Expression of Phosphatidylethanolamine N-Methyltransferase-2 Is Markedly Enhanced in Long Term Choline-deficient Rats*

(Received for publication, August 4, 1995, and in revised form, November 13, 1995)

Zheng Cui and Dennis E. Vance‡

From the Lipid and Lipoprotein Research Group and Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2S2, Canada

When rats are fed a choline-deficient (CD) diet, acute fatty liver develops along with other biochemical changes. However, when choline deficiency is prolonged, the growth rate of CD rats is similar to that of control rats fed a choline-supplemented diet. Furthermore, CD rats maintain their levels of choline-containing lipids, such as phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin. The mechanism for this compensation in CD rats was investigated. We screened the major tissues for the activities of two important enzymes involved in the biosynthesis of phosphatidylcholine, CTP:phosphocholine cytidylyltransferase (CT) and phosphatidylethanolamine N-methyltransferase (PEMT). Only the livers of CD rats had higher specific enzyme activities of PEMT and CT than control animals. The amount of PEMT2, one of two PEMTs in liver, increased 5-fold in CD rats after 6 weeks on the CD diet. A similar increase in the level of PEMT2 mRNA suggested that this activation was due to enhanced expression of the PEMT2 gene in CD livers. The labeling of phosphatidylcholine in isolated hepatocytes from CD rats was consistent with the conversion of PE to PC being increased as a result of a higher expression of liver PEMT. We conclude that activation of PE methylation at the level of gene expression may be the mechanism by which CD rats compensate for the lack of dietary choline.

The CDP-choline pathway for PC² biosynthesis is the major route for choline utilization in all eucaryotic cells. Moreover, the CDP-choline pathway is apparently vital for all mammalian cells (Eagle, 1955). A temperature-sensitive mutation in CT of the CDP-choline pathway of CHO cells leads to cell death at the restrictive temperature (Esko et al., 1981). The rate of PC synthesis is usually regulated at the second step of the pathway catalyzed by CT (Vance and Choy, 1979). CT can be regulated either by translocation of a soluble inactive form to an active membrane-bound enzyme (Vance, 1990a) or by gene expression (Tessner et al., 1991; Hwuveling et al., 1993).

In animals, choline is provided from either diet or endogenous synthesis. A normal diet contains enough choline to satisfy the normal growth of animals (Zeisel, 1981; Zeisel and Blusztajn, 1994). Choline and phosphocholine molecules are also generated endogenously from degradation of PC or other choline-containing lipids by phospholipases C and D. However, phospholipases C and D are part of a recycling pathway, and choline is not generated de novo. The only known endogenous pathway that synthesizes choline molecules with significant capacity is the PEMT pathway in animal liver (Vance and Ridgway, 1988). Methylation of phosphoethanolamine has been shown to occur in some tissues, but at a much lower level than the PEMT pathway in liver (Andriamampandry et al., 1989, 1992). Although tissues other than liver have measurable PEMT activity, these activities are less than a few percent of the activity in liver and are considered to be quantitatively insignificant.

At least two PEMTs that are immunologically distinct are present in rat liver (Cui et al., 1993). PEMT1 is apparently located on the endoplasmatic reticulum, whereas PEMT2 is exclusively associated with a unique liver membrane fraction, the mitochondria-associated membrane (Vance, 1990b; Cui et al., 1993). The cDNA for PEMT2 was cloned from a rat liver library and expressed in several cell types (Cui et al., 1993). The expressed PEMT2 catalyzes all three steps of PE methylation.

