Phosphatidylethanolamine *N*-methyltransferase (PEMT) knockout mice have hepatic steatosis and abnormal hepatic choline metabolite concentrations despite ingesting a recommended dietary intake of choline

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Choline is an essential nutrient for humans and is derived from the diet as well as from *de novo* synthesis involving methylation of phosphatidylethanolamine catalysed by the enzyme phosphatidylethanolamine *N*-methyltransferase (PEMT). This is the only known pathway that produces new choline molecules. We used mice with a disrupted *Pemt-2* gene (which encodes PEMT; $Pemt^{-/-}$) that have previously been shown to possess no hepatic PEMT enzyme. Male, female and pregnant $Pemt^{-/-}$ and wild-type mice ($n = 5$–6 per diet group) were fed diets of different choline content (deficient, control, and supplemented). Livers were collected and analysed for choline metabolites, steatosis, and apoptotic [terminal deoxynucleotidyl transferase-mediated dUTP–biotin nick end-labelling (TUNEL)] positive cells. We found that, in livers of $Pemt^{-/-}$ mice fed any of the diets, there was hepatic steatosis and significantly higher occurrence of TUNEL positive cells compared with wild-type controls. In male, female and pregnant mice, liver phosphatidylcholine concentrations were significantly decreased in $Pemt^{-/-}$ choline deficient and in $Pemt^{-/-}$ choline control groups but returned to normal in $Pemt^{-/-}$ choline supplemented groups. Phosphocholine concentrations in liver were significantly diminished in knockout mice even when choline was supplemented to above dietary requirements. These results show that PEMT normally supplies a significant portion of the daily choline requirement in the mouse and, when this pathway is knocked out, mice are unable to attain normal concentrations of all choline metabolites even with a supplemental source of dietary choline.

Key words: betaine, choline deficiency, liver steatosis, phosphatidylcholine, phosphocholine.

INTRODUCTION

Choline or its derivatives are important for the structural integrity of cell membranes, methyl metabolism, cholinergic neurotransmission, and transmembrane signalling as well as for lipid–cholesterol transport and metabolism [1]. Choline was classified as an essential nutrient for humans [2] and is a dietary component that is crucial for normal function of all cells [1]. The daily requirement for dietary choline is moderated by the capacity to form phosphatidylcholine (PtdCho) via the methylation of phosphatidylethanolamine using S-adenosylmethionine (AdoMet) catalysed by phosphatidylethanolamine *N*-methyltransferase (PEMT; EC 2.1.1.17) [1]. Other important metabolites of choline include acetylcholine, phosphocholine (PCho), glycerophosphocholine (GPCho), and betaine.

PtdCho is a significant component of biomembranes and is required for the formation of the very-low-density lipoprotein (VLDL) particle, which is responsible for delivering the triacylglycerol produced by liver to other tissues [3–5]. It is believed that most PtdCho is formed by the cytidine diphosphocholine (CDP-choline) pathway (Scheme 1), with the alternative pathway (PEMT) contributing approximately a third of total PtdCho synthesis [6]. The latter is the only *de novo* pathway for choline biosynthesis and the PEMT activity is most active in the liver and kidney of mammals [6,7]. These two pathways produce different profiles of PtdCho species: the CDP-choline pathway forms mainly species containing medium chain, saturated fatty acids while the PEMT pathway forms PtdCho containing significantly more long chain polyunsaturated fatty acids [8]. These different species of PtdCho differ in physical properties [8], and in the signalling products they generate when hydrolysed [9].

$Pemt^{-/-}$ mice have two selectively disrupted alleles of the *Pemt-2* gene at exon 2 [10], which encode PEMT, and do not express any PEMT activity in liver. Therefore these mice completely depend on dietary choline intake to meet daily choline requirements. When fed a diet deficient in choline and insufficient in methionine, $Pemt^{-/-}$ mice develop decreased PtdCho concentrations in hepatic membranes, leading to severe liver damage and death; a choline supplemented diet prevents this [11] and, if provided early enough, can reverse hepatic damage [12]. However, we do not know if diets containing control amounts of choline are sufficient to meet the daily requirement for choline in mice that are unable to form choline endogenously. This may be especially important in pregnant animals, in which we have previously observed a greatly increased requirement for choline [13]. For the first time we show that, in the mouse, PEMT activity meets a substantial portion of daily choline requirements, and that even supplementation with four times more choline than

Abbreviations used: AdoMet, *S*-adenosylmethionine; CD, choline deficient; Cho, choline; GPCho, glycerophosphocholine; PCho, phosphocholine; PtdCho, phosphatidylcholine; CDP-choline, cytidine diphosphocholine; CS, choline supplemented; CT, choline control; LC-ESI-IDMS, liquid chromatography–electrospray ionization–isotope dilution MS; PEMT, phosphatidylethanolamine *N*-methyltransferase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP–biotin nick end-labelling; VLDL, very low density lipoprotein.

