Mechanisms of hepatic phosphatidylcholine synthesis in adult rat: effects of pregnancy

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Late pregnancy in the rat (gestational ages 16–21 days) was accompanied by a specific increase in hepatic phosphatidylcholine (PC) and phosphatidylethanolamine (PE) molecular species containing $C_{16:0}$ at the *sn*-1 position and polyunsaturated essential fatty acids (PUFA), in particular $C_{22:6(n-3)}$, at the *sn*-2 position. Incorporation of either CDP:[Me - ^{14}C]choline or CDP:[1,2- ^{14}C]ethanolamine into hepatic microsomal *sn*-1 $C_{16:0}$ PC or PE molecular species *in vitro* was greater at term than in non-pregnant animals, suggesting modifications to the composition of specific diacylglycerol (DAG) pools destined for synthesis of either PC or PE. Also, incorporation of [Me - ^{14}C]choline or [Me - ^{14}C]methionine into hepatic PC *in vivo* over 6 h in term pregnant rats was consistent with decreased phospholipase A_1 -dependent

acyl remodelling of *sn*-1 $C_{16:0}$ to *sn*-1 $C_{18:0}$ molecular species. There was, however, no evidence to support any change to the specificity of acyl remodelling. The rate of PC synthesis by the *de novo* pathway *in vivo* was increased in term liver compared with non-pregnant animals, accompanied by increased choline-phosphotransferase activity *in vitro* in d21 liver microsomes. The rate of PC synthesis by PE *N*-methylation did not appear to change during pregnancy. Changes in composition of plasma PC species at term reflected those of newly synthesized hepatic PC. Our data suggest supply of PUFA to the developing fetal rat is the result of specific adaptations to maternal hepatic phospholipid biosynthesis rather than passive transfer from the maternal diet.

INTRODUCTION

The biosynthesis of choline and ethanolamine glycerophospholipid by rat liver has been extensively characterized (for reviews see [1,2]). The final enzymic steps in the pathways of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) synthesis *de novo* add choline phosphate or ethanolamine phosphate respectively to diacylglycerol (DAG) [1,2]. Consequently the molecular composition of newly-synthesized PC and PE reflects that of their individual substrate pools of DAG. Studies using rat-lung microsomes suggest that the DAG pool for PC synthesis is distinct from and not in equilibrium with bulk microsomal DAG [3]. The final composition of liver PC and PE is determined by the molecular species pattern of this initial synthesis combined with the subsequent processes of acyl remodelling, differential turnover, and inter-conversion between phospholipid classes.

The molecular specificity of PC synthesis has previously been defined in hepatocytes isolated from adult male rats fed a choline-deficient diet [4]. This study analysed the pattern of incorporation of [3H -methyl]choline into PC molecular species using a protocol of 1 h exposure to radiolabel, followed by a chase period of up to 12 h. PC species containing *sn*-1 $C_{16:0}$, particularly PC16:0/18:1 and PC16:0/18:2, were preferentially synthesized, with subsequent conversion to *sn*-1 $C_{18:0}$ species by acyl remodelling involving the sequential actions of either phospholipase A_1 (PLA $_1$) or phospholipase A_2 (PLA $_2$) and then acyltransferase activities. The details of such acyl remodelling mechanisms have been confirmed by the analysis of acyl turnover in rat liver, determined from the incorporation pattern of ^{18}O

from $H_2^{18}O$ into PC species [5]. We have previously demonstrated that comparable acyl remodelling mechanisms in hepatic PC synthesis also occur in other animal species [6]. After 3 h continuous labelling of neonatal guinea-pig liver with [^{14}C]choline *in vivo*, greatest incorporation was measured into *sn*-1 $C_{16:0}$ species, especially PC16:0/18:2. After 18 h, the fractional incorporation of radiolabel into *sn*-1 $C_{18:0}$ species had increased significantly at the expense of *sn*-1 $C_{16:0}$ species, confirming the activity of PLA $_1$ acyl remodelling in PC synthesis by guinea-pig liver.

The studies of the molecular specificity of PC synthesis by rat liver have all used animals fed low-fat diets containing optimal amounts of essential fatty acids. However, the composition of PC in rat liver is readily responsive to hormonal manipulation or to changes in dietary lipid content. For instance, there are distinct sex differences in hepatic PC-composition of male and female rats fed the same diet [7], and there was a rapid depletion of PC polyunsaturated fatty acid (PUFA) content when animals were fed a diet deficient in *n*-6 fatty acids [8]. The molecular mechanisms that underlie such alterations to PC composition have not been studied in detail. It is not clear whether the primary locus for modification of PC acyl composition is at the level of synthesis *de novo*, acyl remodelling or *N*-methylation of PE.

The plasma hyperlipoproteinaemia of pregnancy in the rat represents an extensive modification to lipid metabolism, and secretion is thought to be involved in the supply of lipid nutrient both to the developing fetus and to adipose and mammary triacylglycerol stores [9]. This hyperlipidaemic response in the

Abbreviations used: CPT, CDPcholine:1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2); DAG, diacylglycerol; EPT, CDPethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EC 2.7.8.1); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLA $_1$, phospholipase A_1 ; PLA $_2$, phospholipase A_2 ; PUFA, polyunsaturated long-chain essential fatty acid; S-adenomet, S-adenosylmethionine; TFE, trifluoroethanol; s.d., standard deviation.