It is clear that dietary choline plays a critical role in animals (Zeisel, 1981; Zeisel and Blusztajn, 1994). However, it is not known if dietary choline and endogenously made choline have the same physiological roles. Nor is the contribution known for endogenous and exogenous choline to the total choline pool in animals. In the last few decades, the physiological role of dietary choline has been investigated in several animal models fed a CD diet and in animal cells cultured in CD media (for reviews, see Zeisel (1981) and Zeisel and Blusztajn (1994)). Deprivation of choline from the diet results in serious physiological changes. The rapid accumulation of triacylglycerol in the liver is the most pronounced among other changes in CD rats (Lombardi, 1971). Accumulation of diacylglycerol (Blusztajn and Zeisel, 1989) and activation of protein kinase C (da Costa et al., 1993) were also observed in CD rat livers. These biochemical changes are believed to be factors contributing to a higher incidence of hepatocarcinoma in CD rats in the absence of any known carcinogens (Chandar and Lombardi, 1988).

We were interested in the mechanism by which CD rats compensate for the loss of dietary choline. We hypothesized that the liver PE methylation pathway would be activated in rats fed a CD diet for more than 2 weeks so that more PC would be synthesized which subsequently would be a source of endogenous choline. The results show that CD rats used choline from an endogenous source to compensate for the loss of dietary choline. After 3 weeks on a CD diet, there was a gradual increase of PEMT2 protein (approximately 5-fold after 12 weeks) with a concomitant increase in PEMT2 mRNA. We postulate that the activation of the PEMT pathway as a source for endogenous choline may be responsible for the normal body.
weight gain and normal content of choline-containing lipids in CD rats.

**EXPERIMENTAL PROCEDURES**

Materials—All chemicals were purchased from Sigma unless noted otherwise. Radioactive isotoes and ECL detection kits for immunoblots were obtained from Amersham Corp. BCA protein assay kits were from Pierce Chemicals.

Animals and Care—Three-week-old male Sprague-Dawley rats, with an average weight of 40 g, were used to start the desired diets. The CD diet was purchased from ICN, and 0.4% choline chloride was added for the control diet (CS). Rats were provided with normal 12-h light-dark cycle and free access to the diets and drinking water.

Preparation of Protein Samples of Rat Tissues—The circulatory system was perfused with phosphate-buffered saline until the perfusates were clear. Tissues were dissected and rinsed with ice-cold phosphate-buffered saline. Tissues were cut into small pieces and homogenized in a Polytron homogenizer for 2 × 15 s in homogenization buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1 mM EDTA, 1 mM diethiothreitol, and 1 mM phenylmethylsulfonflyl fluoride). The homogenates were centrifuged for 5 min at 600 × g. The supernatants were collected and used as total cellular protein samples.

Immunoblots—The total liver homogenates from CD and CS rats were separated on SDS-polyacrylamide (12.5%) gels containing 0.1% SDS and transferred to nitrocellulose membranes (Laemmli, 1970; Towbin et al., 1979). The blots were probed with antibodies and immunoreactive material was detected by the ECL system. Rabbit anti-rat protein disulphide isomerase polyclonal antisera (1:1000 dilution) and the cDNA for this enzyme was a gift from Dr. M. Michalak (University of Alberta). The rabbit anti-rat liver PEMT2 antibody was described earlier (Cui et al., 1993).

Labeling of Lipids and Metabolites—Hepatocytes were isolated (Davis et al., 1979) and the cell plating density was 3 × 10⁶/dish (28 cm²). The labeled precursors were [³H]ethanolamine, 5 μCi/dish; [methyl-³H]methionine, 30 μCi/dish; and [methyl-²H]choline, 30 μCi/dish. The cells were plated for 4 h in Dulbecco’s modified Eagle’s medium that contained 17% fetal bovine serum ± 28 μM choline. The cells were subsequently pulsed with various labeled precursors for 8 h in the same medium. The cells were harvested, total lipids extracted (Folch et al., 1957), and phospholipids were separated on silica gel G thin-layer chromatography plates. The solvent system used was chloroform/methanol/acetic acid/formic acid/water at the ratio of 299:128/51/17/4.3 (v/v/v/v).

RESULTS

**Normal Weight Gain and Phospholipid Composition in Long Term Choline-deficient Rats**—CD rats had similar growth rates to control rats. Body weights of both CD and CS rats increased over 13-fold from 40 g at the beginning of the diet to 550 g after 12 weeks on the diets (Fig. 1). CD rats had slightly enlarged, fatty livers with yellow-brown color throughout the 12-week period. No other visible changes were detected in these rats.

