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Transgenic Angiopoietin-Like (Angptl)4 Overexpression and Targeted Disruption of Angptl4 and Angptl3: Regulation of Triglyceride Metabolism

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Lipoprotein lipase (LPL) is a key regulator of triglyceride clearance. Its coordinated regulation during feeding and fasting is critical for maintaining lipid homeostasis and energy supply. Angiopoietin-like (Angptl)3 and Angptl4 are secreted proteins that have been demonstrated to regulate triglyceride metabolism by inhibiting LPL. We have taken a targeted genetic approach to generate Angptl4- and Angptl3-deficient mice as well as transgenic mice overexpressing human Angptl4 in the liver. The Angptl4 transgenic mice displayed elevated plasma triglycerides and reduced postheparin plasma (PHP) LPL activity. A purified recombinant Angptl4 protein inhibited mouse LPL and recombinant human LPL activity *in vitro*. In contrast to the transgenic mice, Angptl4-deficient mice displayed hypotriglyceridemia and increased PHP LPL activity, with greater effects in the fasted compared with the fed state. Angptl3-deficient mice also displayed hypotriglyceridemia with elevated PHP LPL activity, but these mice showed a greater effect in the fed state. Mice deficient in both Angptl proteins showed an additive effect on plasma triglycerides and did not survive past 2 months of age. Our results show that Angptl3 and Angptl4 function to regulate circulating triglyceride levels during different nutritional states and therefore play a role in lipid metabolism during feeding/fasting through differential inhibition of LPL. (*Endocrinology* 146: 4943–4950, 2005)

ANGIOPOIETIN-LIKE (ANGPTL) 3 and Angptl4 are secreted proteins with structural similarity to angiopoietins (1, 2) that have been shown to be potent inhibitors of lipoprotein lipase (LPL) (3, 4). Both proteins contain an N-terminal coiled-coil region and a C-terminal fibrinogen-like domain and are found in plasma both as a native protein and in a truncated form (1, 2, 5, 6). Angptl4 is expressed primarily in placenta and adipose tissue, with detectable expression in several other tissues including heart, liver, and kidney (2, 7, 8). It has recently been shown to be differentially processed, depending on the site of expression (9, 10), and its expression is known to be up-regulated during adipocyte differentiation *in vitro* (7). *In vivo* it is induced by fasting, but its expression is also differentially regulated by all three peroxisomal proliferator-activated receptors (8, 9). Angptl3 is predominantly expressed in liver and is induced by liver X receptor ligands (1, 11). Its mRNA levels in the liver do not seem to be regulated by nutritional state (12). However, its expression seems to be up-regulated in states of insulin resistance or deficiency (12).

LPL is an endothelium-associated enzyme that hydrolyzes the triacylglycerol component of circulating chylomicrons and very low-density lipoproteins (VLDL), resulting in the production of nonesterified fatty acids and 2-monoacylglycerol for tissue use (13, 14). LPL is known to play a central role in overall lipid metabolism and is associated with several pathophysiological conditions such as atherosclerosis, obesity, and diabetes (13–15). Administration of recombinant Angptl3 or Angptl4 protein to mice mediates hypertriglyceridemia, presumably through inhibition of LPL (4, 16). The KK/San mouse is a natural Angptl3-deficient mouse strain due to a stop mutation in the coding region of the *Angptl3* gene (designated as the *hypl* mutation). It is hypolipidemic on an obese, diabetic background (16). Also, these mice have significantly lower plasma free fatty acids, and Angptl3 has been shown to activate lipolysis in adipocytes as a mechanism for this effect (17).

Angptl3 and Angptl4 are thought to represent a new class of molecules that modulate lipid metabolism by regulation of VLDL triglyceride levels through inhibition of LPL activity. In this study, we have taken a genetic approach to evaluate the function of these proteins in mice. We have generated targeted disruptions of both, *Angptl3* and *Angptl4* as well as transgenics overexpressing Angptl4 in the liver and examined the effects of these genotypes on lipid metabolism and postheparin plasma (PHP) LPL activity in both fed and fasted nutritional states. Our data provide strong evidence

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Abbreviations: Angptl, Angiopoietin-like; Apo, apolipoprotein; DEXA, dual-energy x-ray absorptiometry; FPLC, fast protein liquid chromatography; HL, hepatic lipase; KO, knockout; LPL, lipoprotein lipase; mAngptl4-his, recombinant mouse Angptl4; NMR, nuclear magnetic resonance; PHP, postheparin plasma; rh, recombinant human; VLDL, very low-density lipoprotein.

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that these proteins are important regulators of VLDL triglyceride levels *in vivo* and suggest that both proteins play a role in the nutritional regulation of LPL activity.

Materials and Methods

Animal care and maintenance

The protocols used in these studies were approved by the Eli Lilly Research Laboratories Institutional Animal Care and Use Committee. Mice were maintained in a controlled environment (20 ± 2 C, 50–60% humidity, 12-h light, 12-h dark cycle, lights on at 0600 h). Regular chow was Purina Mouse Chow 5015 (9% fat). The access to both chow and water was *ad libitum*. Mice were bled retroorbital under isofluorane anesthesia. PHP samples for LPL assays were collected after injection of 10 U/g body weight of heparin into the tail vein 20 min before blood collection.