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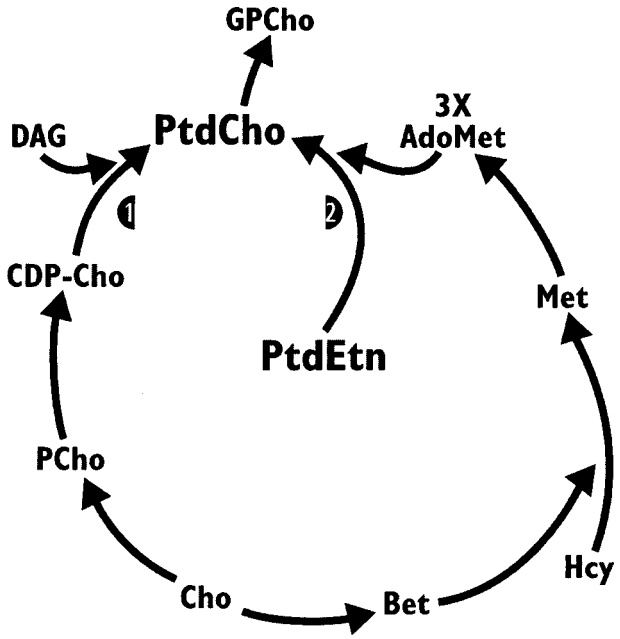

Scheme 1 Pathways for PtdCho biosynthesis

There are two pathways for PtdCho biosynthesis. One via phosphorylation of choline in the CDP-choline pathway (marked 1 on the Scheme) and the alternative via the methylation of phosphatidylethanolamine catalysed by PEMT (marked 2 on the Scheme). Cho, choline; PCho, phosphocholine; CDP-Cho, cytidine diphosphocholine; DAG, diacylglycerol; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; AdoMet, *S*-adenosylmethionine; Met, methionine; Hcy, homocysteine; Bet, Betaine; GPCho, glycerophosphocholine.

contained in the control AIN76A diet, was not adequate to sustain normal concentrations of all choline metabolites in liver or normal liver function.

MATERIALS AND METHODS

Animals

Pemt$^{-/-}$ mice were a gift from Dr Dennis Vance, University of Alberta, Edmonton, Canada. All animals used were housed in cages in a climate-controlled room (24 °C) exposed to light between 06:00–18:00 daily.

Male study

Pemt$^{-/-}$ and wild-type (mixed genetic background of 129/J and C57BL/6) adult male mice (18 of each), weighing 20–25 g at the start of the study, were fed a choline deficient diet (CD, AIN76A diet without choline; Dyets Inc., Bethlehem, PA, U.S.A.) plus choline in their drinking water [choline deficient (CD), 0 mM choline chloride; control (CT), 6.4 mM choline chloride; choline supplemented (CS), 28.9 mM choline chloride; $n = 6$ per group] for 12 days. Mice ingested an average of 3 mg choline/day in the control group, and 12 mg/day in the supplemented group. Livers were collected after mice were anaesthetized with intraperitoneal 200 mg/kg Ketamine (Fort Dodge Animal Health, Fort Dodge, KS, U.S.A.) and 16 mg/kg Xylazine (Ben Venue Laboratories, Bedford, OH, U.S.A.).

Female study

Non-pregnant *Pemt*$^{-/-}$ and non-pregnant wild-type adult female mice (15 of each), weighing 20–25 g at the start of study, were given free access to water and to AIN76A diet (1.1 g choline chloride/kg of diet) before they were randomized. Mice were divided into treatment groups ($n = 5$ animals per group) fed choline control (CT, AIN76A diet containing 1.1 g choline chloride/kg diet), supplemented (CS, AIN 76A containing 4.95 g choline chloride/kg diet) or deficient (CD, AIN76A without choline) diets. Mice ingested an average of 3 mg choline/day in the control group, and 12 mg/day in the supplemented group. These diets were continued for 6 days. Livers were collected after mice were anaesthetized with intraperitoneal 200 mg/kg Ketamine (Fort Dodge Animal Health) and 16 mg/kg Xylazine (Ben Venue Laboratories).

