A Gender-specific Role For Phosphatidylethanolamine N-Methyltransferase-derived Phosphatidylcholine in the Regulation of Plasma High Density and Very Low Density Lipoproteins in Mice*

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Phosphatidylethanolamine N-methyltransferase (PEMT) is involved in a secondary pathway for production of phosphatidylcholine (PC) in liver. We fed Pemt−/− mice a high fat/high cholesterol diet for 3 weeks to determine whether or not PC derived from PEMT is required for very low density lipoprotein secretion. Lipid analyses of plasma and liver indicated that male Pemt−/− mice accumulated triacylglycerols in their livers and were unable to secrete the same amount of triacylglycerols from the liver as did Pemt+/+ mice. Plasma levels of triacylglycerol and both apolipoproteins B100 and B48 were significantly decreased only in male Pemt−/− mice. Experiments in which mice were injected with Triton WR1339 showed that, whereas hepatic apoB100 secretion was decreased in male Pemt−/− mice, the decrease in plasma apoB48 in male Pemt−/− mice was not due to reduced secretion. Moreover, female and, to a lesser extent, male Pemt−/− mice showed a striking 40% decrease in plasma PC and cholesterol in high density lipoproteins. These results suggest that, even though the content of hepatic PC was normal in PEMT-deficient mice, plasma lipoprotein levels were profoundly altered in a gender-specific manner.

Phosphatidylcholine (PC) is synthesized by the Kennedy pathway (CDP-choline pathway), by methylation of phosphatidylethanolamine (PE), or by acylation of lyso-PC (LPC). The Kennedy pathway is the major route for phosphatidylcholine (PC) synthesis in all mammalian tissues and is dependent on the intake of choline (1). In the liver an additional pathway for PC production is catalyzed by phosphatidylethanolamine N-methyltransferase (PEMT), which converts PE to PC via the transfer of three methyl groups from S-adenosylmethionine (2). The liver-specific expression of PEMT suggests that this enzyme might play a role in bile secretion and/or very low density lipoprotein (VLDL) secretion. PC is the primary phospholipid of all classes of lipoproteins in mammals (3) and is required for the secretion of VLDL; other phospholipids cannot substitute (4–7). The Kennedy pathway is required for maintaining normal plasma VLDL levels, because rats fed a choline-deficient diet for 3 days had a 6-fold increase in hepatic triacylglycerol (TG) and decreased plasma TG (6).

The role of PEMT-derived PC for lipoprotein secretion/metabolism has not been clearly established. Treatment of hepatocytes with 3-deazaadenosine, an inhibitor of methylation reactions that utilize S-adenosylmethionine, inhibited PEMT activity by more than 90% but did not decrease VLDL secretion (8). Thus, PEMT activity did not appear to be required for VLDL secretion. Subsequent experiments revealed that one pool of hepatic PC, which was produced by the methylation of PE derived from phosphatidylserine (PS) decarboxylation, was preferentially incorporated into VLDL secreted by hepatocytes (9). Moreover, secretion of this pool of PC labeled by this pathway was not inhibited by 3-deazaadenosine even though this inhibitor reduced the incorporation of labeled serine into PC of hepatocytes by >90% (9, 10). Because 3-deazaadenosine did not inhibit the incorporation of labeled serine into the headgroup of secreted PC, we cannot conclude that PEMT-derived PC is not important for VLDL secretion. More recent studies used bezafibrate to inhibit PEMT activity in rat hepatocytes (11). The secretion of neither apo (apolipoprotein) B48 nor B100 was inhibited, but bezafibrate reduced the lipidation of apoB48 and thus caused a shift in the density of apoB48-containing lipoproteins in the media from that of VLDL to higher densities (11). Neither bezafibrate nor 3-deazaadenosine are specific inhibitors of PEMT (8, 11). Thus, at this juncture the role for PEMT in VLDL secretion was not clear.

A Pemt−/− mouse model now exists (12). Hence, we have assessed more directly the role of PEMT in VLDL secretion. Pemt−/− mice appear normal except when fed a choline-deficient diet, which causes severe liver failure after 3 days (13). Recently, a defect in lipoprotein secretion was detected when hepatocytes from male Pemt−/− mice were isolated, and the secretion of lipoproteins was compared with that from Pemt+/+ hepatocytes (14). These studies showed that a deficiency of PEMT resulted in a 70% decrease in secretion of apoB100 and a 50% decrease in secretion of TG and PC associated with VLDL. It was, therefore, important to determine if this apparent decrease in VLDL secretion also occurred in Pemt−/− mice.
The present study examined plasma lipoprotein and lipid levels, as well as VLDL secretion, in *Pemt*−/− and *Pemt*+/− mice as a function of gender and diet. Male, but not female, *Pemt*−/− mice exhibited a defect in VLDL and apoB100 secretion compared with *Pemt*+/− mice but only when fed a high fat/high cholesterol (HF/HC) diet. In contrast, in *Pemt*−/− mice the amounts of plasma cholesterol and PC in the high density lipoprotein (HDL) fractions was ~40% less in females and 19–25% less in males than in their *Pemt*+/− counterparts.

