Novel GPIHBP1-independent pathway for clearance of plasma TGs in Angptl4<sup>-/-</sup>Gpihbp1<sup>-/-</sup> mice

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Running Title: GPIHBP1-independent TG clearance

**Abbreviations:**

- HFD: high fat diet
- NCD: normal chow diet
- GPIHBP1: glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1
- ANGPTL4: angiopoietin-like 4
- ANGPTL3: angiopoietin-like 3
- sWAT: subcutaneous white adipose tissue
- gWAT: gonadal white adipose tissue
- BAT: brown adipose tissue
Abstract

Mice lacking glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 (GPIHBP1) are unable to traffic LPL to the vascular lumen. Thus, TG clearance is severely blunted, and mice are extremely hypertriglyceridemic. Paradoxically, mice lacking both GPIHBP1 and the LPL regulator angiopoietin-like 4 (ANGPTL4) are far less hypertriglyceridemic. We sought to determine the mechanism by which Angptl4−/−Gpihbp1−/− double knockout mice clear plasma triglycerides. We confirmed that, on a normal chow diet, plasma TG levels were lower in Angptl4−/−Gpihbp1−/− mice than in Gpihbp1−/− mice; however, the difference disappeared with administration of a high-fat diet. Although LPL remained mislocalized in double-knockout mice, plasma TG clearance in brown adipose tissue increased compared with Gpihbp1−/− mice. Whole lipoprotein uptake was observed in the brown adipose tissue of both Gpihbp1+/− and Angptl4−/−Gpihbp1−/− mice, but brown adipose tissue lipase activity was significantly higher in the double-knockout mice. We conclude that Angptl4−/−Gpihbp1−/− mice clear plasma TGs primarily through a slow and non-canonical pathway that includes the uptake of whole lipoprotein particles.

Keywords: lipoprotein metabolism, lipolysis and fatty acid metabolism, lipase inhibition, adipose tissue, chylomicrons, lipoprotein lipase
Introduction

Misregulation of plasma triglyceride clearance is associated with a number of disease states, including diabetes mellitus, atherosclerosis, and hypertension (1, 2). Clearance of plasma triglycerides is primarily mediated by lipoprotein lipase (LPL), which hydrolyzes lipoprotein triglycerides, liberating fatty acids for tissue uptake (3–5). LPL must localize to the vascular lumen to hydrolyze plasma triglycerides. The endothelial transport protein glycosylphosphatidylinositol-anchored HDL-binding protein 1 (GPIHBP1) is required for this localization, transporting LPL across capillary endothelial cells and then anchoring LPL to the capillary wall during the lipolysis of serum triglycerides (6–8). In the absence of GPIHBP1, LPL is trapped in the interstitial spaces and is unable to access triglyceride-rich lipoproteins in the circulation (6, 8). As a result, plasma triglyceride levels in GPIHBP1-deficient mice and humans are dramatically elevated, despite normal production of LPL (7, 9).

ANGPTL4 is a fasting-induced inhibitor of LPL (10, 11). ANGPTL4 deficiency leads to lower triglyceride levels in mice and humans (11–14), and mice overexpressing ANGPTL4 have elevated triglyceride levels (11). Triglyceride tracing experiments in Angptl4−/− mice suggest that one primary role of ANGPTL4 is to inhibit LPL in adipose tissue during fasting, thereby diverting plasma triglycerides away from adipose (15). Preclinical studies suggest that targeting ANGPTL4 can lower plasma triglycerides in primates (14), and humans with homozygous deficiency in ANGPTL4 appear to be protected from cardiovascular disease (13, 14).

Although Angptl4−/− mice have increased LPL activity and reduced triglyceride levels, the necessity of GPIHBP1-mediated trafficking would suggest that Angptl4−/−Gpihbp1−/− double knockout mice share the same severe hypertriglyceridemia as Gpihbp1−/− mice. However, Angptl4−/−Gpihbp1−/− mice have substantially lower plasma triglyceride levels than Gpihbp1−/− (16). The intriguing phenotype of these mice strongly suggests the existence of a GPIHBP1-independent mechanism capable of substantial triglyceride clearance in the absence of vascular LPL. In this study, we investigated the GPIHBP1-independent mechanism by which plasma triglycerides are cleared in mice lacking both GPIHBP1 and ANGPTL4.
Materials and Methods

Mice

All animal procedures were conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were carried out according to guidelines approved by the Institutional Animal Care and Use Committee at the University of Iowa. Mice were group housed (up to 5/cage) in a controlled environment with a 12/12 light/dark cycle, with food and water provided ad libitum during non-fasting conditions. Mice were fed either normal chow diet (NCD) (Envigo, 7913) or high fat diet (Research Diets, D12451) containing 45% kcal/g from fat.

Gpihbp1\(^{−/−}\) mice were obtained from the Mutant Mouse Resource and Research Center (mmrrc.org, strain name: B6;129S5-Gpihbp1\(^{tm1Lex}/Mmucd\))(17, 18). Angptl4\(^{−/−}\) mice were obtained from Mutant Mouse Resource and Research Center (mmrrc.org, strain name: B6;129S5-Angptl4\(^{Gt(OST32973)Lex}/Mmucd\))(17, 19). Both strains were maintained on a mixed C57Bl/6J-129S5 background. The two strains were crossed to generate wild-type, Gpihbp1\(^{−/−}\), Angptl4\(^{−/−}\), and Angptl4\(^{−/−}\)Gpihbp1\(^{−/−}\) littermates.

Plasma Measurements

Littermate mice were fasted for 4 hours (fasted group) or fasted for 6 hours and then allowed to feed on normal chow ad libitum for 2 hours (refed group). Blood was collected via tail-nick. Glucose was assayed using a OneTouch UltraMini glucometer. For triglyceride, insulin, and leptin measurements, blood was collected into EDTA-coated collection tubes (Sarstadt, 16.444.100) and centrifuged at 1500 × g for 15 minutes at 4°C to pellet the cells. For triglyceride measurements, the plasma supernatant from each mouse was combined with Infinity™ Triglyceride Reagent (Thermo Scientific, TR22421) according to the manufacturer’s instructions. Samples were incubated at 37°C for 5 min and absorbance was measured at 500 nm. Triglyceride concentrations were determined by comparison to a standard curve prepared from a triolein standard (Nu-Chek Prep, Lot T-235-N13-Y). Plasma was also assayed for leptin using the Mouse and Rat Leptin ELISA (BioVendor, RD291001200R) and insulin using the Ultra
Sensitive Mouse Insulin ELISA Kit (CrystalChem, 90080). Plasma lipase assays were carried out as described below.

