Overexpression of phosphatidylethanolamine *N*-methyltransferase 2 in CHO-K1 cells does not attenuate the activity of the CDP-choline pathway for phosphatidylcholine biosynthesis

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Chinese hamster ovary (CHO) cells express only a trace amount of phosphatidylethanolamine *N*-methyltransferase (PEMT) activity. CHO cells make their phosphatidylcholine (PC) via the CDP-choline pathway. We investigated whether or not overexpression of PEMT2, an isoform of PEMT, in these cells would down-regulate the activity of the CDP-choline pathway. Transfection of CHO cells with PEMT2 cDNA behind the cytomegalovirus promoter resulted in a series of cell lines that overexpressed PEMT2. Phospholipid metabolism was characterized in cell lines that expressed a medium (281 pmol/min per mg of protein) and a high (1300 pmol/min per mg of protein) level of PEMT activity. The expression of the regulated enzyme (CTP:phospho-

choline cytidyltransferase) in the CDP-choline pathway was increased, not decreased, in these cell lines as judged by immunoblot analysis and enzymic activity. Conversion of phosphatidylethanolamine to PC was enhanced in CHO cells that expressed PEMT2 activity. PC mass was not increased in the transfected compared with the control cells. The rate of PC catabolism made by either the CDP-choline or methylation pathways was unaffected by PEMT2 expression. We conclude that expression of PEMT2 in CHO cells does not down-regulate, but rather enhances, the expression of CTP:phosphocholine cytidyltransferase.

INTRODUCTION

Phosphatidylcholine (PC) is made in mammalian hepatocytes via the CDP-choline pathway and via the transmethylation of phosphatidylethanolamine (PE) catalysed by PE *N*-methyltransferase (PEMT). Other mammalian cells and tissues have at most only 1 % or 2 % of the PEMT activity recovered from liver [1]. Because all nucleated mammalian cells have the CDP-choline pathway, the question arises as to why PEMT has survived in evolution as a liver-specific enzyme. The question became more complicated in 1993 when two different forms of PEMT were discovered in liver, PEMT1 found on the endoplasmic reticulum and PEMT2 recovered on the mitochondria-associated membrane [2]. Why should there be two isoenzymes of PEMT in different subcellular locations of the hepatocyte? Why should there be two pathways for PC biosynthesis?

To gain new insight into the function of PEMT in hepatocytes, we cloned and expressed the cDNA for the PEMT2 isoenzyme in McArdle-RH7777 hepatoma cells in culture [2,3]. Untransfected cells had essentially no PEMT activity even though they were derived originally from a hepatocyte [4]. Unexpectedly, we found that expression of PEMT2 in these hepatoma cells extended the time required for cell division from 20 h to 50 h for the cell lines expressing the greatest amount of PEMT2 activity [3]. Subsequent experiments indicated that PEMT2 expression down-regulated the CDP-choline pathway, specifically the expression of CTP:phosphocholine cytidyltransferase (CT), in the McArdle-RH7777 cells [5]. These results led us to wonder whether PEMT2 expression in a non-hepatocyte cell line would similarly affect the

expression of the CDP-choline pathway. The results presented in this paper demonstrate that expression of PEMT2 in a Chinese hamster ovary (CHO) cell line did not cause decreased expression of CT or down-regulation of the activity of the CDP-choline pathway. Instead there was enhanced expression of CT. Thus the effect of overexpression of PEMT2 on CT expression depends on the cell line used.

MATERIALS AND METHODS

Materials

L-[methyl-³H]Methionine (83 Ci/mmol), [methyl-³H]choline chloride (15 Ci/mmol), [1-³H]ethan-1-ol-2-amine hydrochloride (26 Ci/mmol), L-[³H]serine (32 Ci/mmol), *S*-adenosyl-L-[methyl-³H]methionine (15 Ci/mmol), ACS liquid-scintillation fluid and the ECL[®] Western blotting analysis kit were purchased from Amersham Canada Ltd. (Oakville, ON, Canada). Dishes and flasks for cell culture were obtained from Fisher Scientific (Ottawa, ON, Canada) or VWR Scientific of Canada Ltd. (Toronto, ON, Canada). Ham's F-12 Nutrient Mixture, additional cell culture media and fetal bovine serum were obtained from Life Technologies (Burlington, ON, Canada). Precoated glass and plastic TLC plates (Sil G-25, 0.25 and 0.2 mm thickness) were purchased from E. Merck (Darmstadt, Germany). Phosphatidyl-*N*-monomethylethanolamine (PMME), used for assay of the phospholipid methyltransferase, was obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.), whereas the substrate for

Abbreviations used: CHO, Chinese hamster ovary; CMV, cytomegalovirus; CT, CTP:phosphocholine cytidyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine *N*-methyltransferase; PMME, phosphatidyl-*N*-monomethylethanolamine; SM, sphingomyelin.

