Molecular Distinction of Phosphatidylcholine Synthesis between the CDP-Choline Pathway and Phosphatidylethanolamine Methylation Pathway*

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In addition to the CDP-choline pathway for phosphatidylcholine (PC) synthesis, the liver has a unique phosphatidylethanolamine (PE) methyltransferase activity for PC synthesis via three methylations of the ethanolamine moiety of PE. Previous studies indicate that the two pathways are functionally different and not interchangeable even though PC is the common product of both pathways. This study was designed to test the hypothesis that these two pathways produce different profiles of PC species. The PC species from these two pathways were labeled with specific stable isotope precursors, D9-choline and D4-ethanolamine, and analyzed by electrospray tandem mass spectrometry. Our studies revealed a profound distinction in PC profiles between the CDP-choline pathway and the PE methylation pathway. PC molecules produced from the CDP-choline pathway were mainly comprised of medium chain, saturated (e.g. 16:0/18:0) species. On the other hand, PC molecules from the PE methylation pathway were much more diverse and were comprised of significantly more long chain, polyunsaturated (e.g. 18:0/20:4) species. PC species from the methylation pathway contained a higher percentage of arachidonate and were more diverse than those from the CDP-choline pathway. This profound distinction of PC profiles may contribute to the different functions of these two pathways in the liver.

Phosphatidylcholine (PC) is a major group of phospholipid in all mammalian cells (1). PC is comprised of hydrocarbon chains attached to glycerophosphocholine via acyl, alkyl, or alkenyl linkages. The molecular diversity of PC and other phospholipids is dictated by the combination of different lengths, number of double bonds, and types of linkages of hydrocarbon chains. As a result, a single mammalian cell contains at least a thousand species of phospholipids (2). In most mammalian cells, PC is synthesized mainly via the CDP-choline pathway (3). This pathway uses choline as an initial substrate and is catalyzed by three enzymes: choline kinase, CTP:phosphocholine cytidylyltransferase (CT), and choline-phosphate transferase, with CT as the rate-limiting enzyme (1). Hepatocytes are unique because they also possess a high activity of phosphatidylethanolamine methyltransferase (PEMT) that converts PE to PC via three sequential steps of methylation (4) in addition to a high level of CDP-choline pathway activity. The significance of the PEMT pathway is not completely understood (5).

The PEMT pathway seems redundant because its product, PC, is also synthesized by the CDP-choline pathway in hepatocytes. Therefore, the PEMT pathway is traditionally considered a backup pathway for PC synthesis in hepatocytes (3). However, recent studies indicate that these two pathways apparently have opposite effects on proliferative characteristics of the liver and liver-derived cell lines (6–10). These studies suggest that the higher activity of the CDP-choline pathway favors the faster proliferation of hepatocytes. Conversely, expression of PEMT strongly inhibits the growth of hepatoma cell lines and is negatively associated with the developmental growth of liver and with neoplastic growth of liver tumor induced by chemical carcinogens (6–10).

Recombinant expression of PEMT in cultured cell lines leads to an active synthesis of PC via PE methylation (11). However, the PC synthesized via this methylation pathway does not substitute for the role of PC synthesized from the CDP-choline pathway (12). MT58 is a cell line derived from Chinese hamster ovary (CHO) K1 cells by chemical mutagenesis and carries a temperature-sensitive mutation in the rate-limiting enzyme, CT, of the CDP-choline pathway (13). At the non-permissive temperature, CT becomes inactive, PC synthesis shuts down, and the mutant dies via apoptosis. Recombinant expression of rat liver CT rescues the mutant effectively, whereas recombinant expression of rat liver PEMT fails to rescue MT58 cells at the non-permissive temperature (12). Apparently the roles of the CDP-choline pathway and the PEMT pathway are distinct and not interchangeable. One hypothesis for this distinction is that PC synthesized from different pathways may have different subcellular locations. The ability of exogenous PC to rescue the mutant phenotype (14) suggests, however, that PC synthesized at one subcellular location is unlikely to be restricted from moving freely to another subcellular location. Thus, the hypothesis of distinctive localization of PC is an unlikely explanation for why PEMT fails to rescue the mutant.