Pregnancy study

Timed-pregnant *Pemt*$^{-/-}$ and timed-pregnant wild-type adult female mice, weighing 20–30 g at the start of the study, were given free access to water and to AIN76A diet. On the evening of the eleventh day of pregnancy, mice were divided into treatment groups ($n = 5$ dams per group) and fed control (CT; stayed on AIN76A diet), CS (AIN76A + 4.95 g choline chloride/kg) or CD (AIN76A without choline) diets for 6 days. Mice ingested an average of 3 mg choline/day in the control group, and 12 mg/day in the supplemented group. Livers were collected after mice were anaesthetized with intraperitoneal 200 mg/kg Ketamine (Fort Dodge Animal Health) and 16 mg/kg Xylazine (Ben Venue Laboratories).

Choline metabolite analysis

Briefly, liver samples were frozen at −80 °C and pulverized under liquid nitrogen. An aliquot of 100 mg was taken from each sample and spiked with ^{2}H-labelled internal standards of all the analytes. For a 100 mg sample of tissue, 400 μl of methanol/chloroform (2:1, v/v) was added, vortexed vigorously, and left at −20 °C overnight. Choline compounds were extracted as described previously [14]. At the end of the extraction, tissue samples were centrifuged at 1500 ***g*** for 5 min at 22 °C. The supernatant was transferred to new tubes and the residues were re-extracted with 250 μl of methanol/chloroform/water (2:1:0.8, by vol.). The supernatants from both extractions were combined. To the resulting solution, 100 μl of chloroform and then 100 μl of water were added to form two phases. After centrifugation at 1500 ***g*** for 5 min at 22 °C, the entire aqueous phase (which contained betaine, choline, GPCho and PCho) was separated from the chloroform phase (which contained PtdCho). The aqueous phase was dried by vacuum centrifugation (Speed-Vac) and redissolved in 20 μl of water. To the aqueous solution, 800 μl of methanol was added. This resulted in precipitation of some unwanted compounds from the solution. These insoluble compounds, mostly proteins, were removed by centrifugation at 1500 ***g*** for 5 min at 22 °C. A portion (10 μl) of this final supernatant was analysed using liquid chromatography-electrospray ionization-isotope dilution mass spectrometry (LC-ESI-IDMS); LCQ$^{\text{DECA}}$ quadrupole ion trap mass spectrometer equipped with an API2 electrospray ionization source (Thermo-Quest, San Jose, CA, U.S.A.). PtdCho in the lower organic phase was separated from other lipids using a Bond Elut aminopropyl column (Varian Analytical Instruments, Walnut Creek, CA, U.S.A.) before LC-ESI-IDMS analysis. The column was conditioned with 5 ml of hexane. Samples were constituted in 0.5 ml of chloroform and applied to the column. After washing the column with 4 ml of chloroform/isopropanol (2:1, v/v) and 4 ml of 2% acetic acid in ethyl ether, PtdCho was eluted with 5 ml of methanol. This solution was further diluted based on the

expected contents of the sample so as to fall within the linear range of the assay, and 10 μl was analysed by LC-ESI-IDMS [15].

Assessment of DNA fragmentation and apoptosis

DNA fragmentation was determined using a TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP–biotin nick end-labelling) method [16] in deparaffinized 5 μm thick sections from tissue blocks. This procedure was performed by using an *in situ* cell-death detection kit according to the manufacturer's instructions (ApoTag™, Intergen, Purchase, NY, U.S.A.). The sections were then counterstained with Methyl Green. Hepatic cells were analysed in three different tissue sections per animal. Apoptotic cells were analysed using NIH Image software version 6.1 (NIH Research Service Branch, Bethesda, MD, U.S.A.). For each slide three sections, each with 4.4×10^5 pixels of area, were randomly counted using a light microscope at a magnification of $\times 200$. TUNEL-positive nuclei were scored under a light microscope. The ratio of TUNEL-positive cells to the total number was calculated.

PEMT activity measurement

PEMT was assayed using a modification of the method of Ridgway and Vance [17]. Briefly, PEMT activity was assayed in homogenates of mouse livers in 125 mM Tris/HCl, pH 9.2, and 5 mM dithiothreitol buffer in the presence of 200 μM [methyl-^{3}H] AdoMet (Perkin Elmer Life sciences, Inc., Boston, MA, U.S.A.) and 0.4 mM exogenous phosphatidyldimethylethanolamine (Avanti Polar-lipids, Inc., Alabaster, AL, U.S.A.). The reaction was carried out for 30 min at 37 °C, and was stopped by addition of a chloroform/methanol mixture. An aliquot of the chloroform phase was applied to a silica gel TLC plate [Si250-PA (19C)-Silica Gel, Baker, Inc., Phillipsburg, NJ, U.S.A.] and was developed in chloroform/methanol/acetic acid/water (50:30:5:2, by volume). [^{3}H]PtdCho was determined in bands that co-migrated with authentic standards using liquid scintillation spectrophotometry.

