Disruption of Choline Methyl Group Donation for Phosphatidylethanolamine Methylation in Hepatocarcinoma Cells*

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Despite being widely hypothesized, the actual contribution of choline as a methyl source for phosphatidylethanolamine (PE) methylation has never been demonstrated, mainly due to the inability of conventional methods to distinguish the products from that of the CDP-choline pathway. Using a novel combination of stable-isotope labeling and tandem mass spectrometry, we demonstrated for the first time that choline contributed to phosphatidylethanolamine (PC) synthesis both as an intact choline moiety via the CDP-choline pathway and as a methyl donor via PE methylation pathway. When hepatocytes were labeled with [3-3H]choline containing three deuterium atoms on each of the three methyl groups, [3-3H]PC and [6-3H]PC were detected, indicating that newly synthesized PC contained one or more individually labeled methyl groups from [3-3H]-choline. The synthesis of [6-3H]PC and [6-3H]PC was sensitive to the general methylation inhibitor 3-deazaadenosine and were specific products of PE methylation using choline as a one-carbon donor. While the contribution to the CDP-choline pathway remained intact in hepatocarcinoma cells, contribution of choline to PE methylation was completely disrupted. In addition to a previously identified lack of PE methyltransferase, hepatocarcinoma cells were found to lack the abilities to oxidize choline to betaine and to donate the methyl group from betaine to homocysteine, whereas the usage of exogenous methionine as a methyl group donor was normal. The failure to use choline as a methyl source in hepatocarcinoma cells may contribute to methionine dependence, a widely observed aberration of one-carbon metabolism in malignancy.

In hepatocytes, PC1 is synthesized via two pathways. The CDP-choline pathway is catalyzed sequentially by three enzymes: phosphorylcholine cytidylyltransferase, cholinephosphotransferase (1, 2). The PE methylation pathway is catalyzed by PEMT1 and PEMT2, two distinct hepatic PE methyltransferases that catalyze identical reactions but differ in structure and cellular localization (3). The CDP-choline pathway uses endogenous choline as the initial substrate and generates a pool of PC that is comprised primarily of short chain (16/18 carbons and 18/18 carbons), saturated and monounsaturated fatty acids (4). The PE methylation pathway on the other hand synthesizes a pool of PC predominantly with long chain (18/20 carbons and 18/22 carbons), polyunsaturated fatty acids (4). The two pathways also have opposite roles in liver growth and hepatocyte proliferation. Liver growth and liver carcinogenesis are associated with activation of the CDP-choline pathway (5, 6) and inactivation of the PE methylation pathway (5–7). Increased activity of PE methylation specifically and quantitatively inhibits cellular division of hepatoma cells (8). Inactivation of PEMT via gene knockout is not lethal when choline is present in the diet, but is lethal when choline is absent from the diet (9). During long-term choline deficiency, rat liver PEMT is activated by 5-fold and becomes an essential enzyme to generate the choline moiety endogenously (10).

Methylation of PE in the liver is highly responsive to the dietary content of components that may have metabolic consequences on methylation reactions. PEMT uses the general methyl donor S-adenosylmethionine (AdoMet) in three sequential transmethylation reactions (11). AdoMet is derived from methionine that can be obtained either from extracellular sources or by intracellular methylation of homocysteine. Choline, as a precursor to betaine, is a methyl source for the methylation of homocysteine (see Fig. 1). In the liver, choline is converted to betaine via oxidation steps in the mitochondria by choline dehydrogenase (CDH) (12, 13) and betaine aldehyde dehydrogenase (BADH) (14). A methyl group of betaine is transferred to homocysteine by betaine:homocysteine methyltransferase (BHMT) to generate methionine. Methionine is converted to AdoMet by methionine adenosyltransferase. The liver converts over 60% of free choline into betaine (15), suggesting that choline may play a significant role in methylation reactions. However, an actual contribution of choline to PE methylation has not been demonstrated. This is mainly because many studies of choline metabolism have used choline that contains radioactive methyl groups. Using this radiolabeling technique, the two pools of choline-derived PC, one from the CDP-choline pathway containing the entire choline molecule, and the other from the methylation of PE containing only the methyl groups of choline, could not be distinguished from one another.

