Biochemical and Evolutionary Significance of Phospholipid Methylation*

(Received for publication, August 7, 1998, and in revised form, August 27, 1998)

Christopher J. Walkey§, Liqing Yu, Luis B. Agellon¶, and Dennis E. Vance†
From the Lipid and Lipoprotein Research Group and the Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2S2, Canada

All nucleated mammalian cells synthesize phosphatidylcholine from choline via the CDP-choline pathway. Hepatocytes have a second pathway for the synthesis of phosphatidylcholine, a stepwise methylation of phosphatidylethanolamine, catalyzed by phosphatidylethanolamine N-methyltransferase and encoded by the Pempt gene. We report that when Pempt-deficient mice were fed a choline-deficient diet for 3 days, severe liver pathology occurred apparently due to a lack of phosphatidylcholine biosynthesis. The hepatic concentration of phosphatidylcholine decreased by 50% compared with wild type mice on the diet. The levels of plasma triacylglycerols and cholesterol were decreased by greater than 90% in the Pempt-deficient mice. We suggest that the Pempt gene has been maintained during evolution to provide phosphatidylcholine when dietary choline is insufficient, as might occur during starvation or pregnancy.

Hepatocytes in liver have abundant activity for the phosphatidylethanolamine N-methyltransferase (PEMT) pathway, whereas other cells and tissues express minimal activities for this enzyme (1–4). Since the liver possesses the CDP-choline pathway for PC biosynthesis (5), why PEMT has been retained during evolution is not obvious. One clue to the importance of PEMT function arises from studies on choline deficiency. When fed a choline-deficient (CD) diet, rats survive with minimal adverse effects, but develop fatty livers (6). Therefore, PEMT might function to maintain PC synthesis and generate choline when dietary supply is insufficient. However, the quantitative significance of the PEMT pathway for production of choline as a catabolic product of PC is unclear, and an alternative pathway for synthesis of choline in mammals has been described in which phosphoethanolamine is methylated to yield phosphocholine (7–9).

To gain insight into the physiological significance of PE methylation, we created mice that completely lacked PEMT activity by targeted disruption of the Pempt gene (10). These studies demonstrated that both hepatic isoforms of PEMT, PEMT1 localized to the endoplasmic reticulum and PEMT2 recovered on the mitochondria associated membrane, were encoded by the same gene and absent from Pempt-/- mice. Under standard laboratory conditions, PEMT-deficient mice are indistinguishable from their normal and heterozygous littermates with respect to development, behavior, and fecundity. Microscopic examination of liver slices from 8-week-old mice revealed no obvious histological abnormalities. No significant differences in the composition of plasma lipoproteins or bile, both of which contain large amounts of PC, were observed. Hepatic phospholipid composition, including the level of PC, was minimally affected by the absence of PEMT. Thus, apparently, the PC generated via the CDP-choline pathway can replace PEMT-derived PC, revealing no obvious physiological role for PE methylation under normal conditions.

An important test of the function of the PEMT pathway would be to restrict dietary choline and thereby decrease PC production via the CDP-choline pathway. Would the other pathways that have been reported to make phosphocholine (7–9) compensate for the lack of the PEMT pathway? We now report that severe liver pathology rapidly occurs in PEMT-deficient mice after withdrawal of dietary choline for only 3 days.

EXPERIMENTAL PROCEDURES

Care and Feeding of Mice—The genotypes of the mice were determined by DNA blotting as described (10). Mice of all three genotypes (Pempt+/+, Pempt-/-, and Pempt+/M) had a mixed 129/Sv and C57Bl/6 genetic background. The colony was maintained on a reversed 12-h light/dark cycle. At 8 weeks of age, mice of both sexes were switched to either a CD semisynthetic diet (ICN, catalog number 901387) or a choline-supplemented (CS) diet (CD diet + 0.4% choline chloride) and fed ad libitum for 60 h, then fasted for 12 h before sacrifice.

Histological Analysis—Liver pieces were fixed in 10% formalin, and sections were stained with hematoxylin and eosin according to standard methods. Alternatively, liver pieces were fixed by flash-freezing in liquid nitrogen. Frozen sections were stained with Oil Red O.