Since dietary choline is considered an important source for the CDP-choline pathway of PC synthesis (Zeisel, 1981), we were curious if withdrawal of choline from the diet would result in any significant change in phospholipids. No significant differences occurred in the amounts of PE, phosphatidylethanolamine, phosphatidylcholine, lyso-PC, and sphingomyelin were found in CD compared with CS rats during 12 weeks on the diet (data not shown). Fig. 2 shows that the levels of PC in the major tissues of rats were similar during 12 weeks on CD and CS diets. Since the CD rats receive no choline in their diets, it would be impossible for these rats to maintain normal levels of PC without endogenous synthesis of choline. Since PE methylation is the only known pathway with significant capacity for synthesis of new choline molecules in the form of PC, we speculated that this pathway was activated in CD rats to maintain a normal body weight gain and unchanged levels of choline-containing lipids.

**Activation of Hepatic CT and PEMT in CD Rats**—All major tissues of CD and CS rats were screened for CT and PEMT activities so that we could determine which tissue(s) might be responsible for the increased endogenous synthesis of choline. No significant change of CT or PEMT activities was detected in any tissue except liver (Figs. 3-5). The specific activity of CT increased approximately 90% in the livers of rats fed the CD diet for 3 weeks (Fig. 3) and remained elevated through 12 weeks on the diet. Since CT was assayed at optimal concentrations of activating phospholipids (Yao et al., 1990), the increased CT activity might have been due to more CT protein.

Since an important mechanism for regulating CT activity is the translocation of the enzyme between soluble and particulate fractions, we measured the specific activity of CT in particulate fractions and decreased in soluble fractions during the first 6 weeks (Fig. 4). After 12 weeks, the distribution of CT returned to values similar to those obtained from CS rat livers.

The liver PEMT activity was increased throughout the study period in CD compared with CS rats (Fig. 5). This increase of hepatic PEMT activity suggested that liver may be primarily responsible for the synthesis of endogenous choline required in the CD rats. Since no specific antibody was available for PEMT1, we were unable to identify any possible change in this protein. However, the amount of PEMT2 protein in the livers of CD rats was almost 5 times that from CS rats after 12 weeks on the diet (Fig. 6). The equal loading of liver proteins was confirmed by probing the same blots with a specific antibody to protein disulfide isomerase which was present equally in all samples. The total cellular protein profiles of homogenates from CD and CS livers were also identical when examined by SDS-polyacrylamide gel electrophoresis (data not shown).
PEMT2 mRNA Was Also Increased in Choline-deficient Rat Livers—We analyzed the PEMT2 mRNA of CD and CS rat livers by Northern blotting experiments with the cDNA probe for PEMT2. The total mRNAs were normalized to equal levels of protein disulfide isomerase mRNA. The increases of PEMT2 mRNA were very similar to the changes of the PEMT2 protein in CD rat livers. There was a gradual increase of the PEMT2 mRNA from 3 to 12 weeks on CD diet (Fig. 7). This result suggested that the enhanced expression of the PEMT2 gene was responsible for the increased PEMT2 protein.

Increased Incorporation of Radioactivity into PC in CD Rat Hepatocytes—Hepatocytes were isolated from CD and CS rats and incubated with radioactive precursors of PC synthesis. When [methyl-3H]methionine was used, over 95% of the label in lipid was incorporated into PC and 5% was in lyso-PC and sphingomyelin. At the 3-week time point, labeling of PC by [methyl-3H]methionine was more than 2-fold higher in CD than in CS hepatocytes (Fig. 8). This increase was essentially sustained up to 12 weeks. The labeling of PC from [methyl-3H]methionine in CS hepatocytes was unchanged throughout the dietary period. These data are in agreement with the increased activity of PEMT in livers of rats fed the CD diet. Enhanced conversion of PE to PC was confirmed by [3H]ethanolamine labeling experiments (Fig. 8).

