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Biochemical and Biophysical Research Communications 346 (2006) 234–241
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Inactivation of PEMT2 in hepatocytes initiated by DENA in fasted/refed rats

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Received 15 May 2006
Available online 24 May 2006

Abstract

Phosphatidylethanolamine *N*-methyltransferase (PEMT) is the enzyme that converts phosphatidylethanolamine (PE) into phosphatidylcholine. We have previously shown that PEMT suppressed hepatoma growth by triggering apoptosis. We investigate whether PEMT controlled cell death and cell proliferation triggered by fasting/refeeding and whether it is a marker of early preneoplastic lesions. The induction of programmed cell death and suppression of cell proliferation by fasting were associated with enhanced PEMT expression and activity, and with a decrease in CTP:phosphocholine cytidylyltransferase expression. Refeeding returned the liver growth and expression of CTP:phosphocholine cytidylyltransferase to control levels, while the expression of PEMT decreased to below control values. After DENA administration, PEMT protein, evaluated by Western blotting, slightly increased, but it remained below control levels. The treatment with 20 mg/kg DENA to refed rats induced the appearance of initiated hepatocytes that were negative for PEMT expression. Present findings indicate that PEMT is a novel tumour marker for early liver preneoplastic lesions.

Keywords: PEMT2; Tumour suppressor; Liver cancer; DENA

All eukaryotic mammalian cells synthesise phosphatidylcholine (PC) from choline via the cytidine diphosphate (CDP)-choline pathway. However, hepatocytes have another pathway for the synthesis of PC; i.e., by methylation of phosphatidylethanolamine (PE), catalysed by phosphatidylethanolamine *N*-methyltransferase (PEMT) [1]. Most PEMT activity (PEMT1) has been associated with the endoplasmic reticulum, while a second form of the enzyme (PEMT2) has been localized to the mitochondria-associated membrane [2]. PEMT1 and PEMT2 are encoded by the same gene, *Pemt*. The difference between the two isoform of PEMT, which results in their divergent subcellular localization, must be generated posttranscriptionally [3,4]. The rat liver PEMT2 cDNA has been cloned and characterised. Previous studies have implicated PEMT2 in the regulation of non-neoplastic [5–7] and neoplastic [8–11] liver growth. In all these experimental conditions, PEMT2 was absent when the liver proliferation rates were elevated and was associated with high levels of CTP:phosphocholine cytidylyltransferase (CT) and PKC α, β, and ζ, while quiescent liver showed high levels of expression of PEMT2 and PKC δ accompanied by low levels of CT and PKC α, β, and ζ [5,12,13]. Subsequently, we demonstrated that induction or enhancement of apoptosis in hepatoma cells and host liver, respectively, was associated with a high level of PEMT2 expression and low CT activity [9]. Consistently, choline deficiency induced apoptosis in SV40-immortalized CWSV-1 rat hepatocytes [14] and increased

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doi:10.1016/j.bbrc.2006.05.101

S-adenosylmethionine levels and PC synthesis in primary rat hepatocytes via the methylation pathway [15]. We found also that over-expression of PEMT2 in a McArdle hepatoma cell line by transfection with PEMT2 cDNA markedly increased the cell doubling time by triggering apoptosis without affecting cell division in monolayer cultures [9], and it decreased the number of foci growing in semisolid agar, and the incidence and growth of tumours when inoculated in athymic mice [10]. These studies indicate a role of PEMT2 in the regulation of in vivo hepatoma and hepatocyte cell division and, particularly, of hepatocyte cell death by apoptosis.

We have shown recently that a subnecrogenic dose of diethylnitrosamine (DENA), insufficient per se to initiate liver cancer of fed animals, became able to induce liver cancer in rats refed after 4 days of fasting [16]. This was achieved by triggering cell proliferation as a compensation to fasting-induced cell loss and reducing glutathione *S*-transferase (GST) and lipid peroxidation at the time of DENA administration [17,18]. All these metabolic perturbations likely favour initiation by a subnecrogenic dose of DENA, resulting in a burst of putative-initiated placental form of GST-positive hepatocytes [18].

