Transient inactivation of phosphatidylethanolamine *N*-methyltransferase-2 and activation of cytidine triphosphate:phosphocholine cytidyltransferase during non-neoplastic liver growth

Luciana TESSITORE*, Zheng CUI†‡ and Dennis E. VANCE†§

*Dipartimento di Scienze Cliniche e Biologiche, Università degli Studi di Torino, Torino, Italy, and †Lipid and Lipoprotein Research Group and Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2S2

Phosphatidylethanolamine *N*-methyltransferase-2 (PEMT2) may contribute to the control of hepatocyte cell division, since its inactivation is associated with several types of liver proliferation including tumorigenesis [Cui, Houweling and Vance (1994) *J. Biol. Chem.* **269**, 24531–24533]. To determine if the inactivation of PEMT2 was involved in non-neoplastic proliferation of hepatocytes, we studied the expression of this enzyme in a model of lead nitrate-induced liver proliferation *in vivo* in rats. A maximal decrease in PEMT activity (60 %) and loss of PEMT2 protein (95 %) coincided with maximal DNA synthesis and maximal cytidine triphosphate:phosphocholine cytidyltransferase activity 36 h and 48 h after lead nitrate stimulation in

male and female livers respectively. The decrease in expression of PEMT2 corresponded to a decrease in its mRNA. Compared with males, female rats exhibited a 12 h delay in the peak of DNA synthesis, in cytidyltransferase activity and in the minimum of PEMT2 expression. Supplementation of the rats with dietary choline shifted the female pattern of PEMT2 inactivation, DNA synthesis and activation of cytidyltransferase to 12 h earlier so that it was similar to the time frame of the expression of these activities in males. These results are consistent with the proposal that the inactivation of PEMT2 may have a role in the regulation of non-neoplastic growth of liver.

INTRODUCTION

Phosphatidylethanolamine *N*-methyltransferase-2 (PEMT2) is one of at least two PEMTs in liver and is localized exclusively to a mitochondria-associated membrane [1]. This enzyme catalyses the conversion of phosphatidylethanolamine (PE) into phosphatidylcholine (PC) by addition of three methyl groups from *S*-adenosylmethionine. Recent studies suggest that PC synthesized via the PEMT2 pathway is functionally different from the PC produced by the cytidine diphosphate (CDP)-choline pathway [2]. In these studies mutant Chinese hamster ovary (CHO) cells with a temperature-sensitive defect in the CDP-choline pathway were transfected with the PEMT2 cDNA and grown at the restrictive temperature of 40 °C. PEMT2 activity maintained normal levels of PC at the restrictive temperature but the cells still died.

Other research has linked PEMT2 expression to suppression of liver carcinogenesis and inhibition of cell division ([3]; L. Tessitore, Z. Cui and D. E. Vance, unpublished work). How this liver-specific protein might be involved in tumour cell growth and hepatocyte cell division is not clear. One explanation for cancer suppression and inhibition of hepatocyte cell division might be that PEMT2 down-regulates mitogenic pathways, including the CDP-choline pathway for PC biosynthesis. Several lines of evidence are consistent with this proposal. Firstly, when recombinant PEMT2 was expressed in cultured hepatoma cells in which endogenous PEMT activity was absent, the gene expression of cytidine triphosphate (CTP):phosphocholine

cytidyltransferase (CT), the key enzyme of the CDP-choline pathway, was decreased in a specific and quantitative manner [4]. Secondly, CT activity increased and PEMT2 expression was decreased when liver tumours were induced in rats by carcinogens (L. Tessitore, Z. Cui and D. E. Vance, unpublished work).

To address the question as to whether the inactivation of PEMT2 is also involved in non-neoplastic proliferation of liver, we used lead nitrate to induce transient liver proliferation. In this model, the livers of male rats grow faster and larger than in females [5,6]. Excess dietary choline shifts the proliferative pattern of female livers to that of male livers, but has little effect on proliferation of male livers [5,6]. Moreover, the sexual bias of liver tumour susceptibility towards male humans [7,8] and rats [9] has been well documented. Therefore, liver proliferation induced by lead nitrate is an important model for studying the role of PEMT2 in non-neoplastic growth of liver.

