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Review

Phospholipid methylation in mammals: from biochemistry to physiological function☆

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ABSTRACT

Phosphatidylcholine is made in the liver via the CDP-choline pathway and via the conversion of phosphatidylethanolamine to phosphatidylcholine by 3 transmethylation reactions from AdoMet catalyzed by phosphatidylethanolamine *N*-methyltransferase (PEMT). PEMT is a 22.3 kDa integral transmembrane protein of the endoplasmic reticulum and mitochondria-associated membranes. The only tissue with quantitatively significant PEMT activity is liver; however, low levels of PEMT in adipocytes have been implicated in lipid droplet formation. PEMT activity is regulated by the concentration of substrates (phosphatidylethanolamine and AdoMet) as well as the ratio of AdoMet to AdoHcy. Transcription of PEMT is enhanced by estrogen whereas the transcription factor Sp1 is a negative regulator of PEMT transcription. Studies with mice that lack PEMT have provided novel insights into the function of this enzyme. PEMT activity is required to maintain hepatic membrane integrity and for the formation of choline when dietary choline supply is limited. PEMT is required for normal secretion of very low-density lipoproteins. The lack of PEMT protects against diet-induced atherosclerosis in two mouse models. Most unexpectedly, mice that lack PEMT are protected from diet-induced obesity and insulin resistance. Moreover, mice lacking PEMT have increased susceptibility to diet-induced fatty liver and steatohepatitis. This article is part of a Special Issue entitled: Membrane Structure and Function: Relevance in the Cell's Physiology, Pathology and Therapy.

Contents

Abbreviations: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; ALT, alanine aminotransferase; CD, choline deficient; CT, CTP:phosphocholine cytidylyltransferase; ER, endoplasmic reticulum; Hcy, homocysteine; LCTαKO, liver-specific CTα knockout; LDL, low density lipoproteins; MAM, mitochondria-associated membranes; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine *N*-methyltransferase; TG, triacylglycerol; VLDL, very low density lipoproteins

☆ This article is part of a Special Issue entitled: Membrane Structure and Function: Relevance in the Cell's Physiology, Pathology and Therapy.

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1. Introduction

Phosphatidylcholine (PC) is the quantitatively major membrane phospholipid in mammalian cells. In mammals, PC is also an important constituent of lipoproteins, lung surfactant and bile. PC was first described by Gobley in 1847 as a component of egg yolk and was named lecithin after the Greek word for egg yolk, *lekithos*. Impressively, despite very crude methods available, Strecker demonstrated in the 1860s that PC contained 2 fatty acids linked to glycerol and a third hydroxyl contained phosphocholine [1]. Since that time there have been huge advances in defining the structure of PC, its biosynthesis, its role in membrane structure and more recently its function.

The major pathway for biosynthesis of PC is the CDP-choline pathway described by Eugene Kennedy and co-workers in the 1950s (Fig. 1) [2,3]. The Kennedy pathway is present in all eukaryotic cells with a nucleus. An alternative pathway for PC biosynthesis is the conversion of phosphatidylethanolamine (PE) to PC by PE-methyltransferase (PEMT) (Fig. 2). PEMT is quantitatively important in liver. Estimates are that ~30% of PC made in the liver is via the PEMT reaction [4–6] whereas most of the remaining biosynthetic-derived PC in liver originates via the CDP-choline pathway (Fig. 3). PEMT activity (per mg protein) in other mammalian tissues is usually less than 1% that in liver [7]. Some eukaryotes (e.g., plants and parasites) make phosphocholine via methylation of phosphoethanolamine with AdoMet [8]. The phosphocholine is incorporated into PC via the CDP-choline pathway.

While it has been evident within the past hundred years that PC has a critical role in membrane structure and permeability, the roles for PC and PC biosynthesis in other mammalian functions have been more slowly appreciated. Surprisingly, and not predictably, many additional novel functions are performed by the PEMT pathway in mammals. The objective of this article is to review the highlights of the biochemistry and function of the PEMT pathway.

2. Discovery of the PEMT pathway

In 1940, Vincent du Vigneaud and co-workers showed that methyl groups from methionine could be incorporated into choline in the rat [9,10]. In 1941, DeWitt Stetten provided evidence from studies using ^{15}N that PE could be converted to PC and postulated that the methyl groups originated from methionine [11]. The next key discovery was that of *S*-adenosylmethionine (AdoMet) by G.L. Cantoni [12]. These developments allowed Bremer (photo in Fig. 2) and Greenberg to describe an enzyme activity in rat liver microsomes that used AdoMet to convert PE to PC [13]. Among the observations was the finding that PE methylation had a very high pH optimum (10.3) (Fig. 4). This result has been confirmed for the microsomal activity [14] as well as for the pure enzyme reconstituted in Triton X 100 micelles [15]. The significance of this unusual pH optimum is not clearly understood to this day.

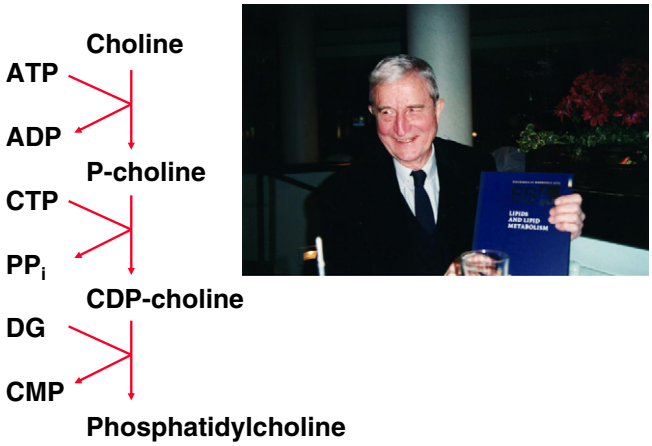

Fig. 1. Outline of the CDP-choline pathway as described by Eugene P. Kennedy in the 1950s. Photo of Professor Kennedy (courtesy of Ms. Denise Wells) when presented with a special issue of BBA in his honor in 1997 [100]. DG, diacylglycerol.

3. Purification of PEMT and cloning of the cDNA and gene

From the studies of Bremer and Greenberg, PEMT was shown to be a membrane-associated enzyme. In the 1970s and 1980s several attempts were made to purify PEMT [16,17]. Purification of integral membrane proteins is challenging and has been called "masochistic enzymology" [18]. It was only the dedicated drive of Neale Ridgway that resulted in the purification of PEMT in 1987 [15]. We are unaware of anyone else who has purified PEMT to homogeneity. PEMT has a molecular mass of 18.3 kDa as determined by SDS-polyacrylamide gel electrophoresis. The molecular mass later calculated from the cDNA sequence of PEMT is 22.3 kDa [19]. The 3 transmethylation reactions (Fig. 2) were shown to be present in the same pure protein, in contrast to the two methyltransferase enzymes in yeast [20], and disproving the previous proposal that two separate proteins in mammalian tissues were involved.

With pure protein available, it was possible to obtain partial amino acid sequence that led to the cloning and expression of the cDNA encoding PEMT [19]. PEMT contains 199 amino acids. A highly specific antibody was generated from a synthetic peptide that encoded the 12 carboxyl terminal amino acids of PEMT. There was a surprising finding when studies were performed with this antibody on subcellular fractions of rat liver; immunoreactivity was detected only in crude mitochondrial fractions whereas 70% of the PEMT activity was associated with the endoplasmic reticulum (ER). When the crude mitochondria

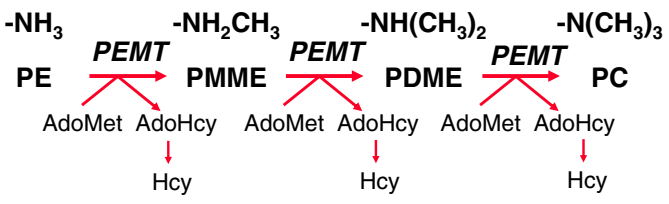

Fig. 2. The pathway for the biosynthesis of phosphatidylcholine (PC) from phosphatidylethanolamine (PE) via the intermediates phosphatidylmonomethylethanolamine (PMME) and phosphatidyldimethylethanolamine (PDME) discovered by Jon Bremer (photographed in 1998) and David Greenberg [13]. AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; Hcy, homocysteine.