In the present study, on the basis of these observations, we investigated whether PEMT might contribute to changes in active cell death and cell proliferation induced by fasting–refeeding by making hepatocytes suitable for initiation by 20 mg/kg of DENA and able to fix the DNA damage by a round of cell division leading to liver cancer when initiated cells were selected by the Solt and Farber promoting regimen. For this purpose, we evaluated PEMT2 expression in rat liver by Northern blot and immunoblot analysis. Since the subnecrogenic dose of DENA was able to induce the appearance of putative-initiated GST-P-positive hepatocytes [18], we also evaluated the PEMT2 expression by immunohistochemistry in the above initiated hepatocytes. We also measured the expression of various isoforms of PKC, because the activation of different PKC isoenzymes is involved in the signal transduction of liver carcinogenesis [19] and because PKC has been proposed to mediate PEMT-associated liver growth perturbations [5,12,13]. We found that down-regulation of PEMT2 expression in the liver of refed animals most likely represents a primary mechanism by which a fasting–refeeding regimen sustains initiation by a subnecrogenic dose of DENA, and it can be proposed to be a tumour marker for liver cancer.

Materials and methods

Reagents. [^{3}H]Thymidine (20 Ci/mmol), [^{3}H]putrescine dihydrochloride (26.3 Ci/mmol), the Enhanced Chemiluminescence System (ECL), the Rapid-hyb buffer, multiprime DNA labelling system kit, and [γ-^{32}P]desoxyCTP were obtained from Amersham Italia (Milan, Italy). Anti-PKC α-δ and ζ were from Santa Cruz. Anti-PEMT was a kind gift from Professor D.E. Vance (Department of Biochemistry, University of Alberta, Edmonton, Canada). Bovine serum albumin was from Sigma (St. Louis, MO). The agarose gel for electrophoresis was from Bio-Rad (Richmond, CA) and all other common reagents were from Merck (Darmstadt, Germany).

Animals. Two-month-old male Fisher 344 rats (Charles River, Como, Italy) were acclimatized for 1 week to an AIN-76 balanced semisynthetic diet (Piccioni, Brescia, Italy) in an animal room under a regular light/dark cycle (light 08:00–20.00), temperature (21 °C), and humidity. After this period, rats were divided into three groups: control rats (day 0; $n = 4$), rats exposed to 1, 2, 3 or 4 days of fasting ($n = 16$), and rats submitted to 4 days of fasting and then refed for 1, 2 or 3 days (refeeding: days 1, 2; $n = 12$) and killed at the end of the fasting or refeeding regimen (Fig. 1A). Sixteen animals were given a single subnecrogenic dose of DENA (20 mg/kg body weight) after 4 days of fasting and 1 day of refeeding, and were killed at different times (1, 3, 5, and 7 days) after treatment with DENA; 4 rats at each experimental time-point (Fig. 1B). During the periods of fasting, a grating obstructed the access of rats to bedding and faeces. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institute of Health.

Biochemical analyses. Livers were removed, weighed, and samples were immediately frozen at −80 °C to determine PEMT and CT activity, phospholipid content, and the expression of RNA and protein (PEMT, CT, PKC).

PEMT and CT activity. 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 Vance et al. [20]. PEMT activity in liver homogenates was assayed as reported by Ridgway and Vance [21]. Protein was measured by the method of Lowry using BSA as standard.

Phospholipid analyses. Phospholipids were isolated from total liver homogenates according to Bligh and Dyer [22] and separated by thin-layer chromatography with chloroform/methanol/acetic acid/water (50:30:8:4, by vol.) as solvent. After the detection of the lipids with I_2 vapour, the respective bands were scraped and phosphorous analysis was performed.