We report that a transient loss of PEMT2 protein and activation of CT coincided with the peak of liver proliferation in response to lead nitrate. The decrease in PEMT2 protein could be explained by a decrease in PEMT2 mRNA. Excess dietary choline shifted the pattern of loss of PEMT2 protein and activation of CT in female livers into a male-like pattern, and the proliferation pattern in female livers was also changed into a male-like pattern. These results suggest that the inactivation of PEMT2, in addition to a possible role in liver tumour suppression [2], may be involved in regulation of non-neoplastic proliferation of liver.

Abbreviations used: PEMT, phosphatidylethanolamine *N*-methyltransferase; PC, phosphatidylcholine; CTP, cytidine triphosphate; CDP, cytidine diphosphate; CT, cytidine triphosphate:phosphocholine cytidyltransferase.

‡ Present address: Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Medical Center Blvd, Winston-Salem, NC 27157, U.S.A.

§ To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

[methyl-³H]Choline chloride (15 Ci/mmol), S-adenosyl-L-[methyl-³H]methionine (15 Ci/mmol), [³H]thymidine (20 Ci/mmol) and the enhanced chemiluminescence (ECLTM) kit were from Amersham International. Picofluor 40 was from Packard Instruments International (Zurich, Switzerland). Choline chloride, BSA and calf thymus DNA were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Silica Gel G60 TLC plates were from Merck (Darmstadt, Germany). Phospho[methyl-³H]choline, the substrate for the CT assay, was synthesized enzymically from [methyl-³H]choline and ATP with choline kinase, as previously described [10].

Animal treatment and labelling of DNA

Wistar rats of both sexes, 6 weeks old (Charles River, Como, Italy), were maintained on a regular light–dark cycle (light 08:00h–20:00h) and fed a balanced semi-synthetic diet (Piccioni, Brescia, Italy). Some groups of animals received choline chloride (1 g/day per kg of body weight) in the drinking water for 3 weeks before a single intravenous dose of lead nitrate (30 mg/kg of body weight). Rats were injected intraperitoneally with [³H]thymidine (500 µCi/kg of body weight) 1 h before killing. Livers were weighed, immediately frozen in liquid nitrogen and stored at –80 °C before measurement of CT and PEMT activities and PEMT2 protein, and at –20 °C before determination of radioactivity in DNA. DNA was assayed by the method of Burton [11], using calf thymus DNA as the standard.

Enzyme assays

Livers were homogenized in buffer containing 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and 1 mM dithiothreitol. CT activity in liver homogenates was determined as described by Weinhold et al. [12] as modified by Yao et al. [13]. PEMT activity in liver homogenates was assayed as reported by Ridgway and Vance [14]. Protein was measured by the method of Lowry et al. [15] using BSA as standard.

Immunoblot analyses

Proteins (50 µg/lane) from the liver homogenates were separated on 12.5% polyacrylamide gel in the presence of 0.1% (v/v) SDS and transferred to Immobilon-P membranes by electrophoretic blotting. The PEMT2-specific antibody was generated against a synthetic C-terminal peptide of PEMT2 and purified by affinity chromatography as previously described [1]. The ECLTM system was used according to the manufacturer's instructions to visualize the proteins on membranes probed with specific antibodies.

mRNA analyses

Total RNA was extracted by homogenizing liver with a Turrax apparatus in guanidine/thiocyanate solution. Poly(A)⁺ RNA was prepared by oligo(dT)–cellulose chromatography [16]. Northern blot analyses were performed with 10 µg samples of poly(A)⁺ RNA by electrophoresis in 1% agarose–formaldehyde gels followed by transfer to nitrocellulose filters [17]. The membranes were probed with PEMT2 cDNA which was labelled with [³²P]dCTP using the Ready-To-Go DNA labelling kit from Pharmacia P-L Biochemicals. Hybridizations were done by the Rapid-hyb method as described by and obtained from Amersham Life Science. The expression of glyceraldehyde phosphate dehydrogenase was used as a control.