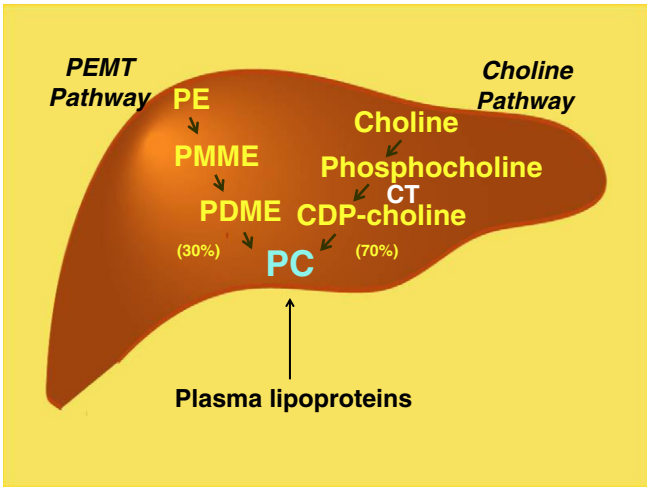

Fig. 3. The major pathways for generation of PC (phosphatidylcholine) in the mammalian liver. The PEMT (phosphatidylethanolamine methyltransferase) pathway is estimated to contribute about 30% of the PC biosynthesized in liver and the choline pathway 70%. Recent estimates are that PC from plasma lipoproteins, at least in mice, contribute an amount equal to biosynthesis of PC in liver.

were further purified, PEMT activity was no longer detected in mitochondria but instead was present only in a membrane fraction that was separated from mitochondria upon ultracentrifugation on a Percoll gradient (Fig. 5) [19]. This membrane fraction is now known as the mitochondria associated membranes (MAM) and is a specific region of the ER that transiently becomes tethered to mitochondria [21]. MAM have been implicated in phospholipid import into mitochondria from the ER [22], as well as regulation of calcium homeostasis, apoptosis and autophagy. Despite enormous efforts, the explanation for why the ER form of PEMT activity is not detected by the antibody that recognizes PEMT on MAM remains unknown. There is no question that the ER and MAM forms of PEMT are derived from the same gene since both forms are eliminated in the *Pemt*$^{-/-}$ mouse [23]. Thus, immunoblotting with this highly specific antibody might not detect all of the PEMT in a sample; nevertheless, enzymatic assay of the PEMT activity should detect all active enzymes present in a sample.

Elucidation of the cDNA sequence encoding PEMT facilitated the characterization of the mouse gene encoding PEMT. This gene was localized to chromosome 11 and contains 7 exons [24]. These studies permitted the generation of mice that lacked PEMT (discussed in Section 6).

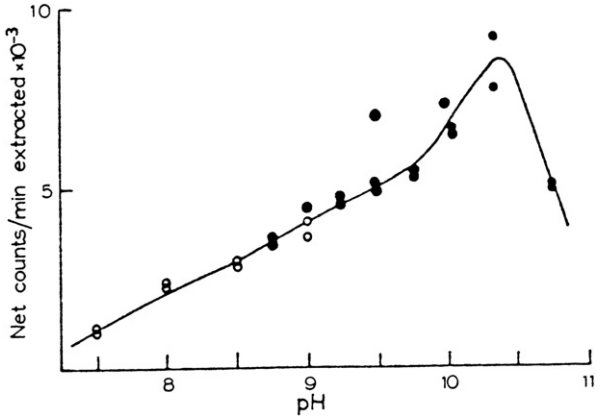

Fig. 4. pH optimum of PEMT in rat-liver microsomes. *S*[*Me*-^{14}C]adenosylmethionine (0.54 μmol, 33,000 cpm) incubated with rat-liver microsomes (11.5 mg protein) for 30 min at 37°. Total volume, 1 ml. Radioactivity incorporated into PC was measured. Open circles, Tris–HCl buffer; closed circles, glycine-KOH buffer. Taken from Fig. 1 in Bremer and Greenberg (1961) *Biochim. Biophys Acta* 46, 205–216.

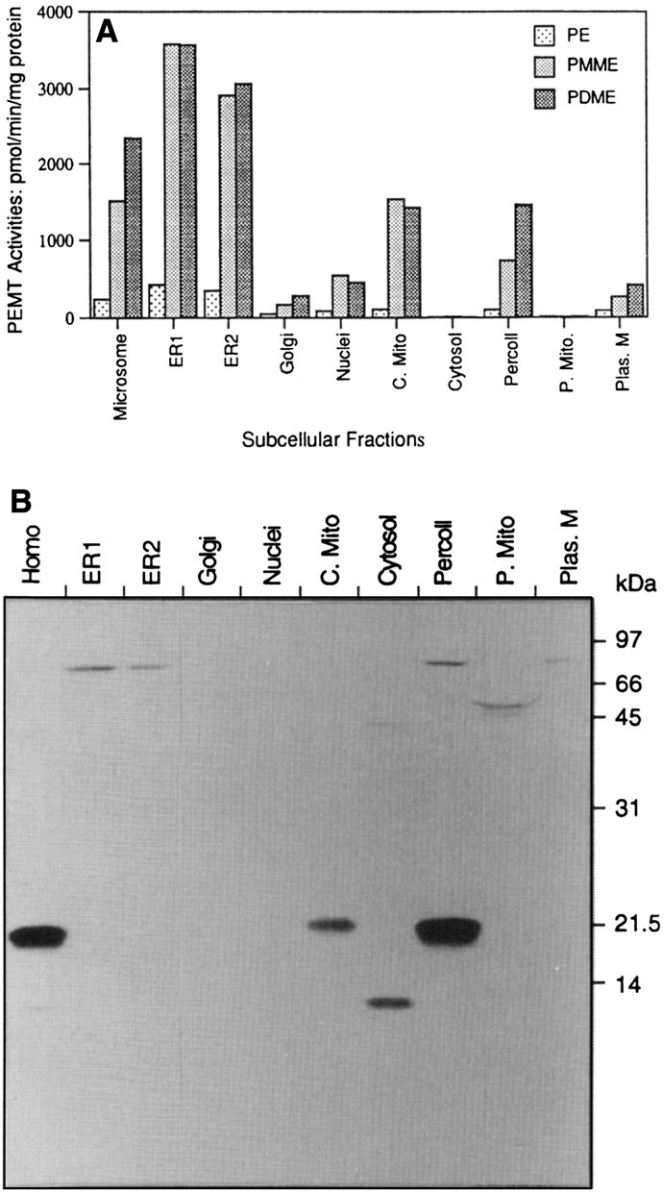

Fig. 5. Subcellular distribution of PEMT activity and protein in rat liver. *Panel A*: subcellular fractions were isolated from rat liver. Twenty-five micrograms of protein in each fraction were used for the PEMT assay. The specific activity of the enzyme with the three different substrates, PE, PMME (phosphatidylmonomethylethanolamine) or PDME (phosphatidyldimethylethanolamine) is given for each fraction. The abbreviations are: *ER1*, heavy endoplasmic reticulum; *ER2*, light endoplasmic reticulum; *C. Mito*, crude mitochondria; *P. Mito*, pure mitochondria; *Percoll*, Percoll gradient fraction now referred to as mitochondria-associated membranes (MAM); *Plas. M*, plasma membrane. *Panel B*: immunoblot analysis of rat liver subcellular fractions probed with a COOH-terminal-specific antibody against PEMT. Fifty micrograms of protein from each fraction were separated on a 12.5% polyacrylamide, 0.1% SDS gel and transferred to a nitrocellulose membrane. PEMT was detected with PEMT antibody via enhanced chemiluminescence. Molecular size markers (in kDa) are indicated at right. The abbreviations are as for *panel A*. Taken from Fig. 6 in Cui et al. (1993) *J. Biol. Chem.* 268, 16655–16663.

