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Adenovirus-mediated Rescue of Lipoprotein Lipase-deficient Mice

LIPOLYSIS OF TRIGLYCERIDE-RICH LIPOPROTEINS IS ESSENTIAL FOR HIGH DENSITY LIPOPROTEIN MATURATION IN MICE*

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Juliane G. Strauss‡, Sasa Frank§, Dagmar Kratky‡, Günter Hämmerle‡, Andelko Hrzenjak§, Gabriele Knipping§, Arnold von Eckardstein¶, Gert M. Kostner§, and Rudolf Zechner‡‖

From the ‡Institute of Molecular Biology, Biochemistry, and Microbiology and §Institute of Medical Biochemistry and Medical Molecular Biology, University of Graz, Graz A-8010, Austria and the ¶Institute of Clinical Chemistry and Laboratory Medicine, University of Münster, Münster D-48149, Germany

Lipoprotein lipase (LPL) is the rate-limiting enzyme for the hydrolysis of triglycerides and the subsequent uptake of free fatty acids in extrahepatic tissues. Deficiency of LPL in humans (Type I hyperlipoproteinemia) is associated with massive chylomicronemia, low high density lipoprotein (HDL) cholesterol levels, and recurrent attacks of pancreatitis when not controlled by a strict diet. In contrast to humans, homozygous LPL knock-out mice (L0) do not survive suckling and die between 18 and 24 h after birth. In this study, an adenovirus-based protocol was utilized for the transient expression of LPL during the suckling period in an effort to rescue L0 mice. After a single intraperitoneal injection of 5×10^9 plaque-forming units of LPL-expressing virus immediately after birth, more than 90% of L0 mice survived the first days of life. 3% of L0 mice survived the entire suckling period and lived for up to 20 months, although LPL activity in mouse tissues and postheparin plasma was undetectable in all animals after 6 weeks of age. Adult LPL-deficient mice were smaller than their littermates until 2–3 months of age and exhibited very high triglyceride levels in the fed (4997 ± 1102 *versus* 113.4 ± 18.7 mg/dl) and fasted state (2007 ± 375 *versus* 65.5 ± 7.4 mg/dl). Plasma total cholesterol levels, free fatty acids, and ketone bodies were elevated in L0 mice, whereas plasma glucose was normal. Most strikingly, L0 mice lacked apoA-I-containing preβ-HDL particles as well as mature HDL resulting in undetectable HDL cholesterol and HDL-apoA-I levels. HDL deficiency in plasma was evident despite normal apoA-I mRNA levels in the liver and normal apoA-I protein levels in plasma, which were predominantly found in the chylomicron fraction. The absence of preβ-HDL and mature HDL particles supports the concept that the lipolysis of triglyceride-rich lipoproteins is an essential step for HDL maturation.

The major function of LPL is the enzymatic cleavage of acyl-glycerol esters in triglycerides (TG)[1] of very low density lipoproteins (VLDL) and chylomicrons. Following its synthesis in parenchymal cells such as adipocytes and muscle cells, the enzyme is translocated and bound to the intimal side of the capillary endothelium by its interaction with sulfated glucosaminoglycans (for a review, see Refs. 1–3). Free fatty acids (FFA), the products of plasma TG hydrolysis, are absorbed by the underlying tissue for storage (adipose tissue) (4) or energy production (muscle) (5). Besides this important enzymatic function, LPL has also been shown to act as a ligand or bridging factor for the receptor-mediated cellular uptake of various lipoproteins (6–8). Additionally, LPL facilitates the selective uptake of lipids and lipophilic vitamins (9–11). Both enzymatic and nonenzymatic LPL-mediated processes greatly affect the metabolism of plasma lipoproteins and energy homeostasis in all vertebrates.

LPL deficiency (type I hyperlipoproteinemia) (12) is a rare autosomal, recessively inherited disease characterized by elevated plasma TG levels, low plasma total cholesterol (TC) levels, and drastically decreased HDL cholesterol (HDL-C) concentrations. Besides these lipid abnormalities, the disorder is associated with the development of hepato- and splenomegaly, eruptive xanthomas, lipemia retinalis, and abdominal pain on a standard diet, which leads to frequent attacks of pancreatitis. The profoundly reduced HDL-C levels in LPL-deficient individuals are based on the role of LPL in HDL biogenesis. In this multistep process, lipid-poor or lipid-free HDL precursors (pre-β-HDL and apoA-I, respectively) are produced and secreted by hepatocytes or enterocytes. Alternatively, these particles are also produced by the LPL-mediated lipolysis of chylomicrons and VLDL or the HDL modification by phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP) (13, 14). HDL precursor particles accept phospholipids and cholesterol from cells through an efflux mechanism that involves the ATP binding cassette transporter 1 (ABC1) (15–18). Subsequently, these particles are converted into mature, large, and spherical HDL-3 and HDL-2 by a process that involves the esterification of cholesterol by lecithin:cholesterol acyltransferase (19), the acceptance of surface remnants from TG-rich lipoproteins (20), and the fusion of HDL particles. The latter two processes are mediated by PLTP (21, 22). According to this pathway, the lack of TG lipolysis in LPL-deficient individuals impairs the generation of HDL precursors and prevents their maturation. More indirectly, the pronounced hypertriglyceri-

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‖ To whom correspondence should be addressed: Rudolf Zechner Institute of Molecular Biology, Biochemistry and Microbiology, University of Graz, Heinrichstrasse 31a, Graz A-8010, Austria.

[1] The abbreviations used are: TG, triglyceride(s); LPL, lipoprotein lipase; Ad-LPL, LPL-expressing adenovirus; HL, hepatic lipase; VLDL, very low density lipoprotein; HDL, high density lipoprotein; TC, total cholesterol; FFA, free fatty acids; apo, apolipoprotein; PHP, postheparin plasma; AT, adipose tissue; PCR, polymerase chain reaction; PLTP, phospholipid transfer protein; FPLC, fast protein liquid chromatography; CETP, cholesteryl ester transfer protein.

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demia observed in these patients additionally results in an enhanced exchange of cholesteryl esters from HDL to VLDL, which thereby also contributes to low HDL-C levels.