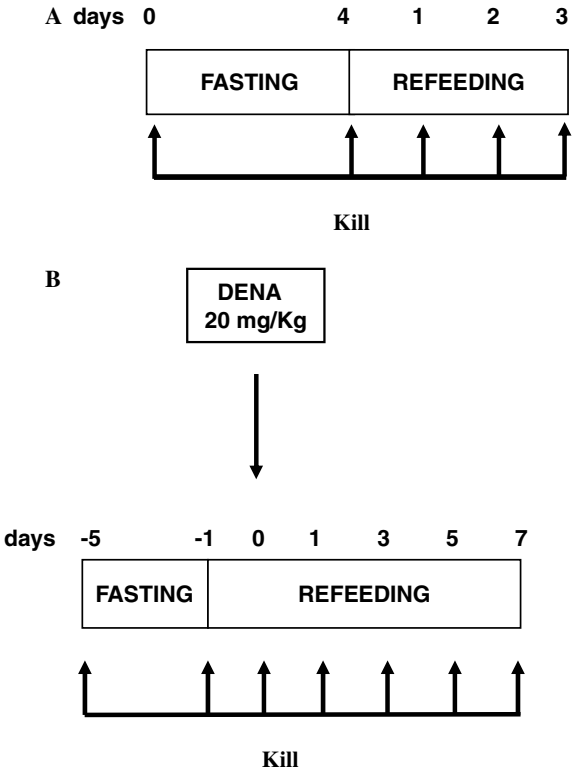

Fig. 1. Experimental protocol: male rats fed with a balanced semisynthetic diet for 2 weeks (A) were exposed to 4 days of fasting followed by 2 days of refeeding; (B) animals were exposed to a fasting period and to a single dose of DENA (20 mg/kg body weight) after 1 day of refeeding. Animals were killed after different periods of treatment.

Northern blot analysis. Poly(A)$^+$ RNA (10 μg) was submitted to 1% (w/v) agarose/formaldehyde electrophoresis and then transferred to a nitrocellulose filter [23]. The sheet was incubated with cDNA PEMT2 or CT (10 ng/ml at 60 °C). Hybridizations were done by the Rapid-hyb method (Amersham Life Science). In order to evaluate the amount of RNA transferred to filters, cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used.

Immunoblot analysis. Proteins from each fraction (100 μg) were separated on 10% and 12.5% (w/v) polyacrylamide gels [24] and transferred to a nitrocellulose membrane by electrophoresis blotting [25]. Transfers were blocked for 2 h at room temperature with 5% non-fat milk in TBS, 0.025% (v/v) Tween 20 and then incubated overnight at 4 °C with the primary antibodies (anti-PEMT2 or anti-PKC α-δ or ζ diluted in non-fat milk). The transfers were washed three times with TBS, 0.025% Tween 20, and incubated for 1 h at room temperature with horseradish peroxidase-goat anti-rabbit or goat anti-mouse diluted 1:4000 in TBS, 0.025% Tween 20. The immunoblots were subsequently washed three times in TBS, Tween 20 and the signal was visualized using the ECL system according to the manufacturer's instructions (Amersham-Biotech).

Histological/histochemical analysis. For histological/histochemical examination, slices taken from different liver lobes were immediately fixed in phosphate-buffered 10% (v/v) formalin (pH 7) for routine embedding in paraffin and staining with hematoxylin and eosin.

Cell death. Section 5 μm thick were stained with an enzymatic in situ label of apoptosis-induced DNA strand breaks, known as the TUNEL technique [17,18]. The terminal deoxynucleotidyl transferase enzyme labels free 3′-OH DNA termini with fluorescein-labelled nucleotides (In Situ Cell Death Detection Kit, POD, Roche Diagnostic GmbH, Mannheim, Germany). Hepatocytes undergoing apoptosis were determined by quantitating the percentage of labelled nuclei by fluorescence microscopy scoring not less than 5000 adjacent hepatocytes and expressed per 100 hepatocyte nuclei (apoptotic index).

Cell proliferation. To calculate the percentage of [^{3}H]thymidine-positive hepatocytes, some samples were processed for autoradiography: sections were coated with NTB-2 Kodak emulsion, dried, and sealed in a dark box at −80 °C for 2 weeks [17,18]. Slides were then developed and counterstained with hematoxylin and eosin. The number of [^{3}H]thymidine-positive hepatocytes was determined by examining not fewer than 3000 hepatocyte nuclei and expressed per 100 hepatocyte nuclei (labeling index).