**RNA Isolation and qPCR Analysis**

Mouse tissues were frozen in liquid nitrogen and pulverized using a Bessman tissue pulverizer. Crushed tissue was resuspended in Trizol (Ambion, 15596-018) and processed according to the manufacturer’s instructions. After assessing mRNA concentration and quality using a NanoDrop spectrophotometer (ThermoScientific, NanoDrop 2000), cDNA was prepared using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Part No. 4368813). qPCR was performed (Invitrogen, SYBR GreenER qPCR Supermix, 11762100) according to the manufacturer’s specifications using an Applied Biosystems 7900HT Fast Real-Time PCR System (Iowa Institute of Human Genetics). Relative expression was calculated with the ΔΔct method (20) using cyclophilin A (CycloA) as the reference gene. Primers used were as follows: TGGCAAGACCAGCAAGAA and CTCCTGAGCTACAGAAGGAATG for CycloA, AGCAGGGACAGAGCACCTCT and AGACGAGCGTGATGCAGAAG for mouse Gpihbp1, CAACTAGCTGGGCCCTTAAT and ATCCACAGACCTACAACAG for mouse Angptl4, and AGCAGGAAGTCTGACCAATAAG and ATCAGCGTCATCAGGAGAAAG for mouse Lpl.

**Plasma and Tissue Lipase Activity Assay**

Mouse tissues were frozen in liquid nitrogen, pulverized using a Bessman tissue pulverizer, and resuspended in LPL assay buffer (25 mM \( \text{NH}_4\text{Cl} \), 5 mM EDTA, 0.01% SDS, 45 U/mL heparin, 0.05% 3-(N,N-Dimethylmyristylammonio) propanesulfonate zwittergent detergent (Acros Organics, 427740050)) containing Mammalian ProteaseArrest protease inhibitors (GBiosciences, cat no. 786-331). The tissue suspension was mixed by vortexing and incubated on ice for 30 min, with intermittent disruption with surgical scissors. The resulting lysate was centrifuged at 15,000 × g for 15 minutes at 4°C to pellet cellular debris. Lipase activity assays were performed on supernatant as previously described (15, 21); samples were combined with a working buffer composed of 0.6 M NaCl, 80 mM Tris-HCl pH 8, 6%
fatty-acid free BSA and an EnzChek lipase fluorescent substrate (Molecular Probes, E33955). Fluorescence was measured over 30 min at 37°C on a SpectraMax i3 plate reader (Molecular Devices). Relative lipase activity was calculated by subtracting background (calculated by reading fluorescence of a sample with no LPL) and then calculating the slope of the curve between the 5 and 13 minute reads. The data were graphed as the average of slopes for each group.

Plasma was collected and prepared as described above. Plasma or recombinant human LPL (21) was combined with molecular grade water (Research Products International) or with 2 M NaCl, then incubated on ice for 2 hours. Samples were then combined with working buffer (as described above) alone or in combination with 10 μM THL. Fluorescence was measured as above using the EnzChek lipase fluorescent substrate. Relative lipase activity was calculated by calculating the slope of the curve between the 1 and 20 minute reads, then subtracting background (activity of THL treated sample). The data were graphed as the average of slopes for each group normalized to plasma from wild-type mice.

**Preparation of Radiolabeled Chylomicrons**

*Gpihbp1*−/− mice were fasted 4 hours and then gavaged with 100 μCi of [9, 10-3H(N)]-Triolein (Perkin Elmer, NET431001MC) or 100 μCi of [9,10-3H(N)]-Triolein, 2 μCi [4-14C]-cholesterol (Perkin Elmer, NEC018050UC) and 20 mg/mL of cholesterol (Chem-Impex International, 50-493-426) suspended in olive oil. After 4 hours, mice were anesthetized and blood was collected by cardiac puncture. Blood was diluted 1:10 with 0.5 M EDTA (pH 8.0) and centrifuged 1500 × g for 15 minutes at 4°C to pellet blood cells. The plasma was then transferred to ultracentrifuge tubes and mixed 1:1 with PBS. After centrifugation at 424,000 × g for 2 hours at 10°C, the chylomicrons form an upper layer. The chylomicron layer was resuspended in fresh PBS and the centrifugation was repeated. Following the second centrifugation, the chylomicron layer was resuspended in PBS to the original plasma volume. Radioactivity was determined in BioSafe II scintillation fluid (RPI, 111195) on a Beckman-Coulter Liquid Scintillation Counter (BCLSC6500).

**Triglyceride Clearance Assay**
Littermate mice were fasted for 4 hours. Mice were anesthetized with isoflurane and injected retro-orbitally with 100 µL of the radiolabeled chylomicron suspension (see above). Proparacaine hydrochloride ophthalmic solution, USP 0.5% (AKORN, 17478-263-12) was used to minimize discomfort both during and after injection. Blood samples were taken via tail-nick at 1, 5, 10, and 15 minutes (for short-term uptake analysis) or at 1, 5, 15, 30, and 60 minutes after injection (for long-term uptake analysis). Blood samples were assayed in BioSafe II scintillation fluid on a Beckman-Coulter Scintillation Counter. After the last blood draw, the mice were anesthetized with isoflurane, and perfused with 20 mL of cold 0.5% tyloxapol in PBS. Tissues were harvested and weighed. Approximately 50 mg of each tissue was then weighed and placed in 2 mL of 2:1 chloroform:methanol overnight at 4°C. 1 mL of 2 M CaCl₂ was then added to each sample to separate organic and aqueous layers. The samples were centrifuged for 10 minutes at 1500 rpm, and the upper aqueous layer was mixed with BioSafe II scintillation fluid and assayed on a Beckman-Coulter Scintillation Counter. The lower organic layer was evaporated overnight to remove chloroform, and the remaining sample was resuspended in scintillation fluid and assayed in BioSafe II scintillation fluid on a Beckman-Coulter Liquid Scintillation Counter. CPM counts from aqueous and organic fractions were combined to obtain the total uptake CPM. CPM were also measured for an aliquot representing 10% (by volume) of the chylomicrons injected into each mouse. This value was used to normalize the radiolabel data across mice.

**Triglyceride Absorption Assay**

Littermate mice were fasted for 4 hours and gavaged with 2 µCi of [9, 10-³H(N)]-Triolein (Perkin Elmer, NET431001MC) suspended in olive oil. Mice were anesthetized with isoflurane and injected retro-orbitally with 500 mg/kg body weight of Triton WR1339 in PBS (n=5-6), or PBS alone for control mice (n=3). Proparacaine hydrochloride ophthalmic solution, USP 0.5% (AKORN, 17478-263-12) was used to minimize discomfort both during and after injection. Blood samples were taken via tail-nick at 30 minutes, 1 hour, 2 hours, 3 hours, and 4 hours following gavage. Blood samples were assayed in BioSafe II scintillation fluid on a Beckman-Coulter Scintillation Counter.
**Evans Blue Permeability Assay**

Littermate mice were fasted for 4 hours and injected via tail-vein with 0.5% Evans Blue in PBS (Fisher, S-13852). After 1 hour, the mice were anesthetized with isoflurane and perfused with 20 mL of PBS to remove unbound dye. Tissues were harvested, snap-frozen in liquid nitrogen and pulverized using a Bessman tissue pulverizer. 50 mg of pulverized tissue was then added to 0.5 mL of formamide (Sigma, F9037) and heated at 55°C for 2 hours to extract dye. Samples were centrifuged briefly to pellet cells and supernatant absorbance was measured at 610 nm. Dye concentration was determined by comparison to a standard curve prepared in formamide from a stock Evans Blue solution. The concentration of dye per mg tissue was calculated for each sample and graphed as percent of wild-type.