4. Structure and characterization of PEMT

As described above, in mice and rats the major enzymatic activity and immunoreactivity for PEMT are in the liver. The minor activity detected in other tissues, particularly in adipose, might also have important functions, as discussed in Section 6. A low level of expression of PEMT in non-hepatic tissues has also been indicated in recent immunoblotting studies (Fig. 6). Importantly, however, a limitation to

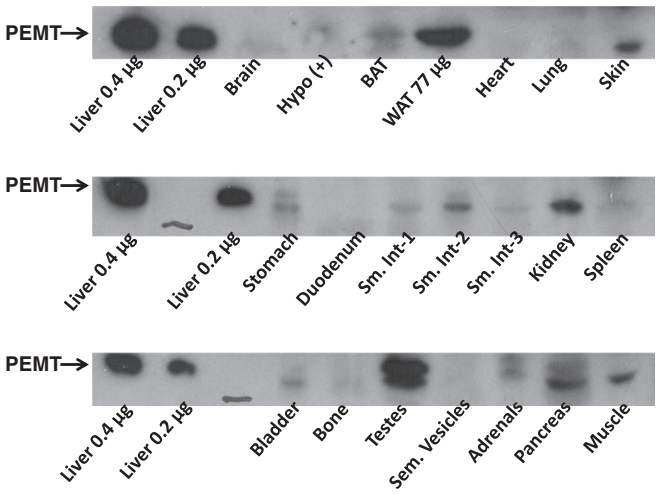

Fig. 6. PEMT expression in mouse tissues. Tissues were collected from C57Bl6 mice. The proteins (100 μg unless otherwise indicated) in homogenates were separated on SDS-gels and immunoblotted for PEMT using antibodies raised against the carboxyl terminal sequence of PEMT. BAT, brown adipose tissue; WAT, white adipose tissue; hypo, hypothalamus; Sm. Int., small intestine; Sem. Vesicles, seminal vesicles.

immunoblotting studies with the PEMT-peptide antibody is that this antibody might not detect all PEMT (Section 3.) In Fig. 6, the amount of liver protein loaded on to the gel was 0.2 to 0.5% of that for the other tissues. Thus, in most of the tissues examined, the immunoreactivity of PEMT is less than 0.5% of that in liver. Only in white adipose tissue and testes is the immunoreactivity somewhat higher. The two immunoreactive bands in testes and pancreas probably result from phosphorylation of PEMT [25]. When the tissue expression profile of the ESTs for PEMT is examined (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Mm.2731), liver appears to have the highest expression (709 transcripts per million). Interestingly, at the morula stage of embryonic development, 460 PEMT transcripts/million were detected. If significant expression of PEMT occurred in the morula, it would be interesting to study this further. However, since this would be purely curiosity-driven research, it might be very difficult to obtain a grant for such research in the current climate for research funding.

4.1. Structure of PEMT, kinetic mechanism and substrate specificity

Even though PEMT is a small protein, a structure by X-ray crystallography or NMR has not yet been reported. However, studies of endoproteinase digestion of epitope-tagged PEMT have been performed [26]. The data suggest a topological model in which 4 transmembrane regions span the ER membrane with both the N and C terminal residues facing the cytosol. A recent model of PEMT spanning the ER membrane is shown in Fig. 7. Additional studies have indicated that residues Gly^{100} and $glutamate^{180}$ are required for binding of AdoMet. Mutations in Gly^{98} and $glutamate^{101}$ significantly elevated the binding constant of AdoMet [27]. At the present time there are no data that define the binding site for PE, phosphatidylmonomethylethanolamine or phosphatidyldimethylethanolamine.

Kinetic analysis of PEMT activity is not simple because there are 3 different phospholipid substrates in the conversion of PE to PC. Nevertheless, the kinetic mechanism for the PEMT-catalyzed reaction has been delineated using pure enzyme reconstituted into phospholipid/Triton X-100 mixed micelles [28]. The study suggests a concerted mechanism that involves a single binding site for the phospholipid substrates. The methylations of phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine follow an ordered Bi-Bi mechanism with the phospholipid substrate being the first molecule to bind and the phospholipid product the last intermediate to be released. The data suggest that PE and the mono- and di-methylated products remain bound to the enzyme while AdoMet is sequentially bound, and that *S*-adenosylhomocysteine (AdoHcy) is released during each of the 3 transmethylation reactions.

Although early reports suggested that PE species that contained polyunsaturated fatty acids were preferred substrates for methylation by PEMT, this is no longer thought to be correct. Rather, methylation of a particular molecular species of PE depends on its concentration and not on its acyl-chain composition [29]. However, once formed, the PC undergoes remodeling of the fatty acyl components by deacylation/reacylation reactions [29,30].

The specificity for the methylation of the phospholipid head group has been studied in primary rat hepatocytes [31]. Incubation of hepatocytes with ethanolamine, monomethylethanolamine and 2-

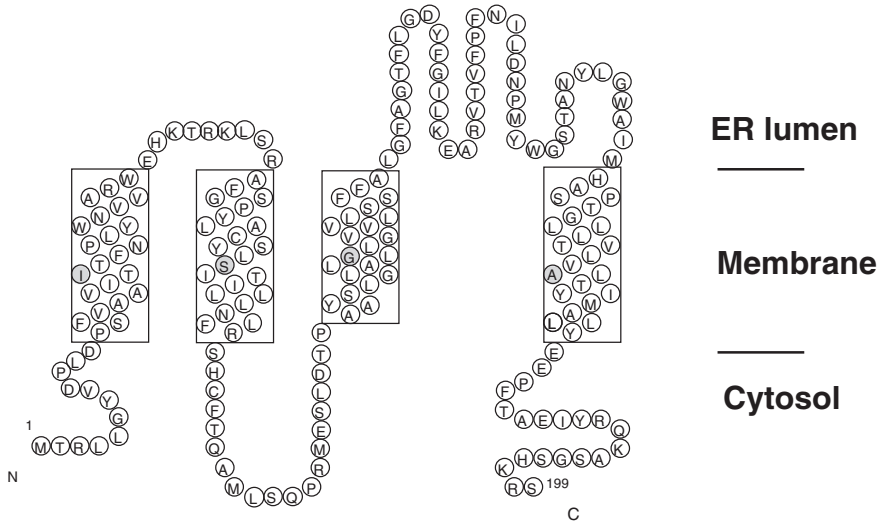

Fig. 7. Proposed orientation of PEMT in the ER membrane. N refers to amino terminus, and C to the carboxyl terminus, of PEMT. The figure was kindly supplied by Dr. Joanne Lemieux and TuoHuang Zhang, University of Alberta. Taken from Fig. 1 in Vance, D.E. (2013) *Biochim. Biophys. Act.* 1831, 626–632.

aminobutanol stimulated the incorporation [CH_3-^{14}C]methionine into the phospholipid fraction. However, stimulation of incorporation of radioactivity did not occur with diethanolamine, diethylethanolamine or dimethylaminoisopropan-2-ol. However, *N*-isopropylethanolamine was converted into phosphatidylisopropylethanolamine that was methylated to form phosphatidyl-*N*-methylisopropanolamine [32]. Thus, the head group specificity of PEMT appears to have some flexibility but whether or not any of these alternative bases would be exposed to PEMT in vivo is doubtful. PE plasmalogen does not appear to be a substrate for methylation by PEMT (M. Hermansson, R. Jacobs, D. E. Vance, unpublished results). Presumably these observations reflect PEMT activity, but experiments with purified enzyme have not been reported.