Generation of Angptl4 transgenic mice

The human apolipoprotein (Apo) E promoter including its hepatic control region (18) was used to express the human Angptl4 cDNA in transgenic mice. B6SJL eggs were injected by standard microinjection methods (19). Transgenic mice were identified by PCR with transgene-specific primers, and transgene expression was confirmed by real-time quantitative PCR from liver RNA. Transgenic mice were mated to C57BL/6 mice (Taconic Farms, Germantown, NY).

Generation of Angptl4-deficient mice

The genomic sequence of Angptl4 could be directly extracted from GenBank (accession no. AF 110520.1). The targeting vector deleted 2.05 kb of the endogenous gene including most of exon 1 containing the start codon and the signal peptide and exons 2 and 3. Homologous recombination in E14–129/Ola embryonic stem cells was performed according to standard procedures as described (20). Homologous recombination was confirmed by Southern blot analysis using a 3′ external probe and a 5′ external probe, respectively. Three positive clones were identified and microinjected into C57BL/6 blastocysts. Male chimeric founders were mated to C57BL/6 females, and agouti-coated offspring were analyzed for germ line transmission of the Angptl4 mutation by Southern blot. 129B6F2 littermates were used for all experiments.

Generation of Angptl3-deficient mice

The sequence of Angptl3 exon 1 (NM_013913) was used as a probe to isolate mouse genomic DNA clones corresponding to the Angptl3 locus from a λ-Fix II phage library prepared from mouse strain 129/SvJ (Stratagene Inc., La Jolla, CA). The targeting vector deleted 1.3 kb of the gene including the exon 1 containing the start codon. Homologous recombination and blastocyst injection were done as described for Angptl4-deficient mice except R1 embryonic stem cells were used for gene targeting. All experiments were performed on 129B6F2 hybrid mice.

Western analysis

Plasma samples for Western analysis were collected in EDTA tubes containing protease inhibitors (Complete mini-protease inhibitor cocktail tablets, Roche Diagnostics, Indianapolis, IN). The polyclonal rabbit antibody was raised against peptide (residues 52–65; GQGLREHAERTRSQ) of the human sequence (2) and purified by affinity chromatography with the antigen.

Northern blot analysis

Livers from wild-type and Angptl3-deficient mice were used for total RNA preparation with the TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Northern blot analysis was performed with PolyA$^+$ RNA, which was obtained from the total RNA using Oligotex (QIAGEN, Santa Clarita, CA). The probe for the Northern blot was a PCR-amplified, 253-bp fragment corresponding to nucleotides 554–806 of the murine Angptl3 cDNA. The primers used were: SF3F2, 5′-AGAACAGCAAGACAACAGCATAAGA-3′ and SF3B2, 5′-TCTGTTATAAACGGCAGAGCAGTCG-3′, respectively. The probe was labeled with ^{32}P d-CTP and hybridization was performed according to the manufacturer's instructions (Clontech, Palo Alto, CA). The same blot was stripped and reprobed with β-actin as a quantity control. Northern blot analysis of Angptl4 knockout mice was performed using total RNA from white adipose tissue isolated from wild-type, heterozygous, and homozygous Angptl4 knockout mice. A 700-bp Angptl4 cDNA fragment spanning exons 1–5 was used as probe. As control the blot was stripped and reprobed with β-actin.

Lipoprotein analysis by fast protein liquid chromatography (FPLC)

The lipoprotein distribution of plasma cholesterol was determined by FPLC. Briefly, plasma samples were injected onto Superose 6 HR column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) at a flow rate of 0.5 ml/min of PBS containing 5 mM EDTA. Cholesterol was assayed post column with the in-line addition of cholesterol reagent (Roche Diagnostics) at a flow rate of 0.12 ml/min. After passing through a 15-m reactor tubing at 37 C, lipoprotein components were detected at 505 nm with a spectrophotometer. Peak quantification was done with Turbochrom Professional software (PerkinElmer LLC, Norwalk, CT).

Analysis of LPL mRNA levels

Real-time quantitative PCR assays of LPL mRNA in murine fat was performed as previously described (21) with the following primer/probe set: AMF104, TGGATGAGCGACTCCTACTTCA; AMF105, CGGATCCTCTCGATGACGAA, AMF106FT CTGGCCCGACTGGTGGAGCAG (probe).

Analyses of serum and plasma samples

Plasma triglyceride and cholesterol levels were determined with a Hitachi 912 (Roche Diagnostics). Cholesterol levels were detected with the cholesterol esterase/cholesterol oxidase enzyme assay (Roche Diagnostics). Triglycerides were determined with the lipase assay enzyme kit (Roche Diagnostics). Blood glucose levels were determined with the Precision G instrument (Abbott Diagnostics, Abbott Park, IL).

Oral glucose tolerance testing

Oral glucose tolerance testing was performed as previously described (22)

Body mass composition analysis by nuclear magnetic resonance (NMR)

A wide-line NMR instrument (Echo-Bruker Instruments; Houston, TX) was used as described elsewhere (22).

Body mass composition analysis by dual-energy x-ray absorptiometry (DEXA) scan

A pDEXA forearm bone densitometer (Norland Medical Systems, White Plains, NY) was used to perform DEXA as previously described (23).