**Immunofluorescence staining**

Littermate mice were fasted for 4 hours and perfused with 10 mL of PBS and 10 mL of 0.4% paraformaldehyde in PBS (Fisher, O4042). Brown adipose tissue was excised and placed in 10% formalin (FormylFixx, ThermoScientific, 9990910) for 1 hour at room temperature. The tissues were rinsed twice in 1× PBS, and placed in 30% sucrose overnight at 4°C. The tissues were then embedded in Tissue-Tek O.C.T. Compound (Sakura, 4583) and frozen. 10 µm sections were prepared from frozen tissues using a cryostat (Leica Microm Cryostat I HM505E), transferred to microscope slides, and stored at -80°C.

For examination of LPL localization, slides were thawed, incubated in ice-cold methanol for 10 minutes, and then washed in 1× PBS 3 times for 10 minutes. Slides were then incubated with 0.2% TritonX100 in PBS 2 times for 30 minutes, and then washed in 1× PBS 3 times for 5 minutes. After incubating in blocking buffer (10% fetal bovine serum in 1× PBS with 0.2% TritonX100) for 30 minutes, slides were incubated overnight at 4°C with Armenian hamster anti-mouse CD31 (1:10; Developmental Studies Hybridoma Bank, University of Iowa, 2H8) and goat anti-mouse LPL (1:50; (22)) in blocking buffer. Slides were then washed once with 0.2% TritonX100 in PBS for 10 minutes and 2× 10 minutes with 1× PBS. Slides were incubated with 1:500 goat anti-hamster-AlexaFluor488 (ThermoFisher Scientific, A-21110) and 1:500 donkey anti-goat-AlexaFluor555 in blocking buffer for 2 hours. Finally,
slides were washed 3 times for 10 minutes with 1× PBS, fixed with Prolong Gold Anti-Fade reagent (Molecular Probes, P36931), and sealed with a coverslip. Slides from 3 mice per genotype were imaged using a (Leica DM5000b, Leica LAS AF software, and 63× lens), and at least ten capillary cross-sections from each mouse were examined for luminal LPL.

For examination of macrophage infiltration, slides were thawed and rinsed briefly in 10× PBS. Slides were then incubated with 0.4% TritonX100 (Fisher, BP151) in 10× PBS at room temperature for 1 hour and rinsed for 2 minutes in 10× PBS. After incubating for 1 hour in blocking solution (5% fetal bovine serum (FBS), 0.4% TritonX100, and 10× PBS), slides were incubated overnight at 4°C with rat anti-mouse FA-11 (1:200; BioLegend, 137001). Following this incubation, slides were washed in 10× PBS with 0.5% Tween20 (Fisher, BP337) 3 times for 10 minutes and then incubated with donkey anti-rat-AlexaFleur488 (1:500; Invitrogen, A-21208) in blocking buffer for 2 hours at room temperature. The slides were then washed 5 times for 10 minutes in 10× PBS with 0.5% tween20, fixed with Prolong Gold Antifade reagent, and sealed with a coverslip. Slides from 3 mice per genotype were imaged using a Leica DM5000b, Leica LAS AF software, and 20× lens. Ten images were taken of each slide, and both nuclei and macrophages were manually counted in ImageJ. Individuals counting were blinded to genotype. The average ratio of macrophages to nuclei was calculated for each slide (with each slide being from a different mouse). The average of the ratio from each mouse was graphed.

Statistics and Outlier Identification

Statistics and outlier identification were performed using GraphPad Prism. Statistical significance was tested using Student’s T-test unless otherwise indicated. Outliers were identified using the ROUT test and were excluded from graphs and from statistical analysis. The number of mice analyzed for each experiment is specified in each figure legend.
Results

Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> Mice Have Reduced Hypertriglyceridemia Compared to Gpihbp1<sup>−/−</sup>Mice

Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice were generated by crossing Gpihbp1<sup>−/−</sup> mice with Angptl4<sup>−/−</sup> mice. Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice were then crossed to generate wild-type, Gpihbp1<sup>−/−</sup>, Angptl4<sup>−/−</sup>, and Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> littermate mice. The expected genetic deficiencies were verified by measuring expression of Gpihbp1 and Angptl4 by qPCR (Figure 1A, B). Consistent with previous reports (16), male Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice were far less hypertriglyceridemic than Gpihbp1<sup>−/−</sup> mice (Figure 1C, D). Interestingly, Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice had significantly higher plasma TGs after refeeding compared to mice fasted for 4 hours (Figure 1D). Female Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice also had significantly less hypertriglyceridemia than female Gpihbp1<sup>−/−</sup> mice (Figure 1E). Body weights for both male and female Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice were normal (Figure 2A, B). We also observed no significant differences in fasted or fed glucose levels (Figure 2C), fasted or fed insulin levels (Figure 2D), or leptin levels (Figure 2E) when comparing Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice with wild-type, Angptl4<sup>−/−</sup>, and Gpihbp1<sup>−/−</sup> mice. Interestingly, food intake was slightly lower for Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice when compared to Gpihbp1<sup>−/−</sup> mice (Figure 2F).

Dietary Fat Absorption Is Normal in Gpihbp1<sup>−/−</sup>Angptl4<sup>−/−</sup> Mice

We tested the possibility that Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice have reduced plasma triglyceride levels compared to Gpihbp1<sup>−/−</sup> mice because they absorb less dietary fat. In combination with the slight decrease in food intake (Figure 2F) a decrease in fat absorption in the gut and the subsequent secretion of triglycerides into the circulation could explain the observed decrease in plasma triglycerides. To measure dietary fat absorption, fasted mice were gavaged with radiolabeled triolein after being injected intravenously with tyloxapol (Triton WR1339) to block LPL-mediated plasma TG clearance (23). Appearance of radiolabel in the circulation was measured over the course of 4 hours. Absorbance was similar in wild-type, Gpihbp1<sup>−/−</sup>, and Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice. Interestingly, dietary fat absorption appeared to be reduced in Angptl4<sup>−/−</sup> single knockout mice (Figure 3A). Whether this is because these...
mice actually absorb less dietary fat or because clearance from the circulation is not completely inhibited in these mice is not clear. As expected, when wild-type and Angptl4−/− mice were gavaged with radiolabel triolein without tyloxapol treatment, radiolabel was cleared from the plasma rapidly enough that little increase in circulating radiolabel was observed (Figure 3B, C). However, the absence of tyloxapol did not result in any observable increase in clearance in Gpihbp1−/− and Angptl4−/−Gpihbp1−/− mice, indicating that both of these genotypes had significantly impaired LPL-mediated TG clearance (Figure 3D, E). Although there appears to be an increase in secretion in Angptl4−/−Gpihbp1−/− mice treated with tyloxapol, the differences between the treated and untreated Angptl4−/−Gpihbp1−/− mice were not statistically significant according to area under the curve analysis (p=0.13).