Homozygous LPL knock-out mice (L0) die shortly after birth (23–25). At birth, these animals have elevated TG and TC levels compared with normal mice. Upon suckling, they become pale, develop severe hypertriglyceridemia due to chylomicron and VLDL accumulation, and die postnatally between 18 and 24 h. Although the exact cause of death has not been elucidated, it is conceivable that the enormous accumulation of large TG-rich lipoproteins in plasma leads to a defective gas exchange in lung capillaries, which causes insufficient oxygen supply, cyanosis, and premature death. Alternatively, an extremely low plasma glucose concentration in newborn L0 animals has also been proposed as a potential cause of death (26).

The transgenic expression of LPL exclusively in skeletal muscle, cardiac muscle (27–29), and liver (26) has been demonstrated to rescue L0 mice from neonatal death. These results suggested that the site of LPL expression is not essential for survival as long as a sufficient amount of the enzyme is present to control excessive TG accumulation and plasma glucose concentrations. In the current investigation, we rescued L0 mice by the transient expression of LPL during the suckling period. The lack of HDL particles in adult LPL-deficient mice indicated that the presence of LPL is essential for the production of mature plasma HDL, which represents the major lipoprotein class in the mouse.

EXPERIMENTAL PROCEDURES

Preparation of Replication-defective Adenovirus Containing the Human LPL cDNA—The recombinant adenovirus coding for human LPL was prepared by cotransfection of pAvCvSv (30) containing 1.6-kilobase pair LPL cDNA and pJM 17 (31) into 293 cells. The 1.6-kilobase pair *Bam*HI-*Kpn*I human LPL cDNA fragment was subcloned into *Bgl*II-*Kpn*I-digested pAvCvSv. The resulting shuttle plasmid (5 μg) was cotransfected with 5 μg of pJM 17 into 293 cells using the calcium phosphate coprecipitation method (32). 2 weeks after transfection, recombinant plaques were picked and propagated on 293 cells followed by the screening for LPL enzyme activity. Positive clones were subcloned twice more by plaque assay on 293 cells, and large scale production of high titer recombinant adenovirus was performed as described (33).

Generation of LPL Knock-out Mice, Adenovirus Treatment, and Genetic Analysis—Interbreeding of heterozygous LPL knock-out mice (L1) resulted in progeny of the following genotypes: 25% L2 (wild type), 50% L1, and 25% homozygous LPL knock-out mice (L0). All offspring were injected with different concentrations of Ad-LPL in 0.9% NaCl intraperitoneally immediately after birth to determine the optimal dose. Genotypes were examined after weaning from tail tip DNA by PCR analysis as reported previously (24). After weaning the mice were kept on low fat diet (2.5% fat).

Growth Curves—All animals from three L1 × L1 matings were weighed at the age of 3, 5, 7, 12, 14, and 21 days after birth, respectively. After weaning, mice were weighed repeatedly at the age of 30, 40, 50, 60, and 80 days.

Determination of LPL and Hepatic Lipase (HL) Enzyme Activity—Postheparin plasma (PHP) from fed animals was collected from the retro-orbital plexus 20 min after the intraperitoneal injection of 1000 units of heparin/kg body weight. The LPL activity was determined in PHP according to an established method (34). Additionally, a monoclonal antibody (5D2) was used to inhibit specifically human LPL activity (kindly provided by Dr. J. Brunzell, Seattle) (35). LPL and HL activities were expressed as μmol of FFA/ml/h. LPL and HL activities were also measured in preheparin plasma of fed animals.

Plasma Lipid and Lipoprotein Analysis—Blood was collected in the morning from fed as well as fasted animals (at least 10 h of nighttime fasting) by retroorbital bleeding. TC, HDL-C, and TG levels were analyzed enzymatically using commercial kits (Sigma). Lipoproteins were isolated by fast protein liquid chromatography (FPLC) using an Amersham Pharmacia Biotech system and a Superose 6 column (Amersham Pharmacia Biotech). Plasma samples were centrifuged 20 min at 13,000 rpm to remove the majority of chylomicrons. Chylomicron-depleted fractions (250 μl) were applied to FPLC analysis and eluted with 10 mM Tris-HCl, 1 mM EDTA, 154 mM NaCl, and 0.02% NaN_3 (pH 7.4). Fractions of 0.5 ml each were collected and enzymatically assayed for cholesterol content. One major lipoprotein peak was identified corresponding to mouse VLDL/chylomicron remnants, and a second smaller peak corresponded to HDL. Low density lipoprotein concentrations were too low to be detected by the FPLC analysis.

Analysis of ApoA-I-containing Lipoproteins by Nondenaturating Two-dimensional Electrophoresis—Plasma samples were analyzed for their contents of preβ-LpA-I and α-LpA-I by nondenaturating two-dimensional polyacrylamide gradient gel electrophoresis. Agarose gel electrophoresis, polyacrylamide gradient gel electrophoresis, and immunoblotting were performed as described previously (36). Briefly, in the first dimension, 40 μl of plasma samples were separated by electrophoresis at 4 °C in a 0.75% agarose gel using a 50 mM merbital buffer (pH 8.7, Serva, Heidelberg, FRG). Bromphenol blue was added to a standard sample to visualize albumin in the native gel. The electrophoresis was stopped when the albumin/bromphenol blue marker had migrated 6 cm. Agarose gel strips containing the preseparated lipoproteins were then transferred to a 3–20% polyacrylamide gradient gel. Separation in the second dimension was performed at 40 mA for 4–5 h at 10 °C. The endogenous plasma albumin was visualized by the addition of bromphenol blue to the cathodic buffer (300 μl/liter of buffer). Electrophoresis in the second dimension was stopped when this band had migrated 10 cm. The proteins separated in the polyacrylamide gradient gel electrophoresis gel were electroblotted onto a nitrocellulose membrane, which was then incubated with biotinylated sheep antibodies against human apoA-I (Roche Molecular Biochemicals). The apoA-I-antibody complexes were visualized by autoradiography after incubation with a streptavidin-biotinylated horseradish peroxidase complex (Amersham Pharmacia Biotech) and a chemiluminescent blotting substrate (Lumilight Plus System; Roche Molecular Biochemicals).

