Insights into the requirement of phosphatidylcholine synthesis for liver function in mice

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Abstract Phosphatidylcholine (PC) is made in the liver by the CDP-choline pathway and via phosphatidylethanolamine *N*-methyltransferase (PEMT), which catalyzes the conversion of phosphatidylethanolamine to PC. Unexpectedly, hepatic apolipoprotein B-100 secretion is inhibited in male, but not female, *Pemt*$^{-/-}$ mice (Noga, A. A., Y. Zhao, and D. E. Vance. 2002. *J. Biol. Chem.* 277: 42358–42365; Noga, A. A., and D. E. Vance. 2003. *J. Biol. Chem.* 278: 21851–21859). To gain further insight into this process, we compared PC metabolism in male and female mice fed chow or a high-fat/high-cholesterol (HF/HC) diet. Immunoblot analyses demonstrated that twice as much PEMT2 was present in livers from female compared with male mice. In contrast, assays of CTP:phosphocholine cytidylyltransferase from livers of *Pemt*$^{+/+}$ mice demonstrated more active cytidylyltransferase in male than in female mice. Secretion of PEMT-derived PC into lipoproteins was examined in vivo by injection of mice with [methyl-^{3}H]methionine in the presence of Triton WR1339. The PEMT-derived PC shifts to smaller-sized particles in response to a HF/HC diet, but only in male mice. Secretion of PEMT-derived PC into bile was enhanced in mice fed a HF/HC diet. These results demonstrate that the synthesis and targeting of PC produced by the PEMT pathway in the livers of mice differs in a gender- and diet-specific manner.—Noga, A. A., and D. E. Vance. **Insights into the requirement of phosphatidylcholine synthesis for liver function in mice. *J. Lipid Res.* 2003.** 44: **1998–2005.**

Supplementary key words phosphatidylethanolamine *N*-methyltransferase • CTP:phosphocholine cytidylyltransferase • gender • diet

Phosphatidylcholine (PC) is the primary phospholipid of eukaryotic cellular membranes and has a crucial role in structural maintenance of the lipid bilayer. In mammals, PC is also the predominant phospholipid in bile, lung surfactant, and plasma lipoproteins, and plays a critical role as a second messenger in signal transduction (1, 2). In all mammalian tissues, PC is made via the Kennedy pathway (CDP-choline pathway), and the activity of CTP:phosphocholine cytidylyltransferase (CT) usually regulates the flux through this pathway (2). In addition, phosphatidylethanolamine *N*-methyltransferase (PEMT) catalyzes the conversion of phosphatidylethanolamine (PE) to PC and is a liver-specific alternative route for PC synthesis (3). Because choline is normally available in mammalian diets, it was not well understood why PEMT activity survived evolutionary selection or why its expression is limited to the liver. Experiments with *Pemt*$^{-/-}$ mice indicated that PEMT survived during evolution to provide PC when dietary choline was limited, such as during starvation (4, 5).

Although the *Pemt*$^{-/-}$ mice appear normal, recent studies with these mice indicate that PEMT contributes to plasma lipoprotein levels in a gender- and diet-specific fashion (6). When *Pemt*$^{-/-}$ mice were fed a high-fat/high-cholesterol (HF/HC) diet, a significant decrease in the secretory rate of apolipoprotein B-100 (apoB-100)-containing VLDL was observed in the male mice (6). The female *Pemt*$^{-/-}$ mice maintained normal VLDL secretion, regardless of diet. We have also observed that hepatocytes from male *Pemt*$^{-/-}$ mice have a defect in the secretion of apoB-100-containing lipoproteins (7), consistent with lower levels of plasma apoB-100 lipoproteins in male *Pemt*$^{-/-}$ mice. Combined, these results suggest that PEMT-derived PC is specifically targeted to, and important for, VLDL assembly in males but not females. In addition, the amounts of plasma PC and cholesterol in HDL were decreased in *Pemt*$^{-/-}$ mice of both genders fed a HF/HC diet (6). This decrease was more pronounced in female (30–40%) than male (20%) mice. Thus, it appears that female mice rely more on the PEMT pathway than do male mice for maintaining PC levels in plasma.

Because of these gender- and diet-specific differences, we initiated studies on the mechanism of these changes. We show that more CT activity is associated with mem-

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Abbreviations: CT, CTP:phosphocholine cytidylyltransferase; ER, endoplasmic reticulum; HF/HC, high-fat/high-cholesterol; MAM, mitochondria-associated membranes; PC, phosphatidylcholine; PEMT, phosphatidylethanolamine *N*-methyltransferase.

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branes (active form of the enzyme) of male than of female mice. We also present data that suggests a gender-specific difference in the intracellular distribution of PEMT. To examine the role of PEMT in VLDL and bile secretion, we performed in vivo radiolabeling studies to determine the amount and distribution of PEMT-derived PC in plasma lipoproteins and bile as a function of gender and diet. In contrast to our expectations, PEMT-derived PC is not specifically targeted for VLDL secretion in male mice fed an HF/HC diet. The HF/HC diet enhanced the utilization of PEMT-derived PC for secretion into bile.