5. Regulation of phospholipid methylation

5.1. Regulation by substrate availability

Probably the first report to examine the regulation of PE methylation demonstrated that incubation of rat primary hepatocytes with 0.2 mM methionine doubled the conversion of PE to PC [4,33]. Subsequently, experiments showed that increasing the concentration of ethanolamine to 0.5 mM from 0.2 mM in the medium of primary hepatocytes doubled the amount of PE in the cells and also doubled the methylation of PE [34]. Related experiments a decade later with rat liver microsomes supported the finding that an increase in the concentration of PE stimulates PE methylation [35]. Furthermore, it appears that only PE newly-made via the CDP-ethanolamine pathway is converted to PC [30].

Not only is the concentration of AdoMet important in the methylation of PE but also the ratio of AdoMet to AdoHcy. A normal ratio of AdoMet to AdoHcy in rat liver was reported to be 5.6 [36]. When livers were perfused with different concentrations of homocysteine and adenosine, the ratio of AdoMet/AdoHcy was decreased from this normal level to 0.6 or 0.3, resulting in a 75% decrease and 99% decrease in PE methylation, respectively. A similar conclusion was reached when the AdoHcy hydrolase inhibitor 3-deazaadenosine, that elevates the concentration of AdoHcy, was used in rat liver [37]. Administration of 3-deazaadenosine to rats decreased PE methylation by 90% [38]. This inhibition was caused by the generation of 2 competitive inhibitors of PEMT, AdoHcy and the 3-deazaadenosine analog of AdoHcy.

5.2. Regulation of enzyme expression

Thirty years ago, the activity of hepatic PEMT was assayed during rat development from the late embryonic stages to adult [39]. PEMT activity before birth was very low (0.03 nmol/min/mg protein). At birth, there was a rapid increase in PEMT activity that peaked at 10 days after birth (0.45 nmol/min/mg protein) and this level of activity was maintained into adulthood. The cause of the striking increase in PEMT expression after birth is not known. In another example, the hepatic expression of PEMT activity was increased 2-fold upon feeding rats a choline-deficient diet for 3 weeks [40]. Moreover, a remarkable 5-fold increase in immunoreactivity of PEMT was observed after 12 weeks of the choline-deficient diet. On the other hand, PEMT activity and immunoreactivity were decreased by >50% one day after rats were subjected to partial hepatectomy and were restored 3–4 days after the operation [41]. The mechanisms underlying these changes have not been determined.

5.3. Transcriptional regulation of PEMT

Estrogen appears to be a positive regulator of the transcription of *PEMT* in primary cultures of human and mouse hepatocytes [42]. In studies in humans, pre-menopausal women who were fed a choline-deficient diet were less likely to develop signs of muscle or liver damage than were post-menopausal women or men suggesting that estrogen might regulate PEMT expression [43]. The idea that estrogen regulates hepatic PEMT expression is consistent with the observation that female mice exhibit higher expression of PEMT than do male mice [44].

A major problem in studies on the transcriptional regulation of PEMT expression has been the lack of a suitable cell line that expresses PEMT. Primary hepatocytes express PEMT but the activity starts to decline upon plating of the cells and ~50% of enzyme activity is lost after 24 h. Moreover, no suitable hepatoma cell line has been identified that has significant expression of PEMT. Nevertheless, this difficulty was overcome when PEMT expression was detected during the differentiation of 3T3-L1 cells into adipocytes [45]. 5′-Deletion analyses for PEMT promoter-luciferase constructs stably expressed in 3T3-L1 cells implicated a regulatory region between −471 and −371 bp relative to the transcriptional start site; a binding site for the transcriptional regulator Sp1 is present in this region. During differentiation of the cells into adipocytes, the level of Sp1 decreased prior to the increased expression of PEMT. These and related experiments implicate Sp1 as a negative regulator of PEMT expression in 3T3-L1 cells [45]. In contrast, Sp1 is a positive regulator of the expression of CTP:phosphocholne cytidylyltransferase, the rate-limiting enzyme in the CDP-choline pathway for PC biosynthesis [46–48]. Whereas the expression of PEMT is decreased during hepatic growth and regeneration, the expression of the cytidylyltransferase is increased [41,49,50]. Hence, it seems that Sp1 reciprocally regulates PC production via the PEMT and the CDP-choline pathways.

5.4. Future directions on regulation of PEMT activity and expression

We now have a general outline of mechanisms that regulate the expression and activity of PEMT in liver and primary hepatocytes. However, much remains to be learned about how the expression of PEMT is regulated during choline deficiency, liver regeneration and during the perinatal period. Mechanisms of post-transcriptional regulation of PEMT represent a virtually untouched area of research. PEMT is modestly phosphorylated by cAMP-dependent protein kinase in vitro but is not phosphorylated in intact hepatocytes treated with a cAMP analog [25]. Does the phosphorylation of PEMT ever become important? What about other post-translational mechanisms such as acetylation?

6. Physiological functions of PEMT

When Bremer and Greenberg described PEMT activity in rat liver microsomes in 1961 [13], they surely and correctly thought that PEMT was important for the biosynthesis of PC, a critical and essential component for membrane structure. It was also probable that PEMT might have a role in the secretion of hepatic very low density lipoproteins since PC is quantitatively the most important phospholipid in plasma lipoproteins [51,52]. However, it is very unlikely that Bremer and Greenberg would have considered that PEMT might have a role in the development of obesity and type 2 diabetes. The next section of this review will describe the now well-established functions of PEMT in whole animal physiology. Previous efforts to understand PEMT function in vivo were severely handicapped by the lack of gene-targeted mice.

In the late 1970s, PEMT activity was identified in various tissues (e.g., bovine adrenal medulla and mammary gland). However, because the activity was very low compared to that in liver, it was surprising that a number of physiological changes (e.g. histamine release from mast cells and leukocyte chemotaxis) were attributed to phospholipid methylation [53–55]. The activity of PEMT in these non-hepatic cells was 0.1% or lower than that in rat liver microsomes [56]. Furthermore, even though changes in microviscosity of the erythrocyte were reportedly due to changes in PEMT activity [55], only a miniscule amount of PE was methylated (0.001% of total PE) [56]. Thus, it seemed unlikely that any of the reported physiological changes could be attributed to changes in PEMT activity. Arguments were presented to rebut these criticisms [56]. Nevertheless, the enthusiasm for a physiological

function of PEMT in cell signaling slowly waned. It was not until mice lacking PEMT (*Pemt*$^{-/-}$ mice) were constructed [23] that it became clearly apparent that the previously observed physiological responses were not due to alterations in PEMT activity.

In other studies, the expression of PEMT in rat hepatoma cells (McA-RH7777) unexpectedly inhibited cell growth whereas expression of other "foreign" proteins via the same vector did not inhibit McA-RH7777 cell growth [57]. Moreover, induction of hepatic tumors in rats with *N*-nitrosodiethylamine coincided with a striking decrease in PEMT activity and immunoreactivity. These observations suggested that PEMT might have a role in regulation of hepatocyte cell growth and possibly the development of liver cancer. However, studies with *Pemt*$^{-/-}$ mice failed to demonstrate an increase in liver cancer in the knock-out mice compared to wild-type mice (Z. Li, D.E. Vance; unpublished results).