GST-P positive hepatocytes. Slices were fixed in ice-cold acetone for double immunohistochemical staining for PEMT2 and the placental form of GST (GST-P) [26]. To evaluate the appearance of GST-P positive hepatocytes, some sections were exposed to GST-P antibody rabbit anti-rat diluted 1:500 for 1 h at room temperature, washed in PBS, and then reacted with goat anti-rabbit IgG diluted 1:200 [18]. After washing, sections were incubated with alkaline phosphatase-conjugated streptavidin. Visualization was achieved by reaction with 5-bromo-4-chloro-3-indoxyl phosphate (BCIP) and nitro blue tetrazolium chloride (NBT). The sites with GST-P antigen were blue. A minimum 2 cm^2 of liver was screened for each sample, using a Universal II microscope with 5× objective lens. The number of GST-P positive cells/cm^2 and the percentage of PEMT-positive cells were determined by means of an interactive image analysis system (Videoplane Zeiss; Kontron, Munich, Germany).

Statistical analysis. All values are expressed as means ± SD. Significance of the differences was calculated using Student's *t*-test.

Results

Fasting was characterised by PEMT2 up-regulation associated with programmed cell death

Four days of fasting were sufficient to induce the appearance of apoptotic features, as demonstrated by an 18-fold increase of the apoptotic index associated with a depression of cell replication evaluated as the labelling index in the rat liver (Table 1). At the end of fasting, PEMT2 mRNA expression was almost doubled while CT mRNA expression was decreased progressively until about 60% (Fig. 2). Immunoblot analysis indicates that the increase of PEMT2 mRNA was reflected in similar changes in PEMT2 protein expression (Fig. 3). Consistent with the above results on PEMT2 and CT expressions, Table 1 shows that PEMT activity was enhanced during fasting about 2-fold, while CT activity was reduced by ∼40%. Analytical assays demonstrated that during the fasting period, PC and PE contents decreased by 70% and 60%, respectively (Table 1). In these conditions, expression levels of PKC isoforms were evaluated: PKC α was doubled and PKC δ was increased by 30% during fasting, while PKC ζ was lowered (Fig. 3).

Refeeding was accompanied by down-regulation of PEMT2 and apoptosis

The refeeding period was characterised by a reduction of the apoptotic index, which returned to control values within 3 days (Table 1). The analysis of the pattern behaviour of PEMT and CT during this period shows that during the first 2 days of refeeding, PEMT mRNA (Fig. 2) and protein levels (Fig. 3) were decreased to below control

Table 1
Effect of fasting/refeeding on CTP:phosphocholine cytidylyltransferase, PEMT, phosphatidylethanolamine, phosphatidylcholine, apoptotic index, and labelling index

	CT (nmol/min/mg)	PEMT (nmol/min/mg)	PC (nmol/mg pt)	PE (nmol/mg pt)	AI	LI
Fasting (days)						
0	2.6 ± 0.14	0.85 ± 0.01	30 ± 1.40	11.0 ± 1.41	0.10 ± 0.01	1.54 ± 0.26
4	1.6 ± 0.21**	1.35 ± 0.03**	9.2 ± 1.72**	4.5 ± 0.23**	1.80 ± 0.12**	0.06 ± 0.01**
Refeeding (days)						
1	2.97 ± 0.02§	0.68 ± 0.02§	12 ± 2.8*	9 ± 1.4§	0.50 ± 0.08*,§§	0.18 ± 0.09*,§
2	3.12 ± 0.14§§	0.51 ± 0.01§	20 ± 1.4*,§	9.80 ± 0.17§	0.27 ± 0.11*,§§	0.45 ± 0.13*,§§
3	3.21 ± 0.14*,§§	0.32 ± 0.01*,§§	32 ± 1.4§§	10.50 ± 1.42§	0.15 ± 0.09§§	0.76 ± 0.38*,§§

CT, CTP:phosphocholine cytidylyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; AI, apoptotic index; LI, labelling index.
Data are expressed as means ± SD, $n = 5$.
§ $p \leqslant 0.05$, §§ $p < 0.01$ versus 4 days of fasting.
* $p \leqslant 0.05$.
** $p \leqslant 0.01$ versus control (day 0).

