Supplementary Materials and Methods

Animals and Diet
Mice with genetic disruption of the Pemt gene \(^1\) were back-crossed for 7 generations to obtain >99.2% homogeneity in a C57BL/6J background. Mice deficient in both PEMT and LDL receptors (\(\text{Pemt}^{-/-}/\text{Ldlr}^{-/-}\) and \(\text{Pemt}^{+/+}/\text{Ldlr}^{-/-}\)), and \(\text{Pemt}^{+/+}/\text{Ldlr}^{-/-}\) mice, were generated by inter-breeding \(\text{Pemt}^{-/-}\) mice with \(\text{Ldlr}^{-/-}\) mice (C57BL/6J, Jackson Laboratory, Bar Harbor, ME). Mice were maintained on a standard chow diet containing 6% (w/w) fat and 0.02% (w/w) cholesterol (LabDiet). At 9 to 12 weeks of age, the mice were fed a HF/HC diet (Basal Diet #TD 84172 from Harlan TEKLAD, Madison WI) containing 20% olive oil (w/w) and 1.25% cholesterol for 16 weeks. All animal procedures were performed in accordance with guidelines provided by University of Alberta’s Health Sciences Animal Policy and Welfare Committee.

Isolation and Culture of Hepatocytes
Hepatocytes were isolated from livers of male mice by perfusion with collagenase (100 U/ml) \(^2\). Hepatocytes were plated on 100 mm collagen-coated dishes (4.5 x 10^6 cells/dish) in Dulbecco’s-modified Eagle’s medium containing 10% fetal bovine serum. Cell viability (typically >90%) was estimated by trypan blue exclusion. After hepatocytes had adhered to the dish (3-4 h) the medium was replaced fresh medium without serum. Cells were incubated for 16 h.

Lipid Analyses
Mice that had been fed the HF/HC diet for 16 weeks were fasted overnight then blood was collected from the vena cava. Total PLs, TG, unesterified cholesterol and CE were extracted from plasma \(^3\) and quantified by gas-liquid chromatography \(^4\).

Isolation of VLDLs from Plasma and Hepatocyte Culture Medium
Lipoproteins were isolated from plasma and hepatocyte culture medium by ultracentrifugation on KBr density gradients \(^5,^6\). The fraction of density < 1.006 g/ml contained VLDLs. PLs were
extracted from VLDLs and separated by thin-layer chromatography in chloroform:methanol:acetic acid:formic acid: water, 70:30:12:4:2 (v:v:v:v). Amounts of PC and PE were determined by phosphorus assay. The distribution of choline-containing PLs, total cholesterol and TG among plasma lipoproteins of different sizes was analyzed by fast protein liquid chromatography (FPLC) with Superose 6 columns, and quantified with enzymatic assay kits for choline, cholesterol (unesterified + esterified) and TG (Sigma).

Apo B in Plasma and Hepatocyte Culture Medium
Proteins in plasma (2 µl) from overnight-fasted mice were separated on 5% or 3-15% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Apo B was detected by immunoblotting with goat polyclonal anti-human apo B antibodies (dilution 1/5,000; Chemicon International) and quantified by densitometric scanning. Apo B secreted into hepatocyte culture medium (2 ml) was concentrated by binding to Cab-O-sil and analyzed by immunoblotting, as above. Loading of equal amounts of protein in lanes of the gel was confirmed by Coomassie blue staining.

Analysis of Atherosclerosis
Mice (8-12 weeks of age) were fed the HF/HC diet for 16 weeks, then euthanized and heart and proximal aorta were excised, embedded in optimal cutting temperature compound (OCT; Tissue Tek) and frozen in dry ice/95% ethanol. Cryosections were stained with Oil Red O and hematoxylin. En face preparations of entire aortas were stained with 0.5 % Sudan IV and lesion area quantified with an image analyzer (AxioCam from Zeiss) using MetaMorph 6.1 (Universal Image Corp.) The extent of atherosclerosis was calculated as Sudan IV-stained area as percentage of total aortic surface area.