**EXPERIMENTAL PROCEDURES**

Materials—The semi-purified diet without fat (TD 84712) was obtained from Teklad (Madison, WI). Diagnostic kits for measurement of total cholesterol, total TG, total glycerol, and aspartate and alanine aminotransferases were purchased from Sigma. Diagnostic kits for choline-derived plasma phospholipids and cholesterol were purchased from Wako Chemicals (Neuss, Germany). Silicon Gel G60 plates for thin-layer chromatography (TLC) were from Merck (Darmstadt, Germany). PC, PE, PS, and treoilein standards were from Avanti Polar Lipids (Alabaster, AL). Sphingomyelin (SM) and LPC standards were obtained from cambridge flash (Cambridge, MA). All other chemicals and reagents were from standard commercial sources.

**Care and Feeding of Mice**—The *Pemt*−/− and *Pemt*+/+ mouse colonies had mixed genetic backgrounds of 129/J and C57BL/6 (12) and were maintained via homozygous breeding in a reversed 12-h light/dark cycle. At the age of 12–14 weeks the animals were fed for 3 weeks *ad libitum* either rodent chow or a high fat/high cholesterol (HF/HC) diet (15). The HF/HC diet consisted of 50% (w/w) semi-purified diet, 19% (w/w) olive oil, 1% (w/w) linseed oil (a source of essential fatty acids), and 1% (w/w) cholesterol (15). During the 3-week dietary regimen, the mice were housed in a non-reversed 12-h light/dark cycle. After 3 weeks, the animals were fasted overnight, weighed, and sacrificed and blood, bile, and livers were collected.

**Protein and Lipid Analysis of Livers**—The livers were excised, weighed, and homogenized with a glass-Teflon homogenizer in 3 ml of homogenization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) followed by sonication for 20 s. Protein was determined using the Coomassie Plus protein protocol from Bio-Rad, which is based on the Bradford assay (16). Bovine serum albumin was used as a standard for all protein assays. Lipids were extracted from the liver homogenates by the method of Bligh and Dyer (17). Phospholipids were separated by TLC using a developing solvent of chloroform/methanol/acetic acid/water (25:15:4:2, v/v), until the solvent reached halfway up the plate. For analysis of phospholipids only, the lipid samples were fractionated by TLC in a developing solvent of chloroform/methanol/acetic acid/water (70:30/12/4, v/v). Lipids were visualized with iodine vapor, scraped, and quantitated (18). Phospholipid mass was measured with the Malachite Green lipid phosphorus assay (18). TG mass was determined by gas-liquid chromatography or the hydroxylamine method (19). Hepatic cholesterol and cholesteryl ester were measured by gas-liquid chromatography.

**Lipid Analysis of Plasma and Bile**—Blood was collected from each mouse via the lower vena cava in the presence of trace amounts of 250 mM EDTA, and plasma was isolated by centrifugation. Sodium azide was added to all plasma samples to a final concentration of 0.01%. Total cholesterol in both plasma and bile was measured using the Sigma Infinity cholesterol reagent modified to a micortiter plate format. Unesterified cholesterol in plasma was analyzed with the Wako Free Cholesterol C kit in a micortiter plate format. Cholesteryl ester in plasma was calculated by subtracting the amount of unesterified cholesterol from the amount of total cholesterol. Plasma TG was measured using the Sigma Triglyceride GPO Trinder kit (protocol 337-B).

The plasma from three to five animals was pooled and separated into lipoprotein fractions using high performance liquid chromatography with an Amersham Biosciences Superose 6 column attached to a Beckman Systems Gold or Nouveau Gold apparatus. In-line assays for total cholesterol (Sigma Infinity cholesterol reagent) and choline-derived phospholipids (Phospholipid B kit, Wako) were performed as previously described (13). For some experiments, phospholipids were extracted from 100 μl of plasma or 4 μl of bile from each animal using a modified Bligh-Dyer protocol (17) and separated by TLC as described above. The phospholipids were scraped from the plates and quantitated (18).

**Measurement of Plasma Aminotransferase Activities**—Aspartate aminotransferase and alanine aminotransferase activities in plasma were measured as previously described (20, 21) using the Sigma GP and GO transaminase kits.

