Disruption of the Murine Lecithin:Cholesterol Acyltransferase Gene Causes Impairment of Adrenal Lipid Delivery and Up-regulation of Scavenger Receptor Class B Type I*

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Lecithin:cholesterol acyltransferase (LCAT) is the major determinant of the cholesteryl ester (CE) content of high density lipoprotein (HDL) in plasma. The selective uptake of HDL-CE is postulated to participate in delivery of tissue-derived cholesterol both to the liver and steroidogenic tissues. Recent studies comparing mice with similarly low levels of HDL, due to the absence of either of the two major HDL-associated apolipoproteins apoA-I and apoA-II, suggest that apoA-I is crucial in modulating this process, possibly through interaction with scavenger receptor class B type I (SR-BI). Because of the central role of LCAT in determining the size, lipid composition, and plasma concentration of HDL, we have created LCAT-deficient mice by gene targeting to examine the effect of LCAT deficiency on HDL structure and composition and adrenal cholesterol delivery. The HDL in the LCAT-deficient mice was reduced in its plasma concentration (92%) and CE content (96%). The HDL particles were heterogeneous in size and morphology and included numerous discoidal particles, mimicking those observed in LCAT-deficient humans. The adrenals of the male Lcat (−/−) mice were severely depleted of lipid stores, which was associated with a 2-fold up-regulation of the adrenal SR-BI mRNA. These studies demonstrate that LCAT deficiency, similar to apoA-I deficiency, is associated with a marked decrease in adrenal cholesterol delivery and supports the hypothesis that adrenal SR-BI expression is regulated by the adrenal cholesterol.

High density lipoprotein (HDL)1 plays a pivotal role in lipid homeostasis by removing cholesterol accumulating in the extrahepatic tissues, a process crucial for maintenance of the structure and function of most cells in the body. The mechanism widely held to explain this process, reverse cholesterol transport (1), describes the metabolic fate of such tissue-derived cholesterol. According to this mechanism, circulating HDL functions as an acceptor of tissue-derived unesterified cholesterol (UC), facilitating efflux of cholesterol from cells. Lecithin:cholesterol acyltransferase (LCAT) performs a central role in this process by catalyzing the conversion of plasma UC, especially that associated with HDL, to cholesteryl ester (CE). It has been demonstrated that the majority of the CE in HDL is delivered to the liver for clearance or recycling. In addition, a fraction of HDL-CE is delivered to the steroidogenic tissues and is utilized as substrate for steroid hormone synthesis. Routes for delivery of HDL-CE to the target organs include: (i) indirectly, by transferring to apolipoprotein (apo) B-containing lipoprotein particles, (ii) directly through a receptor-mediated endocytic process (2, 3), and (iii) via selective uptake pathway where CE in lipoprotein particles is delivered into the cells without simultaneous uptake of the apolipoprotein moieties (4–6). Although numerous patients with a complete deficiency of LCAT have been described, revealing many of the lipoprotein abnormalities and tissue organ damage associated with complete absence of LCAT, there remain many unanswered questions concerning the role of LCAT in the transport of CE to tissues.

To date, much of our knowledge of the selective uptake pathway derives from studies on rodents. In rats, not only is the selective uptake pathway responsible for 65% of HDL-CE delivered to the liver, it is the principal mechanism for delivery of CE to the steroidogenic tissues (4). Murine scavenger receptor class B type I (SR-BI), recently characterized as a potential HDL receptor (7), is believed to participate in mediating the selective uptake of HDL-CE into cells. Key evidence supporting this role for SR-BI includes the tissue expression pattern of SR-BI being coincident with those involved in selective uptake and the observation that SR-BI expression in the adrenal is strongly regulated by intracellular cholesterol content (8). Recently, an in vivo study comparing apoA-I and apoA-II gene-targeted mice, both with similarly low HDL levels, demonstrated that only the lack of apoA-I severely impairs the delivery of CE to the steroidogenic tissues, causing depletion in adrenal CE stores and blunted steroidogenic responses to stress (9). Although the different consequences of the two low HDL states strongly suggest the crucial importance of apoA-I in HDL in mediating selective uptake of CE, the question of whether there are additional independent modulating factors remains unanswered.
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In this study, we tested the hypothesis that HDL resulting from LCAT deficiency, being severely depleted of CE yet containing apoA-I, impairs cholesterol delivery to the adrenals. To investigate this issue, LCAT-deficient mice were created by gene targeting. The effect of complete LCAT deficiency on lipoproteins and cholesterol transport was characterized in these animals.