Analysis of ApoA-I mRNA in the Liver—Livers were removed surgically, weighed, and subsequently frozen in liquid nitrogen. 500 mg of wet tissue were finally homogenized in 5 ml of TRI Reagent (MRC, Karlsruhe, Germany). Total tissue RNA was precipitated with isopropyl alcohol. After centrifugation, the RNA pellet was washed with 75% ethanol, recentrifuged, and dissolved in diethyl pyrocarbonate-treated H_2O. For Northern blot analysis, 10 μg of total RNA were separated by 1% formaldehyde-agarose gel electrophoresis and blotted overnight onto nylon membranes (Hybond N^+; Amersham Pharmacia Biotech). Subsequently, the RNA was cross-linked to the membrane by ultraviolet irradiation. Blots were prehybridized for 4 h at 65 °C in a buffer containing 0.15 M sodium phosphate (pH 7.2), 1 mM EDTA, 7% SDS, and 1% bovine serum albumin. Membranes were hybridized in the same buffer at 65 °C overnight with a specific mouse apoA-I cDNA probe. After hybridization, the blots were washed in 2× SSC and 0.1% SDS for 20 min at room temperature, followed by two additional washing steps in 1× SSC and 0.1% SDS for 10 min at 65 °C each. Specific hybridization was visualized by 3-h exposure to a PhosporImager Screen (Apbiotech, Freiburg, Germany).

The mouse-specific apoA-I cDNA probe was generated by reverse transcription-PCR from isolated mouse liver mRNA using the Advantage RT-for-PCR Kit (CLONTECH). A 665-base pair PCR product was amplified with the forward primer GCACGTATGGCAGCAAGATG and the reverse primer GCATCAGACTATGGCGCAGG. This apoA-I cDNA fragment was inserted into the TA-cloning vector pSTblue (Novagen). The final clone was radioactively labeled with [^{32}P]dCTP (PerkinElmer Life Sciences) using a random priming kit (Prime-a-Gene Kit; Promega, Mannheim, Germany).

Western Blotting Analysis of ApoA-I and ApoA-II in Plasma and Lipoprotein Fractions—Blood was collected from retroorbital plexus, and lipoprotein fractions were isolated by sequential ultracentrifugation as described previously (37). Samples were mixed 1:1 with loading buffer (20% (w/v) glycerol, 5% (w/v) SDS, 0.15% (w/v) bromphenol blue, 63 mmol/liter Tris-HCl, pH 6.8), incubated for 10 min at 95 °C, and fractionated by SDS-polyacrylamide gel electrophoresis (10% for apoA-I, 15% for apoA-II) for 1.5 h at 150 V and transferred to nitrocellulose by conventional blotting procedures. Specific bands were visualized by an ECL assay (Amersham Pharmacia Biotech) after incubation with rabbit anti-mouse apoA-I or apoA-II antibody (Biodesign, dilution 1:2000). As a second antibody, horseradish peroxidase-labeled mouse anti-rabbit antiserum was used (dilution 1:1000; Sigma).

ApoA-I Turnover—ApoA-I was isolated from normolipidemic mouse blood (38) and labeled with ^{125}I (460 cpm/ng of protein) (39). 100 μg were injected into the tail vein, and the disappearance of radioactivity in plasma was monitored over 6 h.

Blood Parameters—Blood samples were collected by retroorbital puncture. Ketone bodies and glucose were determined using commercial kits (Sigma). FFA levels were also measured enzymatically (WAKO

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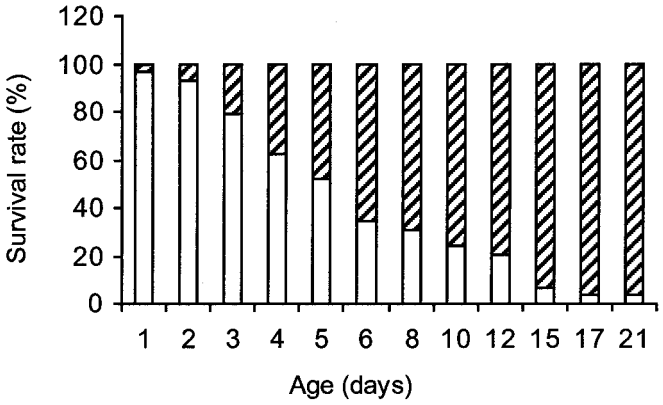

FIG. 1. **Survival curve of Ad-LPL-treated LPL knock-out mice.** Shown is the survival rate (%) of L0 (*open bars*) and L2 (*lined bars*) mice after injection of 5×10^9 plaque-forming units of LPL-expressing adenovirus.

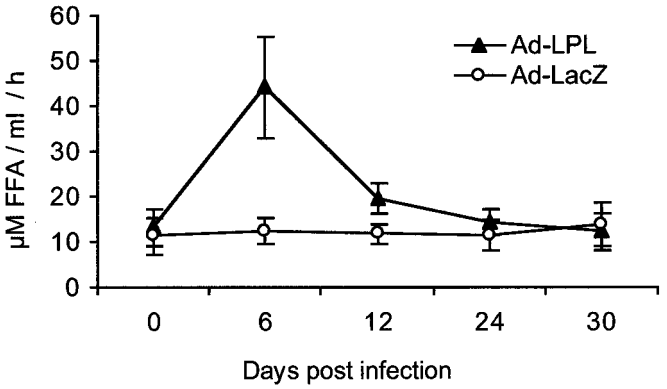

FIG. 2. **Time course of LPL expression after Ad-LPL treatment.** Shown is LPL activity (μmol of FFA/ml/h) in PHP of Ad-LacZ- and Ad-LPL-treated adult control mice ($n = 7$) over a time course of 30 days. The displayed LPL activity includes both human and mouse LPL. Results are presented as means ± S.D. Statistically significant differences ($p < 0.05$) were only observed at 7 and 12 days after treatment.

Chemicals, Neuss, Germany) immediately after the blood was collected. If not otherwise stated, results are given as means ± S.D. Statistical significance was tested by using the two-tailed Student's *t* test.

Histologic Analysis—After killing the mice with Isofluran (Amersham Pharmacia Biotech and Upjohn), various tissues were excised and prepared for analysis. Liver, heart muscle, skeletal muscle, kidney, brain, and spleen were formalin-fixed, embedded in paraffin wax by conventional techniques, hematoxylin-eosin-stained, and examined as previously described (40).