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pregnant rat is accompanied by increased content of docosa-hexaenoic acid ($C_{22:6(n-3)}$) in both hepatic and plasma total phospholipids [10]. We have previously shown that the principle change was an elevated content of PC16:0/22:6 [11], which is possibly associated with the delivery of this fatty acid from the maternal liver to differentiating fetal nervous tissue. Analogous increases in the $C_{22:6(n-3)}$ -content of PC species have also been demonstrated in liver from the pregnant guinea-pig [12] and in plasma in human pregnancy [13]. These studies suggest that specific modifications to the composition of hepatic PC occur during pregnancy. Consequently, we have used the pregnant rat as a good model in which to evaluate mechanisms regulating the specificity of hepatic PC synthesis under altered physiological conditions. Specifically, we have used h.p.l.c. techniques for the resolution of individual PC and PE molecular species to address the question whether the increased PC16:0/22:6 content in maternal rat liver and plasma in late pregnancy is due to modification to the substrate DAG pool(s), acyl remodelling mechanisms, or to the rate of PE *N*-methylation. Knowledge of these mechanisms is important in order to understand the contribution of maternal hepatic phospholipid metabolism to the supply of adequate amounts of $C_{22:6(n-3)}$ to support optimal development of fetal tissues, in particular brain and retina. Our results suggest that a specific increase in PC16:0/22:6 content of maternal rat liver and plasma during late pregnancy is due to modifications to the rate of PC synthesis *de novo* and to the composition of DAG pools used in PC and PE synthesis.

EXPERIMENTAL

Materials

[Methyl- ^{14}C]choline chloride (55 mCi/mmol), L-[methyl- ^{14}C]methionine (55 mCi/mmol), and CDP:[1,2- ^{14}C]ethanolamine (51 mCi/mmol) were obtained from ICN Biomedicals (High Wycombe, Buckinghamshire, U.K.). CDP:[methyl- ^{14}C]choline (57 mCi/mmol) and L-3-phosphatidyl[*N*-methyl- 3H]choline 1,2-dipalmitoyl (81 Ci/mmol) were purchased from Amersham (Buckinghamshire, U.K.). Choline chloride, chloroform and acetonitrile were obtained from Merck (Poole, Dorset, U.K.). H.p.l.c. grade methanol and trifluoroethanol (TFE) were purchased from Rathburn (Walkerburn, Scotland). Other chemicals were from Sigma (Poole, Dorset, U.K.).

Animal procedures

Adult female Wistar rats were obtained from our own colony. Both dated pregnant and non-pregnant rats were fed freely with Rat and Mouse Maintenance Diet number 1 (Special Diet Services, Witham, Essex, U.K.). This diet contained 8.4 g/kg *n*-6 and 0.6 g/kg *n*-3 essential fatty acids (manufacturers' analysis). No $C_{22:6(n-3)}$ was included in the diet.

Non-pregnant or pregnant rats at 16 and 21 days of gestation were injected intraperitoneally with 50 μ Ci of either [^{14}C]choline or [^{14}C]methionine in 1 ml 0.9% (w/v) NaCl and killed 6 h post-injection by cervical dislocation. This incubation period was chosen to permit a degree of acyl remodelling of the newly synthesized hepatic PC [4] and to allow some secretion of labelled PC into plasma. Blood was collected by cardiac puncture and clotting was prevented with 5 mM EDTA. Blood samples were separated into cells and plasma by centrifugation and were stored at $-20^\circ C$. Livers were removed and frozen in liquid N_2 and stored at $-20^\circ C$. One lobe was removed from each liver labelled with [^{14}C]methionine, stored in liquid N_2 and used for

determination of the specific activity of *S*-adenosylmethionine (*S*-adomet).

H.p.l.c. analysis of PC and PE molecular species

Lipids were prepared by extraction of either approximately 100 mg of liver or 200 μ l of plasma with chloroform/methanol (1:1) [14]. 100 or 50 nmoles of PC14:0/14:0 or PE14:0/14:0 in TFE were added as internal standards. The organic phase was dried under N_2 and dissolved in 1 ml of chloroform. Lipid extracts were separated into PC and PE fractions using 100 mg disposable aminopropyl-BondElut cartridges [15], dried under N_2 and dissolved in TFE or chloroform respectively. PC and PE molecular species from liver were resolved by h.p.l.c. on a 25 cm $\times$ 4.6 mm 5 μ APEX II ODS column (Jones Chromatography, Hengoed, Mid Glamorgan, Wales, U.K.) maintained at 50 $^\circ C$ using a mobile phase of methanol/water (925:75) containing 40 mM choline chloride [16] delivered at 1 ml/min. The mass of individual phospholipid molecular species was determined by post-column fluorescence derivatization with 1,6-diphenyl-1,3,5-hexatriene [16]. U.v. absorbance at 205 nm, representing the number of carbon-carbon double bonds, was routinely used to identify molecular species. The incorporation of radiolabels into individual molecular species was determined using a flow-through h.p.l.c. radiochemical detector [6]. Rat-plasma PC molecular species were resolved on a 15 cm $\times$ 4.6 mm 3 μ APEX II ODS column (Jones Chromatography) maintained at 50 $^\circ C$ using a mobile phase of methanol/acetonitrile/water (823/100/77) containing 40 mM choline chloride at a flow rate of 1 ml/min. This gave similar resolution to that obtained with the 5 μ column but permitted substantially shorter run times. Incorporation of radiolabel into plasma-PC molecular species was determined by collecting fractions eluted from the column at 30 s intervals and scintillation counting.