MATERIALS AND METHODS

Creation of the LCAT-deficient Mice—The strategy for construction of the targeting vector pPN2T/LKO is shown in Fig. 1. The targeting vector was linearized and transfected into ES cells by electroporation. Drug selection was performed using both G418 (160 mg/ml) and FIAU (0.5 μM). Genomic Southern blot was used for screening of targeting events using a PCR based probe that hybridizes to exon 6 of the Lcat gene. The targeted ES cells were injected into blastocysts by standard method (10). Chimeric mice were bred with DBA/C57BL/6 F1 hybrids. Genotypes were determined by multiplex PCR. The forward primers either hybridize specifically to the neo-resistant gene (a, 5′-AAG-CAAAAACCATTAGGGG-GC-3′) or to the targeted region (b, 5′-GCTC-CTCAATGTCCTCC-3′) and share a common reverse primer which hybridizes to exon 3 of the gene (c, 5′-GTACCTAACAGATTCCGGTCT-TGC-3′) (Fig. 1A).

Plasma LCAT Activity Assays—LCAT activity was measured on fasting plasma as the rate of synthesis of [3H]cholesteryl esters from unilamellar vesicles prepared with French pressure cell and activated with human apoA-I (Sigma) as described previously (11).

Plasma Lipid and Lipoprotein Analyses—Lipoprotein determinations were performed on 4–8-week-old mice fed a mouse diet. Fasting plasma was analyzed using enzyme end point kits for total cholesterol (Boehringer Mannheim), free glycerol, and triglyceride concentrations (Sigma). Plasma triglyceride concentration was corrected for free glycerol content. Cholesteryl ester lipoprotein fractions were calculated from difference between total and unesterified cholesterol; HDL-cholesterol was measured in plasma after selective precipitation of apoB-containing lipoproteins by polyethylene glycol. Mouse plasma apoA-I levels were quantified by enzyme-linked immunosorbent assay as described previously (12).

Analysis and Characterization of Lipoprotein Subfractions—The relative proportion of HDL with pre-β- and α-mobility on agarose gel in the Lcat(-/-) and Lcat(+/-) mice was determined as described previously (13). Lipoprotein subfractions of d < 1.063 and d = 1.063–1.21 g/ml were obtained from a pool of 200–250 μl of plasma by standard sequential ultracentrifugation techniques. The size distribution of the lipoprotein fractions were obtained by nondenaturing polyacrylamide gradient gel electrophoresis essentially as described by Nichols et al. (14). The morphology of lipoproteins was assessed by negative staining electron microscopy as described previously (15).

Quantification of Tissue Cholesterol Contents—Tissue lipids were measured in the kidney, liver, spleen, heart, lung, skeletal muscle, and adrenal gland of both the Lcat(-/-) and Lcat(+/-) mice. After perfusion of the animal with 0.9% saline, the organs were excised, blotted dry, weighed, and homogenized in 1.15% KCl buffer. Lipids were extracted using the Bligh and Dyer method (16) and reconstituted in the aqueous phase using Triton X-100. Total cholesterol and UC were measured using an enzymatic assay (17).

Tissue Preparation and Light Microscopy—Mice were perfused through the left ventricle with phosphate-buffered saline followed by overnight fixation in 4% formaldehyde. The adrenal glands were infiltrated sequentially with 10, 20, and 30% sucrose in phosphate-buffered saline and quick frozen in Tissue-Tec OCT. 6–10-μm-thick sections were placed on poly-L-lysine coated slides, stained with Oil Red O, and counterstained with hematoxylin (18).

Isolation and Analysis of RNA by Northern Blot Hybridization—RNA was isolated from single adrenals from five male Lcat(-/-) and five male wild type mice using RNA STAT-60 kit (Tel-Test “B”, Inc.) and separated in a denaturing 1% agarose gel containing 2.2 M formaldehyde. RNA was transferred to nylon membranes (Schleider & Schuell), cross-linked, and hybridized with a 554-base pair PCR fragment extending from nucleotides 362 to 915 of the murine SR-BI. The SR-BI mRNA Northern blot signal was normalized to that of the human β-actin probe (CLONTECH Laboratories, Inc., CA) on the same membrane.