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assays of CT, phospho[*methyl*-³H]choline, was synthesized as described by Vance et al. [6]. Unless specified, all other chemicals were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.), BDH (Toronto, ON, Canada) or Fisher Scientific (Ottawa, ON, Canada).

Cell culture

Wild-type CHO-K1 cells (kindly provided by Dr. C. Kent and Dr. C. Raetz) were routinely cultured in Ham's F-12 Nutrient Mixture supplemented with 10% (v/v) fetal bovine serum and maintained at 37 °C, 5% CO₂ in air, and 90% humidity.

Establishment of CHO cell lines that stably express PEMT2

The construction of the expression plasmid for PEMT2 has been previously described [2]. A 10 µg aliquot of the plasmid containing PEMT2 under control of a cytomegalovirus (CMV) promoter was co-transfected with 0.3 µg of pSV-neo plasmid [7] into CHO-K1 cells by precipitation with calcium phosphate [8]. Colony selection was performed in the presence of 0.6 µg/ml G418 (Geneticin®, Life Technologies) in Ham's F-12 supplemented with 10% (v/v) fetal bovine serum. Once selected, colonies were grown in the presence of 0.3 µg/ml G418 and assayed for PEMT activity to confirm expression. Cell lines used as controls were produced by transfection and selection in an identical manner, with an expression plasmid lacking the PEMT2 cDNA insert.

Determination of activities for CT, choline kinase and PEMT2

Samples for enzyme assays were prepared from all cell cultures subjected to the radiolabelling experiments described below. Cells were grown to confluence on 10 cm dishes. After washes with ice-cold PBS, the cells were scraped from the dish into 3 ml PBS. Each dish was washed with an additional 3 ml of PBS; the washings were pooled with the cell scrapings. Cell pellets were collected by centrifugation at 1500 *g* for 5 min at room temperature. The resultant cell pellets were resuspended in fresh homogenization buffer (pH 7.4) consisting of 20 mM Tris/HCl, 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 100 µM leupeptin and 100 µM PMSF. Cells were disrupted by sonication (two continuous bursts of 15 s, 50% duty, less than 10% output; W-385 Heat Systems-Ultrasonics ultrasonic processor). Protein mass was determined by the method of Bradford [9]. CT activity was measured in soluble and membrane fractions as described by Yao et al. [10]. PEMT2 activity in total cellular homogenates was assayed with PMME as substrate, as described by Ridgway and Vance [11]. Choline kinase was assayed by the method of Weinhold and Rethy [12].

Analysis of [³H]serine and [³H]methionine incorporation into phospholipids

Cells were plated in duplicate in 6 cm culture dishes and grown to approx. 70% confluence. The cells were washed with PBS before the addition of radioactive precursors ([³H]serine or [³H]methionine, 5 µCi per dish) in fresh culture medium. Additional 10 cm dishes were set aside for measurement of enzyme activities and analyses of phospholipid mass. The cultures were incubated at 37 °C for 24 h, after which each dish was washed twice with PBS. Cells were scraped in 1 ml of ice-cold PBS with a rubber 'policeman', and each dish was further washed with 1 ml of PBS, which was pooled with the original scrapings in a 15 ml disposable culture tube. The cell pellets were collected by centrifugation at 1500 *g* for 5 min at room temperature and the

supernant was removed by aspiration. The resultant cell pellet was resuspended in 1.6 ml of methanol/water (1:1, v/v) and sonicated twice in 15 s bursts (50% duty, less than 10% output). Each sonicated sample was adjusted to a volume of 1.6 ml and transferred to a glass screw-capped tube, after which aliquots were removed for the determination of protein. The samples were extracted by the addition of 2 ml of chloroform and 80 µl of acetic acid. After separation by centrifugation and removal of the aqueous phase, the organic phase was washed once with 1.5 ml of methanol/water (1:1) containing 0.1% (v/v) acetic acid. The chloroform phase was dried under a stream of N₂. The dried lipids were resuspended in chloroform/methanol (2:1) and aliquots were applied to silica gel TLC plates (in the presence of carrier lipids). Phospholipid classes were separated by using chloroform/methanol/acetic acid/formic acid/water (70:30:12:4:1, by vol.) as the solvent system. After detection of the lipids with I₂ vapour, the respective bands were scraped into scintillation vials. Water (0.5 ml) and 5 ml of ACS scintillant were added to the samples and the radioactivity was quantified by liquid-scintillation spectrometry.