Determination of the specific radioactivity of choline and methionine metabolites

Choline metabolites were isolated from the aqueous phase of chloroform/methanol extracts of liver tissue. Choline was separated from phosphorylated choline metabolites using 100 mg aminopropyl-BondElut cartridges by sequential elution with methanol/0.05% (v/v) ammonia (9:1, v/v) and methanol/0.05% (v/v) ammonia/6% (w/v) NH_4HCO_3 (8:2:2) [17]. The mass of phosphorylated choline metabolites was measured by conversion of phosphorylated choline into betaine by the actions of alkaline phosphatase and choline oxidase in the presence of phenol and the chromogen 4-aminoantipyrene [17]. Incorporation of radiolabel into phosphorylated choline was determined by scintillation counting.

Methionine metabolites were extracted by homogenizing about 100 mg of liver with 0.15 M perchloric acid, partially purified on Dowex 50 ion-exchange resin and resolved by ion-exchange h.p.l.c. [18]. The mass of *S*-adomet was estimated by comparison of u.v. absorption at 254 nm with an external *S*-adomet standard. [^{14}C]methionine incorporation into *S*-adomet was determined by scintillation counting of collected fractions.

Incorporation of CDP:[^{14}C]choline and CDP:[^{14}C]ethanolamine into rat-liver microsomes

Hepatic microsome fractions were prepared from pregnant and non-pregnant rats which had not received radiolabelled phospholipid head-group precursors. Fresh liver tissue was homogenized in 50 mM Tris/HCl (pH 7.2)/0.145 mM NaCl/5 mM EDTA

(disodium salt), and 0.2 mM phenylmethylsulphonylfluoride [6]. After removal of mitochondria by centrifugation at 10000 *g* for 10 min at 4 °C, microsomes were collected by centrifugation of the supernatant at 100000 *g* for 1 h at 4 °C. Microsome preparations were resuspended in 20 ml buffer and stored at -80 °C to minimize degradation of endogenous DAG. The molecular species compositions of endogenous microsomal DAG pools destined for incorporation into either PC or PE were determined by incorporation of CDP:[¹⁴C]choline or CDP:[¹⁴C]ethanolamine respectively. Microsomes (100 μ l containing approximately 12 mg/ml protein) were incubated with either 1.5 μ Ci CDP:[¹⁴C]choline or CDP:[¹⁴C]ethanolamine at 37 °C for 15 min [6]. The reaction was stopped with chloroform/methanol (1:2, v/v) and lipids extracted with chloroform/methanol [14]. The organic phase was dried under N₂ and radiolabel incorporation into individual PC and PE molecular species was measured by h.p.l.c. with a flow-through radiochemical detector.

Determination of rat-liver microsomal cholinephosphotransferase and ethanolaminephosphotransferase activities

CDPcholine:1,2-diacylglycerol cholinephosphotransferase (CPT) and CDPethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EPT) activities were assayed by incorporation of CDP:[¹⁴C]choline or CDP:[¹⁴C]ethanolamine and exogenous *sn*-1,2-dioleoyl DAG into microsomal phospholipids using the method of Cornell [19] modified for use in 1.5 ml Eppendorf tubes. Microsomes (30 μ g protein) were incubated in duplicate with 2.4 mM dioleoyl DAG emulsion in the presence of 0.4 mM CDP:[*methyl*-¹⁴C]choline or CDP:[*methyl*-¹⁴C]ethanolamine, 0.5 mM ethylene glycol bis(β -aminoethyl ether) *N,N,N',N'*-tetra-acetic acid, 10 mM MgCl₂ and 50 mM Tris/HCl, pH 8.5, in a final volume of 200 μ l for 15 min at 37 °C. Substrate blanks and boiled microsomes were included in each batch of assays to correct for non-specific incorporation of radiolabel. The reaction was terminated by addition of 750 μ l chloroform/methanol (1:2, v/v) and 0.001 μ Ci [³H]PC16:0/16:0 was added as internal standard in order to correct for recovery of radiolabelled phospholipids. Phospholipids were extracted into the chloroform phase following addition of 250 μ l of methanol and 250 μ l of water. Incorporation of radiolabel was determined by scintillation counting of the dried chloroform phase.

Statistical analysis

Statistical analysis was carried out using the Mann-Witney U-test.

RESULTS

Hepatic and plasma phospholipid molecular species compositions during pregnancy

H.p.l.c. analysis of adult female rat liver phospholipids resolved nine principal PC molecular species and eight PE molecular species which accounted for > 98 % of the total PC and PE mass (Table 1). The major components of hepatic PC from non-pregnant rats were *sn*-1 C_{18:0}-containing molecular species (Table 1). Pregnancy was accompanied by a number of specific changes in hepatic PC and PE molecular species compositions. During the first 16 days of pregnancy the concentration of PC16:0/22:6 increased 2.4-fold and continued to increase until term (day 21) (Table 1). Similarly, the concentration of PE16:0/22:6 increased

2.6-fold between conception and term (Table 1). The concentration of PC18:0/20:4 increased 1.4-fold between conception and day 16 and then returned to pre-pregnant level by term (Table 1). Similarly, PE18:0/20:4 concentration decreased 1.6-fold between day 16 and day 21 (Table 1). In addition, the concentrations of PC18:0/18:2 and PE18:0/18:2 decreased 2.1-fold and 1.5-fold respectively (Table 1). The concentrations of other hepatic PC and PE molecular species and the total PC and PE concentrations did not change significantly during pregnancy (Table 1).