RESULTS

Creation of the LCAT-deficient Mice—Genomic Southern blot screening of 200 ES cells targeted with pPN2T/LKO yielded a frequency of 1 in 4 for correct homologous recombination event. 2 of the 10 targeted ES cell clones micro-injected gave germ-line transmission of the targeted allele.

Multiplex PCR screening of the targeted mutant is shown in Fig. 1B. Positivity from primer sets b/c alone, a/c plus b/c, and a/c alone identified wild type, heterozygosity, and homozygosity for the targeted Lcat allele respectively. Of 86 offspring from the heterozygote breeding, 25 were wild type, 39 were

| TABLE I

| Genotype | T. Chol. | TG | UC | HDL-C | UCCE | apoA-I |
|----------|---------|----|----|-------|------|--------|
|          | mg/dl (n = 7) | mg/dl | mg/dl | mg/dl |
| +/-      | 111 ± 22 | 67 ± 50 | 25 ± 8 | 84 ± 19 | 0.23 ± 0.06 | 184.7 ± 30.5 (n = 6) |
| +/-     | 19 ± 19 | 78 ± 42 | 21 ± 7 | 50 ± 16 | 0.40 ± 0.17 | 182.6 ± 21.1 (n = 8) |
| -/-      | 33 ± 12 | 117 ± 88 | 24 ± 11 | 7 ± 3 | 2.71 ± 1.11 | 94.9 ± 9.4 (n = 5) |

* p < 0.00003 compared with wild type control.

** p < 0.002 compared with wild type control.
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TABLE II
Lipid analyses of plasma subfractions in Lcat(+/+) and Lcat(−/−) mice

Values are expressed as means ± S.D. T. Chol., total cholesterol
Plasma were pooled from five mice in each group; density subfractions were obtained by sequential ultracentrifugation.

| Genotype | d < 1.063 g/ml | d = 1.063–1.21 g/ml |
|----------|----------------|-------------------|
|          | T. Chol. | UC | CE | T. Chol. | UC | CE |
| +/+      | 15.6     | 10.6 | 5  | 90.4     | 22.1 | 68.3 |
| −/−      | 19.5     | 14.5 | 5  | 16.0     | 13.4 | 2.6  |

Fig. 2. Non-denaturing gradient gel electrophoresis of plasma lipoprotein subfractions pooled from Lcat(+/+) (dashed line) and Lcat(−/−) (solid line) mice. The listed peak size is in nanometers. A, the d < 1.063 g/ml fraction was analyzed on 2–16% gels and stained with Oil Red O; B, the d = 1.063–1.21 g/ml fraction was analyzed on 4–30% gels and stained with Coomassie G250.

Lipoprotein Profiles and Plasma Lipid—Compared with wild type controls, lipid analyses on the Lcat(−/−) mice revealed 70.3 and 91.6% reductions in total and HDL cholesterol, respectively (Table I). Plasma CE was reduced to 9.4% of control, whereas plasma UC level was essentially unchanged. As a result, there was a 11.7-fold increase in the UC/CE ratio compared with the wild type littermates. The Lcat(+/+) mice experienced a more moderate but statistically significant reduction in total cholesterol and CE, resulting in a modest 1.7-fold increase in the UC/CE ratio. The genotype-dependent changes in UC/CE ratio was nearly entirely due to changes in plasma CE levels. Likewise, the reduction in total cholesterol in the Lcat(−/−) mice was largely a result of reduction in HDL cholesterol levels, whereas the non-HDL cholesterol levels for each genotype were essentially unchanged. Plasma apoA-I levels in the mutant mice were also reduced in both Lcat(−/−) and Lcat(+/+) mice to 19 and 86% of their respective controls.