PC can be specifically labeled by [3H-methyl]choline via the CDP-choline pathway. As shown in Fig. 8, there was over a 2.5-fold increase of PC labeling by [methyl-3H]choline in CD hepatocytes at the 3-week time point. This increase was reduced at week 6 and further diminished at week 12.

When cells are labeled with radioactive substrates, the pool size of the precursor substrates in the cells influences the incorporation of radioactivity. We considered that the pool size of choline or phosphocholine in the isolated hepatocytes might be smaller from CD, compared with CS, rats, thus resulting in increased incorporation of label, even though the rate of PC biosynthesis might not be enhanced. To address this concern, we preincubated hepatocytes from CD and CS rats with 200 μM of choline for 4 h before labeling with [methyl-3H]choline. The labeling pattern of PC in both CD and CS hepatocytes was very similar to that obtained without preincubation with choline (data not shown). This result suggested that the increased incorporation of [methyl-3H]choline into PC in CD hepatocytes was not due to a smaller pool size of choline. Moreover, the data are also consistent with activation of CT, the rate-limiting enzyme, in the livers of CD rats (Figs. 3 and 4). The labeling studies with [3H]methionine and [3H]ethanolamine are subject to the same reservations about pool sizes of precursors. However, since both precursors gave similar results (enhanced con-
version of PE to PC), it is unlikely that the increased labeling was due to a pool size effect.

**DISCUSSION**

The major conclusion is that the PE methylation pathway and PEMT2 in liver are activated in rats fed a CD diet for up to 12 weeks. The activation of PEMT2 was achieved largely at the level of enhanced gene expression. The activated pathway was able to synthesize more endogenous choline in the form of PC. This is the first demonstration of an enhanced expression of PEMT2 at the level of the gene. Moreover, to our knowledge, the 5-fold increase of PEMT2 protein in the livers from CD rats is the most significant in vivo activation observed for an enzyme involved in PC biosynthesis in animals. The striking activation of PEMT2 at the level of gene expression in CD rats suggests that PE methylation might play a crucial role in maintenance of normal growth of CD rats.

Earlier studies in which rats were fed a CD diet for only 3 days showed no significant change of PEMT protein as monitored with a polyclonal antibody against purified rat liver PEMT (Ridgway et al., 1989). However, there was a 2-fold increase in PEMT activity in the endoplasmic reticulum fractions from CD compared with CS rat livers. Thus, although there is a doubling of PEMT activity in the livers from CD rats within 3 days after the CD diet is initiated, the increase of PEMT2 protein only occurs after 3 weeks of choline deprivation. Since no specific antibody against PEMT1 is available, we do not know if PEMT1 were activated during long term choline deficiency.

We have recently published evidence that the PE methyltrans-
and transported to other organs which have low PEMT activity (Vance and Ridgway, 1988) and PEMT2 is also exclusively localized to liver (Cui et al., 1993). We postulate that the liver synthesizes choline-containing lipids which are secreted as lyso-PC or as a component of very low density lipoproteins (VLDL) and are transported to other organs which have low PEMT activity. Since the activity for PEMT in non-hepatic tissues is very low and was not enhanced in CD rats, it seems unlikely that significant PE was converted to PC in these tissues. It is also possible that non-hepatic tissues are able to synthesize choline from other pathways. Andriamampandry et al. (1989, 1992) reported that brain and other tissues contain a methyltransferase activities which convert phosphoethanolamine to phosphocholine, although the activities are relatively low.

Even though PE, PC, and other choline-containing lipids are maintained at a relatively normal level in CD rats, the effects of choline deficiency are not totally compensated. The accumulation of triacylglycerol, diacylglycerol, and the activation of protein kinase C still occur (Zeisel and Blusztajn, 1994). The reason for this is not clear.