**Angptl4−/−Gpihbp1−/− Mice Clear More Plasma Triglycerides than Gpihbp1−/− Mice**

We assessed plasma triglyceride clearance using radiolabeled chylomicrons collected from Gpihbp1−/− mice fed 3H-triolein (see Materials and Methods). Wild-type, Gpihbp1−/−, Angptl4−/−, and Angptl4−/−Gpihbp1−/− mice were injected intravenously with radiolabeled chylomicrons, and clearance of radiolabel was measured by taking blood samples 1, 5, 10, and 15 minutes after injection. After 15 minutes, tissues were harvested and the amount of radiolabel was measured to determine uptake into individual tissues. After 15 minutes, both Angptl4−/− mice and wild-type mice had cleared the radiolabel from plasma almost completely, whereas neither Gpihbp1−/− nor Angptl4−/−Gpihbp1−/− mice showed any significant clearance of radiolabel from the plasma (Figure 4A). Despite the apparent lack of plasma clearance, we did observe some increase in tissue uptake in Angptl4−/−Gpihbp1−/− mice compared to Gpihbp1−/− mice (Figure 4B). Uptake was significantly greater in heart, liver, and BAT and trended greater in white adipose tissue.

We reasoned that triglyceride clearance in Angptl4−/−Gpihbp1−/− mice might be too slow to capture adequately with a 15-minute time-course, and therefore we lengthened the observation window to an hour. In this longer time-course, Angptl4−/−Gpihbp1−/− mice cleared significantly more radiolabeled triglyceride than the Gpihbp1−/− mice, though clearance was considerably slower than wild-type or Angptl4−/− mice (Figure 5A). To determine if the clearance we observed in Angptl4−/−Gpihbp1−/− mice
relied on lipase activity, we repeated the clearance assay in Gpihbp1<sup>−/−</sup> and Angptl4<sup>−/−</sup> Gpihbp1<sup>−/−</sup> mice, treating some mice with the lipase inhibitor tetrahydrolipstatin (THL). THL treatment prevented TG clearance in Angptl4<sup>−/−</sup> Gpihbp1<sup>−/−</sup> mice, indicating that TG clearance is driven by lipase activity in these mice (Figure 5B). When we examined tissues after an hour-long clearance assay, radiolabel uptake into brown adipose tissue was considerably greater in Angptl4<sup>−/−</sup> Gpihbp1<sup>−/−</sup> mice compared to Gpihbp1<sup>−/−</sup> mice (Figure 5C). Radiolabel uptake was also somewhat greater in liver, quad, kidney, and white adipose tissue (Figure 5C). Importantly, the greater uptake to all these tissues in Angptl4<sup>−/−</sup> Gpihbp1<sup>−/−</sup> mice, with the exception of kidney, was prevented by treating mice with THL, whereas THL had little effect on uptake in Gpihbp1<sup>−/−</sup> mice. Again, these results suggested that the increased uptake in Angptl4<sup>−/−</sup> Gpihbp1<sup>−/−</sup> mice requires lipase activity.

**LPL Expression, Activity, and Localization in Gpihbp1<sup>−/−</sup> Angptl4<sup>−/−</sup> Mice**

As LPL is largely responsible for plasma triglyceride clearance, we sought to determine if changes in LPL expression or activity might be responsible for the increased TG clearance in Angptl4<sup>−/−</sup> Gpihbp1<sup>−/−</sup> mice. We observed no significant differences in Lpl gene expression among wild-type, Angptl4<sup>−/−</sup>, Gpihbp1<sup>−/−</sup>, and Angptl4<sup>−/−</sup> Gpihbp1<sup>−/−</sup> mice, as determined by qPCR (Figure 6A). We did, however, observe significant difference in lipase activity in several tissues. As we have previously reported (15), lipase activity was significantly increased in the white adipose tissue of Angptl4<sup>−/−</sup> mice compared to wild-type mice (Figure 6B). Angptl4<sup>−/−</sup> Gpihbp1<sup>−/−</sup> mice likewise had increased lipase activity in white adipose tissue compared to Gpihbp1<sup>−/−</sup> mice. Lipase activities in liver and quad were similar across genotypes (Figure 6B). Interestingly, in the heart, GPIHBP1 deficiency, but not ANGPTL4 deficiency, resulted in increased lipase activity, with both Gpihbp1<sup>−/−</sup> and Angptl4<sup>−/−</sup> Gpihbp1<sup>−/−</sup> mice displaying a ~3 fold increase in activity compared to wild-type and Angptl4<sup>−/−</sup> mice (Figure 6B). Lipase activity in brown adipose tissue was particularly interesting as the activity in Angptl4<sup>−/−</sup> Gpihbp1<sup>−/−</sup> mice was significantly higher than either Angptl4<sup>−/−</sup> mice or Gpihbp1<sup>−/−</sup> single knockout mice (Figure 6B), suggesting that the increased lipase activity in BAT required the absence of both GPIHBP1 and ANGPTL4.
Increased LPL activity alone is not sufficient to explain the decreased plasma triglycerides in Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice. Because GPIHBP1 is required for the entry of LPL into the lumen of the vasculature (6), LPL, even if it has increased activity, would still be trapped in the interstitial space in Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice. To determine if Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice are somehow transporting LPL independently of GPIHBP1, we stained brown adipose tissue sections from wild-type, Angptl4<sup>−/−</sup>, Gpihbp1<sup>−/−</sup>, and Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice for LPL, CD31 (an endothelial cell marker), and DAPI. As expected, examination of capillary cross-sections revealed the abundant LPL staining on the luminal capillary surfaces of wild-type and Angptl4<sup>−/−</sup> mice, whereas luminal LPL was largely absent in Gpihbp1<sup>−/−</sup> mice (Figure 7). Likewise, the presence of LPL on the luminal surfaces of capillaries was also minimal in Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice, suggesting that LPL remains mislocalized in these mice (Figure 7).

**Whole Lipoprotein Uptake Is Not Increased in Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> Mice**

Our data suggested triglyceride clearance in Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice is lipase dependent, but that LPL does not efficiently reach the vascular lumen. Therefore, we tested the hypothesis that in Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice, a greater number of whole lipoproteins traverse the capillary wall and reach LPL in the interstitial space. The uptake of whole lipoprotein particles in brown adipose tissue has been described previously (24), and it has been shown that under certain circumstances, such as cold exposure, this uptake is increased (24–26).