Lipid analyses on d < 1.063 and d = 1.063–1.21 g/ml fractions from pooled plasma are shown in Table II. The reduction of HDL-CE in the Lcat(−/−) mice was profound, whereas the HDL-UC reduction in the same mice was less dramatic. Agarose gel electrophoresis of HDL showed that compared with wild type control, the fraction of plasma apoA-I in the α-migrating HDL particles in the Lcat(−/−) mice was decreased from 93.6 ± 0.8% to 78.3 ± 3.5% (p < 0.01) and was increased from 6.4 ± 0.8% to 21.7 ± 3.5% (p < 0.01) in the pre-β-migrating HDL.

Fig. 3. Electron micrographs of negatively stained plasma lipoprotein fractions of Lcat(+/+) and Lcat(−/−) mice. A, VLDL fraction, Lcat(+/+) mice; B, VLDL fraction, Lcat(−/−) mice; C, HDL fraction, Lcat(+/+) mice; and D, HDL fraction, Lcat(−/−) mice. The large sized particles in the Lcat(−/−) HDL fraction represent contaminating VLDL/LDL particles. The arrow in B indicates a notched VLDL particle. The bar markers represent 100 nm.

Lipoprotein Size Distribution and Morphology—The lipoprotein size distribution of the Lcat(+/+) and Lcat(−/−) mice in d = 1.063–1.21 g/ml fractions are shown in Fig. 2. The pattern of the d < 1.063 g/ml fraction of Lcat(−/−) mice, in comparison with that of the wild type, showed absence of the 20.5 nm peak, broadening of the 28.0 nm LDL peak to include intermediate density lipoprotein in the region of 32.1 nm, and preservation of the dominant VLDL peak. Unlike a unimodal size distribution of HDL in the wild types, the Lcat(−/−) mice had a very complex pattern, characterized by a major peak at 7.6 nm and several minor components similar to that found in human LCAT deficiency (19). Electron microscopic evaluation of VLDL from the control mice revealed spherical particles with a mean diameter (d) and a S.D. of 63.5 ± 19.1 nm, whereas VLDL from Lcat(−/−) mice were smaller (d ± S.D. = 51.7 ± 22.8 nm) and were distinguished by the presence of notched particles (Fig. 3). The HDL from Lcat(−/−) mice, compared with control HDL that were homogeneous spherical particles of 10.0 ± 2.1 nm in diameter, were morphologically complex. An important observation, however, is the presence of discoidal particles that form the classical rouleaux structures. Both morphologic features have been described in LCAT-deficient subjects (19, 20). Larger particles were also present and may represent contamination from the d < 1.063 g/ml fraction.

Analysis of the Effect of LCAT Deficiency on the Target Tissues—For the total cholesterol and UC in the nonsteroidogenic tissues examined, no significant difference between the Lcat(−/−) and Lcat(+/+) mice was noted, with the exception of the spleen, which showed a 21% increase in UC content (p = 0.047) but not in total cholesterol (data not shown). After perfusion, the adrenal glands of the Lcat(−/−) mice appeared brown and translucent, in contrast to a pearly white appearance of adrenals from the wild type mice (Fig. 4A), consistent with the depletion of cholesterol in the former. Staining of the adrenal sections with Oil Red O demonstrated an abundance of stored lipid in the adrenal cortex of the wild type mice. In contrast, the Lcat(−/−) mice showed complete absence of lipid staining (Fig. 4B). Quantiﬁcation of tissue lipid in the adrenal glands showed
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FIG. 4. Adrenal histology, lipid content and SR-BI mRNA levels in Lcat (+/+ ) and Lcat (−/−) mice. A, gross appearance of adrenal glands from male Lcat (+/+ ) and Lcat (−/−) mice. The scale shown is in millimeters. B, Oil Red O stained frozen sections of the adrenals showing complete depletion of neutral lipid store in the adrenal cortex of male Lcat (−/−). C, quantification of tissue cholesterol in the adrenals of Lcat (+/+ ) and Lcat (−/−) mice. Bar graph represents the means ± S.D. from three animals in each group. *, p = 0.008, and **, p = 0.09, compared with their respective wild type controls. D, quantification of mouse SR-BI mRNA expression by Northern blot in Lcat (+/+ ) and Lcat (−/−) mice. PhosphorImager quantification of the SR-BI mRNA bands after normalization to the β-actin level. The bar graph represents the means ± S.D. from five animals in each group. *, p < 0.05 compared with wild type control.