Biosynthesis and turnover of ethanolamine- and choline-containing phospholipids and metabolites

Duplicate 6 cm cultures (more than 80% confluent) were pulse-labelled for 1 h with [³H]ethan-1-ol-2-amine hydrochloride (3 µCi per dish) in F-12 medium supplemented with 10% fetal bovine serum. The chase medium consisted of F-12 medium containing serum and 100 µM ethanolamine. At the beginning of the chase period, the radiolabelled medium was removed and cells were washed with 3 ml of PBS before the addition of the chase medium. At the termination of the chase, cells were washed twice with PBS. Cells were harvested as described previously at various times after the addition of the unlabelled medium. After extraction as described above, both aqueous and organic phases were retained. Organic-phase metabolites were separated as described above. After the aqueous phase had been dried, the metabolites were resuspended in methanol/water (1:1, v/v). Water-soluble choline- and ethanolamine-containing metabolites were separated by TLC with methanol/0.6% NaCl/NH₄OH (50:50:5, by vol.) as the solvent system. Choline-containing metabolites were detected after exposure to I₂ vapour; ethanolamine-containing metabolites were detected with ninhydrin (0.1% in ethanol). The bands were scraped and radioactivity was measured.

For pulse-chase experiments with choline, the duration of the pulse was either 1 or 2 h as indicated. [*methyl*-³H]Choline chloride (2–3 µCi per dish) was added to Dulbecco's modified Eagle's medium lacking choline, to prepare the labelling medium. In some experiments, cells were preincubated in medium devoid of choline for up to 20 h to augment the uptake of radiolabelled choline. The chase medium consisted of F-12 medium (with 10% fetal bovine serum) containing 100 or 200 µM choline. The remainder of the experimental protocols were identical with those already described.

The relative rates for turnover of PC generated by the methylation and CDP-choline pathways were determined after the cellular PC pools had been labelled for 24 h with L-[*methyl*-³H]methionine or [*methyl*-³H]choline chloride respectively. Duplicate 6 cm culture dishes (60–70% confluent) were labelled with [*methyl*-³H]choline chloride (3 µCi per dish) for 24 h in Dulbecco's modified Eagle's medium devoid of choline (supplemented with 30 µM methionine, 100 µM leucine, 1 mM arginine, 10 mM glucose, 1 mM sodium pyruvate and 10% fetal bovine serum) or L-[*methyl*-³H]methionine (5 µCi per dish) in Ham's F-

12 medium that contained 100 μ M choline and 10 % fetal bovine serum. Cells labelled with choline were chased for various times with F-12 medium containing 100 μ M choline, whereas samples labelled with methionine were chased with medium (already containing methionine) that included 100 μ M ethanolamine. The radioactivity incorporated during the radiolabelling period, and that lost from [3 H]PC during the chase period, were determined as described for the other pulse-chase experiments.

Analyses of phospholipid mass

Cell pellets from confluent cultures in 10 cm dishes were prepared as described above. Pellets were resuspended in 2.4 ml of methanol/water (1:1) and sonicated. Aliquots were removed for the determination of protein, and 120 μ l of acetic acid and 3 ml of chloroform were added. After separation of the phases by centrifugation, the chloroform phase was washed once with 2.25 ml of methanol/water (1:1) containing 0.1 % acetic acid. After removal of the upper phase, the lower phase was dried under a stream of N_2 . The lipids were resuspended in chloroform/methanol (2:1) and separated by TLC in the absence of carrier lipids as described. Lipid phosphorus in PC and PE was quantified by the method of Zhou and Arthur [13].