Following the rise in hepatic PC16:0/22:6 and PE16:0/22:6 between conception and day 16 of pregnancy, plasma PC16:0/22:6 concentration increased dramatically (2.8-fold) between day 16 and term (Table 2). There was considerably less PC16:0/18:1 in plasma than in liver and, as this species was only poorly resolved from PC18:0/22:6, these two species were reported as a combined peak for plasma analysis. During this period, plasma PC16:0/20:4 concentration increased (1.4-fold) (Table 2). These increases in the concentrations of *sn*-1 C_{16:0} PC molecular species were accompanied by overall decreases in the concentrations of PC18:0/20:4 and PC18:0/18:2 between conception and term (Table 2). The concentrations of other plasma PC species and the total plasma PC concentration did not change significantly during pregnancy (Table 2).

Compositions of diacylglycerol substrate pools utilized in phosphatidylcholine and phosphatidylethanolamine synthesis

One possible mechanism by which the composition of hepatic PC and PE could be modified during pregnancy is compositional changes to DAG substrate pools. In this context, it is important to determine the composition of the relevant DAG pool destined for incorporation into different phospholipid classes rather than the total hepatic DAG pool. We have shown previously that the composition of PC or PE synthesized from either CDP:[¹⁴C]choline or CDP:[¹⁴C]ethanolamine by incubation of microsome preparations *in vitro* reflects the pattern of synthesis *de novo* without acyl remodelling [6]. Thus synthesis of microsome PC or PE under these conditions reflects the composition of the relevant DAG pools. The patterns of CDP:[¹⁴C]choline and CDP:[¹⁴C]ethanolamine incorporation *in vitro* showed pregnancy-associated changes to hepatic DAG pools destined for incorporation into either PC or PE. Incorporation of both CDP:[¹⁴C]choline and CDP:[¹⁴C]ethanolamine into 16:0/22:6 molecular species increased dramatically (1.8-fold and 2.0-fold respectively) during pregnancy (Table 3). However, there were marked differences in the pregnancy-associated changes in CDP:[¹⁴C]choline and CDP:[¹⁴C]ethanolamine incorporation into PC and PE. The newly synthesized PC pool contained significantly more PC18:0/22:6 (2.2-fold) and less PC16:0/18:2 (1.5-fold) and PC16:0/18:1 (2.3-fold) at term than in non-pregnant animals (Table 3), whereas the incorporation of CDP:[¹⁴C]ethanolamine into PE18:0/22:6 (2.1-fold) and PE18:0/20:4 (2.4-fold) was substantially lower at gestational age day 21 (Table 3). These results are consistent with the suggestion that PC and PE are derived from distinct DAG pools. The differences between these total and newly synthesized pools can be accounted for by acyl remodelling and/or differential turnover of individual molecular species.

These changes in DAG-pool compositions were accompanied by increased specific activities of microsomal CPT and EPT activities. CPT activity increased 2.0-fold between conception (14.3 $\pm$ 1.3) and day 16 of gestation (28.7 $\pm$ 5.3 nmoles/min/mg microsomal protein) (*n* = 4, *P* < 0.05). Between day 16 and day 21 of gestation CPT activity decreased, although not significantly,

Table 1 Hepatic PC and PE molecular species compositions

PC and PE molecular species from non-pregnant and pregnant rat-liver were resolved by reverse-phase h.p.l.c. Values are mean $\pm$ standard deviation (s.d.) from eight animals/gestational age. The total PC concentrations were 11.2 ± 1.9 non-pregnant, 15.2 ± 2.3 day 16-pregnant and 12.5 ± 2.5 μ moles/g at term (day 21). The corresponding total PE concentrations were 10.7 ± 0.9 non-pregnant, 12.4 ± 2.3 day 16-pregnant and 11.8 ± 1.4 μ moles/g at term (day 21). * and # indicate values which were significantly different ($P < 0.01$) from non-pregnant animals and from day 16 pregnant animals respectively. ND = not detected.

Molecular species	Phosphatidylcholine concentration (nmoles/g)			Phosphatidylethanolamine concentration (nmoles/g)		
	Non-pregnant	Day 16-pregnant	Day 21-pregnant	Non-pregnant	Day 16-pregnant	Day 21-pregnant
16:0/18:3	110 $\pm$ 32	142 $\pm$ 61	96 $\pm$ 35	ND	ND	ND
16:0/22:6	1042 $\pm$ 460	2471 $\pm$ 566*	3050 $\pm$ 545*#	1521 $\pm$ 222	2875 $\pm$ 639*	3960 $\pm$ 491*#
16:0/20:4	1212 $\pm$ 301	1995 $\pm$ 322*	1545 $\pm$ 442	954 $\pm$ 124	1668 $\pm$ 440*	1252 $\pm$ 421
16:0/18:2	1278 $\pm$ 305	1640 $\pm$ 207	1387 $\pm$ 321	474 $\pm$ 222	584 $\pm$ 184	518 $\pm$ 85
16:0/16:0	230 $\pm$ 87	202 $\pm$ 71	321 $\pm$ 57	ND	ND	ND
16:0/18:1	813 $\pm$ 166	1115 $\pm$ 194	1018 $\pm$ 300	345 $\pm$ 113	436 $\pm$ 133	541 $\pm$ 91
18:0/22:6	1516 $\pm$ 286	2348 $\pm$ 490	1998 $\pm$ 365	2137 $\pm$ 246	2483 $\pm$ 720	2049 $\pm$ 460
18:0/20:4	2798 $\pm$ 712	3972 $\pm$ 687*	2119 $\pm$ 610#	4071 $\pm$ 798	3936 $\pm$ 953	2482 $\pm$ 417*#
18:0/18:2	2111 $\pm$ 373	1805 $\pm$ 257	1001 $\pm$ 276*	868 $\pm$ 269	411 $\pm$ 140*	569 $\pm$ 108

Table 2 Plasma PC composition

Plasma PC molecular species were resolved by reverse-phase h.p.l.c. Values are mean $\pm$ s.d., $n = 8$ samples/gestational age. Total plasma PC concentrations were 3.1 ± 0.6 non-pregnant, 2.8 ± 0.3 day 16-pregnant and 2.9 ± 0.5 μ moles/ml day 21-pregnant. * and # indicate values which were significantly different ($P < 0.01$) from non-pregnant and from day 16-pregnant animals respectively. The species 18:0/22:6+ contained small amounts of poorly resolved PC16:0/18:1 as well as PC18:0/22:6.