Another hypothesis is that the two pathways synthesize different pools of PC molecular species. To test this hypothesis, we devised a novel strategy capable of specifically displaying the PC species derived from the CDP-choline pathway and the PEMT pathway. We report here that the PC species synthesized from the CDP-choline pathway were mainly comprised of medium chain and saturated species, whereas the PC species derived from the methylation pathway are mainly comprised of

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† The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; CT, CTP:phosphocholine cytidylyltransferase; CDP, cytidine diphosphate; PEMT, phosphatidylethanolamine methyltransferase; PAGE, polyacrylamide gel electrophoresis; MEM, modified essential medium.
long chain and highly unsaturated species. Our study suggests that the difference in PC composition contributes to the functional distinction between the two pathways.

**EXPERIMENTAL PROCEDURES**

**Materials**—D4-ethanolamine and D9-choline chloride were from Iso-tech, Inc. [3H]ethanolamine was from American Radiolabeled Chemicals, Inc. Dulbecco's modified essential medium (MEM) and fetal bovine serum were from Life Technologies, Inc. Silica gel H plates were from Analtech, Inc. Phospholipid standards were from Avanti Polar Lipids. All other chemicals and materials were from Fisher Scientific.

**Cell Culture**—Rat primary hepatocytes were obtained by collagenase perfusion (15). Hepatocytes were cultured on collagen-coated culture dishes overnight prior to experiments in Dulbecco's MEM with 10% fetal bovine serum, 10 μg/ml insulin, and 10 mM Hepes buffer. McArdle RH7777 cells were dually transfected with 10 μg of pCMV5/PEMT (11) and 1 μg of pSV2neo plasmid by calcium phosphate precipitation as described (16). G418-resistant colonies were selected with 50 μg/ml G418 and maintained in 200 μg/ml G418.

**Incorporation of Tritium-labeled Precursors into PC**—3.5 × 10⁵ cells were incubated with 1 μCi/ml each, [3H]ethanolamine, or [3H]ethanolamine HCl for 24 h in low choline medium (choline-free MEM + 2% fetal bovine serum). Cells were scraped into methanol, and lipids were extracted according to Bligh and Dyer (17). The lipid extractions were separated on silica gel H plates in a solvent system of 65/35/8 chloroform/methanol/H₂O. Data were acquired using a Micromass Quattro II triple quadruple mass spectrometer (Micromass, United Kingdom) in a solvent system of 45/45/10 methylene chloride/methanol/H₂O. Standards and samples contained 1% formic acid for positive ion analysis, and 1% ammonium hydroxide for negative ion analysis. PC molecular species were detected by precursor ion scanning for m/z + 184, +188, and +193 in the positive ion mode. PE molecular species were detected by neutral loss scanning for m/z 141 or 145 in the positive ion mode. The fatty acid composition of each molecular specie was determined by daughter ion analysis in the negative ion mode. The intensities of PC species with equal concentration decreased significantly as mass increased. To correct spectra for mass discrimination, PC standards were analyzed at equal concentration of total protein from each cell line was prepared by SDS-polyacrylamide gel electrophoresis (19) and transferred to nitrocellulose membrane (20). The membranes were probed with anti-PEMT protein displayed by a reaction with Supersignal Chemiluminescent Substrate (Pierce) and exposure to x-ray films.

**RESULTS AND DISCUSSION**

Electrospray ionization tandem mass spectrometry analysis (18, 21–25) of PC species labeled with specific deuterated precursors allowed us to distinguish the PC species synthesized from the CDP-choline pathway and the PE methylation pathway (Fig. 1A). Different phospholipid classes were specifically detected by the unique mass/charge (m/z) ratio of the molecular fragments produced upon argon-induced collision. All PC molecules produced a fragment with m/z of +184 corresponding to the protonated phosphocholine head group. All PE molecules were detected by the loss of a neutral fragment of m/z 141, corresponding to the uncharged phosphoethanolamine head group. D4-ethanolamine-labeled PC species with m/z +188 head group were exclusively derived from the PE methylation pathway, and D9-choline-labeled species with m/z +193 head group were specifically synthesized from the CDP-choline pathway. The major advantage of this strategy is that the metabolism of major phospholipid groups can be analyzed from total lipid.
Cells were incubated in the presence of 1 μCi/ml 3H-ethanolamine for 24 h, and the lipids were extracted and separated by thin layer chromatography. The PC and PE bands were scraped and counted by a scintillation counter. PEMT2 activity levels were measured by the radioactivity of [3H]PC as a percentage of total radioactivities in both control and PEMT-expressing cell lines and were not affected by the expression of PEMT in principal with the previous report that purified PEMT prefers the long chain polyunsaturated PE as substrate (26) for PC synthesis.