In this paper, we use tandem mass spectrometry to identify specific choline-derived PC pools synthesized by the PE methylation pathway, using choline containing deuterium-labeled methyl groups as a methyl donor. We also describe evidence for the inactivation of this hepatic choline one-carbon donor pathway in hepatocarcinoma cells.
EXPERIMENTAL PROCEDURES

**Materials**—$d_9$-Choline chloride, $d_9$-ethanolamine, and $d_9$-betaine were purchased from Isotec, Inc. $d_7$-Methionine was purchased from Medical Isotopes, Inc. $^{[3]}$H$\text{Etanolamine was from American Radiolabeled Chemicals, Inc.}$ $^{[methyl-^{3}]}$H$\text{S-Adenosylmethionine was purchased from PerkinElmer Life Sciences.}$ Dulbecco’s modified essential medium (DMEM) and fetal bovine serum were purchased from Invitrogen. L-$\text{Methionine, betaine, choline chloride, and ethanolamine were purchased from Sigma.}$ Phospholipid standards were purchased from Avanti Polar Lipids. All other chemicals and materials were purchased from Fisher Scientific.

**Cell Culture**—Rat primary hepatocytes were isolated by a collagenase perfusion procedure (16). Isolated hepatocytes were cultured on collagen-coated culture dishes overnight in DMEM with 20% fetal bovine serum and 10$^{-9}$M insulin. Experiments with hepatocytes were started after overnight culture. Stable McArdle RH7777/PEMT2 cells were established by co-transfection with 10$^{-8}$g of pCMV5/PEMT2 and 1$^{-7}$g of pSV2neo plasmids via calcium phosphate precipitation and selected by G-418 as described previously (8). All experiments were done in serum-, choline-, ethanolamine-, and methionine-free DMEM containing 10$^{-9}$M insulin, 2 mg/ml bovine serum albumin, 20 $\mu$g/ml folate, and 400 $\mu$g/ml serine.

**Deuterium Labeling**—Cells (3.5 $\times$ 10$^5$) were incubated in serum-free DMEM containing 2 mg/ml bovine serum albumin and 500 $\mu$M unlabeled or deuterium-labeled choline, betaine, methionine, and/or ethanolamine, as described under “Results” and in the figure legends. Cell monolayers were washed twice with ice-cold phosphate-buffered saline, scraped into ice-cold water/methanol, and the lipids were extracted according to the method of Bligh and Dyer (17) with 0.3% acetic acid. The lipid extracts were stored in methylene chloride at $-80^\circ$C. Prior to mass spectrometry, the solvent was dried under nitrogen and the lipid extracts were dissolved in 45/45/10 methylene chloride/methanol/water. The aqueous portions of the Bligh and Dyer extracts were stored at $-80^\circ$C until analysis. Experiments were repeated three times with hepatocytes and twice with McArdle-RH7777 cells with identical results. Figures containing mass spectrometric histograms show representative results.

**Lipid Analysis by Electrospray Ionization Tandem Mass Spectrometry**—Lipid extracts were analyzed on a Micromass Quattro II triple quadrupole mass spectrometer (Micromass, United Kingdom). Standards and samples contained 1% formic acid for positive ion analysis. All analyses were performed at a flow rate of 5 $\mu$l/min, argon pressure of 1.8 $\times$ 10$^{-3}$ mBar, and source temperature of 200 $^\circ$C. PC species were detected by a precursor ion scan at collision energy of 25 eV for molecules generating the daughter ion of $m/z$ 184, 187, 188, 190, 191, 193, 194, or 197, depending on the number of deuterium atoms within the head group. The intensities of equimolar PC standards decrease significantly as mass increases (35). A standard curve from equimolar PC standards was used to derive an equation to correct raw results for mass discrimination:

\[
y' = y(xm + b) / (xm + b),
\]

where $y'$ = corrected intensity of each sample peak, $y$ = actual intensity of each sample peak, $s$ = mL of deuterium value of di-14:0 PC standard, $m$ = slope derived from PC standard curve, $b$ = y-intercept derived from PC standard curve, and $x = m/z$. 

Fig. 1. Predicted path of methyl groups from choline to PC in liver. AdoMet, S-adenosylmethionine; AdoHyc, S-adenosylhomocysteine; CDH, choline dehydrogenase; BADH, betaine aldehyde dehydrogenase; MAT, methionine adenosyltransferase; AdoHycH, S-adenosylhomocysteine hydrolase.

