methods

Specificity and rate of human and mouse liver and plasma phosphatidylcholine synthesis analyzed in vivo[S]

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Abstract **Phosphatidylcholine (PC) synthesis by the direct cytidine diphosphate choline (CDP-choline) pathway in rat liver generates predominantly mono- and di-unsaturated molecular species, while polyunsaturated PC species are synthesized largely by the phosphatidylethanolamine-*N*-methyltransferase (PEMT) pathway. Although altered PC synthesis has been suggested to contribute to development of hepatocarcinoma and nonalcoholic steatohepatitis, analysis of the specificity of hepatic PC metabolism in human patients has been limited by the lack of sensitive and safe methodologies. Here we incorporated a deuterated *methyl*-D_9-labled choline chloride, to quantify biosynthesis fluxes through both of the PC synthetic pathways in vivo in human volunteers and compared these fluxes with those in mice. Rates and molecular specificities of label incorporated into mouse liver and plasma PC were very similar and strongly suggest that label incorporation into human plasma PC can provide a direct measure of hepatic PC synthesis in human subjects. Importantly, we demonstrate for the first time that the PEMT pathway in human liver is selective for polyunsaturated PC species, especially those containing docosahexaenoic acid. Finally, we present a multiple isotopomer distribution analysis approach, based on transfer of deuterated methyl groups to *S*-adenosylmethionine and subsequent sequential methylations of PE, to quantify absolute flux rates through the PEMT pathway that are applicable to studies of liver dysfunction in clinical studies.**—Pynn, C. J., N. G. Henderson, H. Clark, G. Koster, W. Bernhard, and A. D. Postle. **Specificity and rate of human and mouse liver and plasma phosphatidylcholine synthesis analyzed in vivo.** ***J. Lipid Res.*** **2011**. 52: **399–407.**

Supplementary key words deuterated choline • lipidomics • multiple isotopomer distribution analysis • phosphatidylethanolamine-*N*-methyltransferase • stable isotope

Integration of lipid metabolism is one fundamental role of the liver, converting the diversity of lipid delivered from the diet as chylomicron remnants and nonesterified fatty acids into a relatively constant composition of lipoprotein lipid for export to extrahepatic tissues. Phosphatidylcholine (PC) synthesis is essential for VLDL secretion from the liver (1), and modulation of PC synthesis and metabolism is directly linked to a number of liver diseases, including alcoholic (2) and nonalcoholic fatty liver (NAFL) disease (3), liver cancer (4), and cystic fibrosis (5). The bulk of chain elongation and desaturation of the dietary essential fatty acids linoleate ($18:2_{n-6}$) and α-linoleneate ($18:2_{n-3}$) to the polyunsaturated fatty acids (PUFA) arachidonate ($20:4_{n-6}$), eiscosapentoneate ($20:5_{n-3}$), and docosahexaenoate ($22:6_{n-3}$) takes place in the liver, and lipoprotein PC is the major vehicle for PUFA exportation from the liver (6).

PC is synthesized by all cell types directly from choline and diacylglycerol by the CDP-choline pathway (7) (**Fig. 1A**), but it can also be synthesized in hepatocytes by three sequential methylations of phosphatidylethanolamine (PE), catalyzed by PE-*N*-methyltransferases (PEMT) (Fig. 1B) (8). This pathway uses *S*-adenosylmethionine (SAMe) as the methyl donor and accounts for about 30% of total liver PC synthesis. The molecular specificity of PC synthesis by both pathways, as defined by the combination of fatty acyl moieties esterified at the *sn*-1 and *sn*-2 positions of the glycerophosphate backbone, has been established in rat and guinea pig livers and hepatocytes by metabolic labeling studies using radioisotopes (9–12). The CDP-choline

Abbreviations: LPCAT, lysophosphatidylcholine acyltransferase; MIDA, multiple isotopomer distribution analysis; MRM, multiple reaction monitoring; NAFL, non-alcoholic liver disease; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, PE-*N*-methyltransferase; SAMe, *S*-adenosylmethionine.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of three equations, one table, and two figures.

This project was supported by an equipment grant from Wellcome Trust, ref. 057405.

Manuscript received 5 October 2010 and in revised form 1 November 2010.