Molecular species	Phosphatidylcholine concentration (nmoles/ml)		
	Non-pregnant	Day 16-pregnant	Day 21-pregnant
16:0/22:6	200 $\pm$ 61	237 $\pm$ 79	666 $\pm$ 90*#
16:0/20:4	245 $\pm$ 51	235 $\pm$ 56	339 $\pm$ 59*#
16:0/18:2	672 $\pm$ 133	654 $\pm$ 38	620 $\pm$ 109
18:0/22:6 +	589 $\pm$ 148	556 $\pm$ 67	581 $\pm$ 126
18:0/20:4	628 $\pm$ 124	498 $\pm$ 89	361 $\pm$ 70*#
18:0/18:2	761 $\pm$ 158	585 $\pm$ 89*	327 $\pm$ 76*#

to 18.6 ± 7.7 nmoles/min/mg. Similarly, EPT activity increased 3.6-fold ($P < 0.05$) between conception (4.3 ± 1.3 nmoles/min/mg microsomal protein) and gestational age day 16 (15.6 ± 5.0 nmoles/min/mg). EPT activity remained elevated at term (12.8 ± 4.1 nmoles/min/mg).

[14 C]choline incorporation into hepatic and plasma phosphatidylcholine

The effects of pregnancy on hepatic PC synthesis and secretion on serum lipoproteins were investigated using [14 C]choline incorporation into hepatic and plasma PC *in vivo* over 6 h. In agreement with the results obtained in hepatocytes by Tijburg et al. [4] *sn*-1 $C_{16:0}$ -containing molecular species were the major components of the newly synthesized PC pool after 6 h incubation (Table 4). The pattern of [14 C]choline incorporation into hepatic PC in pregnant animals showed a dramatic shift towards PC16:0/22:6 and 16:0/20:4, which increased 2.7-fold and 1.5-fold respectively (Table 4). [14 C]choline incorporation into 18:0/22:6 was also increased (1.4-fold) at gestational age day 16 and at term (Table 4). Incorporation into PC18:0/20:4 (1.9-fold) and PC18:0/18:2 (2.2-fold) was increased on day 16 of

Table 3 Molecular species compositions of DAG-pools available for either PC or PE synthesis

The compositions of rat liver microsome DAG-pool utilized in PC and PE synthesis were determined from CDP:[14 C]choline and CDP:[14 C]ethanolamine incorporation. The molecular species compositions of the newly synthesized phospholipid pools were assessed by reverse-phase h.p.l.c. with post-column radiochemical detection. All values are means $\pm$ s.d., $n = 4$ animals/gestational age. *Indicates values which were significantly different ($P < 0.05$) from non-pregnant animals.

Molecular species	CDP:[14 C]choline incorporation (%)		CDP:[14 C]ethanolamine incorporation (%)	
	Non-pregnant	Day 21-pregnant	Non-pregnant	Day 21-pregnant
16:0/22:6	12.2 $\pm$ 1.5	21.9 $\pm$ 1.9*	26.2 $\pm$ 1.6	53.4 $\pm$ 2.3*
16:0/20:4	25.0 $\pm$ 1.4	27.9 $\pm$ 2.8	5.0 $\pm$ 1.0	6.9 $\pm$ 1.7
16:0/18:2	23.8 $\pm$ 0.8	16.4 $\pm$ 2.2*	4.5 $\pm$ 0.9	6.1 $\pm$ 1.7
16:0/18:1	13.5 $\pm$ 1.0	5.8 $\pm$ 2.5*	ND	ND
18:0/22:6	4.6 $\pm$ 1.3	10.1 $\pm$ 4.0*	44.9 $\pm$ 4.9	21.1 $\pm$ 2.6*
18:0/20:4	16.5 $\pm$ 2.0	13.7 $\pm$ 2.4	15.3 $\pm$ 3.5	6.4 $\pm$ 1.5*
18:0/18:2	5.0 $\pm$ 0.7	4.3 $\pm$ 1.3	4.2 $\pm$ 0.5	5.0 $\pm$ 0.5

Table 4 [^{14}C]Choline and [^{14}C]methionine incorporation into rat-liver PC molecular species

The incorporation of [^{14}C]choline and [^{14}C]methionine into rat-liver PC was determined by reverse-phase h.p.l.c. with post-column radiochemical detection. Apparent rates of PC synthesis were derived by correcting radiolabel-incorporation for the specific radioactivity of phosphorylated choline and *S*-adomet substrate pools. The apparent rates of synthesis of the total PC pool from [^{14}C]choline were 9.8 ± 4.1 in non-pregnant animals and 26.6 ± 6.4 $\mu\text{moles/liver/6 h}$ in term pregnant rats. Specific radioactivities of the hepatic phosphorylated-choline pools were 107 ± 19 non-pregnant liver and 36 ± 25 dpm/nmole* in term pregnant liver. Apparent rates of PC synthesis by *N*-methylation of PE were 0.83 ± 0.10 non-pregnant and 0.81 ± 0.25 $\mu\text{moles/liver day}$ 21-pregnant. The specific radioactivities of the hepatic *S*-adomet pool was 1.2 ± 0.3 in non-pregnant animals and 1.2 ± 0.4 dpm/nmole in pregnant animals. All values are means $\pm$ s.d., $n = 4$ animals/gestational age. *Indicates value which were significantly different ($P < 0.05$) from non-pregnant animals. #Indicates values which were significantly different ($P < 0.05$) from day 16-pregnant animals.

