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Hepatic and plasma phospholipid molecular species compositions in the pregnant guinea pig: Effect of chronic ethanol consumption

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The effect of chronic ethanol consumption on maternal hepatic and plasma phospholipid molecular species compositions was investigated by feeding adult guinea pigs ethanol both before and throughout pregnancy. Hepatic phosphatidylcholine (PC)16:0/18:2, PC16:0/16:0, PC16:0/18:1, PC16:0/18:2, and PC16:0/16:0 concentrations were significantly lower in ethanol-fed pregnant animals at 40 days, but not at term (68 days). There was no change to hepatic PC species containing 22:6(n-3) or 20:4(n-6) concentration at either gestational age. Ethanol feeding did not significantly alter maternal hepatic phosphatidylethanolamine composition. Plasma total PC concentration at day 40 was significantly lower in ethanol-fed than control animals, due to a general decrease in all PC species except PC16:0/22:6. However, at term, plasma PC concentration was greater in ethanol-fed animals compared with controls. Pregnancy in the guinea pig is associated with increased hepatic and plasma PC16:0/22:6 content, which is related temporally in 22:6(n-3) accumulation into developing fetal brain phosphatidylethanolamine. In this context, these results suggest that impaired supply of 22:6(n-3) to fetus in the guinea pig is probably not a major mechanism for reduced accumulation of 22:6(n-3) into ethanol-exposed fetal guinea pig brain. (J. Nutr. Biochem. 7:425–430, 1996.)

Keywords: pregnancy; fetal alcohol syndrome; ethanol; docosahexaenoic acid; phospholipids; guinea pig

Introduction

Chronic maternal ethanol consumption during pregnancy results in severely impaired morphological and neurological development of the fetus; fetal alcohol syndrome (FAS).[1,2] Whereas the severity of the characteristic dysmorphia associated with FAS may recede with increasing post-natal age, ethanol-induced damage to the central nervous system (CNS) frequently persists into adulthood. The CNS is particularly sensitive to ethanol exposure during fetal development. Moderate maternal ethanol consumption during pregnancy, about 2U ethanol/day, has been shown to reduce intelligence significantly at 4 years of age,[3] whereas infants born to mothers who habitually consume large quantities of ethanol may exhibit severely impaired intelligence, motor function, and marked hyperactivity.[4,5]

Exposure of non-pregnant adult animals to ethanol as liquid diet,[6–10] in drinking water[11] or by inhalation[12–15] has been shown to decrease 22:6(n-3) content in brain phospholipids in some,[6,13–15] but not all,[8–10] experiments. Because neurological development is critically dependent on adequate accumulation of 22:6(n-3) into fetal brain phospholipids, in particular phosphatidylethanolamine (PE),[16,17] one possible mechanism by which ethanol may impair fetal brain development is by reducing 22:6(n-3) assimilation. Studies in which either rats or mice were fed ethanol during pregnancy have produced conflicting results about the effect of ethanol accumulation of 22:6(n-3) into developing brain such that whereas some studies have shown a reduction in 22:6(n-3) content,[18] others have reported either increased 22:6(n-3) concentration[19] or no significant difference from controls.[20] One possible explanation for the discrepancies

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between the results reported by these studies using animal models with predominantly post-natal brain maturation is that ethanol consumption was terminated before birth and developing brain fatty acid compositions were determined some time after birth. In the developing guinea pig, neurological development[21] and accumulation of 22:6(n-3) into brain PE[22] occurs principally during the prenatal period. Thus the fetal guinea pig represents a model of some aspects of prenatal neurological development in the human. Recently, we have established a model of FAS using the guinea pig. Feeding ethanol chronically to guinea pigs before and throughout pregnancy was associated with a marked reduction both at 40 days gestation and term (68 days) in the concentration of both phosphatidylcholine (PC) (8.8% and 39.5%) and PE (36.3% and 55.0%) docosahexaenoic acid (22:6(n-3))-containing molecular species in fetal brain.[23] This deficit in 22:6(n-3) assimilation into fetal brain was associated with decreased brain growth at 40 days gestation, the time point of maximal neurite outgrowth in the guinea pig,[21] and with impaired motor function at term.[23] These data support the suggestion that impaired 22:6(n-3) accumulation into brain phospholipids may represent one important mechanism of ethanol-induced fetal brain damage.