Immunoblot analyses of PEMT2 and CT

The relative levels of PEMT2 and CT protein were determined by immunoblot analysis. Protein (30–50 μ g) from total cellular homogenates was separated by 0.1 % SDS/PAGE [12.5 % (w/v) gel] on the discontinuous system of Laemmli [14]. Proteins were transferred to nitrocellulose membranes by electrophoretic blotting [15] and probed with specific antibodies against PEMT2 [2] and CT [16]. Reactivity to the antibodies was detected by ECL[®] (Amersham) in accordance with the manufacturer's protocol.

RESULTS AND DISCUSSION

Expression of PEMT2 cDNA in CHO-K1 cells

In an effort to understand the co-ordination of PC biosynthesis by the CDP-choline pathway and the methylation of PE, we have analysed the effects of marked overexpression of PEMT2, an isoform of PEMT [2], on PC metabolism. Transfection of CHO-K1 cells with rat liver cDNA encoding PEMT2 led to the production of a series of cell lines expressing a wide range of methyltransferase activities, two of which are shown in Table 1. The specific activity of PEMT2 in the highest expressor was comparable to the high level of activity (more than 1 nmol/min per mg of protein with PMME as substrate) found in rat liver [2].

Figure 1 Immunoblot analysis of PEMT2 and CT in CHO-K1 cells expressing rat liver PEMT2

Cells were grown to more than 70 % confluence on 10 cm dishes. Cell pellets were homogenized and 50 μ g of the total homogenates from each cell line were subjected to immunoblot analysis with a mixture of specific polyclonal antibodies to PEMT2 [2] and CT [16]. Control refers to a cell line transfected with the pCMV vector lacking the PEMT2 cDNA insert; medium and high refer to cell lines that had been transfected with PEMT2 (specific enzyme activities summarized in Table 1). PEMT2 and CT are identified by the arrows at the right, and the molecular masses of standard proteins are indicated in kDa at the left. The approx. 55 kDa and approx. 75 kDa bands correspond to unknown proteins that interacted with the antibodies to CT and PEMT2 respectively.

The introduction of a high level of constitutively active PEMT2 into cells that already have an intact CDP-choline pathway might conceivably alter the activity of the latter pathway. This is so in McA-RH7777 hepatoma cells, in which expression of PEMT2 leads to the down-regulation of the CDP-choline pathway at the level of expression of CT [5]. However, overexpression of PEMT2 did not attenuate the activity of either soluble or membrane-associated CT when assayed *in vitro* (Table 1). On the contrary, there was an increase in CT activity in these two fractions. Immunoblot analysis also revealed that the mass of CT did not decrease, but rather increased, in response to overexpression of PEMT2 (Figure 1). Densitometric analysis of the CT bands in Figure 1 show that, taking the mass of CT in the control as 100 %, the medium expressor contained 191 % and the

Table 1 Expression of rat liver PEMT2 increases the activity of CT in CHO-K1 cells

Cells were grown to near-confluence on 10 cm culture dishes, harvested and then homogenized by sonication as described in the Materials and methods section. PEMT2 activity was assayed in homogenates by using PMME as phospholipid substrate. CT activity was determined in both soluble and membrane fractions. The results are expressed as the means $\pm$ S.D. of enzyme specific activities, where the numbers in parentheses represent the numbers of independent cultures that were harvested. Control refers to CHO-K1 cells that were transfected with pCMV vector lacking PEMT2. Moderate and high, reflecting activities relative to liver [2], refer to cell lines derived from the transfection of CHO-K1 cells with the rat liver cDNA encoding PEMT2. Statistical significance: * P < 0.001, ** P < 0.02 compared with controls.

Cell line	PEMT2 activity (pmol/min per mg of protein)	CT activity (nmol/min per mg of protein)		Choline kinase activity (nmol/min per mg of protein)
		soluble	membrane	
Control	17.0 $\pm$ 5.9 (19)	10.7 $\pm$ 1.9 (9)	3.7 $\pm$ 1.0 (9)	0.22 $\pm$ 0.06 (5)
Moderate	281.0 $\pm$ 122.2 (18)	21.8 $\pm$ 4.1 (9)*	4.6 $\pm$ 1.0 (9)	0.23 $\pm$ 0.06 (7)
high	1300.5 $\pm$ 537.5 (19)	27.0 $\pm$ 4.8 (9)*	5.2 $\pm$ 1.2 (9)**	0.26 $\pm$ 0.05 (7)

Table 2 Incorporation of [^3H]serine into phospholipids of CHO-K1 cells transfected with PEMT2

Cells were grown to approx. 70% confluence in 6 cm culture dishes and labelled for 24 h with 5 μCi of [^3H]serine per dish, after which the cells were harvested and the lipids were extracted. Radioactivity in phospholipids from the total lipid extract was quantified after separation by TLC. The results are presented as the means $\pm$ S.D. of radioactivity incorporated for the analyses of duplicate dishes from three experiments. Control, medium and high refer to the relative activities of PEMT2 as summarized in Table 1. Abbreviation: SM, sphingomyelin.