To determine whether D4-choline was a viable precursor of PC synthesis, we incubated the RH7777 cells with the labeled choline for various times. A time-dependent accumulation of the labeled PC was observed (Fig. 1D), suggesting that deuterium-labeled choline was indeed a viable precursor for PC synthesis.

To determine the PC profile of the PEMT pathway, we reconstituted the rat liver PEMT into the rat hepatoma cell line McA-RH7777, in which PEMT is absent. Thus, changes in PC synthesis as a specific result of PEMT expression were determined. The stably expressed PEMT was confirmed by Western blot analysis using a rat liver PEMT-specific antibody (9) (Fig. 2A). The reconstituted PEMT pathway was confirmed by measuring the incorporation of [3H]ethanolamine into PC isolated by thin layer chromatography (Fig. 2B). The cells were then labeled simultaneously with excess D9-choline and D4-ethanolamine for 24 h at 37 °C. Total lipids were extracted and analyzed by tandem mass spectrometry. The PC species with head group mass of 188 were derived exclusively from the PEMT pathway and were detected only in the RH7777 cells that expressed PEMT (Fig. 2F) and not in the control cells, in which PEMT activity was absent (Fig. 2E). The PC species with head group mass of 193 from the CDP-choline pathway were present in both control and PEMT-expressing cell lines and were not affected by the expression of PEMT (Fig. 2, C and D).

In the PEMT-expressing RH7777 cells, comparison between PC species newly synthesized from the CDP-choline pathway (Fig. 2D) and that from the PE methylation (Fig. 2F) revealed a clear distinction. The major species of the PC derived from the CDP-choline pathway were diacyl 16:0/18:1, 18:0/18:2, and 18:1/18:1, which together made up a majority of the choline-derived PC. On the other hand, PC derived from the methylation pathway contained significantly more long chain, polyunsaturated PC species (18:1/18:1, 18:0/18:2, 18:2/20:4, 18:1/20:4, 18:0/20:4, 18:0/22:6, 18:1/22:5). These findings were in agreement in principal with the previous report that purified PEMT prefers the long chain polyunsaturated PE as substrate (26) for PC synthesis.

There are additional findings worthy of notice. First, in addition to the readily identifiable PC species, several phosphocholine-containing lipids didn’t match with any deduced structures and m/z of diacyl, 1-alkyl/2-acyl, or 1-alkenyl/2-acyl. The structures of these novel lipids are currently under investigation. Second, because of their biphasic nature, most lyso-PC species were lost in the water phase during lipid extraction according to the method of Bligh and Dyer (17). This problem can be solved by modification of the lipid extraction and lyso-PC species will be a subject of future studies.