MATERIALS AND METHODS

Materials

The semipurified diet (catalog # 84712) was obtained from Teklad (Madison, WI). This diet contained (g/kg): casein, 337.5; dextrose, 260.6; cornstarch, 250; cellulose, 62.5; L-methionine, 3.125; inositol, 7.8; choline chloride, 1.72; Bernhart-Tomarelli mineral mix, 63.6; and vitamin mix, 12.5. Silica gel G60 plates for TLC were from Merck. Lysophosphatidylcholine and sphingomyelin (SM) standards were purchased from Sigma. PC and the phosphatidylmonomethylethanolamine substrate for PEMT assays were obtained from Avanti Polar Lipids (Alabaster, AL). *S*-[methyl-^{3}H]adenosylmethionine, [methyl-^{3}H]methionine, and [methyl-^{3}H]choline were purchased from Amersham-Pharmacia. The polyclonal antibody directed against the C terminal dodecapeptide of rat PEMT2 was raised in rabbits in our laboratory (8). Goat anti-rabbit IgG conjugated to horseradish peroxidase was purchased from Pierce, and the enhanced chemiluminescence detection system was from Amersham-Pharmacia. *S*-adenosylmethionine and Triton WR1339 were purchased from Sigma. All other chemicals and reagents were purchased from standard commercial sources.

Care and feeding of mice

The *Pemt*$^{-/-}$ and *Pemt*$^{+/+}$ mouse colony had a mixed genetic background of 129/J and C57BL/6 and was maintained via homozygous breeding in a reversed 12 h light/dark cycle (4). At the age of 12–14 weeks, the animals were fed ad libitum either a control diet of regular rodent chow (LabDiet, PICO Lab Rodent Diet 20) or a HF/HC diet (9) for 3 weeks, after which the animals were fasted overnight and sacrificed. The HF/HC diet consisted of 80% (w/w) semi-purified diet noted in Materials, 19% (w/w) olive oil, 1% (w/w) linseed oil (a source of essential fatty acids), and 1% (w/w) cholesterol.

Enzymatic assays

Following extirpation, livers were rinsed with PBS then homogenized in a glass-Teflon homogenizer in 3 ml of buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.025% sodium azide] followed by sonication for 20 s. Protein concentration was determined using the Coomassie Plus protein protocol from Bio-Rad that is based on the Bradford assay (10). Bovine serum albumin was used as a standard for all protein assays. PEMT activity was measured in liver homogenates as previously described (11). For some enzymatic assays, total membranes were isolated by centrifugation of the liver homogenates at 600 *g* for 10 min to pellet unbroken cells and nuclei. The supernatant was centrifuged at 100,000 *g* for 1 h. The membrane pellet, containing membrane-associated CT, was resuspended in the above homogenizing buffer, and the supernatant contained the soluble fraction. CT activity was measured in the homogenate, as well as in soluble and microsomal fractions in the presence of PC-oleate vesicles, as previously described (12).

Immunoblotting of PEMT

A sample of liver homogenate (1 mg/ml) was boiled for 5 min in Laemmli buffer (13), and proteins were resolved on a 12% SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes and probed with anti-PEMT2 antibody (8) followed by goat anti-rabbit IgG conjugated to horseradish peroxidase. Immunoreactive bands were visualized by enhanced chemiluminescence according to the manufacturer's instructions.

In vivo radiolabeling of PEMT-derived PC

Male and female mice were fed HF/HC or chow diets for 3 weeks. Subsequently, the animals were fasted overnight, then 200 μl of PBS containing 10% Triton WR1339 (v/v) and 100 μCi [methyl-^{3}H]methionine (5 mCi/ml) was injected into the tail vein. The animals were sacrificed after 2.5 h or 5 h and blood, liver, and bile were collected. Blood was collected via the lower vena cava in the presence of trace amounts of 250 mM EDTA, and plasma was isolated by centrifugation. Two separate 200 μl samples of plasma from each animal were separated by ultracentrifugation into 10 fractions according to density. Briefly, each 200 μl sample was mixed with 1.3 ml of 4.15 M KBr and loaded at the bottom of a 5 ml Quick-Seal tube. Each sample was overlaid to the top with 0.9% NaCl and centrifuged at 416,000 *g* for 1 h in a Beckman VTi 65.2 rotor (14). Lipids were extracted using a modified Bligh-Dyer protocol (15). The phospholipids from each fraction were separated by TLC with a developing solvent of chloroform-methanol-acetic acid-water (25:15:4:2; v/v/v/v). Lipids were visualized with iodine vapor, and the bands corresponding to standard lipids were scraped. From one set of samples, phospholipid mass was analyzed using the Malachite Green lipid phosphorus assay (16). Radioactivity was measured in phospholipids of the second set of samples. Total plasma phospholipid mass and radiolabel were determined as a summation of the values obtained from the 10 density fractions.

Lipids were extracted from 8 μl of bile, and the phospholipids were separated by TLC and analyzed as described above. Livers were homogenized as described above, and lipids were extracted and separated via TLC. Phospholipid mass and the incorporation of radiolabel were determined as described for the plasma samples.