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value of each sample peak.) Histograms of $d_3$-PC were further processed by subtracting out the peak intensities due to isotopic carryover of $d_6$-PC (~8% of $d_6$-PC). All histograms are displayed as relative intensity to the highest peak. In histograms with no significant peaks (defined as 3 times greater than background), the relative intensity is adjusted to background at ~10%. 

**3-deazadenosine (DZA) Inhibition of PE Methylation—**Hepatocytes (3.5 × 10^9 cells) were incubated for 24 h in the presence or absence of 10 μM DZA in regular DMEM containing 1 μCi/ml [3H]methanoline. The extracted lipid was applied onto a silica Gel TLC plate and run in a solvent system of 65/35/8 methylene chloride/methanol/ammonium hydroxide (20% v/v). Bands were visualized by exposure to iodine vapor and the silica Gel bands corresponding to PE and PC standards were scraped into scintillation vials containing 200 μl of MeOH, vortexed, and 3 ml of scintillation fluid was added. Radioactivity was counted on a Beckman LS 5000CE liquid scintillation counter. In 4 parallel experiments, hepatocytes were incubated for 24 h in the presence or absence of 10 μM DZA in serum-free medium containing 500 μM of each component choline, betaine, methionine, and ethanolamine, with each experiment having one deuterium-labeled component and the other three unlabeled components. The lipid extracts were analyzed by ESI-MS/MS as described above.

**Determination of Molecular Species—**The fatty acid composition of individual molecular species was determined in the negative ion mode by daughter ion analysis of the [M-CH3]+ ions of PC in a total lipid extract free from PE. The fatty acid composition of [M-CH3]+ ions of PC that overlapped with the [M-H]- ions of PE or PS in the total lipid extract were verified by daughter ion analysis of high performance liquid chromatography-separated hepatocyte PC (18). Daughter ion analysis was performed at collision energy of 25 eV.

**Detection of Water-soluble metabolites by ESI-MS—**For the detection of choline and betaine, the aqueous portion of the Bligh and Dyer (17) extract from 3.5 × 10^9 cells containing 1% formic acid was analyzed directly by ESI-MS in the positive ion mode at a cone voltage of 35 eV.

**DNA Methylation Assay—**Hepatocytes and RH7777 cells were plated in 100-mm dishes and allowed to attach overnight. The cells were incubated in serum-free DMEM containing 2 mg/ml bovine serum albumin for 24 h at 37 °C. The cells were washed twice with phosphate-buffered saline and trypsinized. The cell pellet was washed once with phosphate-buffered saline and DNA was extracted using the genomic DNA extraction kit from Promega. The DNA concentration was measured on a Beckman DU7500 spectrophotometer. The 260 nm/280 nm ratio for all samples was at least 1.7. Extent of DNA methylation was measured using the DNA methylation assay described by Rampersaud et al. (18). DNA (0.5 μg) was incubated with 2 units of Sss1 methylase (New England Biolabs), 1 × Sss1 methylase buffer, and 3 μCi of [3H]adenosylmethionine for 1 h at 37 °C. Then 15 μl of the assay was loaded onto a DE81 ion exchange filter and washed successively with 0.5 μl phosphate buffer, 70% ethanol, and 100% ethanol. The filter was dried and placed in a scintillation vial with 3 ml of scintillation fluid and counted with a Beckman LS 5000CE liquid scintillation counter. Incorporation of "[M-CH3]+" ions of choline was inversely proportional to the level of DNA methylation.