In vivo Secretion of Lipids
Mice that had been fed the HF/HC diet for 4 weeks were fasted overnight then injected intraperitoneally with Poloxamer 407 (1g/kg body wt). Blood samples were collected and VLDLs/IDLs/LDLs isolated by KBr density gradient ultracentrifugation. Total lipids were extracted from lipoproteins and PLs quantified. TG, CE and cholesterol were quantified by gas-liquid chromatography. The TG content of nascent VLDLs was calculated by subtracting VLDL-TG in plasma before P407 injection from VLDL-TG in plasma at indicated times after P407 injection.
Isolation of Hepatic Microsomes and Luminal Contents
Livers from Pemt+/Ldlr−/− mice and Pemt+/−/Ldlr−/− mice were homogenized in buffer containing 250 mM sucrose and 300 mM imidazole (pH 7.4) 16. The homogenate was centrifuged for 10 min at 10,000 x g and the resulting supernatant was centrifuged at 100,000 x g for 1 h to pellet microsomes. Microsomal luminal contents (containing nascent VLDLs) were released by treatment of microsomes with 0.1 M Na2CO3 (pH 11.0) for 25 min, and microsomal membranes and luminal contents were separated by centrifugation 16. Phospholipids were extracted from intact microsomes and luminal contents and quantified by phosphorus analysis.

Plasma Homocysteine
Total Hcy levels in plasma were measured by reverse-phase high performance liquid chromatography 17.

Clearance of [3H-TG]VLDLs in vivo
[3H]TG-labeled VLDL clearance was assessed as described 18. Briefly, [3H]palmitate (1 mCi) (Amersham) was dissolved in 1.0 ml 0.9% NaCl containing 2 mg/ml albumin. Pemt+/−/Ldlr−/− mice and Pemt+/+/Ldlr−/− mice were injected intravenously with 100 µCi of [3H]palmitate solution. Thirty min later Poloxamer-407 was injected intraperitoneally into the mice (1 g/kg body weight). Blood was collected from the inferior vena cava 4 h later. Plasma was pooled from 8 mice of each genotype and VLDLs isolated by ultracentrifugation (d < 1.006 g/ml) 18 then dialyzed overnight against phosphate-buffered saline; > 90% of radiolabel in VLDLs was in TG. The in vivo clearance of VLDL-TG was assessed after intravenous injection of 50,000 dpm of [3H]VLDL into Pemt+/−/Ldlr−/− mice and Pemt+/+/Ldlr−/− mice. At indicated times, 40 µl blood was collected and radioactivity measured in 10 µl plasma.

Clearance of [125I-apo B]VLDLs in vivo
Blood was collected from 8 Pemt+/−/Ldlr−/− mice and 8 Pemt+/+/Ldlr−/− mice. Plasma VLDLs were isolated by ultracentrifugation (d < 1.006 g/ml) 18 then labeled with 125I using IODO-BEADS iodination reagent according to manufacturer’s instructions (Pierce). Samples were dialyzed overnight against phosphate-buffered saline. Incorporation of [125I] into apoB was confirmed after propan-2-ol precipitation 18. The specific radioactivity of [125I]VLDLs was 150 cpm/ng
protein for VLDLs from \textit{Pemt}^{-/-}/\textit{Ldlr}^{-/-} mice, and 200 cpm/ng protein for VLDLs from \textit{Pemt}^{+/+}/\textit{Ldlr}^{-/-} mice. Mice were injected intravenously with $[^{125}\text{I}]$VLDLs and 30 min later Poloxamer-407 (1 g/kg body weight) was injected intraperitoneally. Blood was collected 4 h later. Clearance of apo B from plasma was measured after injection of $[^{125}\text{I}]$VLDLs (10 $\mu$g tracer in 300 $\mu$l 0.9% NaCl containing 2 mg/ml albumin) from each genotype, separately into tail veins of \textit{Pemt}^{+/+}/\textit{Ldlr}^{-/-} mice and \textit{Pemt}^{-/-}/\textit{Ldlr}^{-/-} mice that had been injected intraperitoneally with Poloxamer-407 30 min previously. Blood (40 $\mu$l) was collected from the tail vein and $^{125}\text{I}$ in apo B measured after propan-2-ol precipitation.

**Plasma Lipase Activity**

Mice were injected with 0.1 U/g body weight heparin (Sigma) to release lipoprotein lipase and hepatic lipase into the circulation. Blood was collected 5 min after injection and plasma stored at -70°C. Post-heparin plasma lipase activity was assayed. Lipase substrate was prepared from 1 mM glycerol trioleate (Sigma) in 0.5 ml gum arabic, mixed with 0.5 $\mu$Ci glycerol tri[9,10($\Delta$)-$^3\text{H}$]oleate (MP Biomedical) in 50 mM Tris (pH 7.5) containing 4 mM CaCl$_2$, 0.15 M NaCl, 200 $\mu$l/ml heat-inactivated serum from fasted humans, 0.5 mg/ml heparin and 1 mg/ml albumin. The substrate was emulsified by sonication on ice for 5 min. Each assay included 100 $\mu$l substrate, 20 $\mu$l mouse plasma, and 80 $\mu$l reaction buffer [0.19 M Tris (pH 8.6), 0.5 mg/ml heparin and NaCl (either 0.19 M or 2.31 M)]. Reactions were incubated at 37 °C for 45 min and terminated by addition of 3.25 ml methanol:chloroform:heptane (28:25:20, v:v:v). Fatty acids were extracted by addition of 1.05 ml of 0.1 M potassium carbonate-borate (pH 10.5). Tubes were vortexed, then centrifuged for 15 min at 3,000 x g. Radioactivity in 1 ml upper phase ($[^3\text{H}]$fatty acids) represented lipase activity. Lipoprotein lipase activity was calculated as the difference between total lipase activity (measured in 0.2 M NaCl) and hepatic lipase activity (measured in 2.3 M NaCl).