6.1. PEMT and liver function

A critical and major step in determining the function of PEMT was the construction of mice that lacked PEMT (*Pemt*$^{-/-}$ mice) [23]. In the 1980s the techniques for targeting specific genes in the mouse were introduced [58]. For disruption of the PEMT gene, the initial step was to characterize the murine gene that encodes PEMT [24]. Subsequently, a section of the gene was disrupted that led to *Pemt*$^{-/-}$ mice. This was the first gene targeted mouse in phospholipid biosynthesis or catabolism.

Generation of *Pemt*$^{-/-}$ mice and subsequent experiments demonstrated that liver was the only organ that produced significant amounts of PC derived from PE [23]. Pulse-chase studies with labeled methionine showed that much of the PEMT-derived PC was exported from the liver and delivered to the lung, kidney, uterus and heart [23]. The *Pemt*$^{-/-}$ mice exhibited no obvious physiological defects. In one sense this lack of phenotype was not surprising since the CDP-choline pathway remained intact and the activity of the important regulatory enzyme in that pathway, CT, was increased in the livers of *Pemt*$^{-/-}$ mice. Thus, an important question was: why did PEMT survive during evolution if it was not apparently essential for making PC? The answer became rapidly evident when the *Pemt*$^{-/-}$ mice were fed a choline-deficient diet [59]. Three days after the diet was initiated, the livers of the *Pemt*$^{-/-}$ mice exhibited end-stage liver failure whereas the *Pemt*$^{+/+}$ mice were normal (Fig. 8). The hepatic concentration of PC was at least 50% lower in *Pemt*$^{-/-}$ mice than in *Pemt*$^{+/+}$ mice or *Pemt*$^{+/-}$ mice. It is noteworthy that the PEMT pathway is the only known mechanism by which mammals can produce a net source of choline (derived from catabolism of PC made by PEMT). The CDP-choline pathway does not generate choline but utilizes choline derived from the diet or from the PEMT pathway. Thus, we concluded that PEMT survived during evolution to provide PC and choline at times when dietary choline was restricted, for example during fasting/starvation.

The rapid induction of liver disease in the CD-*Pemt*$^{-/-}$ mice was unexpected and raised the question of the underlying mechanism. From studies in The Netherlands, it was known that the liver of a 20 g mouse contains ~20 mg of PC and secretes ~23 mg of PC into the bile each day [60]. Therefore, the rapid decay of the livers from CD-*Pemt*$^{-/-}$ mice might be due to the continued secretion of PC into bile. To test this hypothesis, *Pemt*$^{-/-}$ mice were crossed with mice that lacked the multi drug-resistant protein MDR2, a PC-specific protein that is responsible for delivery of PC into bile [61]. When the double knockout *Pemt*$^{-/-}$/*Mdr2*$^{-/-}$ mice were fed the CD diet, the mice survived more than 90 days [62]. Thus, the acute lethality of *Pemt*$^{-/-}$ mice fed the CD diet was due to the rapid depletion of hepatic PC via secretion into bile. Did the 50% lower hepatic PC content of CD-*Pemt*$^{-/-}$ mice lead to liver failure? Apparently not, because after 21 days of choline deficiency, the concentration of PC in *Pemt*$^{-/-}$/*Mdr2*$^{-/-}$ mice was decreased to the same extent as in the CD-fed *Pemt*$^{-/-}$ mice yet the double knock-out mice were still alive. In livers of *Pemt*$^{-/-}$/*Mdr2*$^{-/-}$ mice the PC content decreased by only 20% after 3 days of the CD diet [63]. Thus, the rate of loss of PC was not as rapid in the *Pemt*$^{-/-}$/*Mdr2*$^{-/-}$ mice as in the CD-fed *Pemt*$^{-/-}$ mice. The decrease in PC in *Pemt*$^{-/-}$ mice was not mirrored by a decrease in the hepatic content of other phospholipids. Thus, after 3 days of choline deficiency, the level of PE was unchanged [63]. PC is a bilayer-forming lipid whereas PE can be a non-bilayer forming lipid [64]. Moreover, PC is concentrated in the outer monolayer of the plasma membrane whereas PE resides mainly on the inner monolayer [65]. Thus, we speculated that the decrease of PC in livers of the *Pemt*$^{-/-}$ mice might cause the redistribution of PE into the outer monolayer of the plasma membrane (Fig. 9). Consequently, the presence of the non-bilayer forming lipid (PE) in the outer monolayer might result in membrane permeability leading to the leakage of enzymes and other molecules into the extracellular space. We therefore assessed the presence of the hepatic enzyme alanine aminotransferase (ALT) in plasma since plasma ALT is indicative in humans and rodents of liver pathology. Indeed, there was a striking correlation between the PC/PE ratio in plasma membranes of the *Pemt*$^{-/-}$/*Mdr2*$^{-/-}$ and *Pemt*$^{-/-}$ mice and plasma ALT [63]. As the PC/PE ratio declined, plasma ALT increased. Moreover, PE was detected in the outer monolayer of the plasma membrane of liver cells from *Pemt*$^{-/-}$ mice after 2 days of choline deficiency but not in *Pemt*$^{-/-}$/*Mdr2*$^{-/-}$ mice. Thus, the liver failure in *Pemt*$^{-/-}$ mice appears to be due to a loss of membrane integrity caused by a decrease in the PC/PE ratio of the plasma membrane. Furthermore, manipulation of the PC/PE ratio suggested that this parameter plays a key role in maintaining cell membrane integrity and

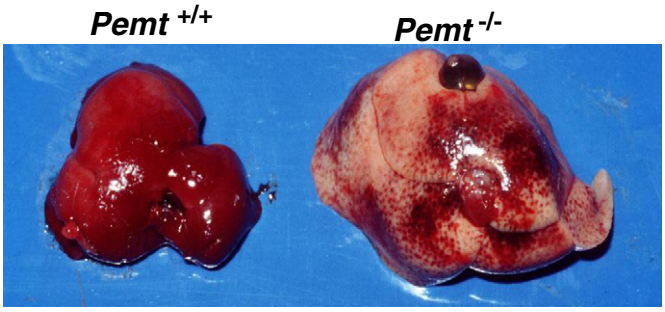

Fig. 8. Photograph of livers from *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice that consumed a choline-deficient diet for 3 days. The gall bladder can be observed adjacent to the liver from the *Pemt*$^{-/-}$ mice. Taken from Fig. 2 in Vance, D.E. (2013) *Biochim. Biophys. Act.* 1831, 626–632.

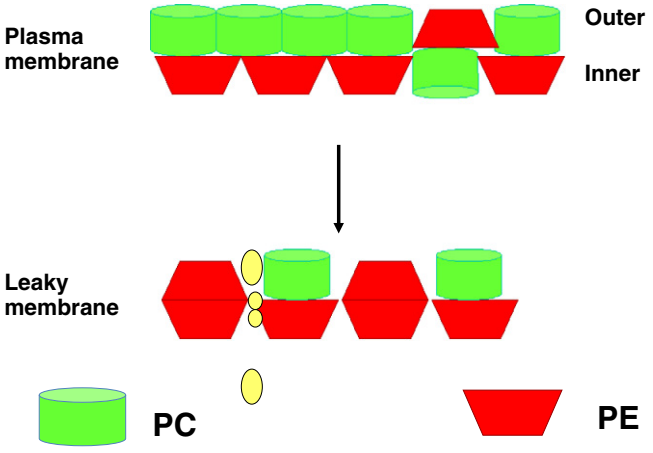

Fig. 9. Proposed model by which replacement of PC in the outer leaflet of the plasma membrane by PE could result in leakage of the membrane.

has a role in the progression of steatosis to steatohepatitis that was observed in *Pemt*$^{-/-}$ mice fed the CD diet for 3 days [59].