RESULTS

Ad-LPL Expression and Survival of L0 Mice—In an attempt to rescue L0 mice from neonatal death, complete litters of newborn mice from 134 L1 × L1 matings were intraperitoneally injected with LPL-expressing adenovirus (Ad-LPL) immediately after birth (2–8 h). The optimal dose of Ad-LPL was determined by injecting four different virus concentrations: 5×10^8, 1×10^9, 5×10^9, and 8×10^9 pfu, respectively. The best results were obtained with an Ad-LPL concentration of 5×10^9 pfu, which was then used in all subsequent experiments. As depicted in Fig. 1, Ad-LPL injection markedly increased the survival rate of L0 mice. Whereas all untreated L0 mice died within 24 h after birth, 97% of treated L0 animals were alive after this time point. During suckling, the mortality of treated animals increased. After 1 week, 35% of the L0 animals were still alive, whereas after 2 weeks only 10% survived. Most importantly, 3% of L0 mice persevered through the suckling period and weaning and lived normally into adulthood. The oldest L0 animals to date are 20 months of age. No correlation was observed between the number of littermates and the survival rate of L0 mice.

LPL and HL Enzyme Activities—Fig. 2 exhibits the time course of LPL expression after Ad-LPL treatment in adult L2 mice. LPL activity measurements in PHP of adult control mice intravenously injected with 5×10^9 pfu of Ad-LPL revealed that a maximum expression level was observed 7 days following virus injection. Afterward, LPL activities decreased sharply and were back to preinjection levels 3 weeks after virus application. Although it was technically not possible to determine LPL activities in PHP of newborn pups, a similar expression pattern can be assumed in Ad-LPL-treated mice during the suckling period. PCR experiments in 3–4-week-old pups demonstrated that 3 weeks after injection, no adenoviral DNA was detectable in liver and other tissues of Ad-LPL-treated mice.

Expectedly, LPL activity in PHP of adult L0 animals (12–14 weeks of age) was undetectable, whereas control animals exhibited normal LPL activity. In contrast, HL activity was increased in L0 mice by 1.7-fold when compared with L2 animals (Table I). In preheparin plasma the activities of HL were identical to those found in PHP, whereas LPL activity was not detectable (not shown).

Growth Curves and Mouse Development—To assess the consequences of LPL deficiency on body weight, all mice from L1 × L1 matings were weighed during suckling at days 3, 5, 7, 12, 14, and 21 and after weaning every 10 days (Fig. 3*A* compares L2 and L0 mice). L0, L1, and L2 mice appeared normal immediately after birth. Upon suckling, Ad-LPL-injected L0 mice became slightly pale and exhibited reduced body weight compared with L1 and L2 mice. The weight differences were most evident just before and after weaning when a 35% weight reduction was observed in L0 mice (Fig. 3*B*). After weaning, L0 mice caught up in weight on a low fat diet, and by 3–4 months of age their body weight was identical to control mice. Gross pathological examination revealed no obvious abnormalities in adult L0 mice.

Plasma Lipids and Lipoproteins—Plasma TG and TC concentrations were determined in L0 and L2 mice at the age of 7 days, 14 days, and 12 weeks (Table II). During the suckling period, plasma TG levels in L0 mice increased gradually and were 2.9- and 68-fold increased in 7- and 14-day-old animals, respectively, compared with L2 mice. After weaning, the hypertriglyceridemia became less severe; however, in 12-week-old animals TG levels of L0 mice were still 44-fold (fed state) and 30-fold (fasted state) higher than in controls. A similar time course was observed for the plasma TC concentrations, although the differences among the mouse genotypes were less pronounced. A moderate 1.9-fold increase in 7-day-old animals was followed by a 14.3-fold increase in 14-day-old mice. At the age of 12 weeks, TC levels of L0 animals were 4.1-fold higher in the fed state and 2.5-fold higher in the fasted state than in L2 mice.

To investigate the lipid distribution among lipoprotein subclasses, fasted plasma samples of adult L0 and L2 mice were subjected to FPLC analysis (Fig. 4). TC measurements in FPLC subfractions revealed a 137-fold increase of the chylomicron-VLDL fraction in L0 mice. In contrast, HDL-C was undetectable in L0 mice, whereas in L2 mice the majority of the plasma cholesterol content was found in the HDL fraction. To independently validate the virtual absence of HDL in L0 mice, HDL-C plasma concentrations were also measured in plasma after precipitation of β-lipoproteins. In contrast to normal HDL-C levels found in L2 animals (84.05 ± 5.3 mg/dl), L0 mice essentially lacked HDL-C (0.9 ± 0.3 mg/dl).

Analysis of ApoA-I-containing Lipoproteins by Nondenaturating Two-dimensional Electrophoresis—Two-dimensional polyacrylamide gradient gel electrophoresis of plasma samples

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TABLE I
Lipolytic activities in postheparin plasma of fed Ad-LPL-treated L0 and L2 mice

Mice were injected intraperitoneally with sodium heparin (1 unit/g body weight), and PHP was taken 20 min after injection. All values represent means ± S.D. Age of the animals at the time of analysis was 12–16 weeks. **, $p \leq 0.01$; ***, $p \leq 0.001$ compared with the controls.

Genotype	*n*	LPL	HL
		μmol FFA/ml/h	
L0	6	0.12 ± 0.17***	2.45 ± 0.38**
L2	7	6.6 ± 1.1	1.43 ± 0.48

from L0 and L2 mice and subsequent immunoblotting using a specific anti-apoA-I antibody identified two apoA-I-containing HDL subclasses in the plasma of L2 animals, one quantitatively major spot with electrophoretic α-mobility (α-LpA-I) and one quantitatively minor with electrophoretic pre-β-mobility (pre-β_1-LpA-I) (Fig. 5). In contrast, plasma samples of L0 mice contained only traces of apoA-I in the HDL region of the gel, indicating not only the virtual absence of α-migrating mature HDL but also a drastic reduction of their precursor preβ_1-LpA-I.

Analysis of ApoA-I mRNA in the Liver—Northern blotting experiments were performed to analyze apoA-I mRNA levels in the liver of L0 and L2 mice. ApoA-I mRNA concentrations were identical in rescued L0 mice compared with L2 mice (Fig. 6). Accordingly, the absence of HDL particles in the plasma of L0 mice is not a result of decreased apoA-I gene expression.

Western Blotting Analysis of ApoA-I and ApoA-II in Total Plasma and Lipoprotein Fractions—ApoA-I protein expression was determined by Western blotting analysis of total plasma, the chylomicron fraction, and the HDL fraction of L0 and L2 animals. As shown in Fig. 7, apoA-I protein was clearly detectable in total plasma of both mouse lines, and the specific signals for apoA-I exhibited similar intensities, suggesting comparable apoA-I concentrations. However, whereas in control (L2) mice essentially all apoA-I was associated with the HDL fraction, L0 mice lacked apoA-I in the HDL density region. In these mice, apoA-I was predominantly found in the chylomicron fraction. Identical results were obtained for the distribution of apoA-II (not shown). These findings indicated that in the absence of LPL-mediated lipolysis, HDL particles cannot be formed, and apoA-I of hepatic origin is retained within TG-rich lipoproteins.