Expression and purification of mouse Angptl4

A HIS tag (sequence DIHHHHHH) was fused to the C terminus of the Angptl4 protein. Media from a stably transfected Chinese hamster ovary cell line were applied to a Ni-loaded iminodiacetic acid column (Amersham Bioscience, Fairfield, CT). The column was washed with 10 mM sodium phosphate, 150 mM sodium chloride buffer (pH 7.5) (PBS). Bound protein was eluted with a gradient from 0 to 0.5 M imidazole in PBS. Fractions were analyzed by SDS-PAGE from which the purity was assessed as greater than 90%. The identity of mAngptl4-his was confirmed by N-terminal sequence analysis.

LPL activity assays

The triolein/gum arabic assay for lipase activity was based on Henderson *et al.* (24). Substrate in the form of triolein emulsion in gum arabic was prepared by sonication in 0.2 M Tris saline buffer (pH 8.5). BSA and heat-inactivated rabbit serum was added after sonication. Free fatty acid released was separated from the substrate and diacylglycerol, according to Borensztajn *et al.* (25). The assay was carried out in the presence and absence of 1 M NaCl to determine hepatic lipase activity. Purified recombinant human LPL and hepatic lipase (HL) were used as standards. Apo CII peptide (residues 44–79, Midwest Biotech, Fishers, IN) was used instead of Apo CII-containing serum. The final concentration of phosphatidylcholine, which is essential for the high potency of the Apo CII peptide, was 1 mM.

Statistics

Results were statistically evaluated with an unpaired Student's *t* test (Microsoft Excel, Microsoft, Redmond, CA). $P < 0.05$ was considered significant.

Results

Angptl4 transgenic mice are hyperlipidemic and have decreased LPL activity

Transgenic overexpression of human Angptl4 in the liver was driven by the Apo E promotor and the Apo E hepatic control region (18). Human Angptl4 protein was detected in plasma from transgenic mice as a single band of approximately 35 kDa by Western blot analysis using an antibody against the N-terminal coiled-coil domain (Fig. 1A). This circulating form of Angptl4 presumably represents the N-terminal truncated protein because the predicted molecular mass of the native protein is 65 kDa. There was no detectable human Angptl4 in plasma of wild-type mice (Fig. 1B). Angptl4 transgenics displayed no overt phenotype or gross abnormalities. There were no significant differences in plasma glucose, insulin, or leptin in the fed or fasted states between transgenic and wild-type mice (data not shown). However, serum triglycerides were increased more than 3-fold in male transgenic mice and 2.6-fold in female transgenics (Fig. 2A). Total cholesterol was 25% higher in both male and female transgenic mice, compared with gender-matched controls (Fig. 2B). The FPLC analysis of the plasma lipoproteins from male mice revealed that the largest portion of the cholesterol increase was found in the VLDL fraction (Fig. 2C), which is expected because it is the major triglyceride-carrying lipoprotein.

To explore the mechanism of the hypertriglyceridemic phenotype, we determined PHP LPL activity in male and female Angptl4 transgenic mice. LPL activity in male transgenic mice was reduced to approximately 6% of the level in wild-type mice and was undetectable in three of four of the female transgenic mice (Fig. 3A), whereas HL activity was unchanged (Fig. 3B). We observed no differences in LPL mRNA levels evaluated by Taqman analysis in adipose tissue of transgenic and wild-type mice (data not shown).

To determine whether liver-specific Angptl4 overexpression altered fat mass, an NMR analysis was performed on 9-month-old mice. There were no significant differences in

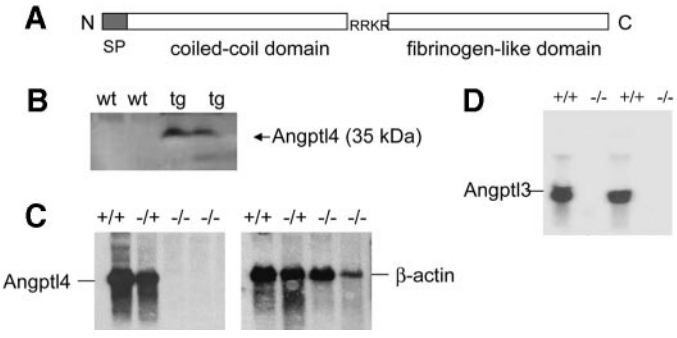

FIG. 1. A, Schematic diagram of the human Angptl4 protein. The RRKR site represents a potential furin cleavage site (1). SP, Signal peptide. B, Western analysis of human Angptl4 in plasma from wild-type (wt) and Angptl4 transgenic (tg) mice. C, Northern blot analysis of *Angptl4* transcript in white adipose tissue from fasted wild-type (+/+), heterozygous (+/−), and homozygous (−/−) Angptl4 KO mice. Twelve micrograms of total RNA were hybridized with a PCR-generated probe containing exons 1–5. The filter was stripped and rehybridized with a mouse β-actin probe (Ambion, Inc., Austin, TX) to control for RNA quantity. D, Northern blot analysis of *Angptl3* transcript in liver tissue from wild-type (+/+) and homozygous (−/−) Angptl3 KO mice. PolyA$^+$ RNA was hybridized with a 253-bp fragment corresponding to nucleotides 554–806 of the murine Angptl3 cDNA.