Molecular species	[^{14}C]choline incorporation (%)			[^{14}C]methionine incorporation (%)		
	Non-pregnant	Day 16-pregnant	Day 21-pregnant	Non-pregnant	Day 16-pregnant	Day 21-pregnant
16:0/22:6	11.4 ± 0.9	$17.1 \pm 1.3^*$	$27.1 \pm 1.6^*\#$	6.6 ± 0.5	$14.3 \pm 3.6^*$	$33.4 \pm 4.9^*\#$
16:0/20:4	16.3 ± 1.7	16.6 ± 2.2	$20.7 \pm 1.9^*\#$	15.0 ± 1.6	18.9 ± 3.8	13.0 ± 3.3
16:0/18:2	18.6 ± 1.4	$22.3 \pm 2.8^*$	$15.6 \pm 1.8^*\#$	9.3 ± 2.1	12.3 ± 3.4	8.0 ± 1.8
16:0/18:1	19.0 ± 4.0	$16.2 \pm 1.8^*$	$9.6 \pm 0.8^*$	ND	ND	ND
18:0/22:6	4.1 ± 0.7	$6.7 \pm 1.8^*$	$9.6 \pm 1.7^*$	15.2 ± 2.7	16.2 ± 2.5	11.7 ± 1.0
18:0/20:4	16.4 ± 3.0	11.4 ± 2.3	10.4 ± 2.1	38.4 ± 2.8	$23.1 \pm 4.9^*$	$18.7 \pm 2.6^*$
18:0/18:2	14.1 ± 4.2	9.6 ± 2.7	$7.2 \pm 1.7^*\#$	15.6 ± 3.8	13.9 ± 1.4	14.7 ± 2.7

Table 5 [^{14}C]Choline incorporation into rat plasma PC molecular species

The incorporation of [^{14}C]choline into rat-liver PC was determined by reverse-phase h.p.l.c. followed by collection of fractions at 30 s intervals and scintillation counting. All values are means $\pm$ s.d., $n = 4$ animals/gestational age. *Indicates values which were significantly different ($P < 0.05$) from non-pregnant animals.

Molecular species	[^{14}C]choline incorporation (%)		Specific radioactivity (d.p.m./nmole)	
	Non-pregnant	Day 21-pregnant	Non-pregnant	Day 21-pregnant
16:0/22:6	7.8 ± 1.8	$22.1 \pm 2.8^*$	22 ± 7	19 ± 8
16:0/20:4	15.3 ± 1.0	$19.8 \pm 2.8^*$	27 ± 5	35 ± 8
16:0/18:2	23.3 ± 1.5	$15.0 \pm 2.7^*$	25 ± 4	21 ± 7
18:0/22:6+	19.0 ± 1.3	$22.1 \pm 1.9^*$	15 ± 3	22 ± 5
18:0/20:4	18.6 ± 2.4	$11.6 \pm 1.8^*$	13 ± 6	15 ± 2
18:0/18:2	16.6 ± 2.7	$9.5 \pm 0.7^*$	13 ± 6	18 ± 2

Table 6 [^{14}C]Methionine-incorporation into rat plasma PC molecular species

The incorporation of [^{14}C]choline into rat-liver PC was determined by reverse-phase h.p.l.c. followed by collection of fractions at 30 s intervals and scintillation counting. All values are means $\pm$ s.d., $n = 4$ animals/gestational age. *Indicates values which were significantly different ($P < 0.05$) from non-pregnant animals.

Molecular species	[^{14}C]choline incorporation (%)		Specific radioactivity (d.p.m./nmole)	
	Non-pregnant	Day 21-pregnant	Non-pregnant	Day 21-pregnant
16:0/22:6	5.1 ± 0.3	$24.5 \pm 2.8^*$	31 ± 5	$15 \pm 8^*$
16:0/20:4	10.5 ± 1.2	$15.0 \pm 3.2^*$	29 ± 5	$17 \pm 5^*$
16:0/18:2	11.1 ± 1.5	11.5 ± 4.0	16 ± 1	$11 \pm 5^*$
18:0/22:6+	15.4 ± 2.2	$23.7 \pm 3.8^*$	19 ± 2	$11 \pm 4^*$
18:0/20:4	35.5 ± 2.9	$16.5 \pm 1.1^*$	27 ± 4	$14 \pm 6^*$
18:0/18:2	22.6 ± 2.8	$8.8 \pm 1.1^*$	22 ± 8	$12 \pm 4^*$

gestation. In contrast, the incorporation of [^{14}C]choline into PC16:0/18:1 was substantially reduced (2.8-fold) in term pregnant animals compared with non-pregnant rats (Table 4).

Correction of [^{14}C]choline incorporation into hepatic PC for the specific activity of the phosphorylated choline substrate pool was used to calculate apparent rates of PC synthesis [17]. Values are quoted for the total amount of PC synthesized over 6 h since it is uncertain whether the incorporation of radiolabel is linear

throughout this period. The apparent rate of PC synthesis was 2.7-fold greater at term than in non-pregnant animals (Table 4).