Published, JLR Papers in Press, November 10, 2010
DOI 10.1194/jlr.d011916

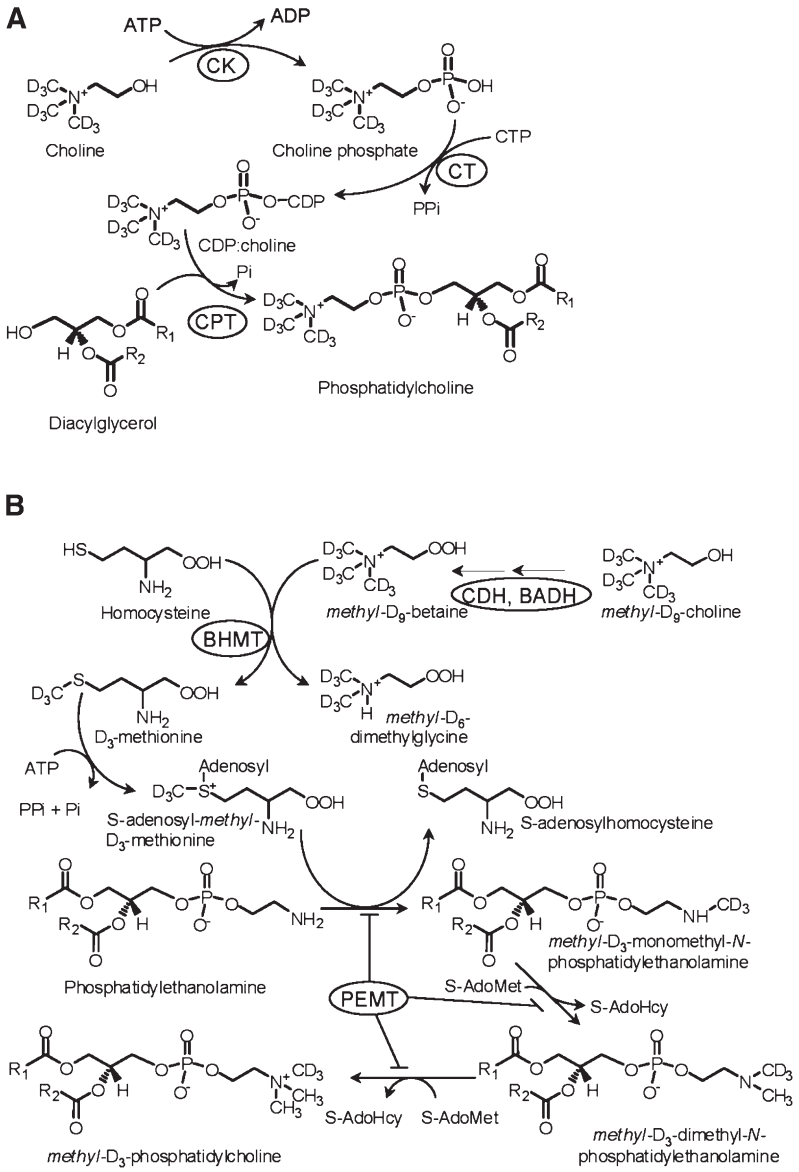

Fig. 1. CDP-choline (A) and phosphatidylethanolamine-*N*-methyltransferase (PEMT) (B) pathways for synthesis of liver phosphatidylcholine. Enzymes indentified are CK, choline kinase; CT, CTP-choline phosphate cytidylyltransferase; CPT, CDP-choline phosphotransferase; CDH, choline dehydrogenase; BADH, betaine aldehyde dehydrogenase; and BHMT, betaine-homocysteine methyltransferase.

pathway forms mainly monounsaturated and di-unsaturated PC species such as palmitoyloleoyl-PC (PC16:0/18:1) and palmitoyl linoleoyl-PC (PC16:0/18:2), whereas the PE-*N* methylation pathway synthesizes PUFA-containing PC species such as palmitoyldocosahexaenoyl-PC (PC16:0/22:6) and palmitoylarachidonoyl-PC (PC16:0/20:4) (in this molecular species nomenclature, e.g., A:a/B:b, A and B represent the total number of carbon atoms in each fatty acid at the *sn*-1 and *sn*-2 positions of the glycerophosphate backbone, respectively, and a and b represent the number of unsaturated double bonds in each fatty acid). The importance of PE-*N* methylation for the supply of PUFA from the liver to peripheral tissues is highlighted in PEMT$^{-/-}$ mice, which have substantially diminished plasma concentrations of docosahexaenoyl-containing ($22:6_{n-3}$) and arachidonoyl-containing ($20:4_{n-6}$) PC species, although fatal liver failure is prevented and total plasma PC is normalized by dietary choline supplementation (13, 14). Considerable evidence suggests that PE-*N* methylation in liver is involved in the regulation of cell division (15). For instance, PEMT expression and activity are low both in human hepatocellular carcinomas (16) and during rat liver growth after partial hepatectomy (17).

PC synthesis in human liver disease has received less attention largely because of the lack of appropriately safe methodologies. Studies using a combination of isotope ratio mass spectrometry (MS) and multiple isotopomer distribution analysis (MIDA) modeling have determined rates of hepatic triacylglycerol synthesis and VLDL secretion by using stable isotopes (reviewed in [18]) but have not investigated the synthesis and turnover of hepatic or plasma PC. An alternative analytical approach (19) analyzes incorporation of stable isotope-labeled substrates into intact PC molecules. Incubation of rat hepatocytes with deuterated *methyl*-D_9-labeled choline chloride, followed by electrospray ionization tandem MS (ESI-MS/MS), enabled determination of the synthesis of individual PC molecular species. Moreover, choline is a major methyl donor for synthesis of SAMe, resulting in transfer of a labeled methyl group to form *methyl*-D_3-SAMe (Fig. 1B) (19).

Consequently, this method is also used to monitor the PEMT pathway flux from the incorporation of the *methyl*-D_3 label (Fig. 1B), as well as to direct incorporation of the *methyl*-D_9 label. Importantly, these analyses of PC biosynthesis fluxes provide information about the individual molecular species that are biologically relevant molecules, and, consequently, analyses of their kinetics are essential to determine the contribution of altered PC metabolism to disease mechanisms.

We previously applied ESI-MS/MS methodology to quantify the synthesis and turnover of individual molecular species of PC in lung surfactant in human volunteers (20) and now use it to describe the synthesis of individual molecular species of hepatic PC in vivo. Initial analyses were conducted in a mouse model to establish whether analysis of the molecular specificity and dynamics of label incorporation into plasma PC can provide a direct insight into liver PC metabolism. This methodology was then applied to human volunteers to show for the first time the molecular specificity of PC synthesis by human liver in vivo, monitored by the appearance of label in plasma PC. Finally, we present a mass isotopomer analysis for the calculation of substrate flux through the PEMT pathway that highlights both the practicality and diagnostic/investigative potential of stable isotope labeling with *methyl*-D_9-choline for the study of diseases involving altered hepatic lipid metabolism in humans in vivo.