To investigate renal clearance of apoA-I-containing particles, Western blotting analyses were performed with concentrated urine of L0 and L2 mice (data not shown). ApoA-I excretion was very low in both mouse lines, arguing against increased apoA-I clearance as a cause of HDL deficiency in L0 mice.

To investigate apoA-I turnover in L2 and L0 mice, the disappearance of radioactivity was followed over 6 h after injection of a single dose of iodinated mouse apoA-I. The decay curves were very similar in both mouse lines (Fig. 8), and the disappearance rates were essentially identical despite the fact that apoA-I was transported on different lipoprotein particles, namely HDL in L2 mice and chylomicrons in L0 mice.

Plasma FFA, Ketone Bodies, and Glucose—To investigate the effects of LPL deficiency on energy metabolism, plasma levels of FFA, ketone bodies, and glucose (Table III) were determined in fed animals during the suckling period (1 and 2 weeks of age) and in fasted adult mice (12 weeks of age). FFA concentrations in plasma of L0 mice were increased in both 2-week-old (3.7-fold) and 12-week-old (3.5-fold) animals. At 7 days of age, L0 mice also exhibited increased FFA plasma levels; however, this difference did not reach statistical significance because of the low number of available L0 mice.

The analysis of ketone bodies in plasma revealed increased

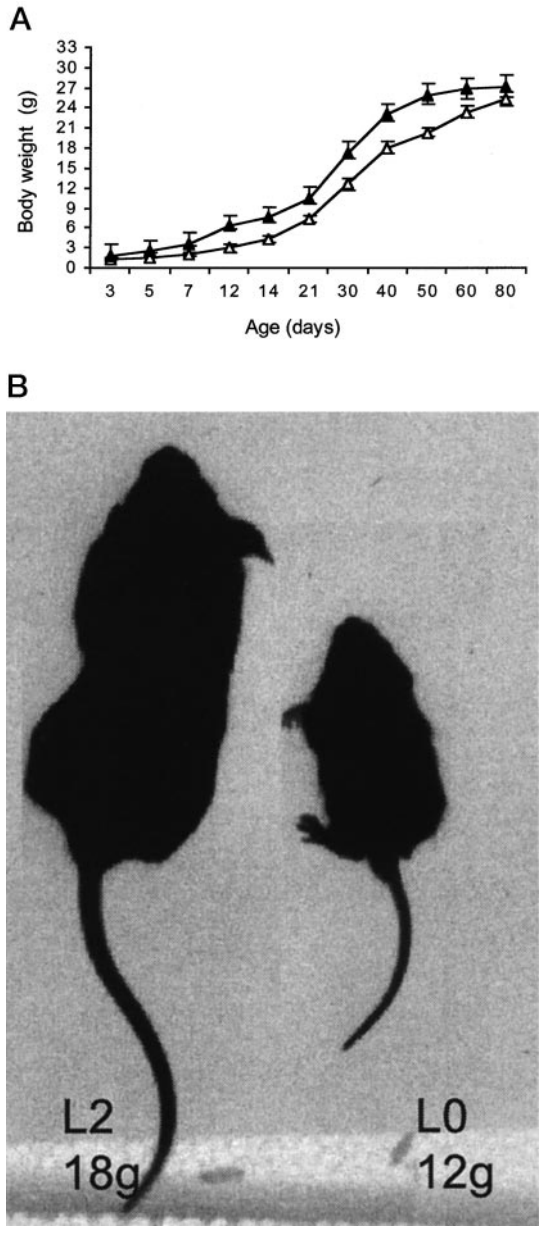

FIG. 3. **Growth of Ad-LPL-treated L2 and L0 mice.** *A*, body weight development over a time course of 80 days. L2 (*closed symbols*) and L0 mice (*open symbols*) were repeatedly weighed in the morning of the indicated days after birth. Results are presented as means ± S.D. *B*, gross appearance of Ad-LPL-treated L2 (*left*) and L0 (*right*) mice. Typical male animals at the age of 4 weeks are compared. The body weights are noted.

concentrations in L0 mice at the age of 7 days (2-fold), 14 days (1.6-fold), and 12 weeks (1.6-fold), suggesting that the increased FFA mobilization in L0 mice led to increased ketone body production in the liver. During suckling, plasma glucose levels were slightly increased with a statistically significant difference at 2 weeks of age (+34%). However, this difference was not seen in fasted adult L0 mice.

DISCUSSION

Mice that lack enzymatically active LPL die prematurely at the beginning of the suckling period. This phenotype was observed in LPL knock-out mice that lack LPL protein (24), as well as in mice carrying the *cld/cld* mutation (25). This genetic defect in a presently unidentified locus on chromosome 17 causes the cellular retention of an incompletely processed LPL protein and the absence of active LPL in the vascular system.

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TABLE II
Triglyceride and total cholesterol levels in the plasma of Ad-LPL-treated L0 and L2 mice

Blood was taken in the morning from 1-week-old, 2-week-old, and 12-week-old male animals of each group having free access to food (fed samples) or after 10 h of nighttime fasting (fasted samples). All values represent means ± S.D. *, $p < 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ compared with the controls.

Age	Genotype	*n*	TG	TC
			mg/dl	
1 week	Fed			
	L0	3	599 ± 132**	121 ± 12
	L2	14	208 ± 79	63 ± 17
2 weeks	Fed			
	L0	3	9453 ± 821**	828 ± 135*
	L2	7	139 ± 42	58 ± 8
12 weeks	Fed			
	L0	7	4997 ± 1102***	396 ± 90**
	L2	5	113 ± 19	97 ± 15
	Fasted			
	L0	7	2007 ± 375***	172 ± 33***
	L2	5	66 ± 7	69 ± 9