RESULTS

Characterization of PEMT activity and subcellular distribution in livers

We first determined whether PEMT activity was dependent upon gender and diet. In vitro PEMT activity assays were performed with liver homogenates from the *Pemt*$^{+/+}$ mice fed either chow or a HF/HC diet for 3 weeks. The specific activity of PEMT was the same in livers of male and female mice and was independent of the diet (**Fig. 1A**). The lack of a difference in in vitro PEMT activity between males and females (Fig. 1A) suggests that total amount of PEMT protein does not depend on gender.

It is well established that in rats, PEMT activity is distributed between two types of subcellular membranes, the endoplasmic reticulum (ER) and the mitochondria-associated membranes (MAM) (8). MAM are ER-like membranes that coisolate with mitochondria (17). An anti-peptide an-

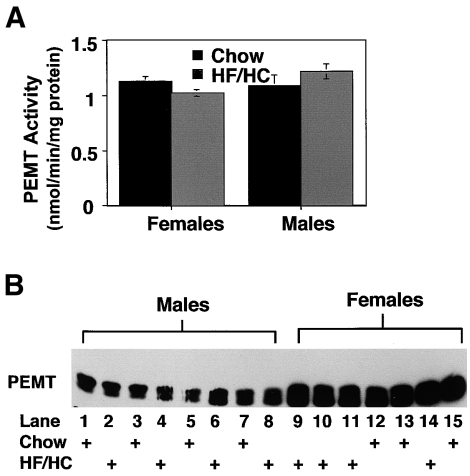

Fig. 1. Amount of phosphatidylethanolamine *N*-methyltransferase (PEMT) activity and protein in livers of mice as a function of diet and gender. Mice (12 to 16 weeks old) were fed either chow or a high-fat/high-cholesterol (HF/HC) diet for 3 weeks. A: Liver homogenates were assayed for PEMT activity. Shown are means ± SEM from at least four separate animals in each experimental group. B: Immunoblotting of liver homogenates of female and male mice. Each lane contained 35 μg of protein. Lanes 1, 3, 5, 7, 12, 13, and 15 are from chow-fed animals, whereas lanes 2, 4, 6, 8, 9–11, and 14 are from mice fed the HF/HC diet.

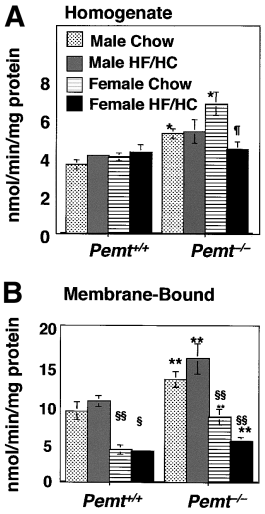

Fig. 2. CTP:phosphocholine cytidylyltransferase (CT) activity depends on *Pemt* genotype and gender. A: Liver homogenates from *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice fed either chow or a HF/HC diet were assayed for CT activity in the presence of phosphatidylcholine (PC)/oleate vesicles. Values are expressed as means ± SEM (n = 3 to 5 mice). Based on a Student's *t*-test, * $P < 0.01$ for *Pemt*$^{+/+}$ mice compared with *Pemt*$^{-/-}$ animals of identical gender and diet. ¶ $P < 0.05$ where *Pemt*$^{-/-}$ chow and HF/HC females are compared. B: Microsomes and cytosol were separated by ultracentrifugation and CT assays were performed. Data are expressed as means ± SEM. ** $P < 0.05$ for comparison of *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice. § $P < 0.01$ and §§ $P < 0.05$ represents a Student's *t*-test comparing females to their male counterparts.

tibody raised against the C-terminal 12 amino acids of rat PEMT recognizes only PEMT protein in the MAM and not in the bulk of the ER (8). Ironically, the majority of PEMT activity is located in the ER. Thus, two forms of PEMT exist that are encoded by a single gene (4). Immunoblotting of PEMT was performed using liver homogenates of female and male *Pemt*$^{+/+}$ mice, using the antibody raised against the C terminus of PEMT (8). Unexpectedly, immunoreactivity to PEMT2 in livers from the female mice was approximately double that of the males (Fig. 1B). These results imply that although total PEMT protein is the same between the genders, more PEMT activity is distributed to the MAM in female mice.

The subcellular distribution of CT activity depends on gender and PEMT genotype

We were curious as to why the CDP-choline pathway does not compensate for a defect in VLDL secretion in the HF/HC-fed male *Pemt*$^{-/-}$ mice (6). **Figure 2A** shows that in both male and female mice fed chow, the total amount of CT activity in the liver homogenate was higher in *Pemt*$^{-/-}$ mice than in the corresponding *Pemt*$^{+/+}$ mice. In female *Pemt*$^{-/-}$ mice, total CT activity was significantly less when the mice were fed the HF/HC diet than when fed chow (Fig. 2A).