We first tested vascular permeability using an Evan’s blue test (27). Increased vascular permeability in Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice might allow both an increase in untethered LPL reaching the circulation as well as an increase in whole lipoprotein particles reaching the interstitial space. However, we observed no differences in vascular permeability across genotypes (Figure 8). To determine if circulating LPL activity increased in Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice, we measured lipase activity in plasma. We observed no increase in the pre-heparin lipase activity of Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice (Figure 8B). When plasma samples were treated with 2 M NaCl, which blocks the activity of LPL but not hepatic lipase (28), we saw no significant decrease in lipase activity in any of the genotypes, indicating that all circulating lipase activity came from
hepatic lipase (Figure 8B). This is consistent with previous observations that there is little active LPL freely circulating in the vasculature (29, 30). We also observed no difference in the macrophage infiltration of brown adipose tissue (Figure 9).

To determine if whole lipoprotein particle transport was specifically increased in Angptl4−/−Gpihbp1−/− mice, we performed a triglyceride clearance assay with chylomicrons which had been labeled with both 3H-triolein and 14C-cholesterol. Classical vascular lipolysis and uptake of the resulting fatty acids would be observed as uptake of only the 3H label, as the 14C-cholesterol would remain with the lipoprotein particle in the vasculature. On the other hand, if whole lipoprotein particles are taken up, uptake of both 3H and 14C would be observed. We observed increased clearance of both 3H and 14C radiolabels from plasma in Angptl4−/−Gpihbp1−/− mice compared to Gpihbp1−/− mice (Figure 10A). As expected, we observed a large increase in the uptake of 3H label in the brown adipose tissue and liver of Angptl4−/−Gpihbp1−/− mice compared to Gpihbp1−/− mice (Figure 10B, C). However, the amount of 14C taken up in BAT was not increased in Angptl4−/−Gpihbp1−/− mice compared to Gpihbp1−/− mice, nor was the amount of 14C uptake increased in any tissue other than liver, consistent with the role of liver in clearing chylomicron remnant particles (Figure 10B, C). These data suggest that, although there is some level of whole lipoprotein transport occurring in brown adipose tissue, this transport is not increased in Angptl4−/−Gpihbp1−/− mice. Of particular note, the level of 3H-triglyceride clearance from the plasma and its uptake into peripheral tissue in wild-type and Angptl4−/− mice is much higher than that of 14C-cholesterol (Figure 11). However, the level of 3H and 14C clearance (as a % of injected dose) are roughly equal in Gpihbp1−/− mice and, outside of brown adipose tissue, also roughly equal in Angptl4−/−Gpihbp1−/− mice (Figure 10). These data suggest that whole lipoprotein particle clearance occurs in GPIHBP1-deficient mice and may be the only type of triglyceride clearance operating in peripheral tissues when GPIHBP1 is absent.

**Triglyceride Clearance in Angptl4−/−Gpihbp1−/− Mice Can Be Overwhelmed By HFD**

Although Angptl4−/−Gpihbp1−/− mice have improved triglyceride clearance compared to Gpihbp1−/− mice, Angptl4−/−Gpihbp1−/− mice still exhibit hypertriglyceridemia, especially in the fed state (see Figure...
1D), and the rate of plasma TG clearance is much slower than in wild-type mice (see Figure 4, 5).

Together these data suggest that triglyceride clearance in Angptl4−/−Gpihbp1−/− mice is relatively inefficient and might be overwhelmed by a high-fat diet. To test this prediction, we carried out triglyceride clearance assays on mice that had been fed a 45% high-fat diet (HFD) for two weeks. Consistent with our previous results (see Figure 1C), before starting the HFD, Angptl4−/−Gpihbp1−/− mice had plasma TG levels that were only 25% of those observed in Gpihbp1−/− mice (Figure 12A). After two weeks on HFD, Angptl4−/−Gpihbp1−/− mice develop a chylomicronemia that rivals that of Gpihbp1−/− mice (Figure 12A). Clearance of radiolabeled triglycerides was slower in Angptl4−/−Gpihbp1−/− mice fed a HFD compared to those fed normal chow (Figure 12B). Moreover, after 2 weeks on a HFD, Angptl4−/−Gpihbp1−/− mice no longer showed a difference in tissue uptake of radiolabeled triglycerides compared to Gpihbp1−/− mice (Figure 12C).
Discussion

In the absence of GPIHBP1, LPL is trapped in the interstitial spaces and is unable to access triglyceride-rich lipoproteins in the circulation (6, 8). As a result, GPIHBP1-deficient mice have dramatically elevated plasma triglycerides, despite normal production of LPL (7). However, Sonnenberg et al. reported that mice deficient in both GPIHBP1 and ANGPTL4 had plasma triglyceride levels that were ~90% less than mice deficient only in GPIHBP1 (16). Our primary goal in this study was to investigate how plasma triglycerides are cleared in the absence of GPIHBP1.

Consistent with the previous report by Sonnenberg et al., we found that Angptl4−/−Gpihbp1−/− mice had plasma triglycerides ~80% lower than those found in Gpihbp1−/− mice after fasting. However, our observations that 1) plasma triglycerides are only 40% lower in refed mice, 2) HFD feeding eliminates the difference in plasma triglycerides between Gpihbp1−/− and Angptl4−/−Gpihbp1−/− mice, and 3) radiolabeled triglyceride clearance in Angptl4−/−Gpihbp1−/− mice is delayed compared to wild-type mice all point to a slow and less robust clearing mechanism than the canonical GPIHBP1-dependent lipolysis observed in wild-type mice.

The severe hypertriglyceridemia in Gpihbp1−/− mice is primarily a result of LPL mislocalization (6, 7). Resolution of LPL mislocalization, either through an alternate transporter or through increased vascular permeability, could potentially explain the decrease in hypertriglyceridemia in Angptl4−/−Gpihbp1−/− mice. Indeed, Dijk et al. (31) proposed that an alternative ANGPTL4-sensitive pathway might allow transport of LPL in the absence of GPIHBP1 and ANGPTL4. In support of this idea, using immunofluorescence, a study by Larsson et al. found some evidence of LPL on the vascular lumen of capillaries in Angptl4−/−Gpihbp1−/− mice (32). In contrast, we found no evidence of increased LPL entry into the vasculature. LPL was still mislocalized in Angptl4−/−Gpihbp1−/− mice as judged by immunofluorescence microscopy. It should be noted that although we did not observe a difference between LPL localization in Gpihbp1−/− and Angptl4−/−Gpihbp1−/− mice, we did sometimes observe trace levels of LPL staining in the capillary lumens of both genotypes (see Figure 7).