An abnormal ratio of PC/PE also can impair other aspects of liver function. In *Pemt*$^{-/-}$ mice, a decreased PC/PE ratio was associated with lower survival rates after partial hepatectomy [66]. Moreover, the ratio of PC to PE was inversely correlated with the development of non-alcoholic fatty liver disease (NAFLD) in mice fed a high fat diet. The reduction in survival after partial hepatectomy and the development of NAFLD were reversed when the PC/PE ratio was normalized by supplementation of the diet with additional choline. In contrast, the PC to PE ratio was increased in livers from obese mice that exhibited ER stress and impaired calcium homeostasis [67]. Moreover, when the activity of hepatic PEMT was attenuated by 50–70% by shRNA, the PC/PE ratio was normalized and calcium transport in the ER was improved. Thus, an optimal level of PC and PE appears to be required for normal hepatic function.

As mentioned in Section 5, the supply of AdoMet can modulate PE methylation. In recent experiments a very large (40-fold) increase in AdoMet stimulated the conversion of PE to PC [68]. This increase in AdoMet occurred in mice that lacked glycine *N*-methyltransferase, an enzyme that normally consumes large amounts of AdoMet. This large increase in AdoMet stimulated the conversion of PE to PC via PEMT. Moreover, the increased activity of PEMT enhanced the hepatic secretion of PC into plasma lipoproteins and stimulated the conversion of PEMT-derived PC to triacylglycerol (TG) leading to steatosis. Supplementation of the mice lacking glycine *N*-methyltransferase with a methionine-deficient diet normalized hepatic AdoMet levels and restored hepatic lipids to control levels [68].

PC can also be a source of hepatic TG as previously reported in rat hepatocytes [69]. More recent studies have demonstrated that PC from both low density and high density lipoproteins can be delivered to hepatocytes [70,71]. Much of this PC is converted into TG. A quantitative estimation of the amount of PC delivered from lipoproteins to the liver in mice suggests that ~50% of hepatic PC is derived from plasma lipoproteins, and remarkably that 30% of this lipoprotein-derived PC is converted into TG [72]. Thus, lipoprotein derived PC is an important source of both PC and TG in the liver.

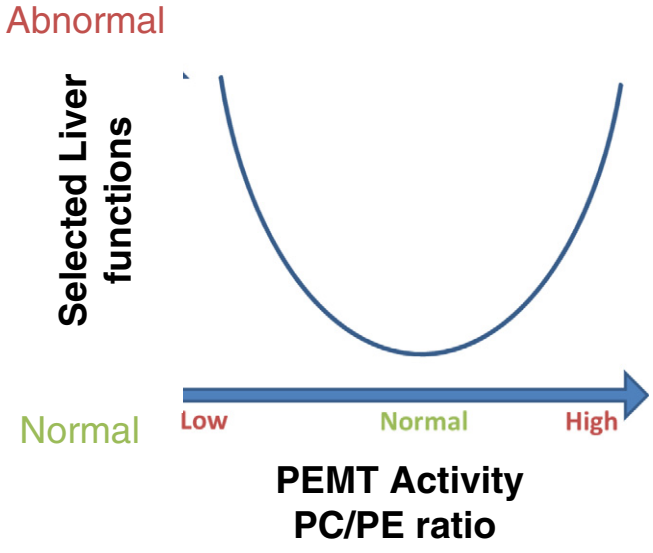

Fig. 10. Graph showing that selected liver functions can be altered when the activity of PEMT or the ratio of PC:PE is lower or higher than normal. Liver functions disturbed at low PC/PE ratio include maintenance of plasma membrane integrity, biosynthesis and secretion of lipoproteins, triacylglycerol homeostasis, biosynthesis of PC and choline. A high ratio of PC/PE can increase triacylglycerol formation, and endoplasmic reticulum stress, and impair calcium homeostasis.

In summary, many hepatic functions appear to be responsive to the activity of PEMT and the ratio of PC to PE in the plasma membrane (Fig. 10). A low PC:PE ratio can adversely affect membrane permeability and decrease lipoprotein secretion (discussed in Section 6.2), whereas abnormally high ratios of PC:PE can lead to steatosis, abnormal calcium homeostasis, ER stress and enhanced lipoprotein secretion.

6.2. PEMT, lipoprotein secretion and atherosclerosis

The requirement for PEMT activity for normal VLDL secretion was first suggested by experiments in primary rat hepatocytes that were incubated for up to 16 h in a medium deficient in methionine and/or choline [73]. When the culture medium lacked both methionine and choline, TG accumulated in the hepatocytes and VLDL secretion was diminished. Supplementation of the hepatocytes with either methionine or choline normalized lipoprotein secretion. Thus, the active synthesis of PC via either pathway satisfied the requirement in cultured rodent hepatocytes for PC for secretion of VLDL. Subsequently, it was demonstrated that VLDL secretion was defective in *Pemt*$^{-/-}$ mice [44,74,75] even though the choline pathway was active. Similarly, the secretion of VLDL from mice that lacked hepatic CT was also attenuated although the PEMT pathway was present [76]. At this juncture it appears that *both* pathways are required for normal hepatic VLDL secretion in mice. Why both pathways are needed is not clear. However, a requirement for both pathways is consistent with the finding that specific pools of PC are used for VLDL secretion from rat hepatocytes [77]. The secretion of PC into VLDL was lower when the PC was derived from PE made via the ethanolamine pathway whereas, PC derived from PE originating from phosphatidylserine decarboxylation was preferentially used for VLDL secretion. Phosphatidylserine-derived PE originates in mitochondria [78] and some PEMT activity is associated with mitochondria associated membranes (MAM) [19] that appear to be involved in VLDL secretion [79]. Thus, it is possible that the proximity of PC biosynthesis in the MAM with VLDL assembly in the ER/MAM lumen might explain the preferential utilization of PEMT-derived PC for VLDL secretion.

In 1954 a possible relationship between phospholipid methylation and atherosclerosis was first reported [80]. The availability of *Pemt*$^{-/-}$ mice permitted us to determine if indeed these mice were protected from atherosclerosis. C57Bl6 mice are resistant to diet-induced atherosclerosis. Hence, studies on atherosclerosis are usually performed with gene-targeted mice that are prone to this disease. Two commonly used models are: LDL receptor-deficient (*Ldlr*$^{-/-}$) mice that are fed a high-fat, high-cholesterol diet, and chow-fed mice that lack apolipoprotein E (*Apoe*$^{-/-}$) [81]. We, therefore, crossed *Pemt*$^{-/-}$ mice with mice of each of these two genetic backgrounds.

PEMT deficiency strikingly protected *Ldlr*$^{-/-}$ mice from atherosclerosis. Compared to *Pemt*$^{+/+}$/*Ldlr*$^{-/-}$ mice fed the high-fat, high-cholesterol diet for 16 weeks, *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ mice developed ~80% fewer atherosclerotic lesions (Fig. 11) [82]. This finding was consistent with the atheroprotective plasma lipoprotein profile in which VLDL/LDL lipids were significantly lower in the *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ mice (plasma TG was lower by ~70%, cholesterol by ~56%, PC by ~34% and cholesteryl esters by ~69%) than in *Ldlr*$^{-/-}$ mice [82]. The levels of plasma apo B100 and apo B48 were also lower in the *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ mice by 30% and 60%, respectively. The clearance of VLDL from *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ mice was more rapid than from *Pemt*$^{+/+}$/*Ldlr*$^{-/-}$ mice, probably due to alterations in VLDL structure caused by an altered lipid content. Thus, the diminished atherosclerosis observed in *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ mice compared to *Pemt*$^{+/+}$/*Ldlr*$^{-/-}$ mice is due to impaired PC biosynthesis in the liver that results in reduced secretion of PC-deficient VLDL that are more rapidly cleared compared to the VLDL produced by *Pemt*$^{+/+}$/*Ldlr*$^{-/-}$ mice.