Our previous results suggest that adequacy of 22:6(n-3) supply to the developing fetus is largely dependent on specific adaptations to maternal hepatic and phospholipid composition and hepatic phospholipid synthesis.[24–26] In the present study, we have used high performance liquid chromatography (HPLC) techniques for resolution and quantification of individual phospholipid molecular species to test the hypothesis that chronic ethanol consumption results in alterations to pregnancy-associated changes in maternal guinea pig hepatic and plasma phospholipid composition. Our results show that chronic maternal ethanol consumption during pregnancy results in specific modifications to individual maternal hepatic and plasma phospholipid molecular species that differ with gestational age.

Materials and Methods

Materials

Analytical grade chloroform, ethanol and choline chloride were purchased from Merck (Poole, Dorset, UK). HPLC-grade methanol was obtained from Rathburn Ltd. (Walkerburn, Scotland). Phospholipiase C from *Bacillus cereus* was from Boehringer-Mannheim (Lewes, Sussex, UK). All other reagents were of analytical grade and were purchased from Sigma (Poole, Dorset, UK).

Animal procedures

The guinea pig model of FAS has been described previously.[23] Briefly, virgin female Dunkin-Hartley guinea pigs (body weight about 700 g) were fed 42 kcal/kg/day (equivalent to 6 g/kg/day ethanol) as a 50% (v/v) solution in two bolus doses per day for 14 days, mated and maintained on this regimen throughout pregnancy.[23] This ethanol feeding regimen resulted in maternal blood ethanol concentration of 254 ± 53 mg/100 mL (n = 8) 1 hour after administration of a single 3 g/kg dose. Both ethanol-fed and control animals were allowed free access to chow diet[23] and water. The chow (FD1; Special Diets Services, Witham, Essex, UK) used was a nutritionally complete diet that contained 3.4% fat by weight. The fatty acid composition of the diet (as percent of total fatty acids) was 22.2% saturates, 30.9% monounsaturates, 22.7% 18:2(n-6), 16.3% 18:3(n-3), and 7.9% polyunsaturated fatty acids (PUFA), principally 20:4(n-6).

At 40 days gestation and at term maternal guinea pigs were anaesthetized with a mixture of Halothane, nitrous oxide (0.1 L/min) and oxygen (1.0 L/min).[27] Blood was collected by cardiac puncture using ethylendaimine-tetraacetic acid (EDTA) as anticoagulant, separated by centrifugation, and the plasma stored at −30°C. Maternal livers were removed immediately, frozen in liquid nitrogen and stored at −30°C.

Phospholipid analysis

Maternal liver samples (about 100 mg) were homogenized in 0.8 mL 0.9% (w/v) NaCl after the addition of PC14:0/14:0 (100 nmoles) and PE14:0/14:0 (50 nmoles) as internal standards. Plasma (100 μL) was added drop-wise to 2 mL ice-cold methanol containing 20 nm PC14:0/14:0 with continuous stirring. Total lipids were extracted with chloroform and methanol.[28] PC and PE were isolated by solid phase extraction on disposable 100 mg aminopropyl BondElut cartridges,[29] PC in the chloroform/methanol (60:40, v/v) wash, and PE in the subsequent methanol wash. Samples were dried under nitrogen at 40°C.

Hepatic PC and PE molecular species were resolved by reverse-phase HPLC on a 25 cm × 4.6 mm 3μ APEX I ODS column thermostatically maintained at 50°C, using a mobile phase of methanol/water (930:70, v/v) containing 40 mM choline chloride at a flow rate of 0.8 ml/min.[22] The mass of eluted intact phospholipid molecular species was determined by post-column derivatization with 1,6-diphenyl-1,3,5-hexatriene and fluorescence detection.[30]