Cell line	Incorporation into ...	$10^{-3} \times$ Incorporation of [^3H]serine (d.p.m./mg of protein)		
		PE	PC	SM
Control		43.7 ± 19.1	8.7 ± 1.3	21.9 ± 5.4
Medium		27.5 ± 9.0	17.3 ± 3.3	16.8 ± 4.4
High		26.8 ± 10.4	33.5 ± 5.7	22.0 ± 7.9

Table 3 Incorporation of [^3H]methionine into phospholipids of CHO-K1 cells transfected with PEMT2

Cells were grown to approx. 70% confluence in 6 cm culture dishes. Cells were labelled for 24 h with 5 μCi of [^3H]methionine per dish, after which the cells were harvested and the lipids were extracted. Radioactivity in phospholipids from the total lipid extract was quantified after separation by TLC. The results are presented as the means $\pm$ S.D. of radioactivity incorporated for the analyses of duplicate dishes from three experiments. Control, medium and high refer to the relative activities of PEMT2 as summarized in Table 1. Abbreviations: SM, sphingomyelin; LysoPC, lysophosphatidylcholine.

Cell line	Incorporation into ...	$10^{-3} \times$ Incorporation of [^3H]methionine (d.p.m./mg of protein)		
		PC	SM	LysoPC
Control		2.9 ± 0.6	1.7 ± 0.5	0.7 ± 0.2
Medium		41.4 ± 12.5	7.9 ± 3.6	3.0 ± 2.0
High		84.5 ± 49.0	12.1 ± 3.6	3.8 ± 1.6

high expressor 189%. Thus the increased activity of CT (Table 1) correlated reasonably well with the immunoblot analyses. The activity of choline kinase (Table 1) was unaffected by PEMT2 overexpression.

To investigate the integrity of the methylation and CDP-choline pathways in the transfected cell lines, we monitored the incorporation of various radiolabelled precursors into phospholipid metabolites. As shown in Table 2, the incorporation of [^3H]serine into [^3H]PE decreased, and increased in [^3H]PC in accordance with the enhanced expression of PEMT2 activity. [^3H]PC accounted for 12%, 28% and 41% of the major radiolabelled phospholipids in the control, moderate and high expressors of PEMT2 respectively. The incorporation of serine into sphingomyelin was not altered by transfection between the various cell lines.

Because serine is incorporated into PC at various levels (glycerol backbone, fatty acid, head group), this would account for the label in [^3H]PC in the control samples that are devoid of PEMT activity. Labelling of the methyl donor *S*-adenosyl-methionine with [^3H]methionine provided a more sensitive index of the relative activities of PEMT2 (Table 3). Little label was incorporated into PC in the control cells. Incorporation of [^3H]methionine into [^3H]PC increased 14-fold and 29-fold over control levels in the moderate and high expressors respectively (Table 3). The incorporation of radioactivity into other products of PC metabolism such as lysophosphatidylcholine and sphingo-

Figure 2 Incorporation of [^3H]ethanolamine into lipid metabolites of CHO-K1 cells transfected with PEMT2

Duplicate 6 cm culture dishes of control cells and stable transfectants expressing PEMT2 were pulsed for 1 h with 3 μCi of [^3H]ethanolamine per dish and radioactivity was chased for the indicated times with unlabelled medium containing 100 μM ethanolamine. At the designated times the chase was terminated and the organic-phase and water-soluble lipid metabolites were extracted and separated as described in the Materials and methods section. The figure summarizes the results of one representative experiment that was repeated twice with similar results. Upper panel, incorporation of radioactivity into PC; lower panel, incorporation of radioactivity into PE.

myelin also rose with increasing levels of PEMT2 activity. Therefore the cell lines transfected with rat liver PEMT2 cDNA did have an intact and active phospholipid methylation pathway.