PEMT deficiency in *Apoe*$^{-/-}$ mice fed a chow diet for 1 year also provided protection from atherosclerosis [83]. In this study, echocardiography demonstrated that systolic function was improved by ~50% in the *Pemt*$^{-/-}$/*Apoe*$^{-/-}$ mice compared to *Pemt*$^{+/+}$/*Apoe*$^{-/-}$ mice. Moreover, TG content of the hearts of *Apoe*$^{-/-}$ mice that lacked PEMT was

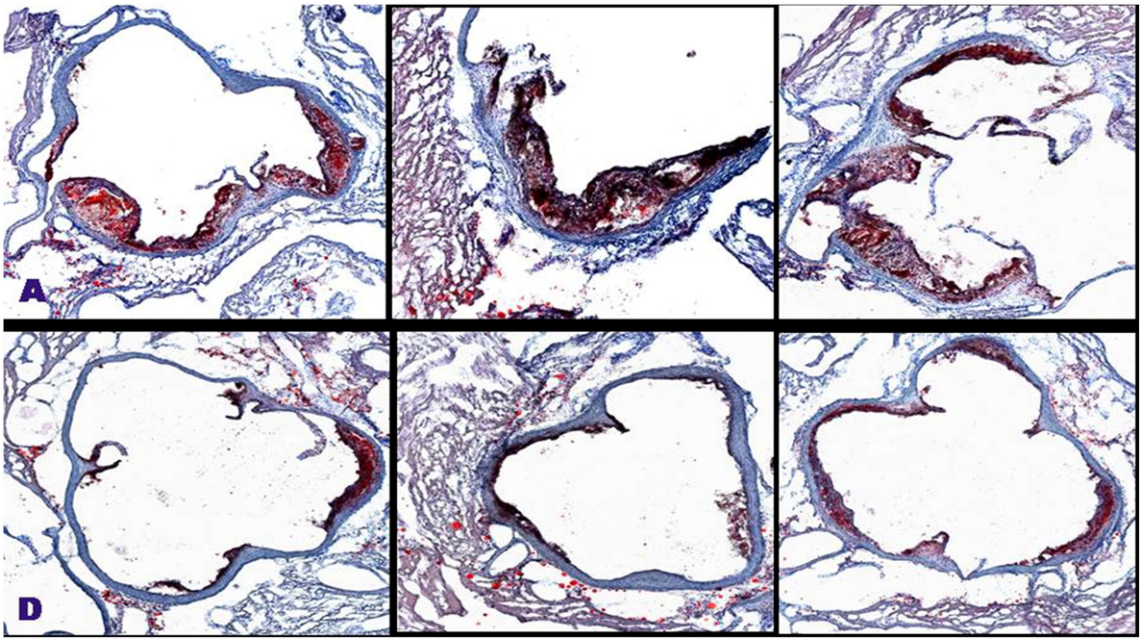

Fig. 11. Atherosclerosis in aortic root regions from *Pemt*$^{+/+}$/*LDLr*$^{-/-}$ mice (top 3 panels) and *Pemt*$^{-/-}$/*LDLr*$^{-/-}$ mice (bottom 3 panels). Staining of the plaques with Oil Red O (red) and hematoxylin (light blue). Taken from Fig. 3 in Vance, D.E. (2013) *Biochim. Biophys. Act.* 1831, 626–632.

significantly lower (by 34%) than in hearts of *Pemt*$^{+/+}$/*Apoe*$^{-/-}$ mice. Thus, our data suggest that disruption of the *Pemt* gene decreases atherosclerosis and attenuates the development of a lipotoxic cardiomyopathy in mice that lack apo E.

6.3. Lack of PEMT protects against obesity and insulin resistance

A possible relationship between PEMT and obesity was not considered until *Pemt*$^{-/-}$ mice were fed a high-fat diet. *Pemt*$^{+/+}$ mice gained ~10 g over 10 weeks when fed a high-fat diet, whereas *Pemt*$^{-/-}$ mice gained little or no weight (Fig. 12) [84]. The *Pemt*$^{-/-}$ mice were also protected against insulin resistance that developed in the *Pemt*$^{+/+}$ mice fed the high-fat diet [84]. On the other hand, the *Pemt*$^{-/-}$ mice developed fatty liver (TG in *Pemt*$^{+/+}$ mice was 184 μg/mg protein compared to 790 μg/mg protein in *Pemt*$^{-/-}$ mice). Moreover, in the *Pemt*$^{-/-}$ mice, plasma ALT (a marker for liver disease) was 10-fold higher than in *Pemt*$^{+/+}$ mice (71.2 units/l compared to 7.2 units/l).

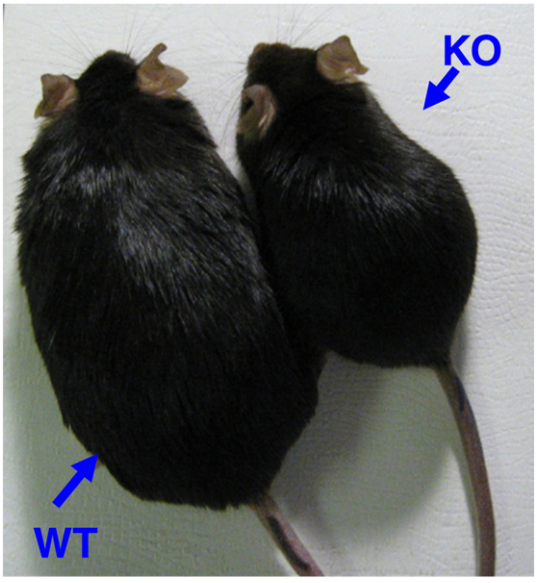

Fig. 12. Photograph of *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice fed a high-fat diet for 10 weeks. WT, *Pemt*$^{+/+}$; KO, *Pemt*$^{-/-}$. Taken from Fig. 4 in Vance, D.E. (2013) *Biochim. Biophys. Act.* 1831, 626–632.

How does the lack of PEMT protect against obesity and insulin resistance (type 2 diabetes)? One possible explanation that was considered was that the lower level of hepatic PC in *Pemt*$^{-/-}$ mice (57 nmol/mg protein) compared to 76 nmol/mg protein in *Pemt*$^{+/+}$ mice might lead to decreased obesity and increased insulin sensitivity. Hence, we determined if reduction of hepatic PC biosynthesis in liver-specific CTα knockout (LCTαKO) mice also protected mice from obesity/insulin resistance. This was a tenable hypothesis since the CT pathway accounts for ~70% of hepatic PC biosynthesis. Hepatic CT activity was decreased by 85% in LCTαKO mice compared to control mice [76]. However, both the control mice and the LCTαKO mice gained a similar 10 g of weight when fed the high fat diet for 10 weeks [84]. Furthermore, the consumption of oxygen and glucose tolerance tests was the same in the control and LCTαKO mice. Thus, diminished PC biosynthesis in the liver does not appear to be a direct factor in protecting *Pemt*$^{-/-}$ mice from obesity/insulin resistance.