Pregnancy in the guinea pig is characterized by a marked hypolipidaemia.[24] To identify accurately and measure plasma PC molecular species, PC was hydrolyzed with phospholipiase C,[31] followed by derivatization of the resultant diacylglycerol species with benzoyl chloride and analysis by HPLC. Briefly, PC was dissolved in 3 mL diethyl ether to which was added 1 mL Tris HCl (pH 7.4) containing 10 mM $CaCl_2$ and 20U phospholipase C. This mixture was incubated at room temperature for 2 hr with continuous shaking. The reaction was stopped by addition of chloroform/methanol/water (2:2:1, v/v). The chloroform phase was collected and dried under nitrogen at 40°C. Diacylglycerol formed was derivatized by incubation with 10% (v/v) benzoyl chloride in pyridine at room temperature for 16 hr. Diacylglycerol benzoates were extracted with 3 mL hexane and 2 mL methanol/water (2:1, v/v) saturated with Na_2CO_3. The organic phase was sequentially washed with methanol/water (2:1, v/v) saturated with Na_2CO_3 and methanol/water (2:1, v/v). Finally, the organic phase was dried under nitrogen at 40°C.

Diacylglycerolbenzoate molecular species were resolved by reverse phase HPLC on a 25 cm × 4.6 mm 3μ APEX I ODS column thermostatically maintained at 25°C using a mobile phase of methanol/acetonitrile/water (89.5:10.:0.5, v/v) at a flow rate of 1.0 mL/min. The mass of eluted molecular species was determined by UV absorbance at λ = 230 nm.

Molecular species were identified according to their retention time relative to the internal standard, collaborated by collection of individual peaks followed by analysis of fatty acid methyl esters by gas chromatography.[24]

Statistical analysis

Statistical analysis was carried out using the Kruskal Wallis test.

Results

Maternal hepatic phospholipid composition

Nine major hepatic PC and seven PE molecular species were consistently identified in samples from both control and ethanol-fed animals, which accounted for >95% of total liver PC and PE. These results were in good general agreement with previous analyses of guinea pig liver phospholipid molecular species compositions.[24] Feeding ethanol to guinea pigs during pregnancy resulted in specific changes in the content of individual hepatic PC molecular species that differed with gestational age. At 40 days gestation ethanol feeding was associated with decreased concentrations of PC16:0/18:2 (44.7%), PC16:0/16:0 (29.8%), PC16:0/18:1 (50.8%), PC18:0/18:2 (64.8%), and PC16:0/18:0 (42.9%) *Table 1.* However, the concentrations of PC16:0/22:6 and PC16:0/20:4 were not significantly different in ethanol-fed animals compared with controls *Table 1.* The changes in the concentration of individual molecular species resulted in an overall 40.6% decrease in hepatic PC concentration *Table 1.* These ethanol-induced changes in maternal liver PC composition at 40 days gestation were not found at term *Table 1.* However, PC16:0/22:6 concentration was 66.9% greater at 68 days gestation compared with control animals *Table 1.*

Analysis of maternal hepatic PE composition indicated that ethanol-feeding has a lower effect on PE composition compared with PC *Table 2.* There were no significant changes to the composition of hepatic PE in ethanol-fed animals either 40 days gestation or at term *Table 2.* In addition, ethanol feeding did not significantly alter the increase in PE concentration associated with late gestation[24] (control 48.8%, ethanol-fed 54.4%) *Table 2.*

Maternal plasma phosphatidylcholine concentrations

Between day 40 of gestation and term there was a significant decrease in maternal plasma PC concentration, consistent with our previous report of a marked hypolipidaemia in pregnant guinea pigs at term.[24] Feeding ethanol to pregnant guinea pigs resulted in a significant decrease (69.1%) in plasma PC concentration at 40 days gestation to a level below that of the maximum hypolipidaemia demonstrated at term *Table 3.* This was due primarily to specific decreases in the concentrations of the major plasma PC molecular species PC16:0/8:2 (69.9%), PC18:0/18:2 (70.0%), PC16:0/18:1 (60.0%), and PC16:0/18:0 (83.3%). The concentrations of the relatively minor plasma PC components PC18:0/22:6 (71.4%), PC16:0/20:4, and PC18:0/20:4 (50.0%) were also significantly decreased *Table 3.* However, there was no significant change in plasma PC16:0/22:6 concentration *Table 3.* In contrast at term, plasma PC concentration in ethanol-fed animals was significantly greater (48.7%) than controls, due to increased contents of PC16:0/18:2 (33.3%), PC16:0/18:1 (40.0%), and PC18:0/18:2 (38.1%) *Table 3.*