MATERIALS AND METHODS

Animal labeling

Eight-week-old C57BL6 female mice (20.8 ± 1.33 g) received intraperitoneal (ip) injections of 100 μl of *methyl*-D_9-choline chloride (10 mg/ml in sterile physiological saline) and were then sacrificed at 1.5, 3, 6, and 24 h (n = 5 mice per time point). Blood was drawn by heart puncture and collected in Microtainer® lithium heparin tubes (Becton Dickinson, Oxford, UK), and plasma was isolated by centrifugation (1,000 *g* for 10 min) and stored at −20°C. Livers and lungs were removed, snap frozen in liquid nitrogen, and stored at –80°C. Animals received appropriate humane care according to National Institutes of Health guidelines. All animal procedures were conducted under United Kingdom Home Office Animal Procedures License PPL30/1661.

Human labeling

Methyl-D_9-choline chloride (3.6 mg/kg of body weight) was infused intravenously over 3 h in 1 male and 3 healthy human female volunteers (22–27 years old; weighing 63–92 kg). Venous blood samples mixed with EDTA were collected at intervals from 6 to 168 h after the start of *methyl*-D_9-choline chloride infusion; samples were centrifuged at 1,000 *g* at 4°C for 15min, and plasma was stored at –20°C. Initial blood samples and those collected at 24, 48, 72, 96, and 168 h were taken after overnight fasting, but volunteers did not fast before all other samples were taken. All volunteers ate their normal diets and consumed a light breakfast before the infusions were started. There was no attempt in this pilot study to control diets before or during the experimental days. The study was approved by the local ethics committee at Hannover Medical School, subjects' written consent was obtained, and standard laboratory parameters showed no signs of impaired renal or hepatic function.

Analysis of plasma and tissue phospholipids and tissue choline phosphate

A total of 100 mg of mouse liver or lung was homogenized (Ultra-Turrax; Janke and Kunkel, Germany) in 3 ml of 0.9% saline on ice. Lipids were extracted from homogenate (800 μl) and plasma (50 μl) by using chloroform and methanol (21), dried under nitrogen gas, and analyzed by ESI-MS. Dimyristoyl PC (PC14:0/14:0) and dimyristoyl PE (PE14:0/14:0) were added as internal recovery quantification standards. PC species were quantified using a Waters Micromass Quattro Ultima triple quadrupole mass spectrometer (Micromass, Wythenshaw, UK) equipped with an ESI interface. Samples were dissolved in methanol-chloroform-water-25% NH_4OH (7:2:0.8:0.2,v/v) and introduced into the mass spectrometer by direct infusion. Collision gas-induced dissociation using argon gas produced protonated phosphocholine head group fragments with m/z of +184, corresponding to endogenous PC, and m/z of +193 for PC synthesized via the CDP-choline pathway (22). Analysis of the products with m/z +187 or +190 (i.e., containing one or two recycled deuterium-labeled methyl groups) revealed indirect incorporation of deuterium into the PC head group via the PEMT pathway (23). PC molecular species were quantified from diagnostic precursor ion scans of the respective fragment ions (P184, P187, P190, P193). A diagnostic neutral loss scan of m/z +141 (NL141) provided analysis of diacyl PE molecular species. Concentrations of infused phospholipids were optimized within the linear response range of the mass spectrometer. Importantly, varying the concentration of infused lipid within this range did not significantly affect the PC or PE molecular species profiles or the fractional incorporation of stable isotope labels. Phospholipid spectra were quantified using MassLynx software and Excel spreadsheets (see Supplementary material).

The aqueous upper phase from tissue extracts was collected and lyophilized, and enrichment of the *methyl*-D_9 label in substrate choline phosphate was determined. Choline phosphate was isolated using aminopropyl solid phase cartridges (Varian Inc., Palo Alto, CA) (24). Endogenous and *methyl*-D_9-choline phosphate was quantified by ESI-MS/MS operated in the multiple reaction monitoring (MRM) mode, monitoring m/z transitions 183.8 → 85.8 and 192.8 → 94.8, respectively.

RESULTS

ESI-MS/MS analysis of molecular specificities of PC synthesis pathways in mouse liver

Incorporation patterns of labeled precursors into mouse liver PC by both the CDP-choline and the PEMT pathways 1.5 h after injection of *methyl*-D_9-choline chloride are illustrated in **Fig. 2**. Spectra have been mass displaced on the x-axis to align PC, newly synthesized PC, and PE with equivalent molecular species components. They show the almost complete segregation of molecular specificities of PC synthesis by the two pathways. Endogenous PC (Fig. 2A, P184 MS scan) was a combination of mono-, di-, and polyunsaturated species. PC synthesized directly by the CDP-choline pathway (Fig. 2B, P193 scan) was preferentially enriched in mono- (PC16:0/18:1) and di-unsaturated (PC16:0/18:2) species. In contrast, PC synthesized by the PEMT pathway (Fig. 2C, P187, containing one recycled *methyl*-D_3 group) was predominantly polyunsaturated, and PC16:0/22:6 was the major species formed. This pattern of molecular species was similar but not identical to that of