**CDH and BADD Activity Assay—**Rat hepatocytes and rat RH7777 hepatoma cells were homogenized with a Branson Sonifier 250 in buffer composed of 0.9 mM CaCl2, 2.7 mM KCl, 1.5 mM KH2PO4, 0.5 mM MgCl2·6H2O, 1.7 mM NaCl, 5 mM NaHPO4·7H2O, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10% glycerol, and 100 μM phenylmethylsulfonyl fluoride. The homogenized samples were centrifuged at 325 × g for 5 min to clear the protein of cell debris. The protein concentration was determined using the Pierce BCA Protein Assay Reagent Kit according to the manufacturer’s instructions. Reaction mixtures for enzymatic assays contained 40 mM glycine (pH 8.5), 130 μM d3-choline, 50 μM of protein extracts, and homogenization buffer to a final volume of 100 μl and were incubated at 37 °C. At desired time points, 10 μl of 1.2 M HCl was added to terminate the reaction. The reaction mixtures were extracted by adding 1 volume of methanol and 2 volumes of chloroform, vortexed, and centrifuged at 117.5 × g for 5 min at 4 °C. The aqueous phase was collected and formic acid was added for a final concentration of 7H2O, 1 mM EDTA, 1 mM ZnCl2, 20 μM folate, and 400 μM serine. Both the hepatocytes and RH7777

**RESULTS**

**Generation of d3-PC and d6-PC from Individually Methylated Methyl Groups of d3-Choline—**To identify the specific products of PE methylation using methyl groups of choline, a labeling strategy was devised to distinguish them from those derived via the CDP-choline pathway. All three methyl groups of choline were expected to be incorporated into PC simultaneously as a whole unit via the CDP-choline pathway. PE methylation, on the other hand, incorporates one methyl group at a time to form PC. If choline is indeed used as a methyl group donor, we expected to detect PC with one deuterated-methyl group (d3-PC) or two deuterated-methyl groups (d6-PC) when hepatocytes were labeled with d5-choline containing three deuterated methyl groups (HO-CH2-CH2-N[3(CD3)2]). To exclude the possibility of d3- and d6-choline as contaminants, the purity of the d5-choline solution was verified by ESI-MS/MS to be 98.7% (result not shown).

Rat hepatocytes were incubated with 500 μM d5-choline for 24 h in serum-free DMEM under standard cell culture conditions as described under "Experimental Procedures." Labeled cells were harvested and total cellular lipids were extracted according to the method of Bligh and Dyer (17). The newly synthesized PC via the CDP-choline pathway was detected in the positive ion mode by a precursor scan for m/z 193, the phosphocholine head group containing 9 deuterium atoms (d5-PC (Fig. 2A)). d3-PC and d6-PC were detected by precursor scans for m/z 187 and 190, respectively. After 24 h labeling with d5-choline, a significant amount of d3-PC and d6-PC was detected (Fig. 2A). The profiles of d3-PC species and d6-PC species were similar to each other and to that of previously reported species from the PE methylation pathway (4). The peaks correspond to the molecular species listed by peak number in Table I as determined by ESI-MS/MS daughter ion analysis of hepatocyte PC. The d3-PC and d5-PC pools of PC were not detected in unlabeled hepatocytes (results not shown).

**Generation of d3-PC and d6-PC from Methylation of PE Using Methyl Groups of Choline—**To verify if the d3-PC and d6-PC were indeed derived via PE methylation, we designed three labeled PC pools of PC. If choline is indeed used as a methyl group donor, we expected to detect PC with one deuterated-methyl group (d3-PC) or two deuterated-methyl groups (d6-PC) when hepatocytes were labeled with d5-choline containing three deuterated methyl groups (HO-CH2-CH2-N[3(CD3)2]). To exclude the possibility of d3- and d6-choline as contaminants, the purity of the d5-choline solution was verified by ESI-MS/MS to be 98.7% (result not shown).

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**Generation of d3-PC and d6-PC from Methylation of PE Using Methyl Groups of Choline—**To verify if the d3-PC and d6-PC were indeed derived via PE methylation, we designed three additional experiments. The first experiment was to determine whether the presence of d5-PE and d6-PC were PEMS-dependent and not due to free radicals and deamination. The second was to determine whether the presence of d5-PE and d6-PC were PEMS-dependent and not due to free radicals and deamination. We chose a well defined hepatocarcinoma cell line, MCA-RH7777, in which PEMS is deficient (19). The presence or absence of PEMS was demonstrated by incubating the cells in serum-free DMEM containing 50 μM d3-ethanolamine (HO-CD3-CD2-NH2), 500 μM choline, 200 μM methionine, 20 μM folate, and 400 μM serine. Both the hepatocytes and RH7777
cells utilized $d_4$-ethanolamine for PE synthesis via the CDP-ethanolamine pathway and produced $d_4$-PE (results not shown). In hepatocytes, the conversion of $d_4$-PE to $d_4$-PC confirmed the presence of the PE methylation pathway (Fig. 2B). In contrast, $d_4$-PC was not detected in RH7777 cells, which confirmed the absence of PEMT in these cells (Fig. 2B). The cells were then labeled in the same medium described above, but with $d_9$-choline and unlabeled ethanolamine. The presence of $d_7$-PC and $d_{13}$-PC were detected in the hepatocytes with active PEMT but not in the PEMT-deficient hepatoma cells (Fig. 2A), suggesting that these deuterated PC pools were produced specifically by PE methylation.