RESULTS

DNA synthesis in rat livers was monitored by measuring the incorporation of [³H]thymidine into DNA. In male rat livers, DNA synthesis increased as the liver progressed from the quiescent state to the proliferative state after the injection of lead nitrate. When male rats were treated with lead nitrate there was an increase in labelling of DNA that reached a maximum (15-fold) 36 h after treatment (Figure 1). This increase was transient, and DNA labelling returned to a level close to that of normal liver by 48 h after treatment with lead nitrate. In female livers the labelling of DNA reached its peak 48 h after the treatment (Figure 1) and returned to near normal at 72 h (results not shown).

The PEMT activity in liver homogenates was measured. PEMT activity represents the sum of all PEMT activities, present on both mitochondria-associated membranes (PEMT2) and endoplasmic reticulum (PEMT1) [1]. Figure 1 displays the inverse relationship between PEMT activity and the highest labelling of DNA in both male and female livers. In male livers, the decrease of PEMT activity was maximal 36 h after the lead nitrate treatment. At this time, PEMT activity was 48% lower than in livers from untreated rats. In livers from female rats, the greatest reduction in PEMT activity (60% decrease compared with control liver) occurred 48 h after lead nitrate treatment, which coincided with the highest rate of DNA synthesis. Both PEMT inactivation and DNA synthesis returned to normal by 72 h (results not shown).

In livers from male rats, the amount of PEMT2 protein decreased after lead nitrate treatment, with a greatly reduced expression (90%) occurring after 36 h (Figure 1). The level of PEMT2 protein approached normal levels by 48 h. The timing of the decrease in PEMT2 coincided with the decrease in PEMT activity and was reciprocal to the pattern of DNA labelling (Figure 1). The decreased expression of PEMT2 protein in female livers was most pronounced 48 h after the lead nitrate treatment (Figure 1) and had recovered to normal levels by 72 h (results not shown). For both male and female rats, the decrease in the amount of PEMT2 protein, as estimated by visual comparisons of immunoblots, was more pronounced than the decrease in PEMT activity. Thus, it is likely that the PEMT activity on the endoplasmic reticulum (ascribed to PEMT1 [1]) was not decreased as much as PEMT2. However, confirmation of this assumption must await the availability of a suitable antibody to PEMT1.

To see if the changes in PEMT2 protein were due to a decreased level of expression of the gene for PEMT2, we measured the amounts of PEMT2 mRNA in female rat livers. A high level of PEMT2 gene expression was observed in the quiescent liver at time zero (Figure 2). This was decreased at 24 h and nearly disappeared at 36 h after the lead nitrate treatment. Thus, as might be expected, the maximal decrease in mRNA preceded the maximal decrease in PEMT2 protein at 48 h (Figure 1). The expression of PEMT2 mRNA increased by 48 h and was at pre-treatment levels by 72 h, at which time the liver weight had returned to normal [6]. The levels of mRNA for glyceraldehyde phosphate dehydrogenase were maintained at 24 and 48 h after lead nitrate treatment. At 48 and 72 h there appeared to be an increased expression of this mRNA.