**Isolation and Immunoblotting of Plasma Apolipoproteins**—Plasma from two to three animals was pooled to a final volume of 200 μl to which was added 1.3 ml of 4.15 mM KBr to a final density of 1.22 g/ml. This sample was put at the bottom of a 5-ml Beckman Quickseal tube and overlaid to the top with 0.9% NaCl. The samples were centrifuged in a VTI 65.2 rotor at 416,000 × *g* for 1 h as previously described (22, 23). Ten 0.5-ml fractions with densities ranging from 1.20 to 1.01 g/ml were collected. To each fraction, 200 μl of a slurry of fused silica (Sigma catalogue no. S-6005, 0.5 g/10 ml of phosphate-buffered saline) was added and mixed for 15 min at 4 °C (24). Afterward, the silica was pelleted by centrifugation for 5 min at 1,000 × *g* and rinsed twice with phosphate-buffered saline. The pellets were boiled in Laemmli sample buffer (25), and proteins were separated on a 3–15% continuous gradient SDS-polyacrylamide gel and transferred onto nitrocellulose membranes.

Immunoblotting was performed using antibody directed against human apoB (dilution 1:5000), the membranes were stripped and immunoblotted for apoA1 (dilution 1:10,000). Quantification of the immunoreactive bands was performed using Image Gauge v3.0 software by Fuji.

**Analysis of ApoB Secretion in Vivo**—Male *Pemt*−/− and *Pemt*+/+ mice were fed an HF/HC diet. Mice (12–16 weeks old) were fed an HF/HC diet for 3 weeks. The mice were fasted overnight and sacrificed, and livers were removed. Sections of each liver were fixed in 10% formalin, sliced, and stained with hematoxylin-eosin (A–D), or the sections were quick-frozen and stained with lipophilic Oil Red O (E and F). The hematoxylin-eosin stained sections are at a magnification of ×10. The livers of two animals were examined for each condition except male *Pemt*−/− and *Pemt*+/+ mice, for which three livers were analyzed. The liver of one mouse for each condition was stained with Oil Red O, and the sections are shown at a magnification of ×40. The images are representative of all livers examined.

![Figure 1](https://example.com/fig1.png)
with Coomassie Blue. The gels were soaked in Amplify, dried, and ethanol (v/v), 0.02% bromphenol blue) (23, 27). Samples were electro-

were fasted overnight and injected with 200 μl of phosphate-buffered saline containing 10% Triton WR 1339 (v/v) and 500 μCi of [3H]Pemt (methionine/cysteine) (Amersham Biosciences) as previously described (26). After 1 h, the animals were sacrificed and blood was collected in the presence of EDTA after cardiac puncture. To 100 μl of total plasma were added 400 μl of phosphate-buffered saline, 55 μl of 10× immunoprecipitation buffer (1.5 μl NaCl, 0.5 μl Tris-HCl, pH 7.4, 50 μl EDTA, 5% Triton X-100 (v/v), and 1% SDS (w/v)), and 10 μl of anti-human apoB antibody and benzamidine (final concentration 1 μl), and all was mixed overnight at 4 °C (23). Protein A-Sepharose CL-4B was added (100 μl of 1:1 mixture with PBS) to each sample and mixed for 1 h at 4 °C. Immunoprecipitates were pelleted by centrifugation, rinsed twice with 1× immunoprecipitation buffer, and boiled in sample buffer (25 mm Tris-HCl, pH 6.8, 4% SDS (w/v), 20% glycerol (v/v), 10% β-mercapto-ethanol (v/v), 0.02% bromphenol blue) (23, 27). Samples were electrophoresed on 5% SDS-polyacrylamide gels, and proteins were stained with Coomassie Blue. The gels were soaked in Amplify, dried, and exposed to film.

**Histological Studies**—Livers were fixed in 10% formalin, and sections were stained by a standard protocol with hematoxylin and eosin. In addition, livers fixed in 10% formalin were quick-frozen, sectioned, and stained with Oil Red O to examine neutral lipid distribution.

**RESULTS**

**Histological Characterization of Livers and Assay of Aminotransferase Activities**—At the age of 9–11 weeks, mice were fed an HF/HC diet or a chow diet for 3 weeks. All mice appeared normal and healthy. The weights of male and female Pemt+/− mice were comparable to those of their counterparts (males, −24–27 g; females, −22–25 g). A small difference was observed in the weight of the liver as a percentage of body weight between male Pemt+/− (4.27 ± 0.18) and Pemt+/+ (3.85 ± 0.08) mice fed an HF/HC diet that approached statistical significance (p = 0.059). Eighty percent of livers from male Pemt+/− mice fed the HF/HC diet were granular in appearance and some were also light in color, suggesting an accumulation of lipid. Histological examination with hematoxylin revealed gross vacuolization in a centrilobular pattern in −50% of the hepatocytes lobules in the male Pemt−/− mice (Fig. 1, A and B). Interestingly, these large vacuoles did not stain with Oil Red O (Fig. 1F), suggesting that they were not engorged with neutral lipid. In contrast, livers of female Pemt−/− and Pemt+/+ mice were indistinguishable in outward appearance. However, histological examination showed that livers of female Pemt−/− mice (Fig. 1B), but not Pemt+/− mice (Fig. 1A), also exhibited a mild vacuolization.