The second experiment was to detect pools of PC that contained deuterium atoms derived from both $d_4$-ethanolamine and $d_9$-choline. The pools of PC from this dual labeling strategy would contain 7, 10, and 13 deuterium atoms. These pools could only be possible if $d_4$-PE were methylated with one, two, or three $d_3$-methyl groups from $d_9$-choline and the remaining methyl groups were from an unlabeled donor. Similar to other labeling experiments, $d_7$, $d_{10}$, and $d_{13}$-PC were detected in hepatocytes but not in RH7777 cells (Fig. 2C). The ability to detect $d_{13}$-PC indicated the use of choline-derived methyl groups for all 3 PE methylation reactions.

The third experiment was to determine whether PE methylation from a choline-derived methyl group was sensitive to methylation inhibition by DZA which is an inhibitor of S-AdoHyc hydrolase and causes an increase in AdoHyc and 3-deaza-AdoHyc (20) and inhibition of PEMT (21). The effectiveness of PEMT inhibition was confirmed by incubating hepatocytes with $[^3]$H]ethanolamine with and without 10 $\mu$M DZA for

TABLE 1  

| Peak number | Molecular species |
|-------------|------------------|
| 1           | 16:0–16:1        |
| 2           | 16:0–16:0        |
| 3           | 16:0–18:2        |
| 4           | 16:0–18:1        |
| 5           | 16:0–20:4        |
| 6           | 18:1–18:2        |
| 7           | 18:0–18:2        |
| 8           | 16:0–22:6        |
| 9           | 18:1–20:4        |
| 10          | 18:0–20:4        |
| 11          | 18:0–22:6        |

Fig. 2. Choline is a one-carbon donor for PE methylation in hepatocytes, but not in hepatoma cells. Primary hepatocytes were incubated with 500 $\mu$M each $d_9$-choline and unlabeled methionine in serum-free DMEM for 24 h. 3.5 $\times$ $10^5$ hepatocytes and RH7777 cells were incubated for 24 h in serum-free DMEM (with 200 $\mu$M methionine) containing 500 $\mu$M $d_9$-choline (A), $d_4$-ethanolamine (B), or $d_4$-choline and $d_4$-ethanolamine (C). Deuterium-labeled PC was detected by analyzing the lipid extracts (2 nmol/ml total lipid phosphorus) by ESI-MS/MS precursor ion scanning in the positive ion mode. Incorporation of labeled methyl groups from $d_4$-choline into PC was detected in the positive ion mode by precursor ion scanning for parents of m/z 187 (d$_5$-PC), 190 (d$_6$-PC), and 193 (d$_9$-PC) (A). The incorporation of $d_4$-ethanolamine into PC to confirm the presence of the PE methylation pathway was detected in parent ions of m/z 188 (B). $d_4$-Ethanolamine-derived PC pools containing methyl groups from $d_4$-choline were detected in parent ions of m/z 191, 194, and 197 (C). Each scan with detectable phospholipid species is normalized to the highest peak in that scan. Other scans are displayed with background at $\sim$10% to demonstrate no detectable peaks. Each prominent peak represents one or more diacyl molecular species (see Table I).
FIG. 3. DZA inhibits methylation of PE and the incorporation of choline-derived methyl groups into PC. 3.5 × 10^5 hepatocytes were incubated for 24 h in DMEM containing 1 μCi/ml [3H]ethanolamine with or without 10 μM DZA. The lipid extracts were separated on Silica Gel H TLC plates in a solvent system of 65:35:8 methylene chloride:methanol/ammonium hydroxide. The PC bands were scraped from the plates and the amount of radioactivity was determined by scintillation counting. In a parallel experiment, 3.5 × 10^5 hepatocytes were incubated for 24 h in DMEM containing 500 μM each d3-choline and d1-ethanolamine with or without 10 μM DZA. Phospholipid extract was analyzed by ESI-MS/MS in the positive ion mode by precursor ion scanning for parents of m/z 191 (d1-PC) and m/z 194 (d3-PC). The resulting histograms were integrated and the total peak areas were determined. The results of the experiments are expressed as percentage of [%HPC. d3-PC, or d6-PC in control hepatocytes, which were in the absence of DZA.