Discussion

Previous analyses of the PUFA content of liver and blood from animals fed ethanol chronically have produced widely conflicting results. For example, in studies in which the 22:6(n-3) content of liver tissue was determined, chronic ethanol consumption resulted in decreased,[32–35] increased[36] or no significant change[16,37] in 22:6(n-3) fractional concentration. Such a wide variation in results may be attributed to differences in duration of ethanol exposure, animal species used, diet, and the subcellular fraction analyzed. One additional source of variation in results, especially in studies in which ethanol did not significantly change hepatic PUFA content, is analysis of total phospholipid fatty acids rather than of molecular species. For example, an equal reciprocal change in PC16:0/22:6 and PC18:0/22:6 concentration would produce a result of no net change in 22:6(n-3) content measured in terms of total fatty acid. Recent studies on rat brain aminophospholipids have indicated that specific molecular species may play distinct and critical roles in the function of the tissue.[38] Thus, to determine accurately changes to membrane phospholipid composition, which may be related to ethanol-induced alterations to tissue func-

Table 1 Maternal hepatic phosphatidylcholine composition

Molecular species	Phosphatidylcholine (nmol/g)			
	D40 Control	D40 Ethanol	D68 Control	D68 Ethanol
16:0/22:6	1,761 ± 293	1,930 ± 215	635 ± 144†	1,060 ± 332*
16:0/20:4	316 ± 85	381 ± 78	205 ± 57	262 ± 107
16:0/18:2	3,235 ± 743	1,788 ± 241*	3,599 ± 423	3,654 ± 844‡
16:0/16:0	647 ± 170	454 ± 58*	537 ± 275	727 ± 298
16:0/18:1	1,250 ± 234	615 ± 78*	1,035 ± 150†	1,432 ± 649‡
18:0/22:6	1,154 ± 414	759 ± 141	685 ± 193†	1,016 ± 589
18:0/20:4	499 ± 126	381 ± 101	434 ± 99	394 ± 166
18:0/18:2	5,773 ± 1,224	2,030 ± 461*	6,190 ± 1,151	5,878 ± 1,672‡
16:0/18:0	569 ± 356	325 ± 166*	668 ± 160	453 ± 409
Total	15,204 ± 3,313	9,035 ± 1,239*	14,006 ± 1,833	14,876 ± 4,272

Values are mean ± S.D. (n = 8/group) of analysis of control and ethanol-fed maternal guinea pig liver PC molecular species.
*Indicates values that were significantly different ($P < 0.001$) between control and ethanol-fed animals of the same gestational age.
†Indicates values that were significantly different ($P < 0.05$) between day 40 and day 68 control animals.
‡Indicates values that were significantly different ($P < 0.05$) between ethanol-fed animals at 40 and 68 days gestation.
Maternal liver weights were: day 40 control 27.47 ± 5.16 g, day 40 ethanol-fed 32.86 ± 3.50 g*, day 68 control 24.1 ± 3.38 g, and day 68 ethanol-fed 32.96 ± 6.61* ($P < 0.05$).

Table 2 Maternal hepatic phosphatidylethanolamine composition

Molecular species	Phosphatidylethanolamine (nmol/g)			
	D40 Control	D40 Ethanol	D68 Control	D68 Ethanol
16:0/22:6	1,070 ± 256	1,322 ± 247	1,691 ± 178†	1,671 ± 206‡
16:0/20:4	196 ± 54	269 ± 62	147 ± 39	130 ± 46‡
16:0/18:2	681 ± 95	579 ± 202	874 ± 239†	705 ± 163
16:0/18:1	193 ± 36	217 ± 41	331 ± 123†	233 ± 52
18:0/22:6	1,692 ± 373	1,594 ± 321	3,081 ± 364†	2,801 ± 367‡
18:0/20:4	894 ± 192	893 ± 174	1,580 ± 110†	1,472 ± 121‡
18:0/18:2	1,818 ± 446	1,210 ± 304	2,005 ± 390	2,383 ± 212‡
Total	6,544 ± 1,136	6,083 ± 950	9,709 ± 1,205†	9,395 ± 659‡

Values are mean ± S.D. (n = 8/group) of analysis of control and ethanol-fed maternal guinea pig liver PE molecular species.
*Indicates values that were significantly different ($P < 0.001$) between control and ethanol-fed animals of the same gestational age.
†Indicates values that were significantly different ($P < 0.05$) between day 40 and day 68 control animals.
‡Indicates values that were significantly different ($P < 0.05$) between ethanol-fed animals at 40 and 68 days gestation.

tion, it is important to analyze phospholipid composition in terms of whole molecular species.