Excess dietary choline shifted the proliferative pattern of female rat livers to that of male rat livers when treated with lead nitrate [5,6]. When supplementary choline was added to the diets of female rats, the changes in DNA synthesis, PEMT activity and PEMT2 protein levels occurred earlier and temporally resembled that seen in livers from male rats. In contrast, in male livers

Figure 1 Amount of PEMT2 protein and PEMT activity decrease, and DNA synthesis and CT activity increase after lead nitrate treatment

Nine-week-old male and female rats were injected intravenously with 30 mg/kg of lead nitrate. For some animals choline chloride (1 g/kg per day) was given in drinking water for 3 weeks before lead nitrate injection. For [^3H]thymidine incorporation into DNA, 500 $\mu\text{Ci/kg}$ was injected 1 h before killing. At the indicated times, rats were killed and liver samples were collected for analysis of DNA labelling (middle panels). For CT and PEMT activities (top panels) and for immunoblot analyses of PEMT2 (bottom panels), livers were homogenized in Tris buffer. Numbers above the top panels refer to time (h) after lead nitrate treatment. All data are averages $\pm$ S.D. from four rats. A representative immunoblot is shown and similar results were obtained in three other experiments. Rats supplemented with choline are indicated as '+ choline'.

Figure 2 PEMT2 expression during lead nitrate-induced liver hyperplasia

Poly(A)⁺ RNA from female rat liver was hybridized with a rat PEMT2 cDNA probe as described in the Materials and methods section. As a control a cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to evaluate the amount of RNA transferred to filters. The molecular mass of hybridizing bands was estimated using ribosomal RNAs as internal standards. This experiment was repeated three times with similar results.

supplementary dietary choline had very little effect on DNA synthesis, PEMT activity and PEMT2 expression.

The reciprocal relationship between PEMT2 and gene expression of CT in cultured hepatoma cells [4] raised the possibility that CT might also be altered in the response of livers to lead nitrate treatment. We therefore measured total cellular CT activity in liver samples after treatment of rats with lead nitrate. Decreased expression of PEMT2 during liver proliferation was

always accompanied by activation of CT. The activity of CT was increased by approx. 80% after 36 h in male livers and 48 h in female livers (Figure 1). The pattern of CT activation followed that of DNA synthesis in both sexes regardless of whether or not supplementary choline was added to the diet.

DISCUSSION

Previous studies in hepatoma cells [3], in regenerating rat liver (M. Houweling, Z. Cui, L. Tessitore and D. E. Vance, unpublished work) and developmental studies (Z. Cui, Y.-J. Shen and D. E. Vance, unpublished work), suggest that PEMT2 might have a role in the control of hepatocyte cell division and might be a candidate liver-specific tumour suppressor. These findings raised the possibility that PEMT2 might be involved in the suppression of non-neoplastic liver proliferation that occurs in lead nitrate-induced liver growth. The results presented in this paper provide evidence in favour of this hypothesis. A striking decrease in PEMT2 gene expression occurred at the same time as there was an increase in DNA synthesis and in the activity of CT.

The previously observed sex difference in DNA synthesis in the livers of rats injected with lead nitrate [5,6] was confirmed in the present investigations. Supplementation of the diet of female rats with choline shifted by 12 h the time of maximal DNA synthesis, CT activity and minimal expression of PEMT2. The molecular basis of the choline effect on these activities in female rats is not understood. These data provide additional evidence to link PEMT2 expression inversely with the division of hepatocytes. Other studies with regenerating rat liver (M. Houweling, Z. Cui, L. Tessitore and D. E. Vance, unpublished work) and rat liver

during development (Z. Cui, Y.-J. Shen and D. E. Vance, unpublished work) show a similar inverse relationship between hepatocyte cell division and PEMT2 expression.

The inverse relationship between CT activity and PEMT2 expression previously described in hepatoma cells [4] was also seen in the lead nitrate-induced hyperplasia. The results add weight to the proposal that there is something special about the CDP-choline pathway that cannot be replaced by PC derived from PEMT2 in mutant CHO cells defective in CT ([2]; F. Tercé, Z. Cui and D. E. Vance, unpublished work). These mutant CHO cells, as well as PEMT2-transfected mutant CHO cells, die at the restrictive temperature via apoptosis ([18]; F. Tercé, Z. Cui and D. E. Vance, unpublished work). Moreover, recent studies with McArdle RH7777 cells have shown that these cells die under conditions of choline deficiency, and transfection with PEMT2 does not protect the cells from death (P. S. Vermeulen and D. E. Vance, unpublished work). The reason why the CDP-choline pathway is specifically required for cell division remains an open question.

