthesis and liver cholesterol content in addition to the increase in triglyceride synthesis. Therefore, although LDLR deficiency may similarly serve to unmask hyperlipidemia in both animal models, the mechanism causing hypercholesterolemia appears to be different.

An intriguing question brought up by these studies is the time course of the hyperlipidemia. Peak TC and TG levels were seen at between 3 and 4 months of age with a rapid drop in TG levels at 5 months and a gradual reduction in TC between 5 and 8 months. The mechanism by which both hypertriglyceridemia and hypercholesterolemia were ameliorated during the later stages of the study is currently unknown. It is probably related to the natural course of energy metabolism in ob/ob mice. To clarify this issue, detailed analysis on individual ob/ob phenotypes such as obesity, glucose/insulin metabolism, and hepatic lipid synthesis in a time course study will be required.

Involvement of Leptin in Plasma Clearance of Non-HDL Cholesterol—It has previously been shown that leptin plays a role in HDL metabolism (15, 20), and we now show that leptin also has strong effects on remnant lipoprotein metabolism. The unexpectedly dramatic hypercholesterolemia in LDLR−/−;ob/ob mice compared with LDLR−/− mice, if not due to hepatic cholesterol overproduction (as discussed above), may possibly be due to the impairment of LDLR-independent lipoprotein clearance. This accumulation of cholesterol-rich remnant lipoproteins appears to take place chronically and cumulatively, as supported by the observation that TC levels increased in an age-dependent manner and short term leptin treatment did not produce a marked effect. While ob/ob mice have long been known to have drastically increased blood glucose and insulin levels because of energy imbalance and insulin resistance, the current study demonstrates that ob/ob mice have the potential for hypertriglyceridemia and hypercholesterolemia and moreover shows a novel potential link between leptin and plasma cholesterol metabolism. This is, to our knowledge, the first observation that leptin might play a role in plasma non-HDL cholesterol metabolism. However, precise kinetic studies are needed to confirm this speculation.

HDL1 Cholesterol in ob/ob Mice Is Not Proatherogenic—It has been known for some time that ob/ob mice have slightly elevated TC levels, which can be completely accounted for by an increase in HDL (3). However, not much emphasis has been placed upon lipoprotein production and metabolism in these animals. Recently, extensive studies on HDL metabolism in ob/ob mice have been reported. These studies have shown that the increase in HDL cholesterol is due to both regular HDL cholesterol and HDL1 cholesterol, a larger particle that shows up in a peak between LDL and HDL, that HDL clearance in ob/ob mice is impaired, and that low dose leptin treatment

significantly reduces HDL levels (15). They further demonstrated that the altered HDL catabolism is due to defective HDL uptake, recycling, and degradation by hepatocytes (20). We have confirmed their observations for increased normal HDL and HDL1 cholesterol in LDLR+/+;ob/ob mice and show a more dramatic increase in these particles in LDLR+/−;ob/ob mice (Figs. 2 and 3). However, it is hard to conclude that HDL1 is cleared through LDL receptors from plasma, since LDLR−/−;ob/ob mice had a slightly decreased HDL1 fraction (Fig. 2 and 3). Of interest is the difference in atherosclerotic lesion development between the LDLR(−/−) and the LDLR+/−;ob/ob mice. While the TC and HDL cholesterol levels between the two groups were nearly identical, there was a noticeable difference in their lipoprotein profiles, with LDLR+/−;ob/ob mice having an HDL1 peak in place of the LDL peak seen in LDLR(−/−) mice. This difference in lipoprotein profiles probably contributed to the differences in atherogenesis noted between the two groups of mice; LDLR(−/−) mice showed signs of lesions in the aortic sinus and the aortic arch, whereas the LDLR+/−;ob/ob mice were completely lesion-free. Therefore, it can be said that this HDL1 is not proatherogenic, although whether it is anti-atherogenic remains to be determined. Future studies comparing these two groups of mice more extensively will help to define the protective effect of this HDL1 pool of lipoproteins in the development of atherosclerosis.

The current studies with LDLR−/−;ob/ob mice demonstrate important findings about remnant lipoprotein metabolism, discussed above, and provide a good model for the contributions of multiple risk factors, including hyperinsulinemia, diabetes, and increased remnant particles, in the development of atherosclerosis. This model also raises the possibility of the therapeutic potential of leptin for treatment of dyslipidemia and atherosclerosis.

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