Measurement of Plasma Aminotransferases and Bile Acids—Blood was extracted from the mice by cardiac puncture and plasma separated by centrifugation. All instruments were pretreated with EDTA to prevent coagulation. Plasma from mice fed either diet was pooled according to genotype and assayed for separate aminotransferase and alanine aminotransferase activities, using the GP and GO transaminase kit (Sigma-Aldrich). Bile acids in individual plasma samples were assayed using diagnostic kit 450-A (Sigma-Aldrich) adapted for microtiter plate analysis.

Analysis of Lipids in the Liver—Livers were homogenized with a motorized glass-Teflon homogenizer in 5 volumes of homogenizing buffer (11). Protein was determined using the BCA assay (Pierce) using bovine serum albumin as a standard. Lipids were extracted from the homogenates in chloroform-methanol (12) and separated by thin-layer chromatography. The bands corresponding to PC and triacylglycerol were scraped and assayed (13, 14), using inorganic phosphate and triolein as standards.

Analysis of Lipids in Plasma—the concentration of triacylglycerol in plasma was measured using a diagnostic kit (Sigma-Aldrich) adapted for microtiter analysis. Plasma samples from mice were pooled according to genotype and lipoproteins were separated by high-performance liquid chromatography on an Amersham Pharmacia Biotech Superox 6
column attached to a Beckman System Gold apparatus as described (15). The relative amounts of total glycerol (primarily derived from triacylglycerol) and total cholesterol were determined with an in-line enzymatic assay by mixing column effluent with assay reagents (SigmaAldrich diagnostic reagent 337-B for glycerol or reagent 352 for cholesterol) and incubation in a postcolumn reactor. Peaks were identified by comparison with lipoprotein standards.

RESULTS

Rapid and Severe Liver Pathology in PEMT-deficient Mice—To examine the importance of PE methylation for production of PC and choline (as a catabolic product of PC) in the absence of dietary choline, we fed mice of all three genotypes (Pempt+/+, Pempt+/−, and Pempt−/−) a CD diet or a CS diet. In Pempt−/− mice fed the CD diet, the net biosynthesis of PC could occur only from residual dietary choline already present in the animal or from endogenously synthesized choline (7–9). After 3 days, 8-week-old mice of all three genotypes fed the CS diet, and mice fed a CD diet and expressing PEMT activity (Pempt+/+ and Pempt−/+), exhibited a 5–10% weight gain. In contrast, Pempt−/− mice fed a CD diet lost between 5 and 10% of their total body weight and appeared indolent and uncoordinated. At necropsy the livers from the Pempt−/− mice were grossly enlarged (~50% by weight), with a distinct pale color. The gall bladders were also engorged (Fig. 1A). Other tissues were not visibly altered. The rapid development of the CD-mediated pathology in the Pempt−/− mice was such that the experiments had to be terminated after 3 days to avoid pain or death. Pempt+/+ and Pempt−/+ mice fed a CD diet displayed slight peripheral discoloration of the liver (Fig. 1A), whereas none of the murine genotypes fed a CS diet displayed any abnormal hepatic morphology.

Liver sections from mice with and without PEMT, fed CS and CD diets, were examined histopathologically. Livers from the Pempt−/− mice fed a CD diet displayed severe vacuolization (Fig. 1D). Much less vacuolization was observed in Pempt+/+ and Pempt−/+ animals fed the CD diet (Fig. 1, B and C). No vacuolization was observed in the livers of any mice fed a CS diet including the Pempt−/− mice (Fig. 1E). Staining of frozen liver sections with lipophilic Oil Red O confirmed the presence of lipids in vacuoles of hepatocytes in all mice fed a CD diet (data not shown).

Alanine aminotransferase and aspartate aminotransferase normally reside in hepatocytes but leak into the bloodstream upon liver injury. Therefore, the activities of these enzymes in the plasma is a useful indicator of hepatic damage (16). The level of alanine aminotransferase activity in pooled plasma was increased 12.5-fold in Pempt−/− mice on a CD diet compared with normal mice on the same diet (Table I). Pempt−/− mice fed a CS diet had an undetectable plasma alanine aminotransferase activity. A similar pattern was observed for aspartate aminotransferase (Table I). The level of bile acids in the plasma, another indicator of liver damage, was elevated 5.6-fold in Pempt−/− mice on a CD diet compared with normal mice on the same diet (Table I). Pempt−/− mice fed a CD diet compared with Pempt+/+ mice on a CD diet (Table I). Moreover, the plasma of Pempt−/− mice fed the CD diet was bright yellow, indicating jaundice. The challenge of PEMT-deficient mice with the CD diet resulted in severe liver damage and clearly demonstrated the importance of PC biosynthesis for proper maintenance of liver function. The presence of low levels of aminotransferases and bile acids in plasma from Pempt+/+ mice on the CD diet is consistent with the observed death of cells in livers from rats fed a CD diet (17, 18).