Histology study

Liver samples were obtained, fixed in 4% neutral buffered formaldehyde (Polysciences, Warrington, PA, U.S.A.), paraffin embedded, cut into 5 μm sections, and stained with Methyl Green. Morphology and steatosis were evaluated using light microscopy.

Analysis of triacylglycerol in the liver

Livers were homogenized with a motorized glass–Teflon homogenizer in 10 vol. of PBS. Lipids were extracted from the homogenates in chloroform/methanol [14]. The concentration of triacylglycerol was measured using a diagnostic kit (Sigma) that was a modification of the enzymic colorimetric method of McGowan et al. [18].

Statistics

To assess the effects of mouse type (knockout and wildtype) and diet (CD, CT, and CS) on each of the study outcomes of interest, an ANOVA for a 2×3 cross-factorial study design was used [19]. The ANOVA model included effects due to mouse type, diet, and interactions. Similarly, to assess the effects of pregnancy status (pregnant and non-pregnant), mouse type and diet on each of the study outcomes of interest, an ANOVA for a $2 \times 2 \times 3$ cross-factorial study design was used [19]. The ANOVA model included effects due to pregnancy status, mouse type, diet, and interactions. In most of the ANOVA models, there were significant interactions. For each outcome and each analysis, several pairwise comparisons of interest were conducted and a Bonferroni correction for multiple comparisons [19a] was used to adjust the significance level ($\alpha = 0.05$). Due to the large number of comparisons for the entire experiment, the Bonferroni correction consisted of adjusting the significance by the number of comparisons for each outcome. For example, three diet comparisons were made for male betaine levels and the Bonferroni correction was $\alpha/3 \approx 0.017$. On the other hand, six pairwise comparisons were made for pregnant versus non-pregnant betaine levels and the Bonferroni correction was $\alpha/6 \approx 0.008$.

RESULTS

Hepatic PEMT activity in *Pemt*$^{-/-}$ and wild-type mice

We investigated whether there is residual PEMT activity in the livers of *Pemt*$^{-/-}$ mice, and whether the remaining activity was influenced by dietary choline intake. After a 12 day dietary treatment, all the animals, including the *Pemt*$^{-/-}$ mice fed on CD diet, survived. The body weights of *Pemt*$^{-/-}$ mice on CD and CT diets decreased by an average of 20% and 10%, respectively, compared with their starting baseline; all other groups gained weight (data not shown). We observed no PEMT activity in *Pemt*$^{-/-}$ mice on any of the diets (Table 1). PEMT activity was detected in livers of wild-type mice and was not influenced by choline in the diet.

Steatosis, DNA fragmentation and apoptosis in *Pemt*$^{-/-}$ mice

On histological examination, liver sections prepared from *Pemt*$^{-/-}$ female mice, treated as described above, had significant steatosis in all livers (Figure 1), while in wild-type female mice, CD, CT and CS groups did not develop histologically detectable hepatic steatosis in the time frame during which we fed the diets (Figure 1). In livers from male, female and pregnant *Pemt*$^{-/-}$ mice on all diets, and in livers of wild-type pregnant mice fed a CD diet, triacylglycerol concentrations were increased. This did not occur in other groups of wild-type mice on any diet (Figure 2).

On histological examination, we observed significant numbers of TUNEL positive hepatocytes with apoptotic morphology in all *Pemt*$^{-/-}$ livers from female mice (Figure 1). These were rarely

Table 1 Significant PEMT activity was not detected in livers of *Pemt*$^{-/-}$ mice

Pemt$^{-/-}$ and wildtype adult male mice, weighing 20–25 g at the start of study, were fed choline deficient (CD) diet (AIN76A diet without choline) plus choline in their drinking water (choline deficient (CD), 0 mM choline chloride; control (CT), 6.4 mM choline chloride; choline supplemented (CS), 28.9 mM choline chloride; $n = 6$ per group) for 12 days. Mice ingested an average of 3 mg choline/day in the control group, and 12 mg/day in the supplemented group. All animals were killed at the termination of experiment and liver tissues were collected. PEMT activity was assayed using a radio-enzymic method as described in the Materials and methods section. Data are expressed as means ± S.E.M., $n = 3$.