In summary, the LCAT-deficient mouse created by gene targeting reproduces the human LCAT-deficient HDL metabolic abnormalities with high fidelity. The noted morphologic abnormalities with high fidelity. The noted morphologic abnormalities

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A complete absence of LCAT activity was observed in the Lcat (−/−) mice, whereas the LCAT activity of the Lcat (+/+ ) mice was reduced to approximately half normal, compatible with autosomal co-dominant inheritance. The same inheritance has been reported in a human LCAT-deficient kindred (21). The alterations of HDL in the Lcat (−/−) mice also resembled those observed in LCAT-deficient humans. This includes a comparable degree of reduction in both plasma HDL cholesterol and apoA-I and the presence of unique discoidal particles with rouleaux formation in the Lcat (−/−) mice. Furthermore, we observed a higher proportion of the HDL particles in the Lcat (−/−) mice with pre-β mobility, a finding also noted in LCAT-deficient patients.

The impact of LCAT deficiency on non-HDL cholesterol is characterized by a modest increase in the UC level. However, gradient gel electrophoresis and electron microscopy studies showed presence of intermediate density lipoprotein-like particles and notching of VLDL, respectively, in the LCAT-deficient mice. In view of the absence of cholesteryl ester transfer activity in mouse plasma, these findings suggest that LCAT may have a direct effect on the metabolism of the triglyceride-rich lipoproteins (22).

Despite its central role in reverse cholesterol transport, the impact of LCAT deficiency on plasma cholesterol transport and delivery to target organs has been minimally explored. In LCAT-deficient humans, the rate of cholesterol efflux into plasma is preserved at 72% of normal. This is in part explained by the relative abundance of pre-β-HDL particles, a subpopulation of HDL believed to be the preferred initial acceptors of tissue cholesterol (23). The relative increase in pre-β-HDL is also observed in the LCAT-deficient mice. However, it has not been determined whether the extremely low level of abnormal HDL associated with LCAT deficiency is able to deliver tissue-derived CE to the target organs. The recent study by Plump et al. (9) demonstrating the crucial role that apoA-I plays in the delivery of CE through the selective uptake pathway and the observation by Wang et al. (8) that murine adrenal SR-BI receptor expression being up-regulated in association with intracellular cholesterol depletion suggest that lipid-receptor interaction between apoA-I and SR-BI may be a major determinant of effective mediation of CE delivery into the tissues. Our similar observation of severe depletion of tissue lipid stores in mouse adrenals and the finding of a 2-fold up-regulation of adrenal SR-BI mRNA in face of adrenal cholesterol depletion in Lcat (−/−) mice suggests that (i) SR-BI is intimately involved in selective uptake of CE and (ii) this same metabolic pathway is defective in the Lcat (−/−) mice despite the presence of apoA-I in HDL. As in apoA-I-deficient mice, it can be inferred that the minor sources of adrenal tissue cholesterol, namely de novo synthesis and LDL receptor mediated cholesterol uptake, are insufficient to compensate for the cholesterol depletion. In Lcat (−/−) mice, the most likely cause for reduced adrenal lipid content is the severe depletion of plasma HDL-CE, the primary source of cellular cholesterol.

The impact of LCAT deficiency on the other tissues examined was significantly less than that observed for the adrenal. A hallmark finding in LCAT-deficient subjects is the marked tissue UC accumulation in a number of solid organs including the liver, spleen, and kidney. With the exception of a slight increase in UC in the spleen, these lipid disturbances were not observed in the nonsteroidogenic tissues in the Lcat (−/−) mice. The mild phenotype in these mice is likely due to the relatively small reservoir of total plasma UC level with the consequent minor impact on tissue lipid homeostasis (24).

In summary, the LCAT-deficient mouse created by gene targeting reproduces the human LCAT-deficient HDL metabolic abnormalities with high fidelity. The noted morphologic abnor-
malities of VLDL suggest that LCAT may have a direct role in the catabolism of triglyceride-rich, apoB-containing particles. Finally, these studies suggest that the reduced cholesterol content of HDL from deficiency of LCAT likely impacts on the flux of cholesterol to the adrenals via the selective uptake pathway.

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