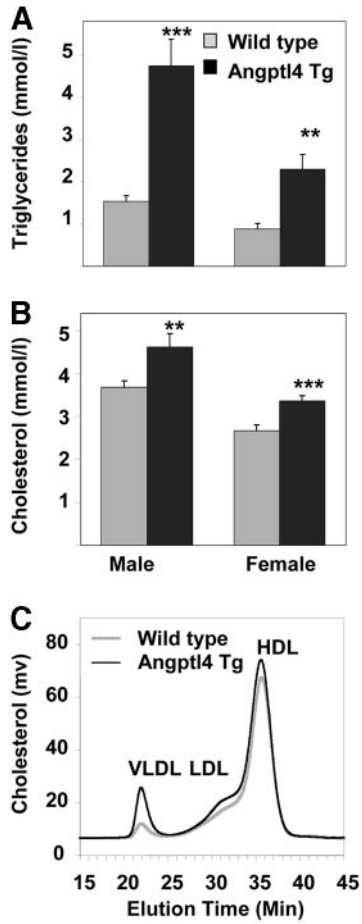

FIG. 2. Angptl4 transgenic mice are hyperlipidemic. Triglyceride (A) and cholesterol (B) levels in fed female and male wild-type (wt) and Angptl4 transgenic mice (Angptl4 tg, n = 8–10). Means ± SE. ***, $P < 0.001$; **, $P < 0.02$. C, Lipoprotein profiles of wild-type and Angptl4 transgenic mice. Chromatograms were generated by FPLC analyses of plasma samples collected from nonfasted male mice. *Lines* represent a composite of eight animals per genotype. HDL, High-density lipoprotein; LDL, low-density lipoprotein.

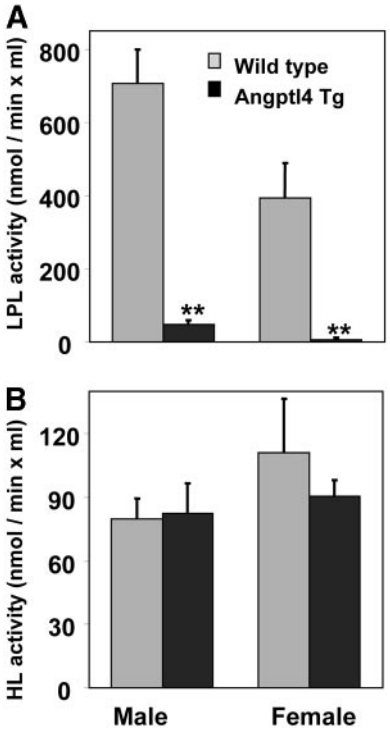

FIG. 3. PHP LPL activity is reduced in Angptl4 transgenic (Tg) mice, whereas HL is unaffected. A, LPL activity from fed male and female wild-type (n = 5 each) and Angptl4 transgenic female and male (n = 4 each) mice. B, HL activity (males, n = 5; females, n = 4). Means ± SE. ***, $P < 0.001$.

lean or fat mass between the transgenics and wild-type mice on chow diet or after feeding a high-fat/high-carbohydrate diet for 15 wk (data not shown).

Angptl4 knockout (KO) mice are hypolipidemic and have elevated LPL activity

To further evaluate the function of Angptl4 *in vivo*, we generated Angptl4 KO mice. Homozygous *Angptl4*$^{-/-}$ mice did not show any detectable *Angptl4* mRNA in adipose tissue, its major endogenous site of expression (Fig. 1C). Angptl4 KO mice were born at the expected Mendelian frequency, were fertile, and had a normal lifespan (up to 24 months) with no apparent phenotype or developmental abnormalities. There were no changes in plasma glucose, insulin, and leptin in KO mice, compared with wild-type mice (data not shown).

Plasma triglycerides in male Angptl4 KO mice in the fed state were reduced by 70%, but there was no change in females (Fig. 4A). The mice were also studied in the fasted state because Angptl4 is up-regulated in wild-type mice by fasting (7), and we expected a stronger phenotype in fasted Angptl4 KO mice. In the fasted state, both male and female KO mice had significantly reduced plasma triglycerides (Fig. 4A). In male KO mice, plasma triglyceride levels were reduced by nearly 90% relative to their wild-type controls, and female KO mice displayed a 65% reduction. Also, plasma cholesterol was decreased significantly in male KO mice in either nutritional state, but in females the decrease did not reach statistical significance (Fig. 4B). Whereas there were no

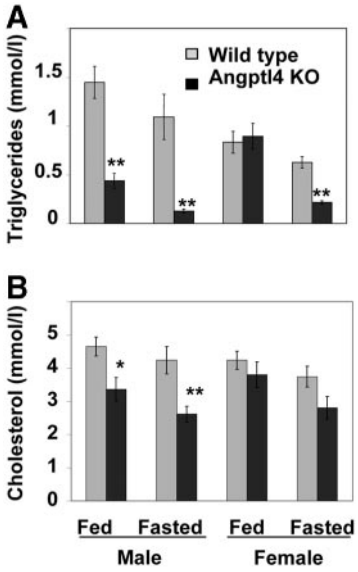

FIG. 4. Angptl4 KO mice are hypolipidemic. A, Triglyceride levels in Angptl4 KO and wild-type mice in fasted (15–16 h) and fed states (n = 4–6). B, Total cholesterol levels in Angptl4 KO and wild-type mice (n = 4–6). Means ± SE. *, $P < 0.05$; **, $P < 0.02$.

apparent differences in body weight of the KO mice at 2 and 6 months of age relative to wild-type mice, the striking reduction in serum triglycerides led us to evaluate body composition of the KO mice by NMR analysis. There were no significant differences in either fat or lean mass of KO mice at 6 months of age, whether fed chow or a high-fat diet for 15 wk (data not shown).