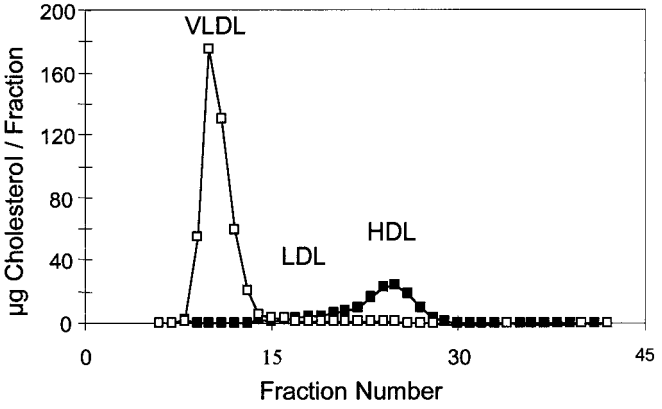

FIG. 4. **Lipoprotein profile.** Shown is a lipoprotein total cholesterol profile by FPLC of fasted plasma from Ad-LPL treated L2 (*filled squares*) and L0 mice (*open squares*) at the age of 10 weeks. Plasma lipoproteins were separated using an Amersham Pharmacia Biotech FPLC system with a Superose 6 column. FPLC fractions 1–40 were collected, and two peaks were identified corresponding to VLDL and HDL. Total cholesterol concentrations were measured in each fraction enzymatically (Sigma).

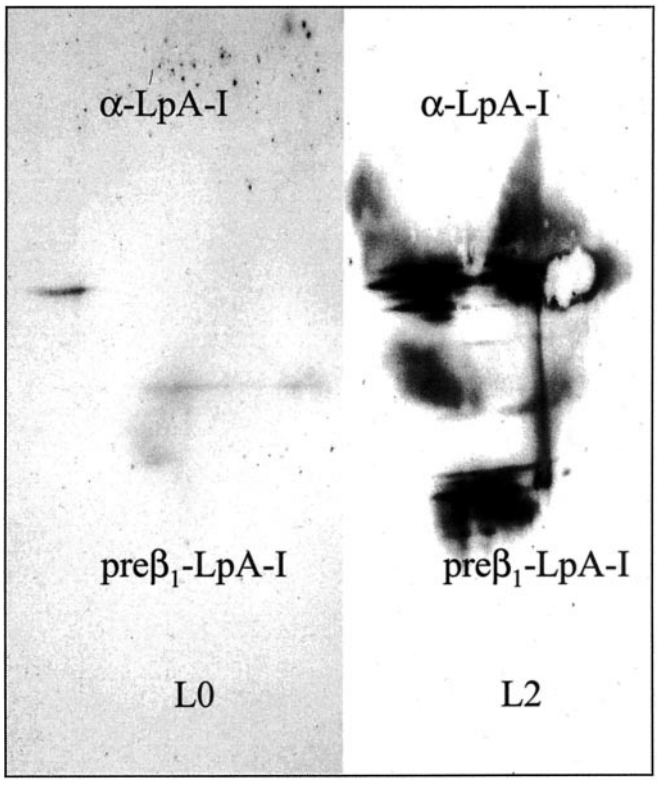

FIG. 5. **Two-dimensional polyacrylamide gradient gel electrophoresis analysis of plasma samples from adult L0 and L2 mice.** Immunoblotting was performed with a specific anti-apoA-I antibody. The locations of preβ_1-LpA-I and α-LpA-I are indicated.

In contrast to mice, LPL deficiency in humans (12), cats (41), and minks (42) is not lethal. This species-specific difference and the actual cause of death in LPL-deficient mice have not been elucidated. Several hypotheses have been proposed: (i) the hypertriglyceridemia following suckling might cause respiratory dysfunction in the mouse because of the higher fat content in mouse milk (10% *versus* 4.5% in human milk) or differences in the anatomy of the lung capillary system; (ii) the abnormally low glucose levels in newborn L0 pups due to the low carbohydrate content in mouse milk (15% *versus* 30% in human milk) might cause lethal hypoglycemia; (iii) the absence of HDL particles might be incompatible with survival, because the majority of plasma lipids are transported in the HDL fraction in mice; (iv) an unidentified, possibly nonenzymatic function of LPL might be essential in mice but is not required in other LPL-deficient species.

Previous studies in which transgenic LPL expression in skeletal muscle, cardiac muscle, or liver (26–29) was achieved in otherwise LPL-deficient mice revealed that these mice can be rescued independently of the site of LPL expression. This suggested that, for survival, the organ in which LPL is expressed is irrelevant as long as sufficient amounts of active enzyme are present in the vascular system. The expression of an enzymatically inactive protein on an L0 background is not sufficient for survival (43). To investigate whether the presence of LPL was obligatory during suckling but dispensable after weaning, the transient expression of LPL by adenovirus-mediated gene transfer was utilized. Ad-LPL was injected into all animals of a litter immediately after birth. LPL expression reached a peak 7 days after injection. Subsequently, enzyme expression declined and was undetectable in weaned animals at 4 weeks of age. A similar expression pattern was observed when LPL-expressing recombinant adenovirus was administered to heterozygous LPL +/− animals (44). The transient expression of LPL after a single virus application resulted in a profound extension of viability in all L0 animals. However, only a small percentage (3%) survived the entire suckling period. These animals were growth-retarded and severely hyperlipidemic. Additionally, they exhibited marked changes in plasma FFA and ketone body concentrations.

Growth retardation was most evident during the suckling period. The effects of complete or partial LPL deficiency during suckling on body weight and development in other species have not been sufficiently studied to date. However, similar trends of decreased body weight have been reported in LPL-deficient cats and minks (41, 42). To our knowledge, data for newborn humans affected with type I hyperlipoproteinemia are not available. Apparently, the decreased availability of TG-derived FFA in suckling L0 mice is not adequately replaced by other substrates in muscle and AT, which might lead to the observed defects in body development. After weaning, when fed a chow diet with 2.5% fat, the animals recovered rapidly and exhibited similar body weight and body composition at 3–4 months of age compared with control mice. Thus, the complete absence of LPL in adult L0 animals did not affect growth. Similar results were obtained in a study of human adults affected with type I hyperlipoproteinemia. These patients were found to have normal

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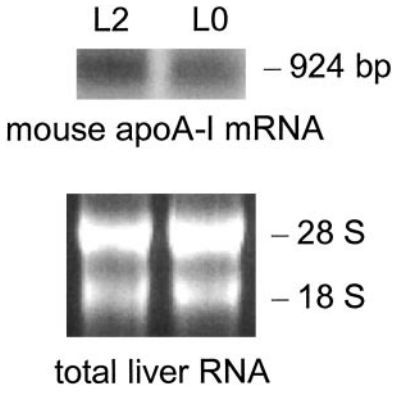

FIG. 6. **ApoA-I mRNA levels in the liver of Ad-LPL-treated L0 and L2 mice.** Total RNA was isolated from liver tissues and subjected to Northern blot analysis. To detect the mouse apoA-I mRNA, a 665-base pair PCR product specific for mouse apoA-I was used as a probe.