Analysis of the hepatic lipids by thin-layer chromatography revealed that the only lipid accumulating in livers of Pempt−/− mice was triacylglycerol. The level of triacylglycerol in the livers from Pempt−/− mice fed a CD diet was increased 3.9-fold (p < 0.05) compared with Pempt−/− mice fed a CS diet (Fig. 2A). Significant amounts of triacylglycerol did not accumulate in the livers of Pempt+/+ mice fed a CD diet compared with a CS diet even though the gross liver morphology and histology indicated what appeared to be fat deposits (Fig. 1, A and B). This result contrasted with previous studies in which hepatic triacylglycerol accumulated by 6.5-fold in rats fed the CD diet.

| Table I | Choline deficiency in Pempt−/− mice elevates aminotransferases and bile acids in plasma |
|---------|-----------------------------------------------|
| Murine plasma was pooled according to genotype (+/+ , +/− , and −/−) and diet, and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were assayed. Plasma bile acids were also measured. ND = not detectable. |
| **Choline-supplemented diet** | **Choline-deficient diet** |
| (+/+) | (+/+) | (+/−) | (−/−) |
| ALT (IU/l) | ND | ND | ND | 33 | ND | 417 |
| AST (IU/l) | <1 | <1 | <1 | 25 | 33 | 150 |
| Bile acids (μM) | <1 | <1 | <1 | 24 ± 9 | 39 ± 13 | 138 ± 43 |
human livers. The hepatic morphology of the latter animals also did not exhibit signs of cell death, which was attributed to massive accumulation of lipid (21). The press a truncated sterol response element binding protein-1a, distinct from that observed for livers from mice that overexpression of hepatic triacylglycerol. *, p < 0.05 for Pempt<sup>−/−</sup> mice on the CD diet compared with the CS diet. The rise in bile acid concentration in the plasma of Pempt<sup>−/−</sup> mice fed a CD diet is not solely a consequence of fat accumulation. Moreover, the gross liver morphology of the Pempt<sup>−/−</sup> mice on a CD diet. The error bars represent S.D. and are too small to be seen for the Pempt<sup>+/+</sup> mice on a CS diet. Statistical analyses were performed with a Student's t test. B, hepatic PC. *, p < 0.05 compared with Pempt<sup>+/+</sup> and Pempt<sup>−/−</sup> mice on a CD diet.

FIG. 2. Hepatic triacylglycerol and phosphatidylcholine content of Pempt<sup>+/+</sup>, Pempt<sup>−/−</sup>, and Pempt<sup>−/−</sup> mice fed either the choline-supplemented (CS) or the choline-deficient (CD) diet. A, hepatic triacylglycerol. *, p < 0.05 for Pempt<sup>−/−</sup> mice on the CD diet compared with the CS diet. p = 0.065 between Pempt<sup>−/−</sup> and Pempt<sup>−/−</sup> mice on the CD diet. p < 0.006 for Pempt<sup>−/−</sup> mice compared with Pempt<sup>+/+</sup> mice on a CS diet. The error bars represent S.D. and are too small to be seen for the Pempt<sup>−/−</sup> mice on a CS diet. Statistical analyses were performed with a Student's t test. B, hepatic PC. *, p < 0.05 compared with Pempt<sup>+/+</sup> and Pempt<sup>−/−</sup> mice on a CD diet.

amount of hepatic PC was even more dramatically decreased (56.4%) in Pempt<sup>−/−</sup> mice (from 63.3 on the CS diet to 27.6 nmol/mg of protein on the CD diet) (Fig. 2B).