We next evaluated PHP LPL activity from fed and fasted Angptl4 KO mice. We found that LPL activity was increased 2-fold in male and female Angptl4 KO under fed conditions. Under fasted conditions a 3-fold increase for females and a 3.5-fold increase for male mice, compared with wild type, was observed (Fig. 5A), which is generally consistent with the changes in plasma triglycerides. The exception was in female KO mice in the fed state, in which the 2-fold increase in LPL activity did not translate into a lowering of plasma triglycerides. HL activity was not significantly different between the wild-type and Angptl4 KO mice of either gender (Fig. 5B).

Recombinant Angptl4 protein inhibits LPL activity in vitro

To determine whether the reduction of LPL activity in transgenic mice was due to direct inhibition by Angptl4, we evaluated the effect of recombinant mouse Angptl4 (mAngptl4-his) on LPL activity *in vitro*. The addition of mAngptl4-his protein to PHP from Angptl4 KO mice at concentrations of 0.4 and 4.0 nM resulted in a dose-dependent inhibition of LPL activity (Fig. 6A). We next determined whether the mAngptl4-his protein inhibited recombinant human (rh) LPL. Using 0.5 μM Apo CII peptide as cofactor, mAngptl4-his inhibited rhLPL in a concentration-dependent manner, with an IC_{50} of 14 nM (Fig. 6C). If Angptl4 were functioning as a competitive inhibitor of Apo CII, we would expect the inhibition to be reversed by a higher concentration of Apo CII. However, we observed that the IC_{50} for inhibition of rhLPL by mAngptl4-his was not significantly

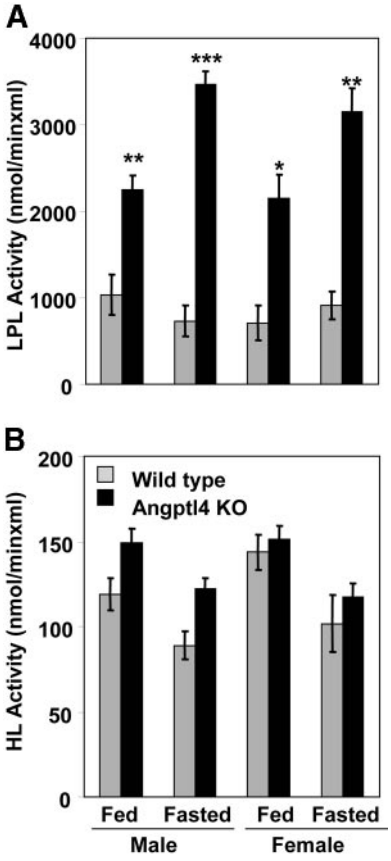

FIG. 5. LPL activity is elevated in PHP from Angptl4 KO mice. A, LPL activity was measured in PHP from overnight fasted (15–16 h) and fed wild-type (n = 5 each) and Angptl4 KO mice (n = 5 each). B, HL activity (n = 5 each). Means ± SE. ***, $P < 0.001$; **, $P < 0.02$; *, $P < 0.05$.

altered (14 *vs.* 20 nM) by increasing the Apo CII peptide concentration from 0.5 to 3.0 μM (Fig. 6C). Thus, Angptl4 appears not to compete with Apo CII in this LPL activity assay.

To confirm the presence of an endogenous inhibitor in the postheparin plasma from Angptl4 transgenic mice, we applied a classical enzyme kinetics approach (26), using LPL in PHP from the transgenic mice. The assumptions for this approach were: 1) the amount of PHP LPL in the transgenic mice was roughly equivalent to that in wild-type mice, only masked by inhibition by Angptl4 overexpression; 2) mouse LPL and human LPL have similar activities *in vitro*; and 3) the endogenous concentration of Angptl3 in the plasma is negligible, compared with the concentration of overexpressed Angptl4. Previously we had determined that the LPL activity in PHP from wild-type mice is equivalent to that of approximately 0.8 nM of rhLPL. We serially diluted PHP from Angptl4 transgenic mice and determined inherent LPL activity at each dilution. For normalization, 0.8 nM of rhLPL was added to serial dilutions of preheparin plasma from Angptl4 KO mice (presumably with no, or very little, endogenous inhibitor) to determine LPL activity at each dilution for comparison. The normalized LPL activities of transgenic/KO were plotted against the dilution factor of the plasma and showed a nonlinear relationship, indicating the presence of an endogenous inhibitor (Fig. 6D). The IC_{50} from this analysis was 12 nM, which was comparable with the IC_{50} that we determined for mAngptl4-his (Fig. 6C).