Because of obvious changes in the livers of male Pemt−/− mice (Fig. 1, D and F), plasma aspartate and alanine aminotransferase activities were measured to assess the possibility of liver damage. Of the 20 Pemt−/− mice on an HF/HC diet examined, only one male had elevated levels of these two aminotransferases (2132 compared with 0–200 Sigma Frankel units/ml for aspartate aminotransferase; 1813 compared with 0 Sigma Frankel units/ml for alanine aminotransferase) suggesting that the observed changes in plasma and hepatic lipids were due to loss of Pemt activity rather than liver damage.

**The Lipid Content of the Liver Is Influenced by Diet, Pemt Genotype, and Gender**—As shown in Table I, the levels of hepatic cholesterol were not influenced by diet, gender, or Pemt genotype (Table I). As expected, the HF/HC diet increased cholesteryl ester in the livers of all mice (Table I). However, this HF/HC diet-mediated increase in cholesteryl ester was more striking in the female mice of both genotypes.

The amount of TG in the livers depended on diet, gender, and Pemt genotype. Disruption of the Pemt gene resulted in an increase of −4-fold in the TG content of livers of male mice fed
the HF/HC diet. In contrast, in female mice the liver TG content was independent of Pemt genotype. Interestingly, the hepatic TG results were similar to those of cholesteryl ester. Female mice fed the HF/HC diet demonstrated an accumulation regardless of genotype, whereas a significant accumulation occurred only in the male Pemt−/− mice.

Hepatic TG accumulation, liver damage, and death occur rapidly when Pemt−/− mice are fed a choline-deficient diet for 3 days (13). In these mice, the content of PC in the liver was 56% less than in Pemt+/+ mice. Because the mice in the current study had been fasted overnight prior to sacrifice, phospholipid analysis was performed to confirm that the mice were not choline-deficient. Although the level of hepatic PC in female Pemt−/− mice on an HF/HC diet was 16% less (p < 0.01) than in their Pemt+/+ counterparts (Table II), 54.8 nmol of PC/mg of protein is within the normal range of hepatic PC (13, 28), indicating that the overnight fast did not induce a choline-deficient state.

Because PE is a substrate for PEMT, one might anticipate that PE levels would be increased in livers of Pemt−/−, compared with Pemt+/+, mice. However, Table II shows that PE levels did not depend on diet, gender, or Pemt genotype. The amounts of PS in the livers were also measured because PS is synthesized by a base-exchange reaction from PC or PE (1). Hepatic PS levels were unaffected by the lack of PEMT activity, gender, or diet (Table II). Similarly, phosphatidylinositol concentrations were unchanged.

**Biliary Lipids Are Unaffected in Pemt−/− Mice—** No major differences were observed between male or female Pemt−/− and Pemt+/+ mice in the volume of bile in the gallbladder (data not shown). The small difference in amount of PC in the bile of male and female Pemt−/− mice (∼11–13 nmol of PC/ml of bile) compared with Pemt+/+ mice (14–18 nmol of PC/ml of bile) was not statistically significant. Cholesterol secretion into bile is closely associated with PC secretion (29). The amount of biliary cholesterol (2.64–3.67 nmol/μl) was also not statistically different between Pemt−/− and Pemt+/+ mice of either gender.

**Plasma Lipids Are Altered in HF/HC Fed Pemt−/− Mice in a Gender-specific Manner—** Choline-derived phospholipids (PC, SM, and LPC) are the major phospholipids in mammalian plasma (30). Plasma PC in female HF/HC-fed Pemt−/− mice was 40% less (p < 0.001) than in female Pemt+/+ mice (Fig. 2A). The amount of plasma PC in male Pemt−/− mice on an HF/HC diet was 19% (p < 0.05) less than in male Pemt+/+ mice (Fig. 2A).

Plasma LPC levels were significantly decreased (p < 0.01) by ∼40% in both male and female Pemt−/− mice, but SM levels were not significantly different between the genotypes of either gender (Table III). PS/phosphatidylinositol and PE did not compensate for the deficit in choline-derived phospholipids in the plasma of Pemt−/− mice. Unexplained is the 2.7-fold higher level of plasma PE in male compared with female mice of both genotypes (Table III).