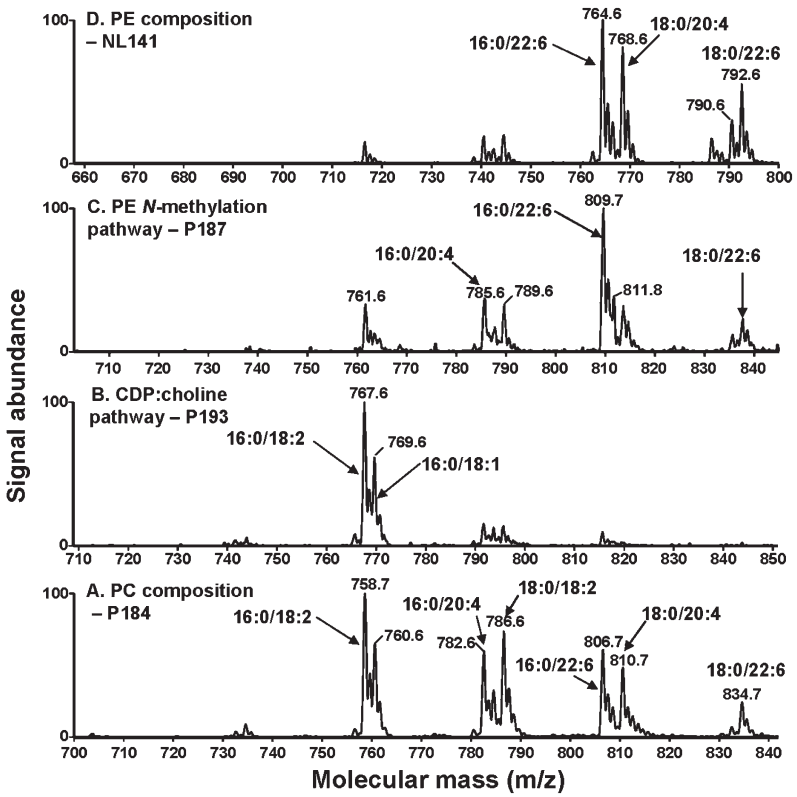

Fig. 2. Incorporation of *methyl*-D_9-labeled choline into mouse liver. *Methyl*-D_9-choline (1 mg) was injected ip, and the mouse was euthanized 1.5 h later. A total lipid extract of liver was analyzed by diagnostic ESI-MS/MS scans. A: precursors of *m/z* 184 (P184) for endogenous PC; (B) precursors of *m/z* 193 (P193) for PC species synthesized by the CDP-choline pathway (*methyl*-D_9-labeled) (C) precursors of *m/z* 187 (P187) for PC species synthesized by the PEMT pathway (*methyl*-D_3-labeled); and (D) neutral loss of *m/z* 141 (NL141) for diacyl PE. Spectra are aligned on the x-axis to enable direct comparisons of phospholipid species with equivalent combinations of fatty acids. The molecular species nomenclature is A:a/B:b, where A and B represent the total number of carbon atoms in each fatty acid at the *sn*-1 and *sn*-2 positions of the glycerophosphate backbone, and a and b represent the number of unsaturated double bonds in each fatty acid.

endogenous PE, the substrate for the PEMT pathway (Fig. 2D, NL141), which was composed mostly of 16:0/22:6, 18:0/20:4, and 18:0/22:6 species.

Kinetics of incorporation of *methyl*-D_9-choline into PC by the CDP-choline and PE-*N* methylation pathways

The totals of all PC species shown in Fig. 2B with abundance proportions greater than 1% of the largest ion peak in each spectrum were quantified for P184, P187, P190, and P183 MS scans in extracts of mouse liver, lungs, and blood plasma and extracts of human plasma. Lung was chosen for comparison, as another organ specialized for PC secretion, in this case, pulmonary surfactant. The fractional incorporation of *methyl*-D_9 label into mouse liver and lung PC and expressed relative to total PC is shown in **Fig. 3A,** and the corresponding incorporation of *methyl*-D_3 label is shown in Fig. 3B. The initial rates of hepatic and lung PC *methyl*-D_9 labeling were already very high by 1.5 h, reached maxima at 3 h, and subsequently declined rapidly. Fig. 3B shows *methyl*-D_3 labeling was almost exclusive for liver PC at 1.5, 3, and 6 h; the lung labeling at 24 h represented PC synthesis by the CDP-choline pathway from recycled *methyl*-D_3-choline and was not associated with PE-*N* methylation (Supplementary Fig. S1).

Appearance of label in mouse plasma PC closely reflected labeling of liver PC by both pathways (Fig. 3C), demonstrating that not only was PC synthesized very rapidly by mouse liver but that it was secreted equally rapidly into the circulation. The comparable pattern of stable isotope labeling of human plasma PC over a more prolonged time scale (Fig. 3D) demonstrated that both pathways were active in the synthesis of lipoprotein PC, with proportionately less PC being synthesized by the PE-*N* methylation pathway in human than in mouse liver.

Molecular specificity of PC synthesis by the CDP-choline and PE-*N* methylation pathways

The composition of endogenous and newly synthesized PC is detailed in Supplementary Table S1 and summarized in **Fig. 4** for the most informative PC species. These species were selected to illustrate the major alterations that occurred over time to the stable isotope incorporation patterns by the CDP-choline (Fig. 4A, C, E) and PE-*N* methylation (Fig. 4B, D, F) pathways into PC from mouse liver (Fig. 4A, B), mouse plasma (Fig. 4CD), and human plasma (Fig. 4E, F). These changes are remarkably consistent across different sample types, although the magnitude of the effect differs. Temporal alteration to mouse liver PC synthesis by the CDP-choline pathway is consistent with acyl remodeling at both the *sn*-1 and the *sn*-2 positions, with initial synthesis as PC16:0/18:2, followed by progressive redistribution of label to PC18:0/18:2 and PC18:0/20:4, and resembles the endogenous PC composition by 24 h (Fig. 4A). The equivalent labeling pattern is essentially identical for mouse plasma (Fig. 4C) and, importantly, very similar for human plasma (Fig. 4E).