Angptl3-KO mice have hypolipidemia and elevated PHP LPL activity

The absence of *Angptl3* mRNA in *Angptl3*$^{-/-}$ mice was confirmed by Northern blot analysis from liver tissue (Fig. 1D). Angptl3 KO mice had no gross abnormalities, were fertile, and exhibited no developmental abnormalities. Body weight and body composition determined by pDEXA scan at 5 months of age were not significantly different between the KO and wild-type mice in (not shown). Plasma cholesterol was significantly lower in males and females in fed and fasted states (Fig. 7B). Furthermore, male and female Angptl3 KO mice displayed severe hypotriglyceridemia in the fed state (Fig. 7A). Whereas female KO mice also displayed this phenotype in the fasted state, we did not observe the severe hypotriglyceridemic phenotype in fasted male Angptl3 KO mice. Because the effects of Angptl3 on plasma triglycerides were previously shown to be caused by an inhibition of LPL (3), we determined whether the differential effects on plasma triglycerides in fasted *vs.* fed Angptl3 KO males were related to changes in LPL activity. PHP LPL activity was approximately 9-fold higher in male Angptl3 KO, compared with male wild-type mice (Fig. 8). In the fasted condition, there was no such increase, and, in fact, LPL activity was slightly decreased in the Angplt3 KO mice.

Angptl3/4 double KO mice exhibit early mortality

We were also interested in examining the phenotype of double-deficient mice. When we crossed double heterozygotes to generate double KO mice deficient for both Angptl3 and Angptl4, we could identify double KO mice at a rate of only 2.9%, which is less than half of the expected rate (6.25%) for this type of crossing and statistically significant different [$P = 0.034$, n = 339 mice genotyped, determined by goodness of fit using StatXact-5 (Cytel Software, Cambridge, MA)]. This indicated that more than half of the double KO mice exhibited pre- or perinatal mortality. Surviving double KO mice were runted and did not survive beyond 2 months of age. The plasma from the surviving mice contained nearly undetectable triglyceride levels and reduced cholesterol, especially in fasted male mice (not shown).

Discussion

In the present study, we generated and performed an initial characterization of mice deficient in either Angptl3 or Angptl4, and mice overexpressing human Angptl4 from the liver. We observed a positive relationship between plasma triglycerides and expression of either Angptl3 or Angptl4. Both the Angptl4 and Angptl3 KO mice displayed a hypotriglyceridemic phenotype. The Angptl3 KO phenotype was similar to that displayed by KK/San mice, which express reduced levels of Angptl3 due to a mutation in the *Angptl3* gene (16). In contrast, the mice overexpressing human Angptl4 had increased plasma triglycerides, consistent with studies in mice administered recombinant Angptl4 or Angptl3 protein (4, 16). These findings clearly demonstrate

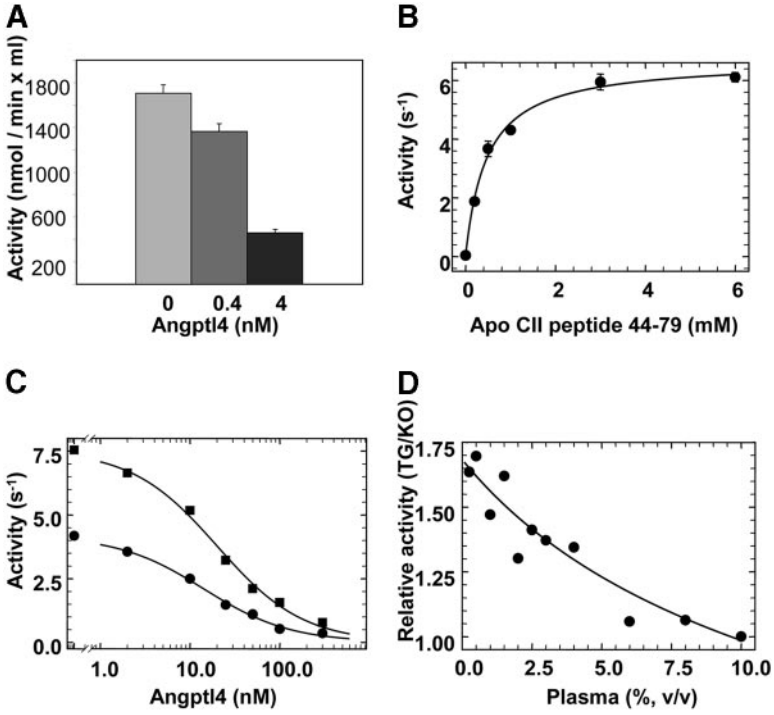

FIG. 6. *In vitro* inhibition of LPL by recombinant Angptl4 protein. A, Addition of recombinant mouse Angptl4 protein inhibits LPL activity in PHP from Angptl4-deficient mice. LPL activity is plotted against the molar concentration of recombinant Angptl4 protein added to the reaction. Each *bar* represents an average from three individuals, each determined in triplicate. B, Concentration dependence of activation of LPL by apoCII peptide. Each *point* represents the average of three determinations ± SE. *Solid line* illustrates the least-square fit of rectangular hyperbola to the data with EC_{50}, 0.5 μM. C, Inhibition of rhLPL by recombinant Angptl4 is independent of apoCII peptide. The activity of 2 nM LPL in the presence of different concentrations of recombinant Angptl4 protein was determined with two different concentrations of apoCII peptide. Concentrations of apoCII peptide, 0.5 μM (●) and 3 μM (■), were approximately equal to the EC_{50} and 6 × EC_{50} for the activation of LPL by this peptide (see B). *Solid lines* represent least-square fit of rectangular hyperbola to the data, with IC_{50} values of 14 and 20 nM, respectively. D, PHP from Angptl4 transgenic (TG) mice contains an endogenous inhibitor of LPL activity. Serial dilutions of PHP from transgenic mice were used. For normalization, preheparin plasma from Angptl4-deficient mice spiked with 0.8 nM rhLPL protein was diluted and assayed in the same way. The resulting activities were compared and plotted against the total amount of enzyme. The *solid line* represents the least-square fit of a model for codilution of enzyme and inhibitor with IC_{50} = 12 nM. Each determination was performed in triplicate.

regulation of plasma triglyceride levels by Angptl3 and 4 *in vivo*.