Plasma Lipoproteins Are Greatly Decreased in PEMT-deficient Mice—PC is an important component of lipoproteins, forming a monolayer around the neutral lipid core of the particles, enhancing their solubility in plasma. The fatty liver observed in rats fed a CD diet could be, at least partially, ascribed to a defect in secretion of triacylglycerol-rich very low density lipoproteins (VLDL) from the liver, resulting in hepatic triacylglycerol accumulation (22, 23). Plasma VLDL from Pempt<sup>−/−</sup> mice contained less triacylglycerol than did VLDL from wild type mice (Fig. 3A), a result that matches the total plasma triacylglycerol values. The amount of plasma triacylglycerol in Pempt<sup>−/−</sup> mice on the CS diet was 43.6 ± 12.3 mg/dl, and the values for Pempt<sup>+/+</sup> and Pempt<sup>−/−</sup> mice on a CS diet, and of Pempt<sup>−/−</sup> mice on a CD diet, were similar. The values for plasma triacylglycerol for Pempt<sup>−/−</sup> mice (30.2 ± 8.0 mg/dl) and Pempt<sup>−/−</sup> mice (28.6 ± 7.3 mg/dl) on the CD diet were significantly different (p < 0.05) from the Pempt<sup>+/+</sup> mice on a CD diet. Pempt<sup>−/−</sup> mice contained very little triacylglycerol in the VLDL fraction. Instead, triacylglycerol was detected in a more dense fraction of lipoproteins having the same density as low density lipoproteins. Whether or not the reduction in circulating triacylglycerol-rich lipoproteins is due to decreased VLDL secretion from the liver or increased clearance of lipoproteins is not known.

As in Pempt<sup>+/+</sup> mice, the majority of plasma cholesterol in Pempt<sup>−/−</sup> mice fed the CD diet is found in high density lipoprotein (HDL) (Fig. 3B). Pempt<sup>−/−</sup> mice had greatly reduced levels of HDL cholesterol, which might be due to defective formation of nascent HDL as a result of decreased availability and/or

C. J. Walkey, L. Yu, L. B. Agellon, and D. E. Vance, unpublished results.
periods of starvation and, therefore, experience choline deficiency. These results also prove that no alternative of PE methylation greatly amplifies the consequences of choline deficiency. The current experiments show that the elimination of PE methylation is important for provision of adequate PC for crucial metabolic processes. The rapid onset of damage to the liver, but not to other tissues, can be ascribed to the high demand for choline and PC for normal hepatic function. Haptic choline can be phosphorylated and enter the CDP-choline biosynthetic pathway or, in liver, can be oxidized to betaine, which is an important source of methyl groups for other metabolic pathways (25). The liver also secretes lipoproteins and bile, which require large amounts of PC as the major phospholipid. For example, the amount of biliary PC that is secreted from the liver is ~23 mg/day for a 20 g mouse (26). The weight of liver in a 20-g mouse is ~2 g, and the total amount of PC in liver would be ~20 mg. Apparently, the mouse liver secretes into bile the equivalent of its entire hepatic pool of PC during a 24-h period. Some of that PC is reabsorbed by the intestine as lyso-PC and is returned to the liver and other tissues (26). Hence, the utilization of PC for bile secretion might be a major reason for the 50% decline in PC levels in liver after only 3 days. In addition, secretion of PC with lipoproteins and oxidation of choline to betaine will also impact on the PC concentrations in liver.

The evolutionary pressure to retain PEMT in liver probably derives from its importance for this enzyme during starvation and during pregnancy/lactation. Humans and other animals store fat and glycogen as energy sources but do not store phospholipids. The concentration of plasma choline in healthy humans decreased from 9.5 ± 0.5 μmol/liter to 7.8 μmol/liter (p < 0.01) after a 7 day fast (27). When animals undergo periods of starvation and, therefore, experience choline deficiency, the function of the Pempt gene is likely to become important for provision of adequate PC for crucial metabolic processes. The current experiments show that the elimination of PE methylation greatly amplifies the consequences of choline deficiency. These results also prove that no alternative pathway, other than phospholipid methylation or the CDP-choline pathway, is of quantitative significance for generating PC or choline in mice. Thus, the decreased PC biosynthesis in Pempt−/− mice fed the CD diet results in the depletion of hepatic PC which rapidly leads to liver failure. We hypothesize that a key function of PE methylation is to sustain both hepatic synthesis of PC and production of the choline moiety derived from PC catabolism during starvation.

Several years ago Zeisel et al. (28) reported that pregnancy and lactation put a severe stress on the levels of choline-containing compounds in the female rat liver. For example, during pregnancy the concentrations of hepatic phosphocholine, betaine, and choline decreased by 50% or more in CD compared with CS rats. The decrease in these metabolites was even more striking in lactating animals. Even though PEMT deficiency does not decrease fecundity of these mice under laboratory conditions, most likely the possession of PEMT would be a significant advantage for survival of mother and pups, especially if choline intake were inadequate.