The increased concentration of plasma *sn*-1 palmitoyl PC species containing $\text{C}_{22:6(n-3)}$ and $\text{C}_{20:4(n-6)}$ in term rats compared with non-pregnant animals was accompanied by a rise in fractional incorporation of [^{14}C]choline into PC16:0/22:6 (2.8-fold) and PC16:0/20:4 (1.3-fold) (Table 5). Conversely, [^{14}C]choline incorporation into other plasma PC molecular

species was decreased in term pregnant rats compared with non-pregnant animals (Table 5). The specific radioactivities of plasma PC species did not change significantly during pregnancy (Table 5).

Incorporation of [^{14}C]methionine into hepatic and plasma PC

The contribution of PE *N*-methylation to the synthesis of hepatic and plasma PC during pregnancy was determined from the incorporation of [^{14}C]methionine over 6 h. The pattern of [^{14}C]methionine-incorporation into hepatic PC (Table 4) resembled the distribution of molecular species in the total PE pool (Table 1), although the ratios between individual molecular species showed slight differences. The proportion of PC16:0/22:6 in the PC pool synthesized by *N*-methylation was higher than in the total PE pool, suggesting some selection of this species for *N*-methylation. During pregnancy the proportion of PC16:0/22:6 in the PC pool synthesized by *N*-methylation increased 5.6-fold (Table 2) accompanied by a 1.9-fold decrease in the synthesis of PC18:0/20:4. The apparent rate of PC synthesis by *N*-methylation over 6 h did not change significantly during pregnancy (Table 4).

The fractional distribution of [^{14}C]CH₃-incorporation into plasma PC in non-pregnant animals (Table 6) closely resembled that of the hepatic PC pool synthesized by *N*-methylation (Table 4). The major pregnancy-associated changes in incorporation of [^{14}C]methionine into plasma PC synthesized by *N*-methylation were similar to those in the hepatic PC pool. Increased [^{14}C]methionine incorporation into PC16:0/22:6 (4.8-fold) and into PC18:0/22:6 (1.5-fold) was accompanied by decreased incorporation into PC18:0/20:4 (2.2-fold) and PC18:0/18:2 (2.6-fold) (Table 6). The specific radioactivities of plasma-PC molecular species synthesized by *N*-methylation were lower in term pregnant rats than in non-pregnant animals (Table 6).

DISCUSSION

Late gestation in the rat presents an ideal opportunity to study mechanisms regulating the specificity of PC synthesis under conditions of physiological modulation of PC composition. The results presented in Table 1 demonstrate a progressive increase in the concentration of PC16:0/22:6 in maternal liver from non-pregnant, through day 16 of gestation, to term, which was accompanied by decreased concentrations of PC18:0/20:4 and PC18:0/18:2 at term. Possible mechanisms underlying such changes in hepatic PC-composition include modifications to DAG substrate-pool composition and/or fatty acid content of acyl donor pool(s), either acyl CoA [20] or lysoPC [21], used in PC acyl remodelling. Also, an increase in the rate of PC synthesis, with no change to the rate of PC acyl remodelling, would result in a compositional shift of total liver PC towards *sn*-1 C_{16:0} species characteristic of synthesis *de novo*, while increased rate of PC synthesis by *N*-methylation of PE, which is relatively enriched in species containing C_{22:6(n-3)} and C_{20:4(n-6)} (Table 1), may produce selective enrichment of the corresponding PC molecular species.

The molecular compositions of newly synthesized PC and PE in non-pregnant and day 21 pregnant-rat liver are detailed in Table 3. Incorporation patterns of CDP:[^{14}C]choline into PC and CDP:[^{14}C]ethanolamine into PE by liver microsomal preparations were not subject to significant acyl remodelling *in vitro*, and thus reflect the composition of endogenous DAG pools [6]. The basic details of these synthetic patterns *in vitro* using microsomes from non-pregnant rats agree closely with

pulse-chase studies of [^{14}C]choline and [^{14}C]ethanolamine into PC and PE respectively in primary hepatocyte preparations from choline-deprived rats [4,22]. CDP:[^{14}C]choline was incorporated preferentially into PC species containing *sn*-1 C_{16:0} rather than *sn*-1 C_{18:0}, while CDP:[^{14}C]ethanolamine was incorporated primarily into PE species containing *sn*-2 C_{22:6(n-3)}. The pregnancy-associated changes to these patterns of phospholipid synthesis *de novo* closely reflected the corresponding alterations to hepatic phospholipid compositions at day 21 of gestation (Table 1). The incorporation of CDP:[^{14}C]choline into PC16:0/22:6 increased significantly, while those into PC16:0/18:2 and PC16:0/18:1, characteristic of PC synthesis *de novo* in hepatocytes from non-pregnant rats [4], decreased at day 21 of gestation (Table 1). The principal change to PE synthesis was a redistribution of CDP:[^{14}C]ethanolamine incorporation from PE18:0/22:6 to PE16:0/22:6 (Table 1), which mirrored the increased concentration of hepatic PE16:0/22:6 at this time in gestation.

Previous studies of CPT from rat liver [23] and lung [24,25] suggest that this enzyme possesses little inherent substrate specificity, and that its substrate DAG pool is metabolically distinct from either bulk microsomal-DAG or exogenous added DAG [3]. In our study, not only were the incorporation patterns of CDP:[^{14}C]choline into PC and CDP:[^{14}C]ethanolamine into PE different in microsomes from non-pregnant rats, but the pregnancy-associated changes were also different (Table 3). For instance, incorporations into PC16:0/22:6 and PC18:0/22:6 were both increased, while those into PE16:0/22:6 and PE18:0/22:6 were inversely related (Table 1), providing further evidence against a common substrate pool.