The mechanism of plasma triglyceride regulation by the Angptl proteins involves the modulation of LPL activity. In mice lacking either Angptl3 or Angptl4, PHP LPL activity was increased, whereas LPL activity was very low in transgenic mice overexpressing Angptl4. Our *in vitro* experiments demonstrated that recombinant Angptl4 protein inhibits LPL activity directly, a property that it shares with Angptl3 (3). The increases in plasma triglycerides and VLDL cholesterol in our Angptl4 transgenic mice were likely due to reduced clearance of VLDL because it has been demonstrated that inhibition of LPL by the Angptl proteins results in delayed clearance of VLDL without impacting the VLDL production from the hepatocytes (3, 27). The Angptl3 and 4 KO mice, in contrast, displayed a hypolipidemic phenotype due to increased LPL activity, which likely increased clearance of VLDL from the circulation. Another potential cause for the detected low plasma

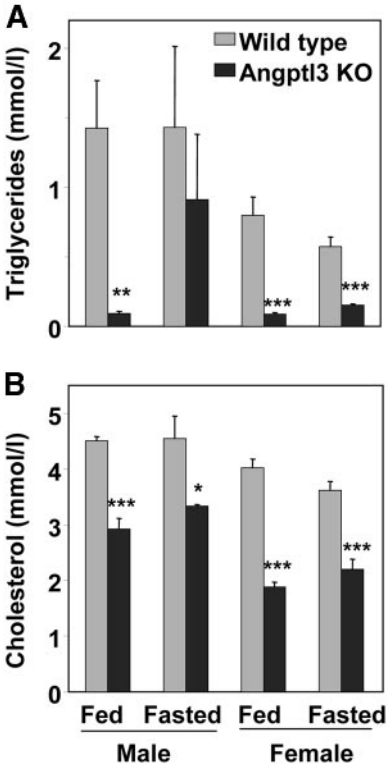

FIG. 7. Lipid analysis of Angptl3 KO mice in fed and fasted states. Triglyceride (A) and total cholesterol (B) levels in fed male (n = 4) and female (n = 7) wild-type and male (n = 4) and female Angptl3 KO (n = 6) mice. Means ± SE. ***, $P < 0.001$; **, $P < 0.02$; *, $P < 0.05$, compared with wild type.

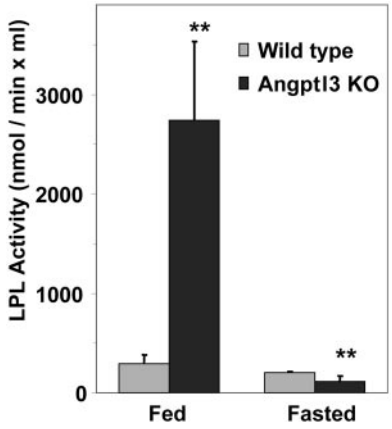

FIG. 8. PHP LPL activity is elevated in Angptl3 KO mice in the fed state only. LPL activity is shown for both KO and wild-type mice under fed and fasted states. Data were obtained only from male mice. Means ± SE. **, $P < 0.02$, compared with wild type.

triglyceride levels could be impaired intestinal lipid absorption. However, we observed that feeding the Angptl4 KO mice a high-fat diet (60% calories from fat) for 15 wk did not cause changes in body weight or body fat relative to wild-type littermates (Köster, A., A. Ford, and P. Eacho, unpublished data), which makes intestinal lipid malabsorption unlikely. Accordingly, both Angptl3 and Angptl 4 regulate circulating levels of triglycerides through inhibition of LPL, the major catabolic enzyme for triglycerides.

The activity of LPL *in vivo* is subject to tissue-specific regulation in response to changes in nutritional state (13). It is upregulated in adipose tissue after feeding to promote the storage of triglycerides, in part through the actions of insulin. During fasting, when insulin levels are low, LPL activity is decreased in adipose tissue to promote the use of triglycerides by peripheral tissues. The short-term regulation of LPL activity in rodents is posttranscriptional in adipose and heart (28, 29), and inhibition of LPL activity in fasting adipose requires the expression of a distinct gene (30). Angptl4 expression is increased in adipose during fasting (8), making it a prime candidate for a gene product Bergö *et al.* (30) described that down-regulates LPL during periods of caloric restriction. This is supported by our observation that Angptl4 deficiency impacts LPL activity and triglycerides more significantly in fasted compared with fed mice. In contrast, the effects of Angptl3 deficiency on triglycerides and LPL activity were greater in the fed state. Thus, Angptl3, whose mRNA expression is not regulated by nutritional state in mouse liver (12), seems to have a more dominant role in LPL regulation during the postprandial period when Angptl4 levels are low. The important role of Angptl4 as a fasting-induced factor is also demonstrated by our observation that in mice lacking both proteins, the hypertriglyceridemic phenotype is most severe in the fasted state. A differential effect of nutritional state on the lipid levels and PHP LPL activity was observed for both KO lines but not the Angptl4 transgenic mice (not shown), most likely due to the already high overexpression of the protein and the lack of endogenous regulation of the Angptl4 transgene. We also observed some gender-related differences in the triglyceride phenotype of the Angptl3 and Angptl4 deficient mice dependent on the nutritional state (Figs. 4 and 7). We have not explored the mechanism of these gender differences, but they may involve estrogen, which exerts transcriptional control over LPL expression (31).