Interestingly, the gender-specific results on plasma cholesterol levels (Fig. 2B) mirrored the results obtained for plasma PC (Fig. 2A). In Pemt−/− female mice fed the HF/HC diet, total plasma cholesterol content was 42% less than in Pemt+/− female mice (p < 0.0001) (Fig. 2B). The difference in plasma cholesterol content between the genotypes of male mice was less pronounced (25% less in Pemt−/− mice than in Pemt+/+ mice, p < 0.05) (Fig. 2B). In all cases, the observed decrease in total plasma cholesterol was due to a decrease in both unesterified and esterified cholesterol (data not shown).

Modulation of the plasma level of TG by PEMT deficiency was quite different from that of PC and cholesterol. The plasma TG content of HF/HC-fed male mice was strikingly (55%, p < 0.05) less in Pemt−/− than in Pemt+/+ mice (Fig. 3). However, the amount of plasma TG was the same in female Pemt−/− and Pemt+/+ mice. These data are consistent with the observed accumulation of TG in the livers of male, but not female, Pemt−/− mice (4.9-fold higher than Pemt+/+ mice) (Table I).

Plasma was separated into lipoprotein components by high performance liquid chromatography (Fig. 4). The content of cholesterol (Fig. 4, A and B) and choline-derived phospholipids (Fig. 4, C and D) in the HDL fractions of both male and female Pemt−/− mice was lower than in Pemt+/+ mice in agreement with the quantitative data of Fig. 2. Because the cholesterol and phospholipid content of VLDL was close to the limit of detection in these experiments (Fig. 4, A–D), it was not possible to evaluate the impact of PEMT deficiency on the content of these lipids in VLDL. Based on the hepatic, total plasma, and lipoprotein distribution of the lipids in the Pemt−/− mice, it is clear that PEMT-derived PC plays a significant role in the distribution of lipids in plasma lipoproteins in a gender-specific manner.

**Plasma ApoB Is Decreased in Male, but Not Female, Pemt−/− Mice Fed an HF/HC Diet—** Plasma collected from two to three animals was pooled, and ten fractions of densities ranging from 1.20 to 1.01 g/ml were separated by ultracentrifugation. Lipid and apoprotein components in each fraction were adsorbed onto fumed silica, and the apoproteins were examined and resolved by SDS-polyacrylamide gel electrophoresis. Immunoblotting of apolB48, apoB100, and apoA1 was performed. The amounts of apoB48 and apoB100 were decreased in the VLDL/low density lipoprotein fractions of Pemt−/−, compared with Pemt+/+, male mice (Fig. 5, B and D) but not in female mice.
Lipids were extracted from 100 μl of plasma and separated by TLC. Phospholipid mass was determined via a Malachite Green lipid phosphorus assay. Shown are the mean values ± S.E. from 8–10 mice for each gender on a HF/HC diet. Statistically significant differences are between Pemt<sup>−/−</sup> and Pemt<sup>+/+</sup> animals of the same gender.

| Genotype | LPC | SM | PS+PI | PE |
|----------|-----|----|-------|----|
| Females  |     |    |       |    |
| +/+      | 19.1 ± 1.9 | 9.4 ± 1.6 | 8.6 ± 1.1 | 2.5 ± 0.1 |
| −/−      | 11.3 ± 1.7* | 5.7 ± 1.4 | 5.6 ± 0.8 | 2.5 ± 0.2 |
| Males    |     |    |       |    |
| +/+      | 19.0 ± 0.7 | 7.3 ± 0.4 | 10.8 ± 0.7 | 7.8 ± 0.4 |
| −/−      | 11.9 ± 0.7* | 6.5 ± 0.8 | 9.4 ± 0.1 | 6.8 ± 0.4 |

* p < 0.01 based on Student’s t test.

Fig. 3. Plasma TG is decreased in male Pemt<sup>−/−</sup> mice fed an HF/HC diet. Plasma TG was measured using the Sigma TG GPO Trinder kit. Values are expressed as means ± S.E. for 9–11 mice for each condition. *, p < 0.05 as determined by a Student’s t test for Pemt<sup>−/−</sup> mice compared with Pemt<sup>+/+</sup> mice.

(Fig. 5, A and C). The difference in apoB100 was more pronounced than in apoB48. Because the majority of plasma TG is present in VLDL, these results are consistent with the analyses of plasma TG (Fig. 3).

In light of the reduced content of cholesterol and choline-containing phospholipids in the HDL fractions of female and male Pemt<sup>−/−</sup> mice (Fig. 4, A and B), we anticipated that there might be a corresponding reduction in the amount of apoA1 in the plasma of Pemt<sup>−/−</sup> mice. However, as shown in Fig. 5 (E and F), the distribution of plasma apoA1 was very similar in Pemt<sup>−/−</sup> and Pemt<sup>+/+</sup> mice of both genders. The phenomenon that HDL cholesterol can be decreased without an accompanying change in the level of apoA1 has been previously observed (31).