The active form of CT in a cell is considered to be associated with membranes, whereas the soluble form of CT is thought to consist of an inactive reservoir (1, 2). Livers of *Pemt*$^{-/-}$ mice had significantly more CT activity associated with total membranes than did livers of *Pemt*$^{+/+}$ mice (Fig. 2B). This result concurs with previous observations (4), and supports the view that *Pemt*$^{-/-}$ mice attempt to compensate for the loss of PEMT activity by up-regulating PC production via the CDP-choline pathway. Surprisingly, however, the specific activity of membrane-bound CT in the liver was 1.5- to 3-fold higher in male than in female mice (Fig. 2B). This result suggests that livers of male mice are more dependent on the Kennedy pathway than are those of the female mice, because a higher percentage of their total CT protein is membrane bound. The specific activity of CT in the cytosolic fraction was similar in male and female mice but was much lower than that associated with membranes (data not shown).

Pilot study of in vivo labeling of plasma PC with [methyl-^{3}H]methionine in the presence of Triton WR1339

The data presented in Figs. 1 and 2 suggest that the activity/subcellular location of key enzymes in the CDP-choline and PEMT pathways for PC biosynthesis depend on gender. Because it appears that the livers of male mice are more dependent on the CDP-choline pathway, we hypothesized that females would be more dependent on PEMT-derived PC. In addition, we have previously shown that male *Pemt*$^{-/-}$ mice fed a HF/HC diet secrete 50% fewer apoB-100-containing lipoproteins compared with male *Pemt*$^{+/+}$ mice fed the same diet (6). Thus, we hypothesized that PEMT-derived PC might be specifically enriched in secreted VLDL of male mice. In order to address both of these hypotheses, we compared the in vivo synthesis and secretion of PEMT-derived PC in male and female mice.

We chose to label the PEMT-derived PC in vivo with [methyl-^{3}H]methionine. However, VLDL secreted by the

liver is rapidly catabolized in mice, and therefore any measurements of PEMT-derived PC in this lipoprotein fraction would be inaccurate. Triton WR1339 inhibits lipoprotein lipase and is commonly used to study the rate of VLDL secretion in vivo (18). However, because this method has not previously been used to examine PC metabolism, it was necessary to perform a pilot study in which the tail veins of four adult C57BL/6 male mice were injected with 200 μl PBS containing 100 μCi [methyl-^{3}H]methionine in the presence or absence of 10% (v/v) Triton WR1339 (19). The animals were sacrificed 2.5 h and 5 h later, and the plasma was fractionated on the basis of density by ultracentrifugation. PC was isolated from each fraction. As expected, Triton WR1339 inhibited the catabolism of VLDL as reflected by an enrichment of radiolabeled PEMT-derived PC in the lower-density fractions (fractions of density 1.0 to 1.025 g/ml) (**Fig. 3**). In the absence of Triton WR1339, radiolabeled PC was not detectable in the VLDL fraction, but was predominant in the higher density fractions (1.075–1.2 g/ml). The 5 h time point was chosen for subsequent experiments, because the radiolabel in all the choline-derived phospholipids [data for lyso-PC (LPC) and SM are not shown] was higher at this time point than after 2.5 h.

Targeting of PEMT-derived PC in the liver as a function of diet and gender

Using the Triton experimental protocol, we compared the in vivo labeling of PC from the PEMT pathway in *Pemt*$^{+/+}$ mice on the basis of gender and diet. *Pemt*$^{+/+}$ mice were chosen for this study, because it was in these animals that we had previously made our observations concerning lipoprotein secretion (6). Each animal was fed chow or the HF/HC diet for 3 weeks and then injected with [methyl-^{3}H]methionine and Triton WR1339 as described above. Liver, bile, and plasma were isolated from all the animals.

First, the livers were examined for any differences in the mass or radiolabeling of choline-containing phospholipids among the experimental groups. No significant differences were observed with respect to the mass of PC, LPC, or SM in any of the livers (**Fig. 4A**). However, the amount of [methyl-^{3}H]methionine-labeled PC was consistently higher in the female chow-fed animals than in female HF/HC-fed animals (Fig. 4B). These data indicate that the HF/HC diet either decreased the biosynthesis of PEMT-derived PC or enhanced its removal from the liver.

Gender also contributed to small differences in labeling of hepatic PEMT-derived PC in the livers; the amount of [methyl-^{3}H]methionine-labeled PC was significantly higher in female mice fed the HF/HC diet compared with the males fed the same diet (Fig. 4B). [Methyl-^{3}H]methionine-labeled PC accumulated more quickly in the females compared with males, and in chow-fed animals over HF/HC-fed animals. As a result of these trends, the specific activity of PC in the livers of male HF/HC-fed animals was significantly lower compared with the chow-fed female mice (Fig. 4C).

Radiolabeled SM and LPC were also analyzed to determine whether or not there were differences in the conversion of PEMT-derived PC to either of these phospholipids in the liver on the basis of diet or gender. No significant differences were observed.

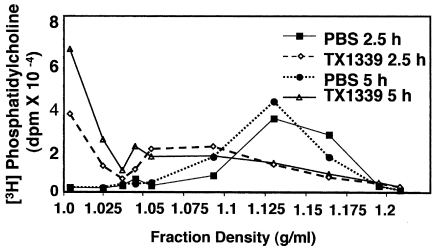

Fig. 3. Pilot study for optimization of conditions for examining PC secretion in vivo. Four adult male C57Bl/6 mice were fasted overnight and then injected with 200 μl of PBS containing [^{3}H]methionine (100 μCi) with or without 10% (v/v) Triton WR1339. Two mice were sacrificed after 2.5 h and two after 5 h, and plasma was separated according to density by ultracentrifugation. Lipids were extracted and resolved via TLC, and radioactivity in PC was measured.