PEMT2 has been implicated in the growth control of liver and suppression of hepatocarcinogenesis (Cui et al., 1994). On the other hand, choline deficiency for very long periods such as one year makes rats much more susceptible to cancer (Lombardi, 1971). Does PEMT2 have a role in liver carcinogenesis induced by choline deficiency? During choline deficiency enhanced PEMT expression could very well be essential for animal survival. If this dietary condition were prolonged for over 50 weeks, the activated PEMT pathway may fail to be sustained in some hepatocytes by either mutation or other mechanisms. Since the absence of PEMT2 is associated with liver tumors and PEMT2 expression is inhibitory for hepatoma cell growth (Cui et al., 1994), loss of PEMT2 may induce the development of liver cancer. If we were able to obtain mice in which the PEMT2 gene has been disrupted, it would be interesting to see if such mice were more susceptible to liver carcinoma.

Acknowledgments—We are very grateful to Sandra Ungarian and Bosco Lo for excellent technical assistance and to Drs. J. van Vance and Martin Houweling for helpful suggestions.

REFERENCES

Andriamampandry, C., Freysz, L., Kanfer, J. N., Dreyfus, H., and Massarrelli, R. (1989) Biochem. J. 264, 555–562

Andriamampandry, C., Massarrelli, R., and Kanfer, J. N. (1992) Biochem. J. 288, 267–272

Blusztajn, J. K., and Zeisel, S. H. (1989) FEMS Lett. 243, 267–270

Chandar, N., and Lombardi, N. (1988) Carcinogenesis 9, 259–263

Cui, Z., Vance, J. E., Chen, M. H., Voelker, D. R., and Vance, D. E. (1993) J. Biol. Chem. 268, 16655–16663

Cui, Z., Houweling, M., and Vance, D. E. (1994) J. Biol. Chem. 269, 24531–24533

Davis, R. A., Engenhor, S. C., Pangburn, S. H., Weinstein, D. B., and Steinberg, D. (1979) J. Biol. Chem. 252, 226–230

Eagle, H. (1955) J. Exp. Med. 102, 595–600

Esso, J. D., Wermuth, M. M., and Raetz, C. R. H. (1981) J. Biol. Chem. 256, 7388–7393

Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957) J. Biol. Chem. 226, 497–509

Houweling, M., Cui, Z., and Vance, D. E. (1990) J. Biol. Chem. 270, 16277–16282

Kukis, A., Myher, J. J., and Geher, K. (1981) Chromatogr. 224, 1–23

Laemmli, U. K. (1970) Nature 227, 680–685

Lombardi, N. (1971) Fed. Proc. 30, 139–142

Reed, K. C., and Mann, D. A. (1985) Nucleic Acids Res. 13, 7207–7221

Ridgway, N. D., and Vance, D. E. (1992) Methods Enzymol. 209, 366–374

Ridgway, N. D., Yao, Z., and Vance, D. E. (1989) J. Biol. Chem. 264, 1203–1207

Rouser, G., Siakoto, A. M., and Fleischer, D. (1966) Lipids 1, 85–86

Sundler, A., and Åkesson, B. (1975) J. Biol. Chem. 250, 3359–3367

Tessner, T. G., Rock, C. O., Kalmar, G. B., Cornell, R. B., and Jackowski, S. (1991) J. Biol. Chem. 266, 16261–16264

Towbã, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354

Vance, D. E. (1990a) Biochem. Cell Biol. 68, 1151–1165

Vance, J. E. (1990b) J. Biol. Chem. 265, 7246–7256

Vanc, D. E., and Choy, P. C. (1979) Trends Biochem. Sci. 4, 145–148

Vance, D. E., and Ridgway, N. D. (1988) Prog. Lipid. Res. 27, 61–79

Weinhold, P. A., Rounsifer, M. E., and Feldman, D. A. (1988) J. Biol. Chem. 263, 5104–5110

Yao, Z., Paml, H., and Vance, D. E. (1990) J. Biol. Chem. 265, 4326–4331

Zeisel, S. H. (1981) Annu. Rev. Nutr. 1, 95–121

Zeisel, S. H., and Blusztajn, J. K. (1994) Annu. Rev. Nutr. 14, 269–296