To determine whether or not apoB secretion was decreased in male Pemt<sup>−/−</sup> mice, we performed in vivo labeling of proteins in the mice in the presence of Triton WR1339, a compound that inhibits the catabolism of TG in plasma VLDL. Male Pemt<sup>−/−</sup> and Pemt<sup>+/+</sup> mice were fasted overnight then injected with 200 μl of 10% Triton WR1339 and 500 μCi of [35S]Promix. The animals were sacrificed 1 h later, and apoB100 and apoB48 were immunoprecipitated from plasma. The amount of apoB100 secreted was significantly lower in Pemt<sup>−/−</sup> mice compared with Pemt<sup>+/+</sup> mice fed the HF/HC diet (Fig. 6, A and B). However, in contrast to the decreased apoB48 in plasma, the amount of apoB48 secreted was not significantly different between Pemt<sup>−/−</sup> and Pemt<sup>+/+</sup> mice. Thus, the smaller amount of plasma apoB48 in male Pemt<sup>−/−</sup> mice, compared with Pemt<sup>+/+</sup> mice (Fig. 5B), is probably the result of increased catabolism rather than decreased secretion.

Role of PEMT-derived PC in VLDL Secretion as a Function of Diet—The results presented above focus on mice fed the HF/HC diet. In addition, Pemt<sup>−/−</sup> and Pemt<sup>+/+</sup> mice fed a chow diet were analyzed. Livers of Pemt<sup>−/−</sup> mice fed the chow diet were not vacuolized (data not shown) in contrast to the HF/HC-fed mice (Fig. 1, B and D). As well, the hepatic accumulation of TG observed in male Pemt<sup>−/−</sup> mice fed the HF/HC diet did not occur when the mice were fed chow (Table I). Moreover, the plasma TG concentration of chow-fed male Pemt<sup>−/−</sup> mice was indistinguishable from that of the Pemt<sup>−/−</sup> males (19.6 ± 3.0 and 20.3 ± 3.0 mg/dl for Pemt<sup>−/−</sup> and Pemt<sup>+/+</sup> mice, respectively). The secretion of neither apoB100 nor apoB48 was affected by the PEMT genotype in mice fed the chow diet (Fig. 6A). Therefore, although our results demonstrate clearly that PEMT-derived PC plays an important role in VLDL secretion, this phenotype becomes obvious only when male mice are challenged with an HF/HC diet.

Requirement of PEMT-derived PC for Plasma HDL as a Function of Diet—In male Pemt<sup>−/−</sup> mice fed the chow diet, plasma PC levels were 30% less than in their Pemt<sup>+/+</sup> counterparts (Fig. 2A). Feeding the HF/HC diet gave similar results. Furthermore, trends for plasma cholesterol in female mice (Fig. 2B) mimicked observations made for plasma PC. Thus, the requirement of PEMT-derived PC for plasma PC and cholesterol in female mice is independent of diet.

Unlike the female mice, chow-fed Pemt<sup>−/−</sup> male mice had normal plasma PC and cholesterol concentrations compared with Pemt<sup>+/+</sup> males (Fig. 2, A and B). The HF/HC diet increased plasma PC and cholesterol in Pemt<sup>−/−</sup> mice but not in PEMT-deficient mice. These changes were also reflected in the HDL in plasma (data not shown). Thus, in male mice the role of PEMT-derived PC on plasma PC and cholesterol is independent of the challenge of the HF/HC diet.

DISCUSSION

The results in this report demonstrate that PEMT is required for normal secretion of VLDL (apoB100, PC, and TG) in male mice challenged with an HF/HC diet. These data are consistent with our previous observations in hepatocytes derived from male Pemt<sup>−/−</sup> and Pemt<sup>+/+</sup> mice in which the secretion of TG, PC, and apoB100 into VLDL was decreased by ~50%, particularly when the hepatocytes were incubated with oleate (14). An additional and provocative observation is that in HF/HC-fed Pemt<sup>−/−</sup> mice, HDL-derived cholesterol and PC were reduced ~40% in females and ~20% in males compared with Pemt<sup>+/+</sup> mice. These data highlight several ways in which PC supply modulates lipoprotein homeostasis in mice and raise the following questions. First, why are these alterations in lipoprotein metabolism gender-specific? Second, why does a lack of PEMT reduce the amount of cholesterol and PC in
plasma HDL? Third, why does a deficiency of PEMT reduce VLDL secretion? Fourth, are these modulations in lipoprotein metabolism dependent upon the route of PC biosynthesis?