	Activity (d.p.m./mg of protein)	
Diet	Wildtype	*Pemt*$^{-/-}$
CD	165 729 ± 83 949	1806 ± 609*
CT	277 764 ± 39 819	691 ± 825*
CS	217 552 ± 62 851	2610 ± 852*

* $P < 0.017$ (Bonferroni correction, 0.05/3) versus wildtype on the same diet.

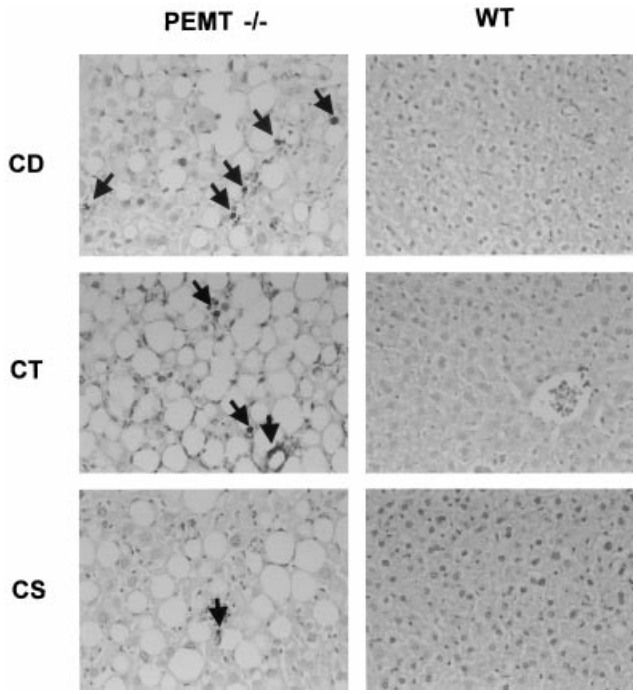

Figure 1 *Pemt*$^{-/-}$ mice develop hepatic steatosis and hepatocyte apoptosis

Non-pregnant female *Pemt*$^{-/-}$ mice and non-pregnant female wild-type mice, weighing 20–25 g at the start of study, were given free access to water and to AIN76A diet (1.1 g choline chloride/kg of diet) before they were randomized. Mice were divided into treatment groups ($n = 5$ animals per group) fed choline control (CT; AIN76A diet containing 1.1 g choline chloride/kg diet), supplemented (CS; AIN 76A containing 4.95 g choline chloride/kg diet) or deficient (CD; AIN76A without choline) diets. Mice ingested an average of 3 mg choline/day in the control group, and 12 mg/day in the supplemented group. These diets were continued for 6 days. All animals were killed at the termination of experiment, liver tissues were collected and sections were prepared from the livers for TUNEL with Methyl Green counterstaining as described in the Materials and methods section. Apoptosis was confirmed using morphological criteria. Representative liver sections are presented from each group at 200 × magnification. Apoptotic cells stain brown and are indicated by arrows in the Figure.

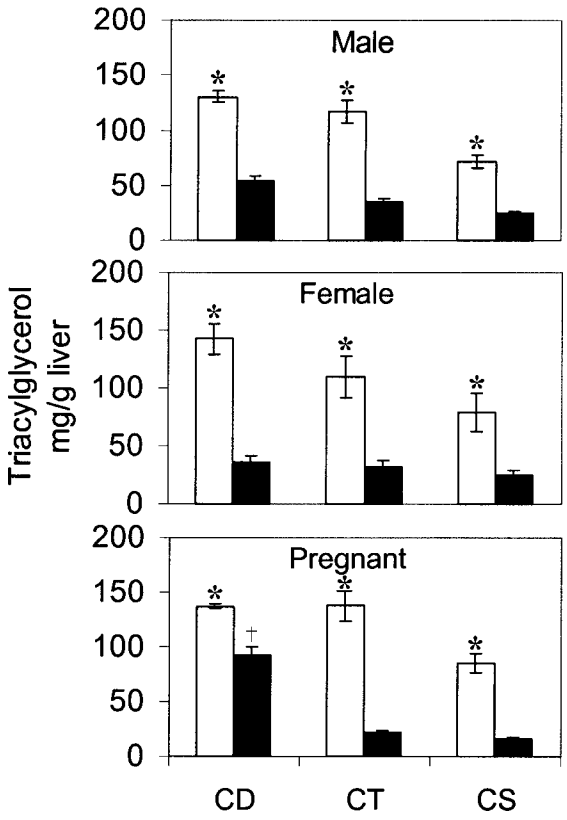

Figure 2 Triacylglycerol concentrations were increased in livers of *Pemt*$^{-/-}$ mice compared with wild-type mice