24 h. The radioactivity in the PC fraction isolated by TLC was measured. There was a 94% decrease in [3H]PC synthesis in the presence of DZA (Fig. 3). When labeled with d3-choline and d4-ethanolamine, the choline-specific PE methylation (d3-PC and d10-PC) was similarly inhibited in hepatocytes treated with DZA (Fig. 3). The detection of d3-PC was too low to obtain reliable numbers. The inhibition results were further confirmed by the similar level of DZA inhibition of d3-PC and d4-PC synthesis from d4-ethanolamine and d5-betaine, respectively, in parallel experiments (results not shown). d3-PC, which was synthesized by the CDP-choline pathway, actually increased 160%, demonstrating that DZA specifically inhibited PE methylation and not the CDP-choline pathway. Taken together, all three labeling strategies in PEMT-defined cells pointed to the conclusion that choline was indeed used as a source of methyl groups for PE methylation.

The Defective Pathway of Choline One-carbon Transfer in Hepatoma Cells—The PEMT dependence of choline-derived PE methylation led to the hypothesis that the inability to form d3-PC and d6-PC could be corrected if PEMT was restored in the hepatoma cells. To test this hypothesis, we expressed PEMT from a full-length cDNA for rat liver PEMT2 in the RH7777 cells and established a stable cell line. The presence of PEMT was confirmed by Western blot analysis (4). The enzymatic activity of PEMT was confirmed by incorporation of d4-ethanolamine into PC (Fig. 4A). Incorporation of d3-methionine into PC in the RH7777/PEMT2 cells (Fig. 4B) suggested that methionine adenylytransferase was active in these cells. However, restoration of PEMT in hepatoma cells failed to incorporate methyl groups from d5-betaine or d6-choline into PC via methylation (Fig. 4, C and D). The synthesis of d6-PC in all three cell populations (Fig. 4D) from d6-choline was primarily due to the CDP-choline pathway. Failure to incorporate d5-betaine methyl groups into PC in RH7777/PEMT2 cells suggested that BHMT and perhaps other upstream steps of the pathway for choline methylation are defective in the hepatoma cells.

To address such a possibility, we used ESI-MS/MS to measure the presence of choline, d5-choline, betaine, and d5-betaine directly in the aqueous phase of the Bligh and Dyer extracts. This strategy offered a direct measurement for all four compounds simultaneously. In unlabeled hepatocytes, the level of betaine was three times as high as that of choline (Fig. 5B, black line, and Fig. 5D). This ratio between choline and betaine is consistent with a previous report that the majority, over 60%, of free choline in the liver is converted to betaine (15). When hepatocytes were labeled with d5-choline in the absence of unlabeled choline, a similar ratio between d5-choline and d5-betaine was observed (Fig. 5B, red line). A complete absence of unlabeled betaine when choline was deficient (in the d5-choline-labeled cells) shows that oxidation of choline is the sole source for betaine in the hepatocytes. In contrast to hepatocytes, hepatoma cells did not have a significant level of betaine above background; neither choline nor d5-choline was oxidized to its product, betaine or d5-betaine, respectively (Fig. 5C).

Newly designed mass spectrometry-based assays for CDH, BADH, and BHMT activities were used to further characterize the choline methyl donation pathways in hepatocytes versus hepatoma cells. The accumulation of products and the disappearance of substrates were measured directly in the reaction mixtures by ESI-MS/MS. The time dependent activity of converting choline to betaine (CDH/BADH) was detected in hepatocytes but not in hepatoma cells (Fig. 6A). Furthermore, the time-dependent BHMT activity of converting betaine to methionine was also detectable in hepatocytes but not in hepatoma cells (Fig. 6B). Together, these results suggest that both choline oxidation and betaine transmethylation are defective in hepatoma cells.