PC is required for at least two different aspects of VLDL secretion. First, PC is the major lipid on the surface monolayer of the VLDL particles. Second, PC is the major phospholipid of the membranes of the secretory pathway (endoplasmic reticulum, Golgi, and plasma membrane). Thus, a deficiency of PC in the secretory pathway or in the nascent particle might limit the secretion of VLDL.

Because the secretion of apoA1 occurs normally in PEMT-deficient mice, it appears that the secretion pathway is operating normally. Therefore, the inhibition of VLDL secretion in the Pemt/H11002/H11002 male mice is likely due to a reduction in the PC content of the surface monolayer of the nascent VLDL particle.

Gender-specific Alterations in Lipoprotein Metabolism in PEMT-deficient Mice—Several independent reports have estimated that ~30% of PC in the liver is made via PEMT (32–34). In female rats a larger fraction of total PC in the liver is made via PEMT (32–34). In female mice, which was analyzed three times. Absorbance readings were taken at 500 nm for cholesterol (A and B) and 505 nm for phospholipids (C and D).
from PE methylation than in male rats (35–38). Our laboratory has preliminary evidence suggesting that the same is true in mice. Yet, countervintually, the present study shows that VLDL secretion is inhibited in male, but not female, Pemt+/- mice. One explanation for why female Pemt+/- mice do not have a defect in VLDL secretion is that female mice might develop an active mechanism for the hepatic uptake of PC from HDL. In support of this theory female Pemt+/- mice demonstrated a decrease in HDL that was independent of diet (Fig. 2). In rats, the hepatic uptake of PC from HDL is a quantitatively important process, because HDL-derived phospholipids contribute up to 38% of total biliary PC (39). If this were also the case in mice, PEMT deficiency might result in an increased uptake of HDL-PC with a corresponding reduction in the amount of PC and cholesterol in the plasma HDL of Pemt+/- mice as observed. Consequently, the female Pemt+/- mice might acquire sufficient PC from HDL to permit a normal level of VLDL secretion, even when confronted with a high fat diet.

The efflux of cholesterol from cells to apoA1 for HDL formation is linked to the efflux of PC (40). Thus, another explanation for the reduction in plasma PC in PEMT-deficient mice might be that mice respond to a lack of PEMT by providing less PC for plasma HDL. In a previous study no differences were observed between the levels of lipids, including PC, in plasma HDL of rats in which the CDP-choline pathway was attenuated by feeding a choline-deficient diet (6). However, in these experiments only male rats were used. Possibly, PEMT-derived PC is specifically required for HDL formation. Alternatively, an experiment in which the CDP-choline pathway is inhibited in female mice might similarly reduce the amount of PC in HDLs. Whether or not the effect is observed only in mice and not rats cannot be addressed due to a lack of a suitable rat model. Our results also raise the question of how much PC in plasma HDL originates from liver.

It is well documented that pre-menopausal women are less susceptible to cardiovascular disease due to low plasma LDL/ HDL ratios (41). The mechanism of this protective effect in women is not well understood. However, the differences we have observed in this study in the regulation of VLDL secretion between the genders might lead to a better understanding of this mechanism.

The Role of Diet—The defect in VLDL secretion in male Pemt+/- mice was observed only with the challenge of an HF/HC diet. Normal VLDL secretion was maintained in male Pemt+/- mice fed a chow diet, as well as in females fed either diet. Thus, in these cases, the loss of PEMT-derived PC did not limit VLDL secretion. The dominant PC biosynthetic pathway in liver is the Kennedy pathway (1), and previous work has shown that it is enhanced in the livers of all Pemt+/- mice (12). Furthermore, the uptake of plasma PC and LPC are other potentially adaptive mechanisms by which the livers of these mice might compensate for the loss of PEMT-derived PC. Therefore, the ability of chow-fed Pemt+/- mice to maintain normal VLDL secretion may have been a result of adaptive mechanisms and redistribution of PC pools.

In the male Pemt+/- mice fed the HF/HC diet, either PC levels or the intracellular distribution of PC has become rate-limiting for the assembly and/or secretion of apoB100-containing VLDL. The results also imply that PEMT-derived PC accommodates the increased demand for PC in response to specific challenges such as an HF/HC diet. Elevated plasma LDL cholesterol, as a result of a high fat diet, is a risk factor for the development of cardiovascular disease in humans (41). Thus, our observation that the loss of PEMT-derived PC restricts VLDL secretion in response to an HF/HC diet may lead to a better understanding of how the liver responds to elevations in dietary fat and cholesterol.

PEMT and VLDL Secretion—The major phospholipid in VLDL, PC, is assembled into the outer surface of VLDL particles. Our laboratory has previously demonstrated that, when the CDP-choline pathway is attenuated in male rats by feeding a choline deficient diet, the amounts of apoB100, apoB48, PC, and TG in plasma VLDL are decreased by 40–60% (6). We attributed these defects in VLDL to a reduction in PC synthesis from CDP-choline and a consequent reduction in the amount of PC in the liver.