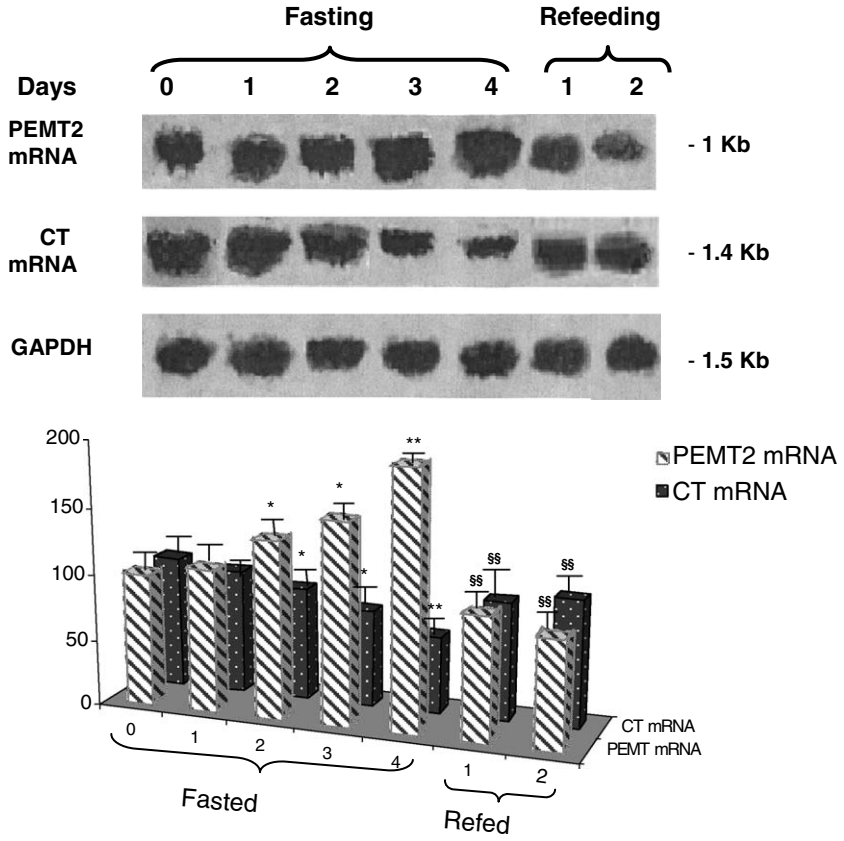

Fig. 2. Northern blot analysis of liver: PEMT2 and CT during fasting/refeeding. cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. The autoradiographs shown originate from one representative experiment. $n = 3$; *$p < 0.05$ versus control (day 0); **$p < 0.01$ versus control (day 0); $^{\S\S}p < 0.01$ versus 4 days of fasting.

levels, while CT appeared to be up-regulated (Fig. 2). We assayed the activity of these two enzymes and observed that during refeeding CT was increased reaching control values, while PEMT was reduced to below basal levels (Table 1). A few days of refeeding were sufficient to normalize PE and PC contents, which returned to basal values (Table 1). During refeeding, the levels of PKC α expression were decreased slightly and those of the δ isoenzyme were reduced until they disappeared; in contrast, levels of PKC ζ were increased to above control values (Fig. 3).

A subnecrogenic dose of DENA induced PEMT2 down-regulation associated with the appearance of putative-initiated GST-P positive hepatocytes

The experimental protocol is shown in Fig. 1B (see Materials and methods for details). Fig. 4 shows the pattern of gene expression of PEMT and PKC isoforms during the hepatocarcinogenesis process induced by a subnecrogenic dose of DENA associated with fasting/refeeding. The expressions during fasting (day 1) and 1 day of refeeding (day 0) matched those reported in Figs. 2 and 3. PEMT protein expression increased during 4 days of fasting and it was lowered on the first day of refeeding when animals received DENA, then it increased slightly after treatment with DENA, even if protein levels remained lower than those in the liver of rats fed regularly. The expression of PKC α increased after 4 days of fasting, then decreased markedly after 1 day of refeeding, to below control levels, and it was still low after treatment with DENA (Fig. 4). The expression of PKC δ, which showed very low levels in the liver of fed rats, increased slightly during the fasting period, decreased after 1 day of refeeding, and disappeared progressively after treatment with DENA (Fig. 4). PKC ζ protein was decreased after fasting, rose after the first day of refeeding, and then decreased progressively to minimum levels after administration of DENA.