Male and female mice were treated as described in Table legend 1 and Figure legend 2. *Pemt*$^{-/-}$ and wild-type timed-pregnant mice, weighing 20–30 g at the start of study, were given free access to water and to AIN76A diet (1.1 g choline chloride/kg of diet). On the evening of pregnancy day 11, mice were divided into treatment groups ($n = 5$ dams per group) and fed: control (CT; stayed on standard AIN76A diet), CS (AIN76A with 4.95 g choline chloride/kg) or CD (AIN76A without choline) diets for 6 days. Mice ingested an average of 3 mg choline/day in the control group, and 12 mg/day in the supplemented group. Liver tissues were homogenized and lipids were extracted in chloroform and methanol. Triacylglycerol concentrations were assayed using an enzymic colorimetric method as described in the Materials and methods section. Open bars indicate *Pemt*$^{-/-}$ mice and solid bars indicate wild-type mice. Data are expressed as the means ± S.E.M. of 5–6 animals per group. $^{*}P < 0.017$ (Bonferroni correction, 0.05/3) versus wild-type on same diet; $\dagger P < 0.008$ (Bonferroni correction, 0.05/6) pregnant versus non-pregnant females from the same mouse type and on the same diet.

observed in the wild-type mouse livers on any diet. The number of cells that were TUNEL positive in livers from *Pemt*$^{-/-}$ female mice on CD, CT and CS diets were $6.0 \pm 0.9\,\%$, $2.8 \pm 0.2\,\%$, and $1.6 \pm 0.3\,\%$, respectively, displaying a dose–response effect. In contrast, the number of cells that were TUNEL positive in livers from wild-type mice on CD, CT and CS diets were $0.1 \pm 0.1\,\%$, $0 \pm 0\,\%$, and $0 \pm 0\,\%$, respectively (data are expressed as means ± S.E.M. of five animals per group); all values for *Pemt*$^{-/-}$ mice were significantly different ($P < 0.017$ Bonferroni correction; 0.05/3) from wild-type on same diet.

Choline and metabolite concentrations in liver were depleted in *Pemt*$^{-/-}$ mice

In male and female mice, hepatic choline concentrations were greatly reduced in *Pemt*$^{-/-}$ mice compared with wild-type mice on CD or CT diets (Table 2). Choline concentrations were similar in both groups on the CS diet. In pregnant mice, both *Pemt*$^{-/-}$ and wild-type mice had diminished hepatic choline concentrations on the CD and CT diets.

Betaine concentrations in livers of male mice were unaffected by PEMT status or by diet (Table 2). In female mice, betaine concentrations in liver increased with increasing choline in the diet, and were significantly lower in *Pemt*$^{-/-}$ mice than in wild-type mice on all diets (Table 2). In pregnant dams we observed a similar pattern, except that both *Pemt*$^{-/-}$ and wild-type mice had diminished betaine concentrations in liver when fed the CD diet (Table 2).

PCho concentrations in liver increased with increasing choline in the diet, and were significantly lower in *Pemt*$^{-/-}$ mice than in wild-type male and female mice (Table 2). In pregnant dams, PCho concentrations were depleted in both *Pemt*$^{-/-}$ and wild-type mouse liver on the CD and CT diets (Table 2). On the CS diet, wild-type mice had much higher liver PCho than did knockout mice (Table 2).

GPCho concentrations in livers of male mice were not changed by diet or by *Pemt* gene status (Table 2). In females GPCho was significantly lower on the CD diet in *Pemt*$^{-/-}$ mice. In pregnant dams, GPCho concentrations were significantly lower in most groups compared with non-pregnant females on the same diet (Table 2).

Table 2 Concentration of choline and its metabolites in livers of wildtype and *Pemt*$^{-/-}$ mice fed choline deficient (CD), control (CT) or supplemented (CS) diets

Mice were treated, and liver tissue processed as described in the Table 1 and Figures 2 and 3 legends. Choline, betaine, phosphocholine (PCho), glycerophosphocholine (GPCho) and phosphatidylcholine (PtdCho) concentrations were assayed using LC-ESI-IDMS as described in the Materials and methods section. Data are expressed as the means ± S.E.M. of 5–6 animals per group.