Because of the common membrane-bound nature of both enzymes and DAG, it was not possible to measure absolute concentrations of the relevant microsomal substrate-pools. Some indication of the relative changes during pregnancy can be provided by comparison of the magnitudes of [^{14}C]substrate incorporations at constant microsomal protein levels in the incubation *in vitro*. Between the non-pregnant and day 21 pregnant rat, CDP:[^{14}C]choline incorporation increased 1.6-fold, while that of CDP:[^{14}C]ethanolamine increased 1.9-fold. Allowance must be made in the interpretation of this comparison for the 2-fold and 3-fold increases respectively in CPT and EPT activities at day 21, measured at saturated concentration of exogenous DAG. It was not possible to calibrate the endogenous DAG pools using exogenously added DAG because of the non-homogeneity between these pools [3]. However, our results *in vitro* suggest that some combination of increased DAG substrate-concentrations and increased enzyme activities may contribute to stimulated synthesis of liver PC and PE in the pregnant rat.

The incubation of rats with [^{14}C]choline for 6 h provided an estimate of the rate of PC synthesis *de novo*. Previous studies show that incorporation of [^{14}C]choline into isolated rat hepatocytes *in vitro* is maintained over 6 h, which suggest that such prolonged incubation times may give a reasonable estimate of the synthetic rate for PC [26]. Although it was not possible to measure initial absolute synthetic rates *in vivo*, this analysis permitted comparisons of relative rates of liver PC synthesis at different times in pregnancy. The increased apparent rate of PC synthesis, from 9.8 $\mu\text{moles/liver/6 h}$ in the non-pregnant rat to 26.6 $\mu\text{moles/liver/6 h}$ at term (Table 4), were consistent with increased microsomal CPT and EPT activities.

The 6 h incubation period with [^{14}C]choline also provided sufficient time for a limited amount of PC acyl remodelling to occur. The general characteristics of this acyl remodelling can be determined by comparison of the pattern of this [^{14}C]choline incorporation *in vivo* (Table 4) both with the pattern of microsomal CDP:choline incorporation *in vitro* (Table 3) and with

the pattern of hepatic PC composition (Table 1). Taking CDP:[¹⁴C]choline incorporation as a measure of the specificity of PC synthesis *de novo* for the non-pregnant rat, this comparison showed loss of [¹⁴C]choline incorporation from PC16:0/20:4 and PC16:0/18:2 and appearance of radiolabel in PC18:0/18:2. This analysis, showing preferential synthesis of *sn*-1 C_{16:0} PC-species and their subsequent conversion to *sn*-1 C_{18:0} PC-species is in good agreement with previous reports [4]. PC16:0/22:6 was apparently subject to minimal acyl remodelling, while that for PC18:0/22:6 and PC18:0/20:4 occurred over a time-period longer than 6 h. Although the extent of acyl remodelling was decreased at term, there was no evidence for any pregnancy-associated modification to these remodelling mechanisms. The dramatically increased incorporation of [¹⁴C]choline into PC16:0/22:6 in day 21 pregnant-rat liver (Table 4) paralleled the increases of both CDP:[¹⁴C]choline incorporation into PC16:0/22:6 (Table 3) and in PC16:0/22:6 mass (Table 1).

The constant rate of [¹⁴C]methionine incorporation into hepatic PC between the non-pregnant and term pregnant rat (Table 4) suggested the increased liver content of PC16:0/22:6 at term was not due to an increased rate of *N*-methylation of PE. It has previously been suggested that PE *N*-methylation may act as a mechanism for the selective synthesis of PUFA-containing PC species [7,27], and we have previously shown that that pathway is obligatory for the synthesis of PC16:0/20:4 by fetal guinea-pig liver [6]. The progressive increased incorporation of [¹⁴C]methionine into hepatic PC16:0/22:6 throughout pregnancy was a direct reflection of the altered composition of the PE substrate pool for *N*-methylation (Table 3). The increased concentration of hepatic PE16:0/22:6 at term (Table 1) was due in turn to its increased synthesis *de novo* (Table 4).

The principle changes to plasma PC composition in pregnancy, increased PC16:0/22:6 and PC16:0/20:4 and decreased PC18:0/20:4 and PC18:0/18:2 concentrations (Table 2), closely reflected corresponding analysis of liver PC. This congruence suggests there was little selection of hepatic-PC molecular-species for export into plasma during pregnancy. Incorporations of [¹⁴C]choline (Table 5) and [¹⁴C]methionine (Table 6) into plasma PC *in vivo* showed similar changes in pregnancy and correlated closely with those of [¹⁴C]choline and [¹⁴C]methionine respectively into liver PC (Table 4). This suggested secretion of newly synthesized PC, rather than of PC subjected to extensive acyl remodelling. There was no suggestion of preferential incorporation of PC derived from PE into plasma PC [28].

The simplest explanation for increased synthesis of liver PC in pregnancy is to support increased hepatic lipoprotein synthesis and secretion required for the supply of lipid nutrients to the

fetus and adipose and mammary storage sites. The specific nature of the increased synthesis of PC16:0/22:6 at term further suggests a role for this individual molecular species in the direct supply of C_{22:6 (n-3)} from maternal liver to the fetus and neonatal pups, possibly to support neurological development.

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