PEMT2 is an unexpected player in hepatocyte cell division. Traditionally, the function of PEMT activity in liver has been thought to be as a back-up pathway for PC biosynthesis. That an isoenzyme of PEMT (PEMT2), localized to mitochondria-associated membranes, might have a regulatory role in hepatic cell division was not anticipated. The initial clue came from transfection of McArdle RH7777 cells with PEMT2 resulting in an extension of the time required for cell division from 18 to 50 h [3]. This cell-culture observation has now been extended to hepatocyte cell division in intact rats in studies on regenerating rat liver, development and lead nitrate-induced proliferation. In each case PEMT2 activity declined and CT activity increased when hepatocyte cell division was induced. Thus it seems that there is 'cross-talk' between the PEMT2 and CT activities, the latter being required for hepatocyte cell division. Moreover, a role for PEMT2 in non-neoplastic proliferation of hepatocytes is

consistent with the possible role of PEMT2 as a liver-specific tumour suppressor (L. Tessitore, Z. Cui and D. E. Vance, unpublished work).

This work was supported by grants from the Medical Research Council of Canada, Consiglio Nazionale Delle Ricerche (Grant No. 95.02456.CT04) and Associazione Italiana per la Ricerca sul Cancro. D.E.V. is a Medical Scientist of the Alberta Heritage Foundation for Medical Research.

REFERENCES

- 1 Cui, Z., Vance, J. E., Chen, M. H., Voelker, D. R. and Vance, D. E. (1993) *J. Biol. Chem.* **268**, 16655–16663
- 2 Houweling, M., Cui, Z. and Vance, D. E. (1995) *J. Biol. Chem.* **270**, 16277–16282
- 3 Cui, Z., Houweling, M. and Vance, D. E. (1994) *J. Biol. Chem.* **269**, 24531–24533
- 4 Cui, Z., Houweling, M. and Vance, D. E. (1995) *Biochem. J.* **312**, 411–417
- 5 Tessitore, L., Perletti, G. P., Sesca, E., Pani, P., Dianzani, M. U. and Piccinini, F. (1994) *Biochem. Biophys. Res. Commun.* **205**, 208–214
- 6 Tessitore, L., Sesca, E., Pani, P. and Dianzani, M. U. (1995) *Chem. Biol. Interact.* **97**, 1–10
- 7 Berman, C. (1958) *Adv. Cancer Res.* **5**, 55–96
- 8 Toh, Y. C. (1973) *Adv. Cancer Res.* **18**, 155–209
- 9 Tessitore, L., Pani, P. and Dianzani, M. U. (1992) *Carcinogenesis* **13**, 385–389
- 10 Vance, D. E., Pelech, S. L. and Choy, P. C. (1982) *Methods Enzymol.* **71**, 576–581
- 11 Burton, K. A. (1956) *Biochem. J.* **62**, 315–320
- 12 Weinhold, P. A., Rounsifer, M. E. and Feldman, D. A. (1986) *J. Biol. Chem.* **261**, 5104–5110
- 13 Yao, Z., Jamil, H. and Vance, D. E. (1990) *J. Biol. Chem.* **265**, 4326–4331
- 14 Ridgway, N. D. and Vance, D. E. (1992) *Methods Enzymol.* **209**, 366–374
- 15 Lowry, O. H., Rosebrough, N. J., Farr, A. R. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 16 Aviv, H. and Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1408–1412
- 17 Maniatis, T. E., Fritsch, E. F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
- 18 Cui, Z., Houweling, M., Chen, M. H., Record, M., Chap, H., Vance, D. E. and Tercé, F. (1996) *J. Biol. Chem.* **271**, 14668–14671