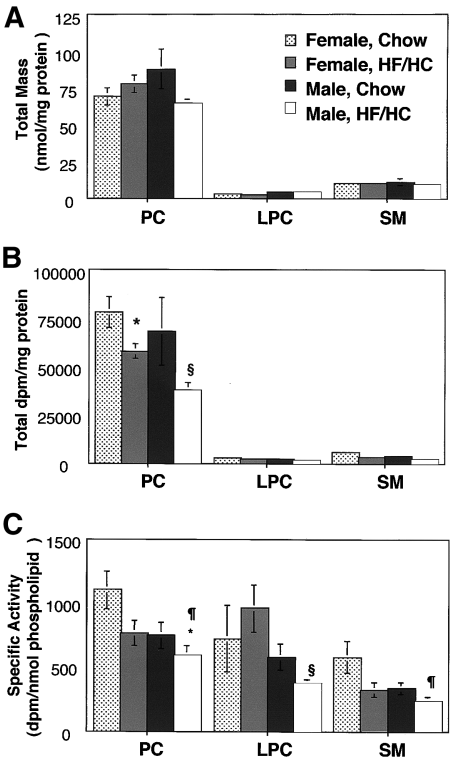

Fig. 4. [^{3}H-CH_3]methionine incorporation into PEMT-derived phospholipids in the liver depends on gender and diet. Female and male mice were fed either a HF/HC or chow diet for 3 weeks then injected with Triton WR1339 and [^{3}H-CH_3]methionine (100 μCi). Four to five animals for each gender and diet were sacrificed after 5 h, the livers were homogenized, and lipids were extracted and separated by TLC. A: Phospholipid mass was determined using a Malachite Green lipid phosphorus assay. B: Radioactivity in PEMT-derived PC, lyso-PC (LPC), and sphingomyelin (SM). Data are expressed as means ± SEM. * $P < 0.05$ for comparison of the HF/HC and chow diet in female mice. § $P < 0.01$ for males versus females fed the HF/HC diet. C: The values in B were divided by the values in A. * $P = 0.05$ compared to males fed HF/HC to those fed chow; § compares the effect of gender between HF/HC-fed mice; and ¶ compares female chow-fed to male HF/HC-fed animals.

PC targeting to bile in response to diet and gender

The amount of PC per microliter of bile was not statistically different between HF/HC- and chow-fed mice (**Fig. 5A**). However, the amount of PEMT-derived PC secreted into bile (Fig. 5B) was significantly increased in mice fed a HF/HC diet compared with chow-fed mice. These results are the opposite of the observations made in the liver, where labeling of PEMT-derived PC in the liver was less when the mice were fed the HF/HC diet than when fed chow (Fig. 4B). Therefore, the HF/HC diet appears to divert hepatic PC for secretion into bile, including PEMT-derived PC. The diversion of PEMT-derived PC into bile for females was significantly higher than males on a HF/HC diet (Fig. 5B).

PC is the predominant phospholipid secreted into bile, while SM and LPC are minor components. Nevertheless, we wished to confirm that neither gender nor diet resulted in PEMT-derived PC being preferentially targeted into these phospholipid pools. Levels of radiolabeled LPC and SM were very low, and no significant differences were observed with regard to diet or sex (Fig. 5).

Targeting of PEMT-derived PC to lipoproteins as a function of diet and gender

The distribution of mass and radiolabeled PC, LPC, and SM in plasma lipoproteins was examined. In the male mice fed a HF/HC diet, data for both PC mass (**Fig. 6A**) and for [methyl-^{3}H]methionine (Fig. 6B) demonstrated a shift from the VLDL fraction (density: 1.009 g/ml) to the HDL-density fractions (densities 1.067–1.118 g/ml). For chow- and HF/HC-fed females, as well as for chow-fed males, 45% of the total PC mass was in the VLDL fraction (Fig. 6A). In the male HF/HC-fed mice, this value was diminished to 25%. Similar decreases in the percent distribution of [^{3}H]PC was also observed in the VLDL fractions of these animals (Fig. 6B). Because the distribution of radiolabeled PC resembled the pattern for total PC, no changes in specific activity were observed in the lipoproteins for any of the mice (data not shown). Secretion of radiolabeled LPC and SM into VLDL was significantly higher in female chow-fed mice than in all the other groups (**Fig. 7A**, **B**).