Initial incorporation into PC16:0/22:6 in mouse liver PC by the PE-*N* methylation pathway (Fig. 4B) reflected the enrichment of corresponding species in liver PE (Fig. 2D). The *methyl*-D_3 label in PC16:0/22:6 decreased with time, accompanied by increased fractional labeling of PC16:0/18:2 and PC18:0/18:2, and again resembled the endogenous PC composition by 24 h. The distribution of *methyl*-D_3 label in mouse plasma PC over the 24 h period

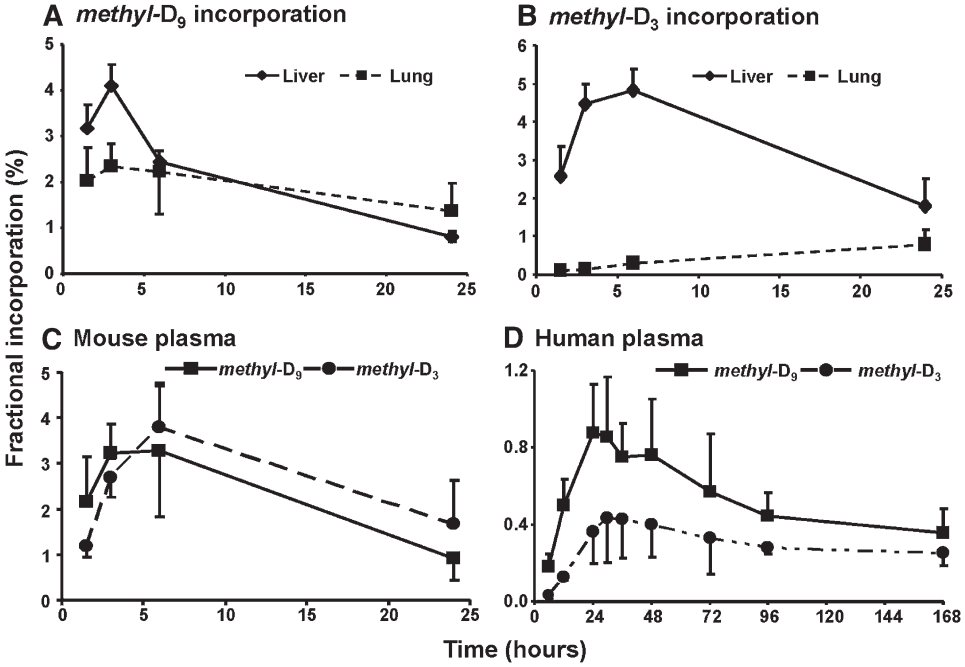

Fig. 3. Fractional enrichment of *methyl*-D_9 and *methyl*-D_3 labels in liver, lung, or plasma total PC in vivo. A: Direct incorporation of *methyl*-D_9 label by the CDP-choline pathway into mouse liver (circles) and lung (squares) total PC over a 24 h period after ip injection of 1 mg of *methyl*-D_9-choline chloride ($n = 5$ mice/group). B: Incorporation of *methyl*-D_3 label in SAMe mice, demonstrating the absence of the PEMT pathway in mouse lung. C and D: Time courses of incorporation of the *methyl*-D_9 (squares) and *methyl*-D_3 (circles) labels into, respectively, mouse an human plasma. Results were calculated as a percentage of total PC, given by the sum of P184, P187, P190, and P193 for all selected species (means ± SD).

essentially mirrored that in liver PC (Fig. 4D). The corresponding distribution of *methyl*-D_3 label in human plasma PC molecular species followed a very similar pattern, again with an initial incorporation into PC16:0/22:6 and PC18:0/20:4 and a subsequent redistribution to 18:2-containing species (Fig. 4F). This analysis demonstrates for the first time that the PEMT pathway in human liver is highly specific for the synthesis of 22:6- and 20:4-containing PC molecular species destined for lipoprotein secretion.

Importantly, the similar labeling patterns of mouse liver and plasma PC by both pathways at each time point clearly show that, although newly synthesized liver PC undergoes a substantial process of acyl remodeling after its initial synthesis, this is not required for secretion of lipoprotein PC. Instead, these results suggest that newly synthesized PC is secreted from the liver into the circulation with minimal modification to the initial synthesis pattern. While liver PC labeling data are not available, the human plasma labeling results also strongly suggest a similar mechanism for synthesis and secretion of human plasma lipoprotein PC.

Turnover of individual PC species synthesized by PE-*N* methylation

The *methyl*-D_3 fractional incorporations illustrated in Fig. 3 represent the mean turnovers of all selected PC species, but Fig. 4 shows how the distribution of incorporated label into two selected PC species changes with time. PC16:0/22:6 and PC16:0/18:2 are the characteristic PC species synthesized, respectively, by the PE-*N* methylation and CDP-choline pathways. The *methyl*-D_3 fractional enrichment results presented in **Fig. 5** demonstrate a high flux of PC16:0/22:6 through the PE-*N* methylation pathway with no initial synthesis of PC16:0/18:2 via this route.

Rate of mouse liver PC synthesized by the CDP-choline pathway

The maximal rate of PC synthesis by the CDP-choline pathway at 3 h was calculated based on the assumptions that *1*) all *methyl*-D_9 label was incorporated during the previous 1.5 h; *2*) PC labeled between 0 and 1.5 h was already secreted; *3*) enrichment of the choline phosphate substrate pool could be accurately estimated; and *4*) the whole system was at steady state. The fractional enrichment of *methyl*-D_9-choline phosphate (**Fig. 6A**) in liver followed a nonlinear distribution, with a rapid initial decline to 3 h, followed by a slower decrease to 24 h. Although these data are limited, they suggest a biphasic response, with the latter period representing uptake of lipoprotein by the liver and recycling of labeled choline (25). Given that at 3 h the mean enrichment of *methyl*-D_9-PC was 4.15% and that of *methyl*-D_9-choline phosphate was 5.97% between 1.5 and 3 h, an estimate of the maximal rate of synthesis by this pathway was calculated as $(4.15 \times 100)/(5.97 \times 1.5) = 46.3\%$ of total liver PC/h.