Biosynthesis of PC by the methylation pathway was also assessed in pulse-chase experiments with [^3H]ethanolamine. The results of these experiments are summarized in Figure 2. After a 1 h pulse, the incorporation of radioactivity into [^3H]PC quickly reached a peak and remained elevated in cells expressing PEMT2 (Figure 2, upper panel). In contrast there was a decrease in the amount of radioactivity in [^3H]PE during the chase period (Figure 2, lower panel). The observation that the level of [^3H]PC did not continue to increase over the duration of the chase, in spite of a decline in [^3H]PE, might suggest that the methyltransferase preferentially used only PE that was newly synthesized as the phospholipid substrate. This phenomenon has been reported previously for the metabolism of PE in rat hepatocytes [17]. Additionally, there was evidence for the catabolism of the radiolabelled PC because the accumulation of radioactivity in PC was paralleled by a gradual accumulation of intracellular

Figure 3 Incorporation of [³H]choline into lipid metabolites of CHO-K1 cells transfected with PEMT2

Duplicate 6 cm culture dishes of control cells and stable transfectants expressing PEMT2 were pulsed for 2 h with 3 μ Ci of [³H]choline per dish in choline-deficient medium. The radioactivity in the cells was chased for the indicated times with unlabelled medium containing 100 μ M choline. Organic-phase and water-soluble metabolites were separated from a total lipid extract as described in the Materials and methods section. The figure shows the results of one experiment that was repeated three times with similar results. Upper panel, incorporation of radioactivity into PC; lower panel, incorporation of radioactivity into phosphocholine.

[³H]glycerophosphocholine in the PEMT2 transfectants (results not shown). In comparison with the PEMT2 transfectants, it was also apparent that the control cell line incorporated less radiolabel into [³H]PE after the 1 h pulse. Possibly ethanolamine transport is stimulated in the PEMT2-expressing cell lines. Interestingly, after the 1 h pulse, the control cell line lacking PEMT2 showed a much greater level of accumulation of radioactivity in breakdown products of PE. At the end of the pulse, [³H]glycerophosphoethanolamine in the control cells accounted for $20.3 \pm 8.1\%$ (means $\pm$ S.D.) of the total radioactive metabolites recovered by TLC, compared with $2.3 \pm 0.4\%$ and $1.0 \pm 0.2\%$ for the moderate and high expressors respectively.

Although the apparent activity of CT increased in response to increased activity of PEMT2 (Table 1), similar levels of [³H]choline were incorporated into [³H]PC during pulse-chase experiments with the medium expressor, and less incorporation was observed in PC in the highest expressor of PEMT2 (Figure 3, upper panel). The latter result is probably due to less radioactivity's being taken up by cells with high PEMT activity than by control cells or cells that express medium amounts of PEMT2. Furthermore in cells with the highest PEMT2 activity, less label

Table 4 Phospholipid mass in CHO cells transfected with rat liver PEMT2

CHO-K1 cells were grown to near-confluence on 10 cm culture dishes. Cells were harvested and lipids were extracted as described in the Materials and methods section. PC and PE were separated by TLC and the phospholipid mass was determined. The results are expressed as the means $\pm$ S.D. for three separate experiments for the control and high expressors of PEMT2, and as the mean of two experiments for the moderate PEMT2 expressor.

Cell line	PC mass (nmol/mg of protein)	PE mass (nmol/mg of protein)
Control	105.2 ± 12.3	47.7 ± 10.0
Moderate PEMT2	102.1	30.0
High PEMT2	94.8 ± 13.7	32.4 ± 10.7

Figure 4 Turnover of PC generated by the CDP-choline pathway in CHO-K1 cell lines transfected with PEMT2

To estimate the rate of turnover of PC generated from the CDP-choline pathway, duplicate 6 cm cultures were labelled with 3 μ Ci of [³H]choline for a period of 24 h. The radioactivity in the cells was chased for up to 8 h with medium containing 100 μ M unlabelled choline. The radioactivity remaining in PC was measured after lipid extraction and separation of phospholipids by TLC. The $t_{1/2}$ for PC was determined from the slope of the graphs of $\ln(\text{d.p.m./mg})$ against time. The results for control cells and medium and high expressors are shown and represent the averages from two experiments.

was recovered in [³H]phosphocholine (Figure 3, lower panel) and [³H]glycerophosphocholine (results not shown). However, because choline uptake is not normally a limiting factor in the biosynthesis of PC via the CDP-choline pathway, we would not expect the rate of uptake to affect the overall rate of PC biosynthesis.