			nmoles/g of wild-type liver			nmoles/g of *Pemt*$^{-/-}$ liver		
Mouse group	Assay	Diet ...	CD	CT	CS	CD	CT	CS
Male	Choline		188 ± 28	219 ± 11	220 ± 29	52 ± 2.3*	56 ± 4.2*	251 ± 54
	Betaine		225 ± 19	293 ± 26	363 ± 60	317 ± 42	273 ± 58	428 ± 140
	PCho		160 ± 20	637 ± 183	937 ± 159	42 ± 12	42 ± 4.4*	70 ± 12*
	GPCho		287 ± 22	309 ± 20	219 ± 10	270 ± 23	353 ± 29	337 ± 27
	PtdCho		17449 ± 1016	17786 ± 1327	18731 ± 804	8741 ± 252*	10178 ± 450*	17833 ± 506
Female	Choline		105 ± 15	110 ± 9.3	116 ± 17	24 ± 1.7*	45 ± 9.2*	104 ± 14
	Betaine		436 ± 76	526 ± 63	705 ± 109	115 ± 16*	176 ± 21*	426 ± 43*
	PCho		314 ± 45	517 ± 45	830 ± 22	120 ± 2.4	168 ± 11*	255 ± 32*
	GPCho		581 ± 45	653 ± 77	669 ± 141	293 ± 19*	454 ± 75	685 ± 48
	PtdCho		16729 ± 398	17208 ± 230	16896 ± 275	8652 ± 433*	11947 ± 552*	16126 ± 429
Pregnant	Choline		14 ± 1.9†	18 ± 1.6†	72 ± 3.9	20 ± 7.3	14 ± 3.4	87 ± 33
	Betaine		298 ± 53	489 ± 68	851 ± 34	214 ± 25	208 ± 39*	417 ± 45*
	PCho		137 ± 17	184 ± 11	1256 ± 428	106 ± 11	115 ± 5.7	287 ± 80*
	GPCho		248 ± 38†	388 ± 82†	371 ± 11†	138 ± 12	221 ± 28	788 ± 381
	PtdCho		12609 ± 303†	15752 ± 241	16419 ± 283	5439 ± 142*†	9490 ± 467*†	15262 ± 594

*$P < 0.017$ (Bonferroni correction: 0.05/3) versus wildtype on same diet; †$P < 0.008$ (Bonferroni correction: 0.05/6) pregnant versus female from same mouse type and on same diet.

PtdCho concentrations in liver were not influenced by diet in wild-type mice (males, females and pregnant; Table 2). In *Pemt*$^{-/-}$ mice (males, females and pregnant) on the CD or CT diets, PtdCho concentrations in livers were significantly diminished compared with that measured in wild-type mouse livers (Table 2). In pregnant dams PtdCho concentrations were significantly lower in CT and CD groups compared with non-pregnant females on the same diet (Table 2).

DISCUSSION

Taken together, our studies demonstrate that *Pemt*$^{-/-}$ mice are unable to maintain concentrations of important choline compounds in liver, and develop liver dysfunction despite consuming adequate, or, for some metabolites, supplemented amounts of choline in the diet. This is unexpected, as the PEMT pathway for PtdCho biosynthesis had previously been considered to be a minor source of PtdCho [6]. Though it was previously reported that hepatic steatosis in *Pemt*$^{-/-}$ mice can be reversed to a great extent by treatment with choline [12], we find that dietary intake of choline at the amounts recommended for rodents did not reverse steatosis in livers of these mice, and though supplemental choline diminished hepatic triacylglycerol concentrations, it did not restore values to normal in *Pemt*$^{-/-}$ mice.

This is the first time that choline metabolism and steatosis have been characterized in male, female and pregnant *Pemt*$^{-/-}$ mice (Table 2). PEMT activity is increased by oestrogen [20], thus it is harder to induce choline deficiency in females than in males [1]. Though female rats are resistant to choline deficiency, pregnant rats are as vulnerable to deficiency as are males [13]. During pregnancy, large amounts of choline are delivered to the fetus across the placenta and this depletes maternal stores of choline. As expected, in wild-type mice the CD diet was associated with diminished concentrations of several choline compounds in liver. In *Pemt*$^{-/-}$ mice, females were no longer resistant to choline deficiency, and pregnant mice were extra-sensitive to a CD diet (as assessed by PtdCho concentrations in liver). Choline concentrations (Table 2) and PtdCho concentrations (Table 2) in *Pemt*$^{-/-}$ mice were depleted on CD and CT diets, but were rehabilitated by the CS diet. It is interesting that PCho concentrations were diminished in *Pemt*$^{-/-}$ mouse liver and were not repleted by the CS diet. We suggest that that there was a more rapid conversion of PCho into PtdCho via cytidylyltransferase activity. This is consistent with previous reports that choline deficiency causes translocation of CTP:phosphocholine cytidylyltransferase from cytosol to endoplasmic reticulum in rat liver [21], and that cytidylyltransferase activity is increased in *Pemt*$^{-/-}$ mice [10]. PCho is the most labile of the choline compounds in the liver [22] and it is a novel second messenger that acts as a mitogen in 3T3 cells [23]. In females (both non-pregnant and pregnant), *Pemt*$^{-/-}$ mice had diminished betaine concentrations on all diets. Diminished availability of betaine should result in increased dependence on methyltetrahydrofolate as a source of methyl groups for the conversion of homocysteine into methionine [24,25].