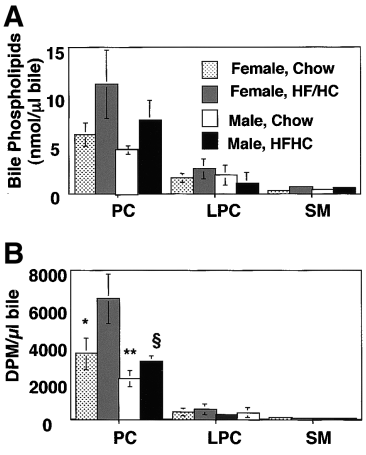

Fig. 5. Incorporation of [^{3}H-CH_3]methionine into PEMT-derived PC secreted into bile. Four to five animals for each gender and diet were used. A: Mass of phospholipids secreted into bile. B: Incorporation of [^{3}H]methionine into PEMT-derived phospholipids. Data are means ± SEM. * $P = 0.051$ and ** $P < 0.05$ for comparison of mice of the same gender fed chow versus the HF/HC diet. § $P = 0.05$ for comparison of female and male mice.

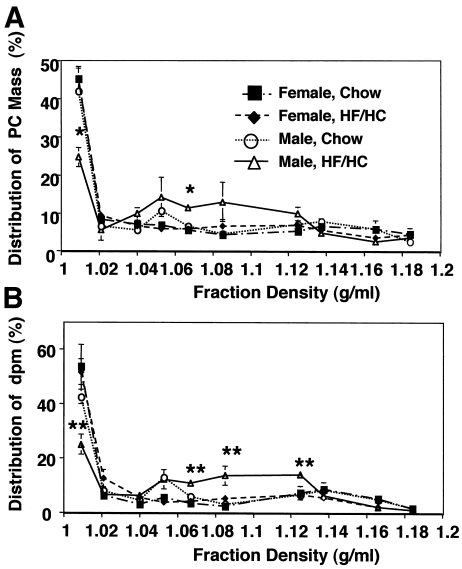

Fig. 6. Incorporation of [^{3}H-CH_3]methionine into PEMT-derived PC is decreased in plasma VLDL in male HF/HC-fed mice. Two 200 µl samples of plasma from each animal injected with [^{3}H]methionine and Triton WR1339 were separated into density fractions. One sample was used for phospholipid mass measurements and the other for radiolabel measurements. Four to five animals for each gender and diet were used. A: Distribution of PC mass was determined by calculating the percent of the total PC mass in each density fraction. Data are displayed as the mean of values ± SEM. * $P < 0.01$ where the male HF/HC-fed animals were compared with all the other study groups (ANOVA). B: Distribution of [^{3}H]PC was determined by calculating the percent of the total radiolabel in each density fraction. Values are expressed as mean percentage ± SEM. ** $P < 0.05$ where the male HF/HC-fed animals were compared with all the other study groups via ANOVA. Densities represent the following fractions: 1.010 g/ml VLDL, 1.020–1.060 g/ml LDL, and 1.061–1.185 g/ml HDL.

The amount of PEMT-derived PC recovered in the liver, bile, and plasma

The above results strongly support the hypothesis that there is an increased amount of PEMT-derived PC utilized for bile secretion in mice fed a HF/HC diet. We have also addressed this hypothesis by calculating the total amount of PEMT-derived PC that is recovered in the liver, bile, and plasma (**Table 1**). The results show that at 5 h after injection of labeled methionine, of the label recovered, ~90% of the PC made via the PEMT pathway is found in the liver. Moreover, there is twice the percent of PEMT-derived PC in the bile of HF/HC compared with chow-fed mice that agrees well with the dpm of PC per microliter of bile (Fig. 5B). The distribution of [methyl-^{3}H]methionine-labeled PC to plasma does not appear to be influenced by either diet or gender. This agrees with the finding that the amount of labeled PC per 100 µl of plasma

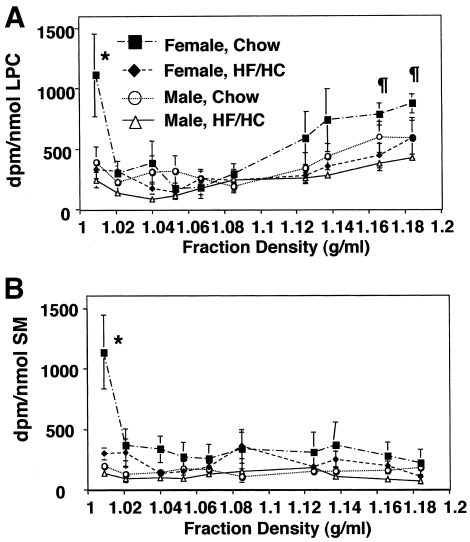

Fig. 7. PEMT-derived LPC and SM are targeted for VLDL secretion in female chow-fed animals. For the experiments described in the legend of Fig. 6, the specific activity for each density fraction was determined by dividing the ^{3}H dpm values by total phospholipid mass for LPC (A) and SM (B). Significance of differences was tested by comparing the female chow values to numbers attained from all other study groups (ANOVA, * $P < 0.05$). ¶ $P < 0.01$ as determined by a Student's *t*-test comparing female chow-fed mice to male HF/HC-fed animals.

was not affected by either diet or gender (data not shown). As observed in Fig. 3, there is no accumulation of label in the HDL fraction in the presence of Triton WR3119. The lower levels of radioactivity in the HDL fraction in the presence of Triton WR3119 compared with no detergent could be due to decreased conversion of VLDL PC into the HDL fraction or increased metabolism of HDL stimulated by the detergent. Thus, the calculations in Table 1 could underestimate the amount of labeled PC that was contributed to the plasma compartment. Similarly, the recovery of labeled PC in bile could also be an underestimate because of secretion of bile into the intestine.