The induction of putative-initiated hepatocytes by a single dose of 20 mg/kg of DENA i.p. measured as GST-P positive cells is shown in Table 2. Single GST-P positive hepatocytes appeared a few days after i.p. administration of 20 mg/kg of DENA in the liver of refed rats following 4 days of fasting (Table 2), while very rare GST-P positive cells were found in the liver of regularly fed rats receiving the same dose of DENA (data not shown). It is of interest that, following injection of DENA, the expression of PEMT2, evaluated by immunohistochemical analysis, disappeared in GST-P positive hepatocytes, while only a few GST-P negative hepatocytes did not express PEMT2 (Table 3).

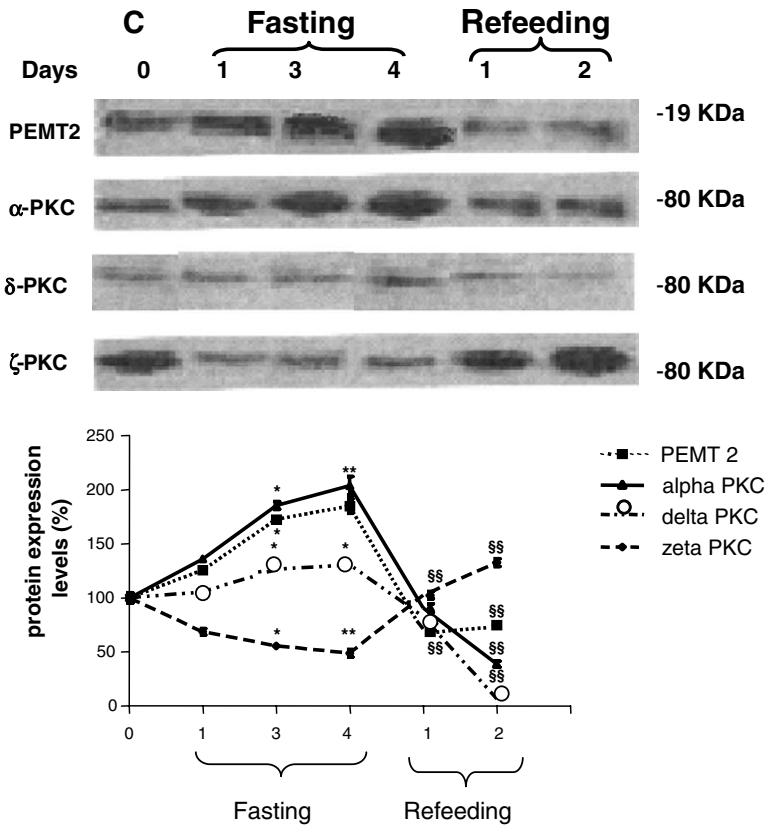

Fig. 3. Immunoblot analysis of liver: PEMT2 and PKC isoforms during fasting/refeeding. The immunoblots shown originate from one representative experiment. $n = 3$; $^{*}p < 0.05$ versus control (day 0); $^{**}p < 0.01$ versus control (day 0); $^{\S\S}p < 0.01$ versus 4 days of fasting.

Discussion

Liver cancer is one of the most common causes of tumour death in the world [27], the primary risk factors being chronic infection with hepatitis B and C viruses, and dietary exposure to aflatoxin B1 [28]. The heterogenicity of gene lesions suggests that hepatocellular carcinoma may be produced by selection of both genomic and epigenetic alterations that compromise many regulatory pathway [29]. Here, we found that modulations of PEMT2 expression induced by fasting/refeeding dietary regimen were likely to make the liver prone to cancer initiation by a ''non-initiating'' dose of DENA, and PEMT2 inactivation appeared to be a novel marker of putative-initiated GST-P positive hepatocytes.

Exposure of rats to fasting is known to suppress liver cell proliferation and to induce apoptosis, which is characterised by up-regulation of bax and TGFβ expression as well as down-regulation of Bcl2 [18,30]. However, refeeding inhibited the apoptotic process and triggered a wave of cell division with opposite gene changes in bax, bcl2, and TGFβ, associated with down-regulation of GST and lipid peroxidation [17,18,30], so that cycling hepatocytes can be susceptible to DENA and, thereafter, DENA-initiated cells could easily undergo another cell-cycle favouring fixation of the DNA lesion. In this context, PEMT2 up-regulation associated with enhanced expression of PKC α and δ during fasting, followed by their down-regulation after 1 day of refeeding at the time of DENA administration, could make the hepatocytes suitable for initiation by a ''non-initiating'' dose of DENA. Thus, PEMT2 appears to act synergistically with bax, TGFβ, and PKC α and δ, and to counteract the action of CT, bcl2, and PKC ζ. At other times it seems that there is a ''cross-talk'' between PEMT2, CT, and PKC isoforms for either hepatocyte cell-cycle progression or induction of programmed cell death [5,12,13].