FIG. 2. Reconstitution of the PE methylation pathway in the rat hepatocyte-derived cell line. McA-RH7777 cells were co-transfected with pCMV5/PEMT (rat liver) and pSV2-neo plasmids and selected for stable G418-resistant cell lines. The expression of functional PEMT was confirmed by Western blot analysis (A). Fifty μg of cell lysates from each cell line was separated on a 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane. The proteins were probed with a specific antibody to PEMT2 and visualized by enhanced chemiluminescence. Conversion of PE to PC in the cell lines was determined by incorporation of tritium-labeled ethanolamine into PC (B). Cells were incubated in the presence of 1 μCi/ml 3H-ethanolamine for 24 h, and the lipids were extracted and separated by thin layer chromatography. The PC and PE bands were scraped and counted by a scintillation counter. PEMT2 activity levels were measured by the radioactivity of [3H]PC as a percentage of total radioactivities in both 3HPE and 3HPC. Cells were labeled with D4-ethanolamine and D9-choline as described in Fig. 1. PC species labeled by D9-choline (C and D) and D4-ethanolamine (E and F) in control cells (C and E) and PEMT-expressing cells (D and F) were analyzed by electrospray tandem mass spectrometry. The x axes are offset by a mass of 5 to line up vertical positions of the corresponding PC species with different head group masses (188 versus 193).
To determine whether these distinct profiles of PC in RH7777 cells reflected the physiological status of PC synthesis in the liver in which the CDP-choline pathway and PEMT pathway are endogenously co-existent, we performed similar studies in freshly prepared hepatocytes. The primary hepatocytes were labeled with the D4-labeled ethanolamine and D9-choline for 24 h. The total lipids were then extracted and analyzed. The distinction of the PC species from the two pathways was much more profound in primary hepatocytes than that of RH7777/PEMT cells (Fig. 3 and Table I). The PEMT pathway in primary hepatocytes produced more 38-carbon PC species of 18:2/20:4 and 18:0/20:4 than that in RH7777/PEMT cells. The differences between RH7777/PEMT and primary hepatocytes may reflect the fact that RH7777 cells are fast dividing cells in which bioactive PC species are turning over faster than those in the non-dividing hepatocytes.

Although primary hepatocytes did not divide in cultures, we wondered to what degree the endogenous PC was replaced by the newly synthesized PC when excess D4-ethanolamine and D9-choline were present in the culture media. We labeled the cells for 24 h with both deuterated precursors. Comparison of unlabeled PC between labeled cells and unlabeled cells revealed that over 75% of endogenous PC was replaced by the newly synthesized PC in 24 h (Fig. 4, A and B). Of the labeled PC, 70% was from the CDP-choline pathway and 30% was from the PE methylation pathway. The turnover of PC was surprisingly active even when hepatocytes were essentially in a quiescent state. Comparison of the residual PC species to that in the unlabeled cells suggests that the turnover of 16:0/18:2, 16:0–20:4, 18:1/18:1, 18:2–20:4, 18:0/20:4, and 18:0–22:6 species were much more active than that of 16:0/18:1 (Fig. 4, C and D). PC species derived from PE may be more actively metabolized than the choline-derived PC species. Additionally, PC species from the methylation pathway contained a higher per-

FIG. 3. Distinct species of PC from the CDP-choline pathway and from the PE methylation pathway. Primary hepatocytes were incubated with D4-ethanolamine and D9-choline at 50 μg/ml for 24 h at 37 °C. Extracts of total lipids were analyzed by the electrospray tandem mass spectrometry as described in Fig. 1. A, PC species derived from the CDP-choline pathway; B, PC species derived from the PE methylation pathway. The x axes are offset by a mass of 5 to line up vertical positions of the corresponding PC species with different head group masses (188 versus 193).

TABLE I
Quantitation of PC species in primary hepatocytes

Total chloroform extracts of primary hepatocytes were prepared as described under “Experimental Procedures.” Lipids with head group mass of 184 (unlabeled), 188 (from the PE methylation pathway), and 193 (from the CDP-choline pathway) were identified by electrospray tandem mass spectrometry. Fatty acid composition was determined by daughter ion analysis. m/z values of all major unlabeled PC peaks are shown in column 1. The percentages of all m/z peaks in the total PC species derived from the CDP-choline pathway are listed in column 2 and in the total PC species derived from the PEMT pathway are shown in column 3. Multiple species in each m/z peak, if present, are shown in columns 4–7 in decreasing order of prevalence. “A” indicates alkyl or alkenyl-linked fatty acid residue at sn-1 position.

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<th>m/z</th>
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Exogenous PC with medium side-chains and high degree of saturation can rescue the mutant cells at the non-permissive temperature (14). However, long-chain and highly unsaturated PC species are “toxic” to the cells. Expression of PEMT in RH7777 displayed a strong and quantitative inhibition of cell growth (6). Our results suggest that the inhibitory effect of PEMT expression in RH7777 cells may be because of the production of long-chain highly unsaturated PC species that are “toxic” to the cells.