DNA Hypomethylation in Hepatoma Cells—It is therefore reasonable to hypothesize that defective utilization of choline as a methyl source may reduce other cellular methylation reactions, such as DNA methylation, in addition to the abrogated PE methylation. To test this hypothesis, hepatocytes and RH7777 cells were incubated in the presence of 500 μM choline, methionine, and serine. The DNA was isolated and methylated DNA was assayed using a procedure previously described by Balaghi and Wagner (22). This assay involves incubating genomic DNA with a bacterial methylase in the presence of [3H]AdoMet. Sss1 methylase methylates cytosine residues at the 5'-position in CG sequences in both hemi- and unmethylated DNA (23). Thus, the extent of [3H]-methyl incorporation is inversely proportional to the methylation status of the DNA. In the presence of all three methyl donors, RH7777 DNA had twice as much 3H-methyl incorporation, and therefore was only 50% methylated, compared with hepatocyte DNA.

DISCUSSION

This study demonstrates for the first time that choline is capable of donating methyl groups for lipid methylation in the liver. A more important finding, however, is that this pathway is completely abrogated in hepatocarcinoma cells. This study also provides an explanation for why hepatoma cells become “methionine dependent,” a widely observed phenotype for many tumor cells in cell culture. This is the first study to show directly that choline methyl groups can be used for PE methylation. The combination of phospholipid stable-isotope labeling and detection by ESI-MS/MS allowed us to follow the metabolic fate of choline-derived methyl groups independently of the intact molecule. We demonstrated that d3-PC and d6-PC were specific products of one-carbon metabolism using choline as a methyl source. The production of d3-PC and d6-PC were dependent on the presence of PEMT and sensitive to DZA, a universal inhibitor of methylation. The dual labeling of PC with d4-ethanolamine and d6-choline provided a pool of d3-PC that could only have been synthesized by PE methylation. The sensitivity and versatility of ESI-MS/MS also allowed the detection of the aqueous metabolites of the choline one-carbon transfer pathway by direct analysis of the water-soluble portion of the Bligh and Dyer extract.
FIG. 4. Comparison of multiple steps of choline transmethylation in primary and hepatoma cells. $3.5 \times 10^5$ hepatocytes, RH7777, or RH7777/PEMT2 cells were incubated for 24 h in serum-free DMEM containing 500 $\mu$M $d_4$-ethanolamine + 500 $\mu$M each methionine and choline (A), 500 $\mu$M $d_3$-methionine + 500 $\mu$M each ethanolamine and choline (B), 500 $\mu$M $d_3$-betaine + 500 $\mu$M each ethanolamine and methionine (C), or 500 $\mu$M $d_9$-choline + 500 $\mu$M each ethanolamine and methionine (D). Deuterium-labeled PC was detected by analyzing the lipid extracts (2 nmol/ml total lipid phosphorus) by ESI-MS/MS precursor ion scanning in the positive ion mode. The incorporation of $d_4$-ethanolamine into PC to confirm the presence of the PE methylation pathway was detected in parent ions of m/z 188 (A). The incorporation of labeled methyl groups from $d_3$-methionine (B) was determined by analyzing for the presence of $d_3$-, $d_6$-, and $d_9$-PC (precursor ion scans of m/z 187, 190, and 193). The incorporation of a labeled methyl group from $d_3$-betaine (C) and $d_9$-choline (D) into PC was also determined by analyzing for the presence of $d_3$-, $d_6$-, and $d_9$-PC.
AdoMet is the universal one-carbon donor for cellular methylation reactions. There are three sources for generating hepatic pools of methionine required for conversion to AdoMet: 1) exogenous methionine; 2) choline, via CDH, BADH, and BHMT; and 3) 5-methyltetrahydrofolate, via methionine synthase (MS). The third source, 5-methyltetrahydrofolate, contains a serine-derived one-carbon group which is incorporated into the precursor 5,10-methylenetetrahydrofolate via serine hydroxymethyltransferase (39). However, we could not detect any transfer of serine-derived methyl group to PC. The use of choline-derived, but not serine-derived, methyl groups for PE methylation was surprising. The enzymes in the choline and 5-methyltetrahydrofolate one-carbon transfer pathways that catalyze the transmethylation steps to convert homocysteine to methionine, BHMT, and MS, respectively, are reported to utilize homocysteine equally in a recombinant in vitro system (24). Thus, the difference in the utilization of choline-derived and serine-derived methyl groups in our experiments may reflect a difference in substrate utilization in cells versus an in vitro system. However, it may also reflect the possibility that the liver generates distinct pools of AdoMet from different precursors that are used for different transmethylation reactions. The evidence that a choline-devoid diet can cause DNA hypomethylation even in the presence of other methyl sources supports this idea (25–27). Together with the fact that the majority of free choline is converted to betaine, our data also suggest that choline may be a critical factor for general methylation reactions in hepatocytes. The contribution of methyl groups from exogenous methionine versus exogenous choline toward PE methylation was estimated from experiments in Fig. 4. In this experiment, hepatocytes were incubated in the presence of equimolar amounts of $d_5$-methionine and unlabeled choline (Fig. 4B) or unlabeled methionine and $d_9$-choline (Fig. 4D) in medium with all other components equal. The total labeled PC derived from $d_5$-methionine in Fig. 4B was added to the total labeled PC derived from $d_9$-choline in Fig. 4D. Based on the total of these pools, the exogenous methionine contributed to the majority of PE methylation, while exogenous choline contributed ≈5%.