As mentioned above, PEMT makes PC from PE; degradation of the PC made by the PEMT pathway results in de novo formation of choline. Thus, LCTαKO mice (that contain PEMT) can biosynthesize choline whereas *Pemt*$^{-/-}$ mice cannot. The high-fat diet normally contains 1.3 g of choline/kg diet. Hence, wildtype mice fed this diet are not choline-deficient. However, we considered the possibility that the *Pemt*$^{-/-}$ mice lacked sufficient choline. We, therefore, supplemented the high-fat diet with an additional 2.7 g of choline/kg diet and found that the protection against obesity and insulin resistance induced by PEMT deficiency was abolished. Thus, *Pemt*$^{-/-}$ mice fed the high-fat/high-choline diet gained weight, normalized hepatic PC, developed glucose intolerance and showed other metabolic features characteristic of *Pemt*$^{+/+}$ mice fed the high-fat diet [84]. Hence, deficiency of PC in the liver did not provide the protection observed in *Pemt*$^{-/-}$ mice fed the high-fat diet. In contrast, the deficiency of choline biosynthesis provided the beneficial effect. Moreover, when *Pemt*$^{+/+}$ mice were fed a choline-deficient diet for 12 weeks, these mice were also protected from diet-induced obesity/insulin resistance [84]. These data are consistent with the finding that when obese mice were fed either a CD or a choline-

supplemented/high fat diet for 4 weeks, insulin resistance was lower in the CD mice [85]. Furthermore, when male ob/ob mice (that lack leptin and become obese) were fed a high-fat diet ± choline for two months, the choline-deficient mice gained less weight and exhibited improved insulin and glucose tolerance compared to the choline-supplemented mice [86].

What is the mechanism by which a decreased supply of choline produces an anti-obesity/anti-insulin resistance phenotype in mice? One clue is that the levels of glucagon are lower in *Pemt*$^{-/-}$ mice fed the high-fat diet than in *Pemt*$^{+/+}$ mice [87]. When the diet was supplemented with extra choline, glucose and insulin intolerance in the *Pemt*$^{-/-}$ mice and plasma glucagon were normalized to the level in *Pemt*$^{+/+}$ mice. Moreover, a glucagon receptor antagonist (2-aminobenzimidazole) blunted the choline effects. Thus, it seems likely that choline induces glucose and insulin intolerance in *Pemt*$^{-/-}$ mice via modulation of plasma glucagon and its action in liver. Nevertheless, a great deal more research is required to understand how choline modulates glucagon levels and reverses the beneficial phenotype of *Pemt*$^{-/-}$ mice.

6.4. PEMT and lipid droplets

While current data strongly suggest that the liver is involved in protecting against atherosclerosis/obesity/insulin resistance in *Pemt*$^{-/-}$ mice fed the high-fat diet, studies with 3T3-L1 adipocytes suggest that PEMT in adipose tissue might also be involved in regulating some of these phenotypes [88]. PEMT protein/activity was not detected in 3T3-L1 fibroblasts. However, 4 days after the cells were differentiated into adipocytes, PEMT was expressed. Subsequent experiments demonstrated that PEMT had an important role in the stabilization of lipid droplets in 3T3-L1 adipocytes [88]. Hence, although the activity of PEMT is relatively low in adipose tissue compared to liver, the conversion of PE to PC might be important for normal fat deposition in adipocytes. Thus, lack of PEMT in adipose tissue from *Pemt*$^{-/-}$ mice might contribute to the lower fat mass in mice fed the high-fat diet for 10 weeks.

6.5. PEMT regulation of plasma homocysteine levels

Homocysteine (Hcy) is a non-protein amino acid that is a catabolic product of AdoHcy generated by transmethylation reactions in which AdoMet is the methyl donor [89]. Mild hyper-homocysteinemia is an independent risk factor for atherosclerosis and cardiovascular diseases [89–91]. Even a 50% higher-than-normal level of plasma Hcy has been linked to a 60% increase in risk of coronary artery disease for men, and 80% increase for women. A significant contributor of plasma Hcy is hepatic AdoHcy generated by the 3 methylation reactions catalyzed by PEMT during the conversion of PE to PC. Thus, the plasma levels of Hcy are 50% lower in *Pemt*$^{-/-}$ mice than in *Pemt*$^{+/+}$ mice and hepatocytes isolated from *Pemt*$^{-/-}$ mice secrete 50% less Hcy than do *Pemt*$^{+/+}$ hepatocytes [92]. Furthermore, the culture medium of McArdle RH7777 hepatoma cells stably transfected with a cDNA encoding PEMT contained 3 times as much Hcy after 24 h compared to hepatoma cells transfected with vector alone [92]. We also studied Hcy formation in mice that lacked hepatic CTα (LCTαKO). In these mice, PEMT activity is double that in livers of mice that express CTα [76,93], and plasma Hcy is 20–40% higher in the LCTαKO mice than in control mice [93]. Moreover, hepatocytes from LCTαKO mice secrete 40% more Hcy than do control hepatocytes. Consequently, it seems likely that PEMT is important not only for producing PC in the liver but is also a significant source of plasma Hcy, as well as choline, in mice.

7. PEMT in humans

Biochemical studies on humans are always a challenge but the ease of DNA sequencing has allowed some progress. Zeisel and co-workers found that a Val-to-Met substitution at residue 175 of the human PEMT protein is associated with non-alcoholic fatty liver disease [94]. When cDNAs encoding either the Val-175 isoform or the Met-175 form of PEMT were expressed in McArdle hepatoma cells, the Met isoform of PEMT had ~40% lower specific activity than did Val-175 PEMT [94]. When the polymorphism for residue 175 was examined in 59 control subjects and 28 humans with non-alcoholic liver disease, Met/Met at residue 175 occurred in 68% of the subjects with non-alcoholic liver disease but in only 41% of subjects without non-alcoholic liver disease. Thus, the V175M polymorphism was suggested to confer susceptibility to non-alcoholic liver disease in humans. In agreement with this conclusion, the V175M variant is more frequent in patients with non-alcoholic steatohepatitis than in healthy subjects [95]. However, no association between the V175M mutation and steatosis was observed in the Dallas Heart study [96]. When only Caucasians were considered in the Dallas Heart study a correlation between the V175M polymorphism and liver disease was, however, detected [97]. It is possible that the V175M polymorphism is associated with fatty liver only if the secretion of TG into plasma VLDL is decreased and/or TG synthesis is increased [97].

A G to C polymorphism was detected in the promoter region of the *PEMT* gene [98]. Of the 23 subjects carrying the C allele, 18 developed "organ dysfunction" when fed a low choline diet. A gene dose–response relationship was suggested between the C allele and organ dysfunction in subjects fed a very-low-choline diet. In pre-menopausal women, those with two C alleles were more likely to develop organ dysfunction than were women with one or no C alleles [99]. Moreover, post-menopausal women who received estrogen were less likely to exhibit organ dysfunction when fed a low-choline diet [99]. Thus, it appears that post-menopausal women with low estrogen levels have a higher dietary requirement for choline than do pre-menopausal women. Moreover, the C allele in the PEMT gene increases the requirement for choline in both groups of women [99].

8. What next?

Since we have not been very successful in the past in predicting the future of PEMT research, perhaps we should just write "whatever will be, will be; the future is not ours to see". On the other hand, while we can expect the unexpected, there are a few clear directions for PEMT research in the future. First, what needs to be explained is the mechanism(s) by which large supplements of choline can override the protection from obesity/insulin resistance observed in *Pemt*$^{-/-}$ mice fed a high-fat diet. Second, it is possible that pharmaceutical inhibition of PEMT might protect humans from atherosclerosis, obesity and type 2 diabetes. Nevertheless, at this juncture, development of PEMT as drug target has some challenges because *Pemt*$^{-/-}$ mice fed a high-fat diet exhibit steatosis/steatohepatitis [84]. Thus, it will be important to determine if inhibition of PEMT results in steatosis in other animal models, such as the guinea pig or hamster, that have a lipid metabolic profile more similar to that of humans. If steatosis occurred in these animals, we would need to think creatively about mechanisms by which steatosis might be relieved when PEMT is inhibited.

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