If LPL reaches the circulation, either through increased vascular permeability or an alternative transporter, but is not tethered to the vascular wall, it would not be visible when staining tissues, but could
still act to reduce plasma triglycerides. In fact, a reduction of plasma triglycerides is precisely what is observed in \textit{Gpihbp1}^{−/−} mice when LPL is released from the interstitial space by infusion of heparin (22). Dijk et al. reported that circulating LPL mass was increased in \textit{Angptl4}^{−/−} mice (33), though the activity of the circulating LPL was not measured. We observed no change in vascular permeability, nor did we observe an increase in plasma lipase activity. Moreover, if free circulating LPL were the main contributor to plasma clearance, we would predict a diffuse increase in fatty acid uptake across multiple tissues, rather than primarily an increase in uptake in brown adipose tissue. Thus, we conclude that unbound circulating LPL is unlikely to be the only source of lipolysis.

Despite persistent LPL mislocalization in \textit{Angptl4}^{−/−} \textit{Gpihbp1}^{−/−} mice, LPL may still play a role in the triglyceride clearance in these mice. We observed increased whole-tissue LPL activity in the adipose tissue of \textit{Angptl4}^{−/−} \textit{Gpihbp1}^{−/−} mice compared to \textit{Gpihbp1}^{−/−} mice. The increases in LPL activity in white adipose tissue were consistent with what has been observed for \textit{Angptl4}^{−/−} single knockout mice ((15) and Figure 6), but the activity in brown adipose tissue of \textit{Angptl4}^{−/−} \textit{Gpihbp1}^{−/−} mice is markedly higher than that observed in wildtype, \textit{Angptl4}^{−/−}, or \textit{Gpihbp1}^{−/−} mice. Importantly, treatment with the lipase inhibitor THL completely abolished the increased triglyceride clearance in \textit{Angptl4}^{−/−} \textit{Gpihbp1}^{−/−} mice. Although it is formally possible that a different THL-sensitive lipase is responsible, our results suggest that LPL remains necessary for triglyceride clearance in \textit{Angptl4}^{−/−} \textit{Gpihbp1}^{−/−} mice.

If LPL is mislocalized to the interstitial space in \textit{Angptl4}^{−/−} \textit{Gpihbp1}^{−/−} mice, and yet continues to contribute to triglyceride uptake into tissues, the implication is that the triglycerides are themselves transported into the interstitial space where they can be acted upon by LPL. One possible mechanism by which this could occur is the uptake of whole lipoprotein particles. If whole lipoproteins are able to traverse the endothelial layer of capillaries, LPL, despite its mislocalization, would be able to act on these particles and release fatty acids for uptake. Our data suggest that there is a significant amount of whole lipoprotein uptake in \textit{Angptl4}^{−/−} \textit{Gpihbp1}^{−/−} mice. However, the whole lipoprotein uptake in \textit{Angptl4}^{−/−} \textit{Gpihbp1}^{−/−} mice was not any greater than that observed in \textit{Gpihbp1}^{−/−} mice. This result is consistent with the recent report from Larsson et al. (32), who also found that at room temperature, TG uptake, but not lipoprotein particle uptake (as measured by uptake of radiolabeled retinol) was increased.
in the brown adipose tissue of BAT \textit{Angptl4}\textsuperscript{−/−}\textit{Gpihbp1}\textsuperscript{−/−} mice compare to \textit{Gpihbp1}\textsuperscript{−/−} mice. Although there was no increase in whole lipoprotein uptake, the already substantial uptake in \textit{Angptl4}\textsuperscript{−/−}\textit{Gpihbp1}\textsuperscript{−/−} mice may explain the improved triglyceride clearance in these mice. We propose a model in which whole lipoprotein particles are taken up primarily in brown adipose tissue in both \textit{Angptl4}\textsuperscript{−/−}\textit{Gpihbp1}\textsuperscript{−/−} mice and \textit{Gpihbp1}\textsuperscript{−/−} mice, but the higher LPL activity in \textit{Angptl4}\textsuperscript{−/−}\textit{Gpihbp1}\textsuperscript{−/−} mice results in greater hydrolysis of lipoprotein triglycerides. Reduction of triglycerides in lipoproteins in turn allows more efficient uptake of remnant particles by the liver. The increased uptake of triglycerides in brown adipose tissue and in liver leads to reduced plasma triglyceride levels in \textit{Angptl4}\textsuperscript{−/−}\textit{Gpihbp1}\textsuperscript{−/−} mice.

The uptake of whole triglyceride-rich lipoprotein particles into brown adipose tissue has been described previously for brown adipose tissue (24–26), but in normal mice the contribution of whole lipoprotein uptake to brown adipose fatty acid uptake appears to be far less than that from classical LPL lipolysis (24). Importantly, reduction of ANGPTL4 is important for the increase in whole lipoprotein uptake into brown adipose tissue that occurs with cold exposure (26), and deletion of ANGPTL4 in \textit{Gpihbp1}\textsuperscript{−/−} mice increases cold-induced whole lipoprotein uptake compared to deletion of GPIHBP1 alone (32). Our model implies not only that whole lipoprotein particles pass through the capillary wall and enter the interstitial space, but also that these lipoprotein particles also can return to the circulation, a concept that has not yet been tested. Our model also suggests that \textit{Gpihbp1}\textsuperscript{−/−} mice have some ability to clear plasma triglycerides. This notion is supported by the fact that plasma triglycerides do not increase throughout the life of \textit{Gpihbp1}\textsuperscript{−/−} mice, but plateau once mice reach adulthood (7).

Angiopoietin-like 3 (ANGPTL3), like ANGPTL4, inhibits LPL activity, and ANGPTL3-deficiency decreases plasma triglyceride levels. Interestingly, unlike \textit{Angptl4}\textsuperscript{−/−}\textit{Gpihbp1}\textsuperscript{−/−} mice, mice deficient in both GPIHBP1 and ANGPTL3 have only modestly decreased plasma triglycerides (16). Our model is consistent with this observation and the current paradigms for ANGPTL4 and ANGPTL3 action. There is strong evidence that ANGPTL4 in adipose tissue acts on LPL before its transport to the vasculature (15, 21, 33). In the absence of ANGPTL4, LPL activity is increased in adipose tissue, resulting in increased plasma triglyceride clearance and fatty acid uptake into adipose (15). In the absence of GPIHBP1, LPL activity is still increased in adipose tissue (as shown in Figure 6), but LPL becomes mislocalized to the
interstitial space. Any lipoprotein particles that make it to the interstitial space would be subject to increased LPL activity. ANGPTL3, however, is an endocrine factor produced almost exclusively in the liver (34, 35). In the absence of GPIHBP1, LPL does not reach the vasculature, does not interact with circulating ANGPTL3, and thus tissue LPL activity in \textit{Angptl3}^{−/−} \textit{Gpihbp1}^{+/−} mice would be little different than that in \textit{Gpihbp1}^{−/−} mice.