DISCUSSION

We have determined whether diet and/or gender alter PC metabolism in livers of mice. This interest was prompted by observations on lipoprotein metabolism in *Pemt*$^{-/-}$ mice (6) and on lipoprotein secretion from hepatocytes of these mice (7).

PEMT activity

The specific activity of PEMT in the livers of mice was identical regardless of diet and gender. This result suggests that the total PEMT protein in the livers of all the animals is the same. Nonetheless, immunoblotting using an anti-PEMT antibody that recognizes only PEMT2 protein [previously identified as being localized to the MAM (8, 20)] implied that female mice contained at least twice as much PEMT2 protein as did male mice. Our lab developed a method to separate these two fractions using a DEAE-Seph-

TABLE 1. Estimation of total radioactivity recovered in phosphatidylcholine from livers, bile, and plasma from mice 5 h after injection into the tail vein of 100 μCi [methyl-^{3}H]methionine

			dpm × 10^{-4}		
Sex	Diet	Liver Weight	Liver[a]	Bile[b]	Plasma[c]
		g			
Female	chow	0.82 ± 0.02	1,150 (90%)	40 (3%)	90 (7%)
Female	HF/HC	0.9 ± 0.04	945 (87.5%)	70 (6.5%)	65 (6%)
Male	chow	0.97 ± 0.05	1,190 (93%)	25 (2%)	70 (5%)
Male	HF/HC	1.01 ± 0.06	710 (88%)	35 (4%)	58 (7%)

HF/HC, high fat/high cholesterol.

[a] It is estimated that 1 g of liver has 175 mg protein. The total dpm of labeled phosphatidylcholine (PC)/liver was calculated from the dpm values presented in Fig. 4B.

[b] The amount of bile secreted into the bile duct during the 5 h of the experiment is estimated to be 100 μl. This value was multiplied by the dpm in PC per microliter of bile as shown in Fig. 5B.

[c] The amount of plasma in a 25 g mouse is estimated at 1 ml. Because Triton WR1339 was injected with labeled methionine, much of the secreted PC is expected to still be in the plasma compartment. Hence the dpm in PC in 100 μl of plasma was measured and multiplied by 10 to give the values presented.

arose column (unpublished observations). From this separation, we know that the MAM-associated PEMT2 is more positively charged than the ER-localized PEMT1. A number of potential phosphorylation sites exist in the PEMT sequence, one of which is within the last 12 residues. However, it has not been demonstrated that PEMT is phosphorylated in vivo (21). We have not identified consensus sites for any other forms of posttranslational modification of PEMT. Northern blotting of murine PEMT mRNA revealed a single band, implying that the difference between the two forms of PEMT is likely not due to alternative splicing (22).

CT activity and distribution

The predominant pathway for PC synthesis in the liver is the de novo Kennedy pathway (23–25). In this study, we investigated the activity of the rate-limiting enzyme, CT, in murine liver. Our data showed that total CT activity was modestly elevated in *Pemt*$^{-/-}$ mice compared with *Pemt*$^{+/+}$ mice. It is noteworthy, however, that this increase in CT was seen only in mice fed chow, but not in mice fed the HF/HC diet. The specific activity of CT on cellular membranes was also increased in the *Pemt*$^{-/-}$ mice, as previously demonstrated (4). We hypothesize that these animals are attempting to compensate for the loss of PEMT activity, because PEMT is an important contributor to PC in the liver.

The purpose of the HF/HC diet was to challenge hepatic PC levels by increasing its secretion into both bile and lipoprotein particles. However, neither total CT nor membrane-bound CT activity was enhanced in response to this increased demand for PC production (Fig. 2). It is conceivable that increased consumption of fatty acids from the diet might have increased the requirement for CT activity (1, 2, 9).

In our previous study, plasma lipoprotein levels were decreased in the *Pemt*$^{-/-}$ mice fed a HF/HC diet (6). From this study, it appears that the Kennedy pathway is unable to compensate for the loss of PEMT activity with the challenge of the HF/HC diet. Therefore, the decrease in

plasma PC observed in all *Pemt*$^{-/-}$ mice on the HF/HC diet was likely due to insufficient PC production. This also suggests that PEMT activity was necessary for producing the excess PC required when the mice were placed on a HF/HC diet. Previous experimentation on rat hepatocytes demonstrated that the levels of PEMT protein are in excess of PE substrate (26). If this were the case in mice as well, it would not be necessary to increase protein levels of PEMT in response to the HF/HC diet.