We report that increased DNA fragmentation and morphologic changes characteristic of apoptosis were observed in livers of *Pemt*$^{-/-}$ mice even when adequate or excess amounts of choline were provided in their diets. This has previously been described in choline deficient rat liver [26] and the apoptotic pathway involves both a p53-dependent, NFk-B dependent pathway [27] as well as a p53-independent apoptotic pathway [28–30].

Steatosis in choline deficiency and in the absence of PEMT occurs, in part, because VLDL cannot be exported from liver in the absence of the synthesis of PtdCho [3], and dietary choline deficiency causes steatosis in mice, rats, baboons and humans [1,3–5,31]. In addition, PEMT activity is important for secretion of apolipoprotein B100-containing VLDL via a yet-to-be defined mechanism that is independent of PtdCho concentration in hepatocytes [32]. In previous reports, adding choline to the diet of *Pemt*$^{-/-}$ mice reversed hepatic steatosis [12]. We found that steatosis was not resolved by feeding a diet adequate in choline, and was only partially resolved by feeding a CS diet. In addition, Walkey and colleagues [11] found that *Pemt*$^{-/-}$ mice died on a

CD diet after 3 days, while in our study animals survived for at least 12 d. Perhaps this apparent disagreement is due to differences in experimental methods. We studied male, female and pregnant female mice, they studied male mice. The period that the mice were exposed to treatment was different; we fed experimental diets for a longer period (6 or 12 days compared with 3 or 4 days). We fed our mice the night before they were killed; Waite et al. fasted their mice overnight [12]. The diets that the mice were fed in the two experiments were different. In our study, AIN76A purified diet was used and in the Waite et al. study ICN (Cosa Mesa, CA, U.S.A.) purified diet was used. Our diets contained 8.2 g/kg diet methionine. Their diets contained 3 g/kg diet methionine. In addition, the fat sources were not the same (50 g/kg diet corn oil in our diet, 100 g/kg safflower oil in the Waite diet [12]). In addition, we used deficient, control (0.11 % choline chloride) and supplemented choline diets (0.495 % choline chloride); Waite et al. [12] used only deficient and supplemented (0.4 % choline chloride) diets in their study. Thus, they did not determine whether the recommended amounts of dietary choline met the needs of *Pemt*$^{-/-}$ mice. These dietary composition differences also may account for the increased survival time of our mice, as methionine methyl groups can spare the use of choline as a methyl donor (Scheme 1; [33]).

In summary, PEMT activity in liver provides an important source of choline, and reduces the amount of choline that must be consumed in the diet to maintain normal liver function.

Pemt$^{-/-}$ mice appear to need more choline in their diet to sustain normal hepatic concentrations of some important choline compounds. Liver steatosis seen in *Pemt*$^{-/-}$ mice is reduced but not fully resolved by supplementation of the diet with choline. In our studies, pregnant mice were the most vulnerable to dietary availability of choline and to deletion of PEMT activity. The fetus derives choline from maternal blood across the placenta [34], and fetal brain development is very sensitive to the availability of choline [35–37]. We are currently conducting studies to determine if brain development is altered in *Pemt*$^{-/-}$ mice. The *Pemt* gene is polymorphic in humans, with more than 100 single nucleotide polymorphisms present in 40 subjects studied [38]. If any of these mutations disturb PEMT function, these humans are likely to have increased dietary requirements for choline. This would be especially apparent during pregnancy. We are currently conducting experiments to determine which single nucleotide polymorphisms in PEMT are functionally significant.

This work was supported by the National Institutes of Health (DK55865, AG09525). Support for this work was also provided by grants from the NIH to the UNC Clinical Nutrition Research Unit (DK56350) and the Center for Environmental Health (ES10126). We thank Dr Dennis E. Vance for kindly providing us with *Pemt*$^{-/-}$ mice.

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Received 30 September 2002/20 November 2002; accepted 5 December 2002
Published as BJ Immediate Publication 5 December 2002, DOI 10.1042/BJ20021523