Rate of mouse and human liver PC synthesis by PE-*N* methylation

A similar estimate for PEMT pathway flux was calculated using MIDA to determine the fractional enrichment of the

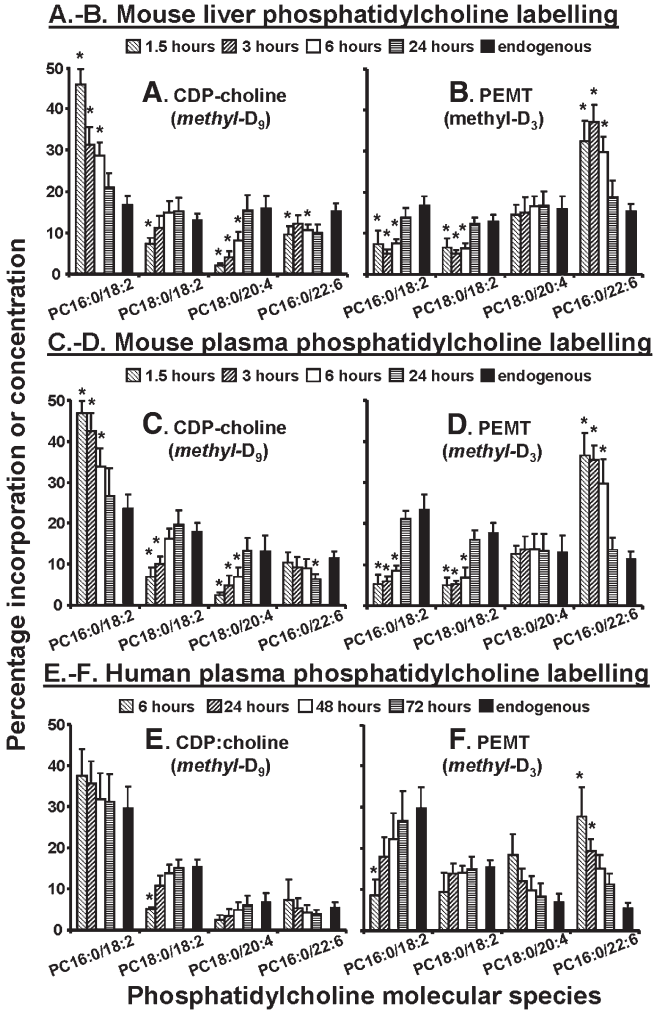

Fig. 4. Molecular specificity of incorporation of *methyl*-D_9 (CDP-choline pathways, panels A, C, and E) and *methyl*-D_3 (PEMT pathway, panels B, D, and F) into PC from mouse liver (A, B), mouse plasma (C, D), and human plasma (E, F). Incorporations into four individual PC species were chosen as representative of PC synthesis by both pathways. Results are percentages of incorporations into each PC species at each time point, calculated relative to total incorporation. Solid bars represent the fractional concentration of the SAMe species in endogenous PC.

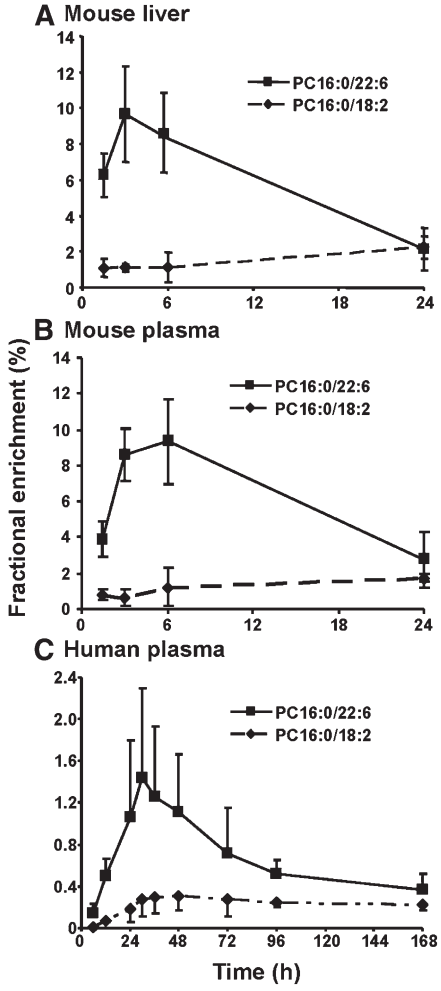

Fig. 5. Incorporation of *methyl*-D_3 label into selected PC species in mouse liver (A), mouse plasma (B) and human plasma (C). Fractional incorporation of *methyl*-D_3 into PC16:0/22:6, the major product of the PEMT pathway, and PC16:0/18:2, the major product of the CDP-choline pathway.

methyl-D_3-SAMe pool in the liver (26). Under these experimental conditions, PE can incorporate up to three *methyl*-D_3 groups during its conversion to PC by PEMT. Incorporation of one *methyl*-D_3 group will be detected by a P187 MS scan and that of two *methyl*-D_3 groups by a P190 scan, with comparable patterns of incorporation (Supplementary Fig. S2). Enrichment of *methyl*-D_3-SAMe is then calculated with the equation $P190/\Sigma(P187 + P190)$ (see Supplementary Equation S3).

Importantly for clinical applications, very similar enrichment values can be calculated from the analysis of both mouse plasma and liver PC (Fig. 6B). By analogy, an equivalent calculation can then be proposed using human plasma PC data to provide a surrogate measure of the *methyl*-D_3-SAMe enrichment in human liver (Fig. 6C).