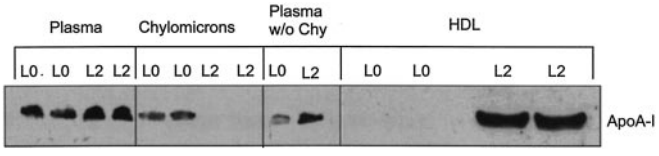

FIG. 7. **ApoA-I levels in plasma and lipoprotein fractions.** For Western blotting analysis samples were mixed 1:1 with loading buffer (5% SDS), incubated for 10 min at 95 °C, and fractionated by SDS-polyacrylamide gel electrophoresis. Bands were visualized by an enhanced chemiluminescence assay after incubation with rabbit anti-mouse apoA-I antibody. As a second antibody, horseradish peroxidase-labeled mouse anti-rabbit antiserum was used.

AT and body weight (45). Additional evidence for normal fat mass development in the absence of LPL in AT was obtained from induced mutant mouse lines that expressed LPL exclusively in muscle but lacked the enzyme in AT (27). These animals had normal body weight and AT mass. However, their AT exhibited a profound change in fat composition. Essential fatty acids were drastically decreased and replaced by saturated and monounsaturated fatty acids in the AT lipid moiety.

Weaned animals on a chow diet (2.5% fat) live for over a year. However, in the absence of LPL the animals are severely hypertriglyceridemic. Plasma cholesterol levels are also increased as a consequence of the drastic increase in the cholesterol content in the TG-rich lipoprotein fraction. In contrast, low density lipoprotein-C and HDL-C were essentially absent in L0 mice. The absence of HDL-C is particularly remarkable, since in normal mice more than 75% of the plasma cholesterol moiety is found in HDL. HDL-C concentrations are also drastically reduced in human LPL deficiency (80–95%), although there is considerable variation. This heterogeneity is partially due to the large number of different mutations in the LPL gene, some of which cause partial absence of LPL activity in PHP, while others result in complete loss of activity (12). Similarly, both of the other existing animal models for familial chylomicronemia syndrome, namely cats and minks, were shown to produce small amounts of active LPL. In contrast to the complete LPL-deficiency in L0 mice, the residual level of LPL activity in these models might explain the essentially normal HDL-C levels (41, 42).

Low HDL levels in LPL deficiency have been attributed to the reduced formation of HDL precursors, the disturbed maturation of these precursors by acquisition of surface remnants (20), the enhanced CETP-mediated transfer of cholesteryl esters from HDL onto TG-rich lipoproteins (46), and the lack of FFA (47). In our study, the absence of CETP in mouse plasma and the increased levels of FFA (Table III) rule out the latter two explanations. The analysis of apoA-I-containing HDL in

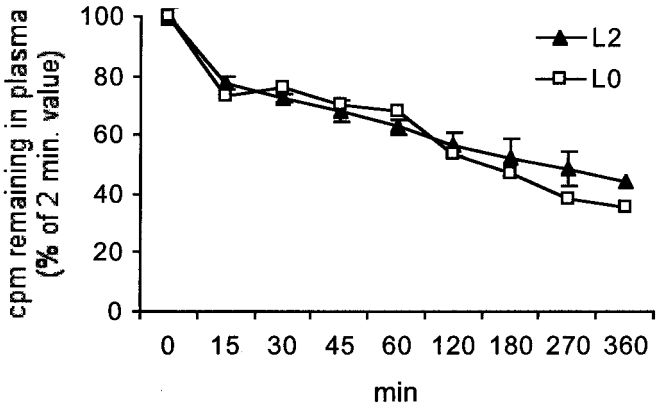

FIG. 8. **ApoA-I turnover.** ApoA-I was isolated from mouse plasma, labeled with ^{125}I, and injected into the tail vein. The percentage of radioactivity remaining in plasma was determined at the times indicated.

TABLE III
Glucose, FFA, and ketone body levels in the plasma of Ad-LPL-treated L0 and L2 mice

Blood was taken in the morning from 1-week-old, 2-week-old, and 12-week-old male animals of each group having free access to food (fed samples) and after 10 h of nighttime fasting (fasted samples). All values represent means ± S.D. *, $p < 0.05$; **, $p \leq 0.01$ compared with the controls.

Age	Genotype	*n*	Glucose	Ketone bodies	FFA
			mg/dl		
1 week	Fed				
	L0	3	129.3 ± 18	18.4 ± 4.6*	34 ± 9
	L2	14	102.2 ± 14	9 ± 2.4	21 ± 6
2 weeks	Fed				
	L0	3	161.6 ± 19.7*	17.4 ± 4.1	63.2 ± 9.2**
	L2	7	120.5 ± 11.7	10.3 ± 3.2	17 ± 3.2
12 weeks	Fasted				
	L0	5	119.8 ± 8	13.3 ± 1.2**	76 ± 11.3**
	L2	6	122.6 ± 5	8.4 ± 0.8	22 ± 6.6

mouse plasma in two-dimensional electrophoresis revealed that both preβ_1-LpA-I and α-LpA-I are absent from LPL-deficient mice despite normal apoA-I mRNA levels in the liver and normal apoA-I protein levels in plasma. ApoA-I in L0 mice, however, was predominantly associated with the chylomicron fraction. Current evidence from liver perfusion studies and experiments with isolated hepatocytes suggests that either free apoA-I (48) or poorly lipidated apoA-I ("nascent " HDL) (49, 50) is secreted by the liver and is subsequently remodeled by PLTP (22) and lecithin:cholesterol acyltransferase (51). Our observations indicate that these HDL precursor components fuse with TG-rich lipoproteins before they are converted to more mature pre-β_1-LpA-I or α-LpA-I particles. An alternative explanation for the lack of HDL, namely that mature HDL particles are first formed normally in LPL knock-out mice but subsequently are absorbed by TG-rich lipoproteins, is unlikely for the following reasons. First, other hypertriglyceridemia models in the mouse that result in high plasma TG levels and have either low or normal LPL expression exhibited reduced HDL levels, but the essential disappearance of HDL, which would be expected if mature HDL would simply be absorbed by TG-rich lipoproteins, was not observed (43, 52). Second, HDL precursor particles (pre-β_1-LpA-I) should be detected in the plasma of LPL knock-out mice if only mature HDL particles (α-LPA-I) were absorbed by TG-rich lipoproteins. However, careful analysis by two-dimensional polyacrylamide gradient gel electrophoresis revealed that pre-β_1-LpA-I particles are barely detectable in the plasma of LPL knock-out mice. This finding suggests that either these particles fuse with TG-rich lipoproteins before they