The profound distinction in PC molecular species from the two pathways was unexpected. Nevertheless, given the opposite effects of the two pathways on hepatocyte proliferation, this distinction in PC species may account for most, if not all, of the functional difference of the two pathways. This profound distinction may also reflect a possibility that both the choline-phosphotransferase and the PE methyltransferase have preference for fatty acid side-chains in addition to the head group specificity. However, it cannot be excluded that the distinct function of the PEMT pathway may also be because of a selective depletion of PE species as the initial substrates of methylation. PC species turn over at fast and yet different rates even in non-dividing cells. With our current strategy of labeling and detection, all molecular species of PC can be cleanly resolved and readily quantitated. Not only can the PC species from different pathways be distinguished unequivocally via labeling with distinctive precursors, but the newly synthesized PC can be compared with the pre-existing PC species. The latter feature of this strategy offers a unique advantage allowing a precise estimation of turnover rates of all subclasses and all species of phospholipids. Mass spectrometry offers a resolution of at least 0.01\% m/z (or 0.1 dalton for PC species). Such a resolution is capable of detecting changes of one proton at either head group or side-chains. Tandem mass spectrometry eliminates any requirement for separating phospholipid subclasses before analysis. Thus, chloroform extracts of cells can be directly analyzed. This advantage appears critical for analysis of phospholipid species because many separation procedures, such as the thin layer chromatography, introduce preferential loss of certain species (results not shown). The sensitivity of this strategy requires more than 5,000 cells for analyzing the profiles of phospholipid species. With the addition of a nanospray device, lipids from as few as 50 cells will provide ample amount of materials for analysis. With such unprecedented resolution, sensitivity, and simplicity of tandem mass spectrometry, appropriate labeling with stable isotope precursors shall reveal many aspects of lipid metabolism that cannot be clearly addressed by radioactive labeling.

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FIG. 4. Differential displacement of PC by newly synthesized PC. Primary hepatocytes were labeled with D9-choline and D4-ethanolamine as described in Fig. 1. Equal amounts (500 pmol of lipid phosphorus) of lipid extracts for each sample were analyzed. PC species (A) in unlabeled cells and unlabeled PC species (B) in the cells after 24-hour labeling with 50 μg/ml at 37 °C were analyzed by electrospray tandem mass spectrometry. To compare the relative composition of PC within each spectrum (A and B), the intensities (y axes) were adjusted to obtain close-up views of the profiles. Panel C is the close-up view of panel A, and panel D is the close-up view of panel B.

centage of arachidonate (Fig. 3B). Given the widely spread involvement of arachidonic acid in cellular regulations, the PC species derived from the PEMT pathway in the liver may serve as an important source for generating lipids active in cellular regulation.

The biochemical significance of the PEMT pathway in hepatocytes has been of great interest because the CDP-choline pathway is already present in all mammalian cells and is involved in the pathway synthesized only two or three major species of PC in the liver. As an important source for generating lipids active in cellular regulations, the PEMT pathway may also be because of a selective depletion of PE species as the initial substrates of methylation. PC species turn over at fast and yet different rates even in non-dividing cells. With our current strategy of labeling and detection, all molecular species of PC can be cleanly resolved and readily quantitated. Not only can the PC species from different pathways be distinguished unequivocally via labeling with distinctive precursors, but the newly synthesized PC can be compared with the pre-existing PC species. The latter feature of this strategy offers a unique advantage allowing a precise estimation of turnover rates of all subclasses and all species of phospholipids. Mass spectrometry offers a resolution of at least 0.01% m/z (or 0.1 dalton for PC species). Such a resolution is capable of detecting changes of one proton at either head group or side-chains. Tandem mass spectrometry eliminates any requirement for separating phospholipid subclasses before analysis. Thus, chloroform extracts of cells can be directly analyzed. This advantage appears critical for analysis of phospholipid species because many separation procedures, such as the thin layer chromatography, introduce preferential loss of certain species (results not shown). The sensitivity of this strategy requires more than 5,000 cells for analyzing the profiles of phospholipid species. With the addition of a nanospray device, lipids from as few as 50 cells will provide ample amount of materials for analysis. With such unprecedented resolution, sensitivity, and simplicity of tandem mass spectrometry, appropriate labeling with stable isotope precursors shall reveal many aspects of lipid metabolism that cannot be clearly addressed by radioactive labeling.
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