These values for *methyl*-D_3-SAMe enrichment were then used to correct corresponding *methyl*-D_3-PC enrichments to provide estimates of total PC synthesis by PE-*N* methylation at each time point for mouse liver and plasma (Fig. 7A) and human plasma (**Fig. 7B**). Initial incorporation phases for each plot were linear, providing estimates of flux though this pathway. The similarity in synthetic rates for mouse liver (5.04%/h) and plasma (4.71%/h) strongly suggests that the value of 0.53%/h calculated for human plasma can be used as a surrogate for human liver PC flux by the PEMT pathway in vivo. The specificity of this synthesis (**Fig. 8**) for five representative PC species (PC16:0/22:6, PC18:0/22:6, PC18:0/20:4, PC16:0/18:2, and PC16:0/18:1) showed that synthesis rates varied over 1 order of magnitude for all three sample types, with essentially identical rank orders, and was greatest for 22:6-containing PC species, intermediate for PC18:0/20:4, and lowest for PC16:0/18:1 and PC16:0/18:2. This similarity in synthetic rates further suggests that this stable isotope labeling approach can be used to probe mechanisms regulating the specificity of hepatic PEMT activity in vivo in human subjects as well as in animals experiments.

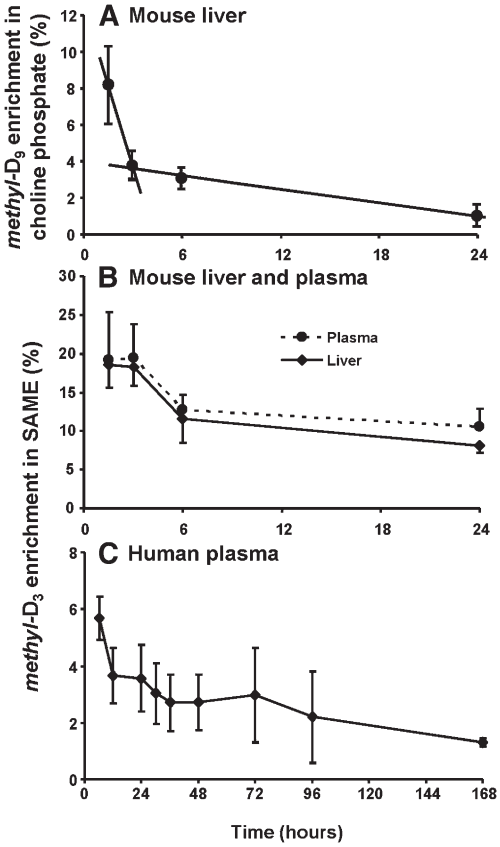

Fig. 6. Enrichment of stable isotope labels in liver choline phosphate and *S*-adenosylmethionine (SAMe). A: *Methyl*-D_9 enrichment in mouse liver choline phosphate was calculated from the ratio of the MRM transitions from 183.8 → 85.8 and 192.8 → 94.8. *Methyl*-D_3 enrichment of liver SAMe was calculated by MIDA from the P187 and P190 scans from mouse liver and plasma (B) and human plasma (C).

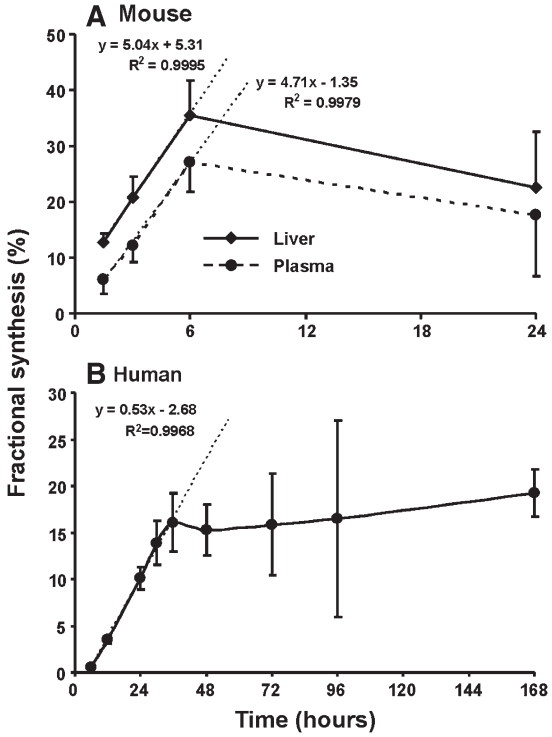

Fig. 7. Absolute rates of liver PC synthesis by the PEMT pathway. Results were derived from MIDA-corrected *methyl*-D_3 incorporations into mouse liver and plasma (A) and human plasma (B) and are presented as fractional synthetic rates of the pool of total liver PC.

DISCUSSION

Incorporation of *methyl*-D_9-labeled choline has previously been used to characterize the molecular specificity of PC synthesis by isolated rat hepatocytes and hepatoma cells, but the results presented here are the first demonstration of the specificity of human plasma PC synthesis. Strong similarities between the specificities of mouse liver and plasma PC synthesis further suggest that incorporation of label into human plasma PC can be used as a surrogate for human liver PC synthesis in vivo. This experimental approach is relatively inexpensive, rapid, and straightforward and eliminates the use of substrates labeled with radioactive isotopes. In particular, use of MIDA for estimating *methyl*-D_3-SAMe enrichment enables absolute rates of synthetic flux through the PEMT pathway to be estimated, in turn providing a noninvasive method for determining liver PEMT activity in vivo in patients. It is important to emphasize that quantification of *methyl*-D_3-SAMe enrichment by this experimental approach is independent of the initial source of the methyl donor and would be equally applicable, for instance, to studies of the incorporation of *methyl*-D_3-methionine.