Regardless of the mechanisms involved, the relevance of PEMT in the development of the hepatocellular carcinoma is supported strongly by the frequent gene inactivation in rat liver during chemical carcinogenesis induced by either Solt and Farber [8] or aflatoxin [10] models, in transplantable hepatoma [9] as well as in human hepatocellular carcinoma [11], primarily due to reduced levels of mRNA transcripts and protein. In particular, the loss of PEMT mRNA expression in human liver cancer is associated with high proliferative rates and histological grade, as well as with severe outcome of patients, suggesting that clones lacking PEMT expression might be selected during liver tumourigenesis and progression, and PEMT expression could be proposed as a novel prognostic marker [11]. Our present results indicating that the putative-initiated GST-P positive hepatocytes are negative for PEMT expression largely

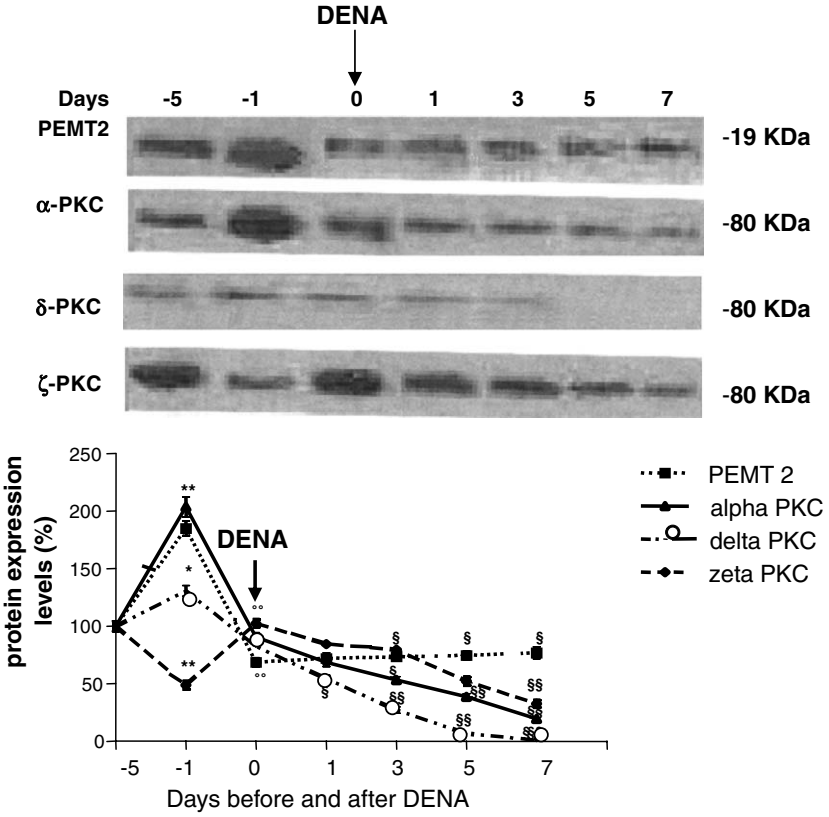

Fig. 4. Immunoblot analysis of liver: PEMT2 and PKC isoforms during hepatocarcinogenesis. Day 5, fed controls; day 1, days fasted; day 0, day refed and DENA administration; days 1, 3, 5, and 7, after treatment with DENA (refed rats). $n = 3$; $^{*}p < 0.05$ versus control (day 0);$^{**}p < 0.01$ versus control (day 0); $^{\circ\circ}p < 0.01$ versus 4 days of fasting; $^{\S}p < 0.05$ versus 1 day refed rats and administration of DENA; $^{\S\S}p < 0.01$ versus 1 day refed rats and administration of DENA.