When the liver homogenate was separated into fractions containing membranes and cytosol, we found that the membrane-bound CT activity was 1.5- to 3-fold higher in male mice compared with females (Fig. 2B). These results in mice agree with previous studies in male rats that were shown to rely more heavily than female rats on the Kennedy pathway for production of hepatic PC (27–30). While it is unclear which CT isoform is responsible for the higher activity of CT, we presume it to be CTα, which is the most highly expressed isoform in hepatic tissue (31). Because the active form of CT resides on membranes, we conclude that the activity of CT in the liver is higher in male mice than in female mice. The activity of CT in the soluble/cytosolic fraction of the livers was not markedly different between *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice, nor was this activity markedly altered by diet or gender. These results might have been complicated by the CTα isoform being present in the nucleus as well as on the nuclear membrane (32). A previous study has shown that the CTα protein shuttles among the nucleus, ER, and cytosol (33). Unbroken nuclei are lost during processing of the livers, so some CT activity in the nucleus was likely lost. Difficulty in interpreting measurements of CT activity in the soluble fraction of cells has been encountered in other studies (34).

In vivo targeting of PEMT-derived PC to bile

To understand if gender and/or diet played any role in the production of PC for bile, we radiolabeled PEMT-derived PC with [methyl-^{3}H]methionine in vivo. When mice were fed the HF/HC diet, the level of PEMT-derived PC in the livers of both male and female mice was less than in chow-fed mice. Our data suggest that this alteration was due to an increased secretion of PEMT-derived PC and PC mass from the liver into the bile of the HF/HC-fed mice. Moreover, male mice secreted less biliary [^{3}H]PC than did the females. Thus, it appears that the HF/HC diet increases the demand on PC for bile secretion, resulting in movement of PEMT-derived PC from the liver to the bile. Because the specific activity of PEMT-derived PC in the bile was approximately the same as in the liver, it appears that PEMT-derived PC is not specifically targeted for bile secretion. These results are consistent with those of a previous study aimed at determining which of the two PC biosynthetic pathways was responsible for contributing PC for bile secretion (35). The results indicated that, although mice depend more on the CDP-choline than on the PEMT pathway for secretion of PC into bile, the fatty acid composition of the secreted PC was similar irrespective of the source of PC.

In vivo targeting of PEMT-derived PC to the plasma

Total PC as well as PEMT-derived PC in plasma lipoproteins could be influenced by a combination of gender and diet. Earlier studies with *Pemt*$^{-/-}$ mice showed that VLDL secretion was impaired in males fed a HF/HC diet (6, 7). One conclusion from these results would be that male mice rely on PEMT-derived PC for VLDL secretion when challenged with a HF/HC diet. The present results do not support that conclusion and demonstrate that the total amount of PEMT-derived PC recovered in the plasma does not appear to be influenced by the HF/HC diet or gender. However, it is clear that the HF/HC diet causes both total and PEMT-derived PC to be decreased in the VLDL fraction and increased in higher-density fractions. The mechanism for this change in distribution is unknown. Perhaps male mice rely more on smaller apoB-48-containing particles for secretion of neutral lipid from the liver under the HF/HC conditions compared with the other dietary and gender conditions. Using smaller particles to remove hepatic TG would be more demanding on PC levels, because more lipoprotein surface area would be required for the secretion of smaller quantities of core neutral lipids. Thus, less PC might be available for VLDL assembly and secretion. In agreement with this hypothesis, the HF/HC-fed male mice had the highest level of PC mass in their plasma (data not shown).

Interestingly, [methyl-^{3}H]methionine-labeled LPC and SM were enriched for VLDL secretion in female chow-fed animals (Fig. 7). Specific activity for these phospholipids in the plasma decreased in response to both the HF/HC diet as well as the male gender. These results support our hypothesis that gender and diet differences can alter either the synthesis or the fate of PEMT-derived PC in mice.

In mice, HDL is the predominant plasma lipoprotein and constitutes the majority of plasma PC. It is not yet known how much HDL-derived PC comes from the liver; however, our previous study also indicated that HDL levels are influenced in mice by diet, gender, and the presence or absence of PEMT (6). The objective of this study was to examine the distribution of PEMT-derived PC to apoB-containing lipoproteins, but the potential that PEMT-derived PC is also utilized for HDL does exist.

Previously, we estimated that a mouse secretes the equivalent of its entire pool of hepatic PC into bile in a 24 h period (5). Because of the metabolism of PC in the plasma, it has not been possible to estimate how much hepatic PC is secreted into the plasma. Nevertheless, the results in Table 1 suggest that, at least for PEMT-derived PC, significantly more PC is secreted into the plasma than into bile. Thus, the demand on hepatic PC for plasma lipoproteins and for bile is enormous.

Conclusions

Based on our observations, we propose the following mechanistic model of PC metabolism as a function of both diet and gender. Male mice have more hepatic CT activity distributed to the active, membrane-bound fraction than do females, and therefore male livers are more dependent on the Kennedy pathway for PC synthesis. Al-

though the Kennedy pathway is still the predominant pathway for PC synthesis in female mice, they produce more PC via the PEMT pathway than do male mice. With the challenge of a HF/HC diet, enhanced PC production is required by the liver for increased production of PC associated with bile and lipoproteins. Because CT activity is not influenced by the HF/HC diet, the data suggest that the PEMT pathway provides the necessary PC under these conditions. If the amount of PEMT protein were in excess, it would be unnecessary to increase the levels of the protein.

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