Although lipid absorption assays did not detect any decrease in lipid absorption in \textit{Angptl4}^{−/−} \textit{Gpihbp1}^{−/−} mice, these assays did result in two interesting observations. First, in both \textit{Gpihbp1}^{−/−} mice and \textit{Angptl4}^{−/−} \textit{Gpihbp1}^{−/−} mice, treatment with tyloxapol had no effect on the accumulation of radiolabeled triglycerides in the circulation. This observation supports the model wherein no vascular lipolysis occurs in the absence of GPIHBP1. Thus, tyloxapol has no ability to further decrease vascular lipolysis. The second observation was that \textit{Angptl4}^{−/−} mice had an apparent defect in lipid absorption. This observation runs counter to previous studies where it has been shown that ANGPTL4 can inhibit intestinal lipases, and thus, the absence of ANGPTL4 increases lipid absorption (36). We postulate that the apparent decrease in lipid absorption is actually the result of incomplete blockage of triglyceride clearance by tyloxapol. Thus, the lower rate of radiolabel accumulation in the circulation of \textit{Angptl4}^{−/−} mice is the result of increased clearance from the circulation, not a decrease in absorption.

Although our study helps illuminate the mechanism by which \textit{Angptl4}^{−/−} \textit{Gpihbp1}^{−/−} mice have decreased hypertriglyceridemia compared to \textit{Gpihbp1}^{−/−} mice, there are some limitations to our data and a few outstanding questions. Although data was collected from both male and female mice, not all experiments were done in both genders. Therefore, we may have missed some gender-specific differences. However, given the results from experiments that were done in both genders and our previous studies with \textit{Angptl4}^{−/−} mice (15), we believe the gender-specific differences are likely to be minor. Another limitation is that our study was performed in mice. Thus far, GPIHBP1-independent triglyceride clearance has not been described, or even examined, in humans. Human individuals have substantially less brown adipose tissue than mice. Thus, even if the same mechanism of clearance is present in human brown or beige adipose tissue, it may be insufficient to substantially reduce plasma triglycerides.
It will be important to further investigate the mechanism by which triglyceride-rich lipoprotein particles are trafficked across the capillary wall. A previous study from Bartelt et al. showed that the increase in whole lipoprotein uptake to brown adipose tissue observed after cold exposure depended on the fatty acid receptor CD36 (25), but how CD36 might mediate uptake of whole lipoproteins is unclear. CD36 binds a wide range of lipoproteins (37), but whether direct binding of CD36 to chylomicrons facilitates uptake of these particles has yet to be determined. Bartelt et al. also found that inhibition of LPL by THL or release of LPL by heparin greatly reduced cold-induced whole lipoprotein uptake, suggesting the necessity of LPL for this uptake (25). The mechanism by which LPL facilitates whole lipoprotein uptake has not been elucidated, nor is it clear if LPL trapped in the interstitial space, as is the case with Angptl4−/−Gpihbp1−/− mice, participates in this uptake beyond hydrolyzing lipoprotein TGs in the interstitial space. Addressing these questions will further illuminate the nature of non-canonical triglyceride clearance.

We observed that after two weeks on a high-fat diet, Angptl4−/−Gpihbp1−/− mice had triglyceride levels that were not significantly different from those of Gpihbp1−/− mice. One possible explanation for this observation is that on a HFD, the increase in chylomicrons entering the circulation overwhelms the slow clearance of triglyceride-rich lipoproteins and subsequently, TG levels rise. Another intriguing possibility is that whole lipoprotein particle uptake is more efficient with smaller liver-derived VLDL particles. On high-fat diet (or in refeeding experiments), the larger, intestinally-derived chylomicron particles are less efficiently taken up and thus less TG clearance occurs. As the mechanism of GPIHBP1-independent triglyceride clearance is studied further, it will be important to determine if there is a distinction between the clearance of VLDL- and chylomicron-derived triglycerides.
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Figure 1

**A-B)** Angptl4 (A) and Gpihbp1 (B) expression in 10-12-week old male wild-type, Angptl4<sup>−/−</sup>, Gpihbp1<sup>−/−</sup>, and Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice (n=5-6) as measured by qPCR and normalized to wild-type (±SEM). C) Representative plasma from 10-12 week male mice. D) Plasma triglycerides (±SEM) of fasted (4 h) and refeed (6 h fast, 2 h refeed) 15-19-week old male mice (n=6-7). E) Plasma triglycerides of 4 h fasted 28-32-week old female mice (n=6-7). (*p<0.05, ***p<0.001)