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accumulate to detectable levels or the precursor of these particles (apoA-I) has already been absorbed, thus preventing pre-β_1-LpA-I formation. Precedence for the accumulation of HDL precursor particles but not mature HDL was reported in a recent publication describing an ABC-A1-deficient mouse model (53). This study demonstrated, using two-dimensional polyacrylamide gel electrophoresis, that in ABC-A1-deficient mice pre-β_1-LpA-I particles are formed normally, yet mature HDL particles (α-LpA-I) are absent. In LPL-deficient mice, not even these HDL precursor particles accumulate detectably, which argues for a defect in an early step of HDL maturation in these mice. Presumably, that step is the dissociation of apoA-I-containing surface remnants as a result of LPL-mediated hydrolysis of the TG core of TG-rich lipoproteins. The importance of surface remnants and other lipolysis products for the maturation of HDL was previously shown in *in vitro* experiments (47, 54, 56) and PLTP knock-out mice, which are defective in the transfer of phospholipids from VLDL to HDL and which also show marked decreases in the serum levels of HDL-C, apoA-I, preβ-LpA-I, and α-LpA-I (22).

Increased clearance of apoA-I into the extravasal space (57, 58) was not observed in L0 mice, because apoA-I concentrations were very low in the urine of both LPL-deficient and control mice. Additionally, the disappearance rates of apoA-I from plasma were identical in L2 and L0 mice, indicating similar turnover kinetics despite the fact that apoA-I was transported in HDL in control mice and in chylomicrons in L0 mice. Taken together, our results support the following conclusions. HDL components are produced normally but are rapidly absorbed by TG-rich lipoproteins at an early stage of HDL maturation before pre-β_1-LpA-I particles are formed. This results in an essential absence of HDL precursor particles, absence of mature HDL, and absence of HDL cholesterol in LPL-deficient mice. These observations are important because recently it has become widely accepted that the major factors responsible for HDL biogenesis are apoA-I expression by the liver, the subsequent cholesterol absorption from peripheral tissues facilitated by ABC-A1, and finally particle remodeling mediated by lecithin:cholesterol acyltransferase, CETP, hepatic lipase, and PLTP (15–19, 21). We show that these processes, at least in mice (which lack CETP), are not sufficient for normal HDL maturation and HDL cholesterol levels when LPL is not present. Thus, LPL, together with apoA-I and ABC-A1, is required for the formation of mature HDL particles.

Recently, the partial absence of LPL in heterozygous LPL knock-out mice has been shown to affect insulin secretion from β-islets, leading to decreased plasma glucose levels in these animals (59). Accordingly, the complete absence of LPL in L0 animals was expected to have a much more profound effect on plasma glucose metabolism. Unexpectedly, however, both glucose and insulin (data not shown) concentrations were identical in adult L0 mice compared with control animals. During the suckling period when adenovirus-derived LPL was produced predominantly in the liver, plasma glucose levels were slightly increased. These results suggested that, first, hypoglycemia in LPL-deficient mice does not occur during suckling or in adult animals and is therefore unlikely to cause the premature death in more than 95% of the Ad-LPL-treated L0 animals. Second, the absence of LPL in islet cells of L0 animals did not affect plasma glucose or insulin levels and is therefore unlikely to markedly affect carbohydrate metabolism. These results are in accordance with previous data from transgenic mouse lines that expressed LPL exclusively in muscle. Although these mice also lacked LPL in β-islets, they exhibited normal to slightly increased blood glucose levels. In humans, the situation is less clear. Although one study reported decreased glucose levels in heterozygous patients with type I hyperlipoproteinemia, glucose tolerance and blood glucose were normal in a large number of homozygous patients with LPL deficiency when compared with normal subjects (60, 61).

Interestingly, FFA and ketone body concentrations were increased in rescued L0 mice. This observation was made in suckling animals as well as in adult mice and has not been reported for other LPL-deficient species. The mechanism behind this observation has yet to be elucidated; however, one could speculate that the observed increase in HL activity might be responsible for the increased FFA concentrations. Alternatively, it is conceivable that inadequate incorporation of FFA into adipose tissue and muscle of L0 mice might contribute to the plasma FFA pool. This phenotype was recently observed in mice expressing human LPL defective in heparin binding (55). Increased FFA transport to the liver combined with decreased VLDL synthesis as a result of the presence of large amounts of TG-rich lipoproteins in plasma could cause hepatic lipid accumulation. This hypothesis is consistent with increased hepatic lipid levels and hepatomegaly commonly observed in LPL-deficient humans and mice (12, 24). Accordingly, the increased availability of FFA in the liver might result in increased β-oxidation rates and increased ketone body formation in L0 mice.

In conclusion, the life span of LPL-deficient mice was prolonged by adenovirus-mediated gene therapy during the suckling period. A small fraction of these animals survived the entire suckling period and developed into normal adulthood with a life expectancy of at least 20 months when kept on a low fat diet. The absence of HDL-C and HDL-apoA-I suggested an essential role for LPL in the maturation of HDL. Regarding energy metabolism, the complete absence of LPL in all tissues including β-islets in rescued, adult animals had no effect on plasma glucose and insulin levels; however, FFA and ketone body concentrations were increased. Adult LPL-deficient mice will be extremely useful for the investigation of the role of LPL and LPL mutants in lipid, lipoprotein, and energy metabolism.

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LIPIDS AND LIPOPROTEINS:

Adenovirus-mediated Rescue of Lipoprotein Lipase-deficient Mice: LIPOLYSIS OF TRIGLYCERIDE-RICH LIPOPROTEINS IS ESSENTIAL FOR HIGH DENSITY LIPOPROTEIN MATURATION IN MICE

Juliane G. Strauss, Sasa Frank, Dagmar Kratky, Günter Hämmerle, Andelko Hrzenjak, Gabriele Knipping, Arnold von Eckardstein, Gert M. Kostner and Rudolf Zechner

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