The rapid disappearance of label from phosphocholine (Figure 3, lower panel) is in agreement with enhanced CT activity (Table 1). That this is not reflected by increased incorporation of label into PC could be due to effects on pool sizes of PC precursors such as phosphocholine or choline. Quantification of these precursors would probably not be enlightening because of the apparent compartmentation of these pools in cells [18].

Overexpression of PEMT2 does not alter PC mass or turnover

The cellular masses of PC and PE in the various cell lines were measured; the results are summarized in Table 4. PC mass did not increase in cells harbouring PEMT2; there was very little difference in PC mass among the cell lines. By comparison, PE mass decreased in cell lines expressing moderate and high levels of PEMT2 activity, but neither of these changes was statistically significant.

Figure 5 Turnover of PC generated by the methylation of PE in CHO-K1 cells transfected with two different amounts of PEMT2

Duplicate 6 cm dishes of cells expressing PEMT2 were labelled with 5 μ Ci of [3 H]methionine for 24 h. The cells were then chased for up to 8 h with unlabelled medium containing 100 μ M ethanolamine. The $t_{1/2}$ for PC was determined as in Figure 4. The results are the averages from two experiments for each cell line expressing medium or high PEMT2 activities.

In previous studies by Walkey et al. [19], overexpression of CT in COS cells resulted in enhanced degradation of PC. We were curious to see whether overexpression of PEMT2 would increase the rate of degradation of PC derived from the CDP-choline and/or phospholipid methylation pathway because there was no significant difference in the mass of PC despite the introduction of PEMT2. Previous investigations have supported the idea that PC made by the CDP-choline pathway and the methylation of PE are not functionally interchangeable in CHO cells [20]. Thus transfection with the cDNA of PEMT2 into a mutant CHO cell line, with a temperature-sensitive defect in CT, failed to allow the mutant to grow at the restrictive temperature despite the normalization of the levels of PC. Therefore the turnover rates of PC might be different because the products of the two pathways might be functionally distinct or represent different compartments.

The turnover time for PC generated from the CDP-choline pathway was determined from the slopes of the plots of the logarithm of the d.p.m. in [3 H]PC/mg of protein against time after labelling with [3 H]choline (Figure 4). The half-lives ($t_{1/2}$) for PC were 10.8, 11.0 and 12.2 h in control cells and cells that expressed moderate and high levels of PEMT2 respectively. Parallel experiments were performed in which PC synthesized via the methylation of PE was selectively labelled with [3 H]methionine and the degradation of [3 H]PC was monitored during a chase period (Figure 5). The $t_{1/2}$ for PC made by the methylation pathway was 9.9 h for the moderate expressor of PEMT2 and

14.1 h for the high expressor. The $t_{1/2}$ for the very small amount of PC generated by methylation of PE in control cells was estimated at 10.7 h (results not shown).

Conclusion

The results from this study clearly document an increase in CT as a result of PEMT2 transfection as indicated by both immunoblot studies and enzyme assays. This contrasts with previous observations in rat hepatoma cells where overexpression of PEMT2 diminished the activity of the CDP-choline pathway at the level of CT [5]. Why was there no down-regulation of CT in the transfected CHO cells, whereas there was in McArdle RH7777 cells? We speculate that there might be liver-specific factors that co-ordinate the regulation of CT and PEMT2 activities, and that these factors are absent from CHO fibroblasts. The first step in addressing this hypothesis will be to clone and characterize the PEMT2 gene. This will then allow the identification of regulatory elements in the gene, after which the identification and isolation of transcription factors can begin.

We thank Susanne Lingrell, Sandra Ungarian and Peter Tai for their technical support, Dr. Zheng Cui for the PEMT2 cDNA and antibody to PEMT2, Dr. Martin Houweling for helpful discussion of experimental results and protocols, and Dr. Jean Vance for helpful discussions. This work was supported by a grant from the Medical Research Council of Canada. M.W.L. was a recipient and M.B. is currently a recipient of Fellowships from the Medical Research Council and the Alberta Heritage Foundation for Medical Research. D.E.V. is a Medical Scientist of the Alberta Heritage Foundation for Medical Research.

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