**VLDL diameter**

Blood was collected from \textit{Pemt}^{-/-}/\textit{Ldlr}^{-/-} mice and \textit{Pemt}^{+/+}/\textit{Ldlr}^{-/-} mice. Plasma VLDLs were isolated and extensively dialyzed against phosphate-buffered saline. Particle diameter was measured 55 times over a 6 min period in 500 $\mu$l aliquots of each sample diluted in water using a Zetasizer Nano ZS analyzer (Malvern).
Statistical Analysis

Values are means ± S.D. Statistical significance of differences between Pemt\(^{-/-}\)/Ldlr\(^{-/-}\) mice and Pemt\(^{+/-}\)/Ldlr\(^{-/-}\) mice, and between Pemt\(^{-/-}\)/Ldlr\(^{-/-}\) mice and Pemt\(^{+/-}\)/Ldlr\(^{-/-}\) mice, were analyzed by the Student’s \(t\) test unless otherwise indicated. Differences were considered statistically significant at \(P < 0.05\).

References


Supplementary Table 1. Lipid profiles of $\text{Pemt}^{+/+}/\text{Ldlr}^{-/-}$, $\text{Pemt}^{+/+}/\text{Ldlr}^{-/-}$ and $\text{Pemt}^{-/-}/\text{Ldlr}^{-/-}$ mice after 16-week of HF/HC diet

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{Pemt}$</td>
<td>$\text{Ldlr}$</td>
<td></td>
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<tr>
<td>$^{+/+}$</td>
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<td></td>
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<tr>
<td>$^{-/-}$</td>
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<tr>
<td>$^{-/-}$</td>
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</tr>
<tr>
<td>(n=14)</td>
<td>(n=5)</td>
<td>(n=16)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>30.4 ± 4.9</td>
<td>26.3 ± 2.6</td>
</tr>
<tr>
<td>Liver Weight (g)</td>
<td>1.44 ± 0.25</td>
<td>1.68 ± 0.16</td>
</tr>
<tr>
<td>Plasma (mg/dl)</td>
<td></td>
<td></td>
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<tr>
<td>Cholesterol</td>
<td>356 ± 123</td>
<td>274 ± 124</td>
</tr>
<tr>
<td>Cholesteryl Ester</td>
<td>236 ± 83.2</td>
<td>137 ± 67.5*</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>498 ± 173</td>
<td>229 ± 179*</td>
</tr>
<tr>
<td>Liver Homogenate (µg/mg protein)</td>
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<td></td>
</tr>
<tr>
<td>Phospholipid</td>
<td>110 ± 10.4</td>
<td>114 ± 7.0</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>22.7 ± 4.2</td>
<td>17.5 ± 3.1*</td>
</tr>
<tr>
<td>Cholesteryl Ester</td>
<td>127 ± 21.2</td>
<td>147 ± 34.8</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>356 ± 105</td>
<td>989 ± 97.1*</td>
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<tr>
<td>Liver Microsomes (µg/mg protein)</td>
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<tr>
<td>Phospholipid</td>
<td>134 ± 6.4</td>
<td>109 ± 12.2*</td>
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<tr>
<td>Cholesterol</td>
<td>27.6 ± 3.7</td>
<td>17.0 ± 2.9*</td>
</tr>
<tr>
<td>Cholesteryl Ester</td>
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<tr>
<td>Triacylglycerol</td>
<td>60.9 ± 11.2</td>
<td>33.8 ± 7.7*</td>
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</tbody>
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Values are means ± S.D.  $^*$ P < 0.01 vs. $\text{Pemt}^{+/+}/\text{Ldlr}^{-/-}$; $^+$ P < 0.05 vs. $\text{Pemt}^{+/+}/\text{Ldlr}^{-/-}$; $^\ddagger$ P < 0.01 vs. $\text{Pemt}^{-/-}/\text{Ldlr}^{-/-}$; $^\S$ P < 0.05 vs. $\text{Pemt}^{-/-}/\text{Ldlr}^{-/-}$.