We conclude that PEMT provides PC and choline when dietary choline is insufficient. The PEMT-deficient mice are also a valuable model for generating liver dysfunction, since the condition is experimentally controllable and, therefore, will allow the onset of disease to be precisely defined.

Acknowledgments—We thank Sandra Ungarian, Ross Waite, and Dr. P. N. Nation for technical assistance. We are grateful to Drs. Jean Vance and Steven Zeisel for helpful comments and discussion.

REFERENCES

1. Bremer, J., and Greenberg, D. M. (1960) Biochim. Biophys. Acta 37, 173–175
2. Bremer, J., Figard, P. H., and Greenberg, D. M. (1960) Biochim. Biophys. Acta 43, 477–488
3. Vance, D. E., and Ridgway, N. D. (1988) Prog. Lipid Res. 27, 61–79
4. Vance, D. E., Walkey, C. J., and Cui, Z. (1997) Biochim. Biophys. Acta 1348, 142–150
5. Kennedy, E. P. (1989) in Phosphatidylcholine Metabolism (Vance, D. E., ed) pp. 1–8, CRC Press, Boca Raton, FL
6. Zeisel, S. H. (1990) J. Nutr. Biochem. 1, 332–349
7. Andriamampandry, C., Freysz, L., Kanfer, J. N., Dreyfus, H., and Massarelli, R. (1989) Biochem. J. 264, 555–562
8. Andriamampandry, C., Freysz, L., Kanfer, J. N., Dreyfus, H., and Massarelli, R. (1991) J. Neurochem. 56, 1845–1850
9. Andriamampandry, C., Massarelli, R., and Kanfer, J. N. (1992) Biochem. J. 288, 267–272
10. Walkey, C. J., Donohue, L. R., Bronson, R., Agellon, L. B., and Vance, D. E. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12880–12885
11. Houweling, M., Cui, Z., Tessitore, L., and Vance, D. E. (1997) Biochim. Biophys. Acta 1346, 1–9
12. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–917
13. Barlelt, G. R. (1959) J. Biol. Chem. 234, 466–468
14. Snyder, F., and Stephens, N (1959) Biochim. Biophys. Acta 34, 244–245
15. Kieft, K. A., Bocan, T. M. A., and Krause, B. R. (1991) J. Lipid Res. 32, 859–866
16. Marshall, W. J. (1995) Clinical Chemistry, p. 231, Mosby, London
17. Shin, O. H. (1997) J. Cell Biochem. 64, 196–208
18. Albright, C. D., Liu, R., Bethea, T. C., Da Costa, K.-A., Salganik, R. I., and Vance, D. E. (1997) Biochim. Biophys. Acta 1338, 555–562
19. Yao, Z., and Vance, D. E. (1998) Biochem. J. 338, 65–70
20. Torchia, E. C., and Agellon, L. B. (1997) Eur. J. Cell Biol. 74, 190–196
21. Shimano, H., Horton, J. D., Hamner, R. E., Shimomura, I., Brown, M. S., and Goldstein, J. L. (1996) J. Clin. Invest. 98, 1575–1584
22. Haines, D. S. M., (1966) Can. J. Biochem. 44, 45–57
23. Yao, Z., and Vance, D. E. (1988) J. Biol. Chem. 263, 2998–3004
24. Glickman, R. M., and Sabesin, S. M. (1994) in The Liver: Biology and Pathobiology (Arias, I. M., ed) pp. 391–414, Raven Press, Ltd., New York
25. Zeisel, S. H., and Bluettajn, J. K. (1994) Annu. Rev. Nutr. 14, 209–296
26. Kuipers, F., Efferink, R. P. J. O., Verkade, H. J., and Groen, A. K. (1997) in Subcellular Biochemistry (Bittman, R., ed) Vol. 28, pp. 295–318, Plenum Press, New York
27. Savendahl, L., Mar, M.-H., Underwood, L. E., and Zeisel, S. H. (1997) Am. J. Clin. Nutr. 66, 622–625
28. Zeisel, S. H., Mar, M.-H., Zhou, Z., and da Costa, K. A. (1995) J. Nutr. 125, 3049–3054