Although both Angptl3 and Angptl4 inhibit LPL activity, they do not appear to be redundant proteins. We observed decreased triglyceride levels in mice deficient in either gene, which would not be expected if one gene assumed the function of the other. Thus the differential effects of nutritional status observed in the KO mice suggest distinct roles for the proteins. Angptl3 and Angptl4 would also be expected to have distinct roles in regulating triglyceride metabolism based on their sites of expression. Angptl4 is expressed primarily in adipose, which is rich in LPL, whereas Angptl3 is mainly expressed in the liver, an organ normally low in LPL. Yu *et al.* (10) speculated that proteolytic processing of Angptl4 into a truncated, active N-terminal coiled-coil domain, which circulates in plasma, is delayed in LPL-rich tissues like adipose due to a tethering effect to LPL. As a result, Angptl4 may not be readily released into the circulation and thus may function mostly in an autocrine/paracrine manner, regulating LPL locally in adipose. Consistent with this proposal was the finding that overexpression of Angptl4 in the heart, an LPL-rich organ, resulted in inhibition of LPL activity only in the heart (10). PHP LPL activity was not decreased by heart-specific Angptl4 overexpression, even though plasma triglycerides were increased by about the same magnitude we observed in our liver-specific Angptl4 transgenics. These findings demonstrate that the level of expression of LPL in the heart is sufficient to regulate systemic triglyceride levels and, apparently, to cause transgenically expressed Angptl4 to be retained in the heart to function locally in an autocrine/paracrine fashion. This is in contrast to the situation in our mice, in which transgenic Angptl4 was made in the LPL-low liver, presumably processed readily, and released into the circulation as a truncated, 35-kDa N-terminal fragment. This concept is consistent with the observations of Mandard *et al.* (9) that human adipose seems to produce exclusively unprocessed, full-length Angptl4, whereas human liver mainly produces the truncated protein. Thus, liver-expressed Angptl4 may function in an endocrine fashion and contributes to the regulation of LPL in adipose and potentially other tissues. It has been shown that Angptl3 is processed in a manner similar to Angptl4 (5). Thus, endogenous Angptl3, which is primarily made in mouse liver, may also be processed into the truncated form and function in an endocrine fashion.

The inhibition of LPL activity in Angptl4 transgenic mice resulted in a 2.6- to 3-fold increase in plasma triglycerides. Administration of Angptl4 by injection or adenoviral expression also resulted in increased plasma triglyceride levels, although the increase after adenoviral expression was much higher, probably due to higher transient expression levels of Angptl4. In all cases, the effect was modest, compared with the 30- to 50-fold elevation of plasma triglycerides observed in LPL knockout mice (32), suggesting only a partial inhibition of total LPL activity by Angptl3 or Angptl4.

Because adipose LPL facilitates the storage of postprandial triglycerides within adipocytes, we anticipated that Angptl4 transgenic mice, with reduced LPL activity, would have reduced fat storage and adipose mass. However, there was no significant change in adipose mass of the Angptl4 transgenic mice. Likewise, despite the elevated LPL activity and a reduction in plasma triglycerides in the Angptl3 and Angptl4 KO mice, body weights and fat content were not significantly different from wild-type mice. Moreover, Angptl3 deficiency in KK/San mice also had no effect on body weight (16). The lack of a significant change in the fat mass of our transgenic and knockout mice suggests that compensatory mechanisms exist in adipose to stabilize fat mass under conditions of variable fatty acid delivery by LPL. Increased adipose lipogenesis is one likely mechanism at play in the Angptl4 transgenic mice because lipogenesis is activated in LPL-deficient adipose tissue (33, 34). In addition, adipocytes can modulate intracellular fat content by regulating hormone-sensitive lipase activity, as was observed in human LPL transgenic mice (35). Other lipases in adipose tissue may compensate for decreased LPL to maintain input of fatty acids from lipoproteins. Indeed, endothelial lipase, a phospholipase whose expression is undetectable in adipose of normal mice, is abundantly expressed in adipose of LPL-deficient mice (36). We feel it is likely that the maintenance of normal fat mass in mice overexpressing or deficient in Angptl3 and Angptl4

results from compensatory metabolic changes in adipose triglyceride metabolism.

In conclusion, we have shown that: 1) hepatic overexpression of Angptl4 in transgenic mice results in hypertriglyceridemia, concomitant with reduced PHP LPL activity; 2) Angptl4 protein is an inhibitor of LPL activity; 3) Angptl3 and Angptl4 deficiency results in hypotriglyceridemia associated with elevated PHP LPL activity, the magnitude of which is dependent on the nutritional state of the mice; and 4) mice deficient in both Angptl3 and Angptl4 exhibit severe hypotriglyceridemia and early death. Taken together, our data support the hypothesis that these molecules are key regulators of triglyceride metabolism that function by modulating LPL activity.

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