We have now deleted the PEMT pathway for PC synthesis in mice and have found a defect in VLDL secretion in male mice challenged with an HF/HC diet. Interestingly, in Pemt+/- mice the secretion of only apoB100, but not apoB48, was inhibited. Whereas when the CDP-choline pathway was inhibited in rats, the plasma levels of both apoB48 and apoB100 were reduced (6). These observations are consistent with experiments from several laboratories that show when neutral lipid supply (e.g. oleate, TG, or microsomal TG transfer protein) is limited, the secretion of apoB100 is inhibited to a greater extent than that of apoB48 (42–44). One possible explanation for the different dependence of apoB100 and apoB48 secretion on PC biosynthetic pathways is that a reduction in PC synthesis by either the PEMT or CDP-choline pathways inhibits the secretion of apoB100. Possibly, apoB48 secretion does not require the PEMT pathway but specifically requires PC made by the CDP-choline pathway. In addition, our data do not exclude the

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**A. A. Noga and D. E. Vance, unpublished observations.**
possibility that a deficiency of PC at a specific intracellular site in the livers of *Pemt<sup>−/−</sup>* mice is responsible for the reduced secretion of apoB100-containing VLDL even though the total mass of PC in the liver is not significantly decreased. This possibility seems unlikely, because the defect in *Pemt<sup>−/−</sup>* mice is seen only in males.

An alternative explanation, and the one that we favor, is that secretion of both apoB48 and apoB100 into VLDL by the liver is inhibited when PC in the liver decreases below a threshold level as a result of a deficiency of PC made by either pathway. The level of PC in the liver of rats was significantly (25%) reduced when they were fed a choline-deficient diet for 3 days (6), whereas the amount of PC in the livers of *Pemt<sup>−/−</sup>* mice was not significantly reduced when the Pemt pathway was eliminated. Our laboratory has previously established that the CDP-choline pathway for PC synthesis is increased in livers of *Pemt<sup>−/−</sup>* mice (12), presumably as compensation for the lack of PC made from Pemt. Thus, the deficit in PC in Pemt-deficient mice is probably not as severe as in rats fed the choline-deficient diet for 3 days. Other potential adaptive mechanisms by which *Pemt<sup>−/−</sup>* mice might compensate for the decrease in PC synthesis is by increasing the uptake of PC and/or lyso-PC from plasma and/or by decreasing PC degradation (45). We also speculate that during VLDL assembly more PC is required to assemble the VLDL and bile secretion. The experiments presented in this report illustrate the relative importance of PC for membrane integrity, lipoprotein secretion, and bile secretion in hepatocytes. Membrane phospholipid composition is tightly regulated (46, 47), a finding that is supported by our observation that PC synthesis from CDP-choline is increased in response to elimination of Pemt activity (12). One would predict that a supply of PC for maintaining cellular membrane integrity would be more important than providing PC for lipoprotein secretion. However, it is not clear why in male *Pemt<sup>−/−</sup>* mice the apparently limited supply of PC is used to maintain a normal secretion of PC into bile, whereas the secretion of TG in VLDL is compromised. Maintenance of normal bile secretion is extremely demanding on the PC pool in the liver. It has been estimated that in mice an amount of PC equivalent to the total mass of PC in liver is secreted into bile each day (13, 48). Bile is required for the normal uptake and digestion of dietary fat, an important source of energy for the body. On the other hand, hepatic VLDL secretion is a mechanism by which liver exports lipoprotein lipid for distribution of fat/energy among other tissues. Our data are consistent with the hypothesis that an efficient intake of an energy source from the diet, in a process involving bile, is more important to the mouse than lipoprotein secretion by the liver.

**CONCLUSION**

We generated *Pemt<sup>−/−</sup>* mice to determine the function of PEMT-derived PC in the liver. Because phospholipid metabolism is tightly regulated, and more than one pathway exists for production of all the major phospholipids including PC (49), it is particularly challenging to unravel the function of the Pemt pathway. The present study suggests that Pemt activity plays an important role in regulating the levels of plasma lipoproteins. One major finding is that in male mice Pemt-derived PC normally accommodates the increased need for PC in response to a challenge, such as an HF/HC diet. Under these "stressful" conditions, the requirement of PC for VLDL secretion cannot be met in *Pemt<sup>−/−</sup>* mice by the CDP-choline pathway alone. Whether or not the Pemt pathway specifically provides a pool of PC for VLDL assembly is an intriguing question that needs to be addressed. Equally important, the levels of plasma cholesterol and PC in HDL are also modulated in mice by Pemt activity in liver.

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