This ability to quantify PEMT metabolic flux in vivo has considerable potential clinical application to a wide range of metabolic and hepatic diseases. Liver PEMT is a significant methylation activity in the body (27) and is a principal determinant of plasma homocysteine concentration (28, 29), an established risk factor for coronary heart disease (30). Activity of PEMT is decreased in liver biopsies from patients with cirrhosis (31); the PEMPT pathway loss-of-function V175M polymorphism is associated with NAFL (3, 32); $PEMT^{-/-}$ mice develop fatal steatohepatitis when placed on a choline-deficient diet (33); and decreased PEMT pathway flux is an essential component of the choline-methionine-deficient dietary mouse model of NAFL (34). $PEMT^{-/-}$ mice on a choline-sufficient diet have a normal liver PC content but decreased concentration of plasma PC and PC species containing 20:4 and 22:6 (13). Results shown in Figs. 4 and 8 highlight the importance of PEMT activity in hepatic lipoprotein exportation of PUFA-containing PC to extrahepatic tissues and strongly suggest that virtually all newly synthesized PUFA-containing PC species secreted by mouse liver are derived from PE-*N* methylation and not from the CDP-choline pathway. Equivalent distributions and kinetic changes of *methyl*-D_9 and *methyl*-D_3 labels in human compared with mouse plasma PC suggest similar mechanisms for the metabolism and secretion of lipoprotein PC in humans, although this remains to be confirmed in future studies using human liver biopsies. A small proportion of VLDL is synthesized by enterocytes, but, while de novo synthesis from choline

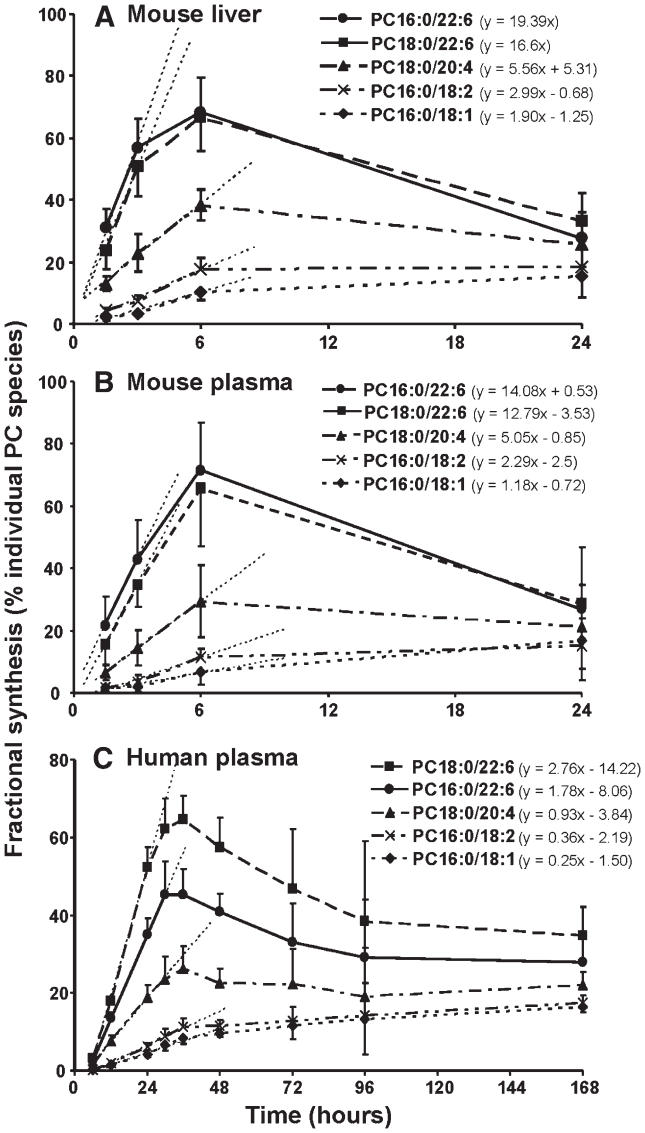

Fig. 8. Absolute rates of synthesis by the PEMT pathway of selected molecular species of liver PC. Rates of PC synthesis were calculated from MIDA-corrected *methyl*-D_3 incorporations into five individual PC molecular species for mouse liver (A), mouse plasma (B), and human plasma (C). Results are presented as fractional synthetic rates for each individual PC species.

is essential for PC secretion into the intestinal lumen (35), the contribution of de novo PC synthesis to enterocyte plasma lipoprotein packaging and secretion is not known. The methodology presented here does not directly address this potential enterocyte contribution.

The similarity of molecular specificities of newly synthesized PC synthesis in mouse liver and plasma has significant implications for mechanisms regulating assembly and secretion of plasma lipoproteins. Previous studies have shown that rat liver PC (9, 11) is synthesized by the CDP-choline pathway largely as PC16:0/18:2 and PC 16:0/18:1 and is then acyl remodeled to the mature composition by a variety of phospholipase and lysophosphatidylcholine acyltransferase (LPCAT) activities (36, 37). Results presented in Fig. 4 clearly demonstrate that liver PC, newly synthesized by either pathway, undergoes little acyl remodeling before being secreted. As acyl remodeling apparently occurs over a period of 3–6 h (9, 11) and as our estimate of the combined flux rate through both pathways is approximately 50%/h for both mouse liver and plasma PC, PC acyl remodeling must be only one of a number of activities that determine liver PC molecular species composition. In this context, the relative contributions to the equilibrium composition of liver and plasma PC of LPCAT (37), lecithin-cholesterol acyltransferase (38), recycling of extrahepatic PC to the liver (25), and secretion of PC in bile (39) have yet to be fully evaluated.

Our data have strongly suggested that modulation of the N methylation pathway provides capacity for the directed supply of PUFA from the liver to extrahepatic tissues, which is largely independent of PC synthesis by the CDP-choline pathway. Use of MIDA to calculate absolute rates of PC synthesis by PE-*N* methylation enables this pathway flux to be calculated in vivo in human patients and consequently has considerable potential for the investigation of a range of important liver diseases, including hepatic cancers, alcoholic and nonalcoholic fatty liver disease, and cystic fibrosis.

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