Detection of $d_9$-choline-methylated PC products by ESI-MS/MS and aqueous choline metabolites allowed us to compare the steps of choline transmethylation between primary hepatocytes and hepatoma cells. In agreement with reports of choline and betaine levels in rat liver (28, 34) the level of betaine found in hepatocytes was much higher than that of free choline, by 3-fold in our experiments. However, in RH7777 cells the level of betaine was less than 1/20 of free choline (Fig. 5, C and D). Although capable of using methionine as a methyl source for PE methylation (Fig. 4B), RH7777/PEMT2 cells still could not use exogenous betaine as a methyl group donor (Fig. 4C). Thus, the defective mechanism in hepatocarcinoma cells for PE methylation includes at least CDH/BADH, BHMT, and PEMT1/PEMT2.

Like RH7777 cells, the human hepatoma cell line HepG2 has defects in the oxidation of choline to betaine and in the incorporation of choline methyl groups into PC via PE methylation.2

In addition, HepG2 has been shown to have down-regulated

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2 C. J. DeLong and Z. Cui, unpublished results.
BHMT expression compared with liver (29). Human liver methionine synthase has been found inactive in hepatocarcinoma cells (30). The inactivation of choline oxidation and betaine transmethylation in RH7777 cells therefore contributes to the growing evidence that aberrations in one-carbon metabolism play a significant role in carcinogenesis. The absence of both the choline and the 5-methyltetrahydrofolate one-carbon transfer pathways provides a logical explanation for “methionine dependence,” a widely observed phenomenon in malignancy. Additionally, these findings provide a mechanistic explanation for the long-held observation that choline deficiency leads to liver cancer in rats (25, 31–33). With choline deficiency studies, it is difficult to determine how each role of choline contributes to the carcinogenic process. We have developed a novel approach that allowed us to isolate the role of choline as a methyl group source from that of its role as a precursor to de novo PC synthesis. Given the unique roles of the CDP-choline pathway and PE methylation pathway in liver growth, the disrupted contribution of choline to PE methylation and other methylation reactions in hepatocarcinoma cells would favor cellular growth. Conversely, the contribution of choline to methylation would play a significant role in the control of hepatocyte proliferation.

Liver is the major organ in which PE can be converted to PC via methylation reactions (3). It has been postulated that maintenance of the PE to PC ratio is critical for liver functions (36) such as secretion of lipoproteins and control of the homocysteine level which, if disregulated, may have serious consequences such as heart and coronary diseases (37). It is not surprising that BHMT is present in the liver (38), where there is a high requirement for methylation of lipids and other macromolecules and for regulation of homocysteine homeostasis. BHMT is also present in kidney (38) in which the level of betaine may also be regulated to achieve optimum osmotic pressure. Nevertheless, the implication of a defect in PE methylation in carcinogenesis (5–8, 40–43) is further strengthened by the finding that the only endogenous supply for methyl groups used for phospholipid methylation is completely abolished in the hepatoma cells.

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