Table 2
Effect of DENA treatment on appearance of placental form of glutathione-*S*-transferase (GST-P) hepatocytes

Days	GST-P + hepatocytes/cm^2	
	−DENA	+DENA
0	0.20 ± 0.17	0.18 ± 0.16
3	0.17 ± 0.13	$78.03 \pm 14.03^{**}$
5	0.21 ± 0.18	$112.22 \pm 26.14^{**}$

All rats were exposed to fasting for 4 days, then refed. DENA (20 mg/kg) has been administered after 1 day of refeeding (day 0, see Fig. 1). Data are expressed as means ± SD, $n = 5$.
** $p \leqslant 0.01$ versus control (day 0).

Table 3
Effect of DENA treatment on PEMT2 expression in placental form of glutathione-*S*-transferase (GST-P) hepatocytes

After DENA (days)	PEMT2 − GST-P + cells (%)	PEMT2 + GST-P − cells (%)
0	1.32 ± 0.11	95.2 ± 3.41
3	$3.15 \pm 0.94^{*}$	87.3 ± 5.14
5	$2.78 \pm 0.78^{*}$	91.4 ± 6.23

Data are expressed as means ± SD, $n = 5$.
* $p \leqslant 0.05$ versus control (day 0).

confirmed the previous clinical data [11] as well as the reduced expression of PEMT in the focal proliferative lesions [8].

The finding that the decrease in expression of the PEMT2 gene was reversible during non-neoplastic liver growth suggests that regulation of PEMT2 expression levels is required for cell proliferation [5–7]. PEMT2 appears as an unexpected player in hepatocyte cell division and programmed cell death, since PC is required for normal progression through the cell-cycle. However, PEMT has been implicated recently in neurogenesis. Zhu et al. [31] have reported perturbations in the brain development of Pemt −/− mouse as enhanced cell proliferation and reduced differentiation in the foetal hippocampus associated with hypermethylated DNA due to increased concentrations of *S*-adenosylmethionine. Interestingly, modulation of DNA methylation related to protooncogenes and growth suppressor genes is an epigenetic event leading to cancer development [32,33], thus PEMT inactivation could result in hypermethylation of tumour suppressor genes and, consequently, their reduced gene expression could contribute to cell growth and carcinogenesis.

Suppression of PC biosynthesis by either genetic defect [15] or removal of choline from the medium of SV40-immortalized rat hepatocytes [14] triggered apoptotic cell death. On the other hand, both the stationary phase of in vivo hepatoma growth and host liver hypoplasia were achieved by induction of apoptosis and enhancement of PEMT2 expression [9]. However, a direct involvement of

PEMT2 in the apoptotic process derives from our studies in which over-expression of PEMT in a hepatoma cell-line following transfection with PEMT2 cDNA induced apoptosis, resulting in a decreased cell doubling time [9]. In line with these observations, exposure of MLP-29 to either serum deprivation or retinoic acid induced apoptosis as well as PEMT expression [34]. Consistent with our results, more recently, Zou et al. [35] have reported that hepatoma cell-lines transfected with PEMT2 cDNA down-regulated Akt, PI3K, c-Met, PDGF-R, and Bcl-2 to induce apoptosis for suppressing cell growth. Further experiments will be performed to clarify the relation of Pemt with genes regulating the cell-cycle. Of interest, since the primary cellular localization of PEMT2 appears to be mitochondria and both the intrinsic pathway of apoptosis and autophagic cell death are known to involve mitochondria [36] it is not surprising that PEMT can have a role in the regulation of cell death.

Walkey et al. [37] have suggested that the Pemt gene may have been conserved during evolution as a protective mechanism to provide PC when dietary choline is insufficient; e.g., during starvation, pregnancy or lactation. While Pemt −/− mice fed a choline supplemented diet display no liver damage and lead a normal life, Pemt −/− mice fed a choline-deficient diet developed fatal liver disease, which can be rescued rapidly by the addition of dietary choline [38], but not by the addition of dietary dimethylethanolamine [39]. It is well known that dietary choline deficiency is per se able to induce liver cancer in rodents [40,41], and PEMT expression is markedly enhanced in long-term choline-deficient rats, suggesting that Pemt-deficient mice are more prone to liver cancer.

Acknowledgments

We are grateful to Professor D.E. Vance for helpful discussions. This work was supported by MIUR, Italian Ministry of Health and University A. Avogadro, Faculty of Pharmacy.

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