Figure 1. Plasma triglyceride levels in Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup>Mice. A-B) Angptl4 (A) and Gpihbp1 (B) expression in 10-12-week old male wild-type, Angptl4<sup>−/−</sup>, Gpihbp1<sup>−/−</sup>, and Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice (n=5-6) as measured by qPCR and normalized to wild-type (±SEM). C) Representative plasma from 10-12 week male mice. D) Plasma triglycerides (±SEM) of fasted (4 h) and refeed (6 h fast, 2 h refeed) 15-19-week old male mice (n=6-7). E) Plasma triglycerides of 4 h fasted 28-32-week old female mice (n=6-7). (*p<0.05, ***p<0.001)
Figure 2. Metabolic phenotypes of Angptl4<sup>−/−</sup> Gpihbp1<sup>−/−</sup> Mice. A-B) Body weight over time for male (A) and female (B) wild-type, Angptl4<sup>−/−</sup>, Gpihbp1<sup>−/−</sup>, and Angptl4<sup>−/−</sup> Gpihbp1<sup>−/−</sup> mice (n=5-8 for male, 10-18 for female). C-D) Plasma glucose (C) and insulin (D) levels of fasted (4 h) and refed (6 h fast, 2 h refeed) 15-19-week old male mice (n=6-8). E) Plasma leptin levels of fasted (4 h) 15-19-week old male mice (n=5). F) Average food intake of 15-19 week-old male wild-type, Angptl4<sup>−/−</sup>, Gpihbp1<sup>−/−</sup>, and Angptl4<sup>−/−</sup> Gpihbp1<sup>−/−</sup> mice as measured over 7 days (n=6-7). (*p<0.05)
Figure 3. Dietary fat absorption appears normal in Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice. Mice were injected with tyloxapol (T-WR1399) to block lipid absorption and gavaged with radiolabeled triolein. A) Appearance of radiolabel in the circulation (±SEM) over time in male wild-type, Angptl4<sup>−/−</sup>, Gpihbp1<sup>−/−</sup>, and Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice (28-32 weeks; n=5-6/group). B-E) Appearance of radiolabel in the circulation (±SEM) of wild-type (B), Angptl4<sup>−/−</sup> (C), Gpihbp1<sup>−/−</sup> (D), and Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> (E) mice that were untreated (n=3/group) or treated with T-WR1339. Values shown as % gavaged dose/ml plasma. T-WR1339 treated samples in B-E are the same as those shown in (A).
Figure 4. Triglyceride clearance is impaired in $\text{Angptl}^{4/-} \text{Gpihbp}^{1/-}$ mice. Female wild-type, $\text{Angptl}^{4/-}$, $\text{Gpihbp}^{1/-}$, and $\text{Angptl}^{4/-} \text{Gpihbp}^{1/-}$ mice (30-34 weeks; n=5-6/group) were injected retro-orbitally with chylomicrons labeled with $^3$H-triolein. A) Chylomicron clearance from plasma. Radioactivity was measured in plasma samples taken 1, 5, 10, and 15 minutes after injection ($\pm$SEM). Graph shows radioactivity normalized to the 1 min time point. B) Tissue uptake. After the final bleed, tissues were harvested and radioactivity was measured. Radiolabel uptake into the indicated tissues is expressed as percent of injected dose per mg of tissue ($\pm$SEM). (*p<0.05)
Figure 5. *Angptl4*^−/−* Gpihbp1*^−/−* mice demonstrate lipase-dependent clearance. A) Chylomicron clearance from plasma. Fasted male wild-type, *Angptl4*^−/−*, *Gpihbp1*^−/−*, and *Angptl4*^−/−*Gpihbp1*^−/−* mice (15-20 weeks; n=6-7/group) were injected retro-orbitally with chylomicrons labeled with ³H-triolein. Radioactivity was measured in plasma samples taken 1, 5, 15, 30, 45, and 60 minutes after injection. Graph shows radioactivity normalized to the 1 min time point. B-C) Chylomicron clearance and tissue uptake in the presence and absence of THL. Fasted male *Gpihbp1*^−/−* and *Angptl4*^−/−*Gpihbp1*^−/−* mice (29-34 weeks; n=5-6/group) were injected retro-orbitally with chylomicrons labeled with ³H-triolein. Radioactivity was measured in plasma samples taken 1, 5, 15, 30, and 60 minutes after injection. Radioactivity measurements are normalized to the 1 min time point (B). (C) Radioactivity in harvested tissues as percent injected dose/mg of tissue (±SEM). (*p<0.05, **p<0.01)
Figure 6. *Lpl* expression and lipase activity in Angptl4<sup>−/−</sup>Gpihbp1<sup>−/−</sup> mice. A) *Lpl* expression in 4 h fasted male mice (10-12 weeks; n=5-6/group) as measured by qPCR. Graphs show fold change (±SEM) compared to wild-type. B) Lipase activity in the tissues of 4 h fasted male mice (15-19 weeks; n=6/group). Graphs show fold change (±SEM) compared to wild-type. (*p<0.05, **p<0.01, ***p<0.001)
Figure 7. LPL localization in Gpihbp1<sup>−/−</sup>Angptl4<sup>−/−</sup> mice. Immunofluorescence staining of brown adipose tissue in female mice (10-14 weeks). Sections were stained with an antibody against LPL, as well as with antibodies against CD31 (green) to identify endothelial cells and with DAPI (blue) to identify nuclei. The luminal face of endothelial cells is marked by arrowheads.
Figure 8. Vascular permeability is unchanged in Angptl4<sup>−/−</sup> Gpihbp1<sup>−/−</sup> mice. **A**) Vascular permeability in female wild-type, Angptl4<sup>−/−</sup>, Gpihbp1<sup>−/−</sup>, and Angptl4<sup>−/−</sup> Gpihbp1<sup>−/−</sup> mice (10-14 weeks; n=5-14/group). Mice were injected with Evan’s Blue dye. After 1 h, mice were extensively perfused and tissues were collected and uptake of dye was quantified. Graphs show levels of dye/mg tissue normalized to wild-type (±SEM). **B**) Pre-heparin plasma lipase activity in the tissues of 4 h fasted male mice (15-19 weeks; n=4/group) and recombinant LPL (rLPL) with or without treatment with 2 M NaCl (to block LPL activity). Graphs show fold change (±SEM) compared to untreated wild-type.
Figure 9. Macrophage infiltration is not increased in BAT of $\text{Angptl4}^{+/−}\text{Gpihbp1}^{+/−}$ mice.

Immunofluorescence staining of brown adipose tissue in female wild-type, $\text{Angptl4}^{−/−}$, $\text{Gpihbp1}^{−/−}$, and $\text{Angptl4}^{−/−}\text{Gpihbp1}^{−/−}$ mice (10-14 weeks; n=3/genotype). Sections were stained with an antibody against FA-11 (green) to detect macrophages and DAPI (blue) to identify nuclei. A) Average ratio of macrophages/nuclei (±SEM). For each genotype 10 images/mouse (30 images/genotype) were scored for nuclei and macrophage staining. B) Representative images from each genotype.
Figure 10

Figure 10. Whole particle uptake in Angptl4−/− Gpihbp1−/− mice. Female Gpihbp1−/− and Angptl4−/− Gpihbp1−/− mice (20-24 weeks; n=6/group) were injected retro-orbitally with chylomicrons labeled with both 3H-triolein- and 14C-cholesterol. A) Chylomicron clearance from plasma. Both 3H and 14C radiolabel were measured in plasma samples taken 1, 5, 15, 30, and 60 minutes after injection. Graph shows radioactivity normalized to the 1 min time point (±SEM). B-C) Tissue uptake. After the final bleed, tissues were harvested and uptake of both 3H (B) and 14C (C) radiolabel was determined. Graphs show radiolabel uptake into the indicated tissues expressed as percent of injected dose per mg of tissue (±SEM). (*p<0.05, **p<0.01)
Figure 11. Whole particle uptake in wild type and Angptl4⁻/⁻ mice. Female wild-type and Angptl4⁻/⁻ mice (18-22 weeks; n=6-7/group) were injected retro-orbitally with chylomicrons labeled with both $^3$H-triolein and $^{14}$C-cholesterol. A) Chylomicron clearance from plasma. Both $^3$H and $^{14}$C radiolabel were measured in plasma samples taken 1, 5, 10, and 15 minutes after injection. Graph shows radioactivity normalized to the 1 min time point (±SEM). B-C) Tissue uptake. After the final bleed, tissues were harvested and uptake of both $^3$H (B) and $^{14}$C (C) radiolabel was determined. Graphs show radiolabel uptake into the indicated tissues expressed as percent of injected dose per mg of tissue (±SEM).
Figure 12. *Gpihbp1*−/− *Angptl4*−/− mice are unable to maintain increased triglyceride clearance in the face of HFD feeding. Female *Gpihbp1*−/− and *Angptl4*−/− *Gpihbp1*−/− mice (20-24 weeks; n=6/group) were fed a normal chow (NCD) or 45% high-fat diet (HFD) for two weeks. A) Fasting (4 h) plasma triglycerides (±SEM) of all mice before diet switch and after 2 weeks on the indicated diet. (*p<0.05, **p<0.01) B-C) Chylomicron clearance and tissue uptake after 2 weeks on diet. Fasted (4 h) mice were injected retro-orbitally with chylomicrons labeled with 3H-triolein. (B) Radiolabel was measured in plasma samples taken 1, 15, 30, and 60 minutes after injection. Graph shows radiolabel normalized to the 1 min time point (±SEM). (C) Radiolabel in harvested tissues. Graphs show percent injected dose/mg of tissue (±SEM).