In the present study we report for the first time analysis of the effect of chronic ethanol ingestion on maternal guinea pig hepatic and plasma phospholipid molecular species compositions. Whereas both maternal hepatic and plasma PC showed changes in specific molecular species content after ethanol feeding, there was no significant ethanol-induced change in hepatic PE composition.

We have shown previously that pregnancy in the guinea pig is associated with a specific increase in hepatic PC16:0/22:6 and PC16:0/20:4 contents between 25 and 35 days gestation, which subsequently decreased to near non-pregnant levels at term.[24] We have proposed that such changes to PC content may represent adaptations to hepatic phospholipid metabolism to ensure adequate supply of PUFA to the developing fetus during the period of maximal 22:6(n-3) accumulation into developing fetal brain PE.[24] The present results show that maternal ethanol consumption during pregnancy does not significantly alter such adaptations to liver PC16:0/22:6 and PC16:0/20:4 content at day 40, suggesting that mechanisms responsible for increased hepatic PC 22:6(n-3) and 20:4(n-6) concentration at critical time points in pregnancy are not significantly affected by ethanol consumption. One possible implication of these data is that impaired supply of 22:6(n-3) from mother to fetus is probably not a major mechanism for ethanol-associated reduced 22:6(n-3) assimilation into developing brain PE.[23] However, such measurements of PC concentration do not eliminate the possibility of reduced flux of 22:6(n-3) through maternal hepatic and plasma PC pools, which may result in decreased supply of 22:6(n-3) to the fetus. In contrast, hepatic PC16:0/22:6 concentration in ethanol-fed animals was markedly greater at term compared with controls, and, although lower, was not significantly different from values at day 40 (*Table 1*). The PC16:0/22:6 content of control maternal liver, however, decreased substantially between day 40 and term *Table 1*.[24] This suggests that ethanol may alter some aspects of PC16:0/22:6 metabolism in late gestation.

Despite the lack of alteration to maternal hepatic PC16:0/22:6 content at day 40, ethanol feeding substantially decreased PC16:0/18:2, PC18:0/18:2, PC16:0/16:0, and PC16:0/18:1 content of hepatic PC species at 40 days gestation, but not at term *Table 1*. One possible explanation for this differential effect of ethanol on these PC molecular species, but not those containing 22:6(n-3) and 20:4(n-6), is the relative contribution of different pathways for PC syn-

Table 3 Maternal plasma phosphatidylcholine composition

Molecular species	Phosphatidylcholine (μM)			
	D40 Control	D40 Ethanol	D68 Control	D68 Ethanol
16:0/22:6	2 ± 1	2 ± 1	1 ± 1	1 ± 2
16:0/20:4	1 ± 1	0 ± 0*	0 ± 0†	1 ± 1*‡
16:0/18:2	16 ± 6	5 ± 2*	9 ± 2†	12 ± 3*‡
16:0/18:1	10 ± 4	4 ± 3*	5 ± 3†	12 ± 2*‡
18:0/22:6	7 ± 1	2 ± 1*	2 ± 1†	1 ± 1
18:0/20:4	2 ± 1	1 ± 1*	1 ± 2†	0 ± 0
18:0/18:2	30 ± 9	9 ± 4*	13 ± 5†	21 ± 9*‡
16:0/18:0	12 ± 3	2 ± 1*	7 ± 3†	9 ± 4‡
Total	81 ± 17	25 ± 9*	39 ± 10†	58 ± 12*‡

Values are mean ± S.D. (n = 8/group) of analysis of control and ethanol-fed maternal guinea pig liver PC molecular species.
*Indicates values that were significantly different ($P < 0.001$) between control and ethanol-fed animals of the same gestational age.
†Indicates values that were significantly different ($P < 0.05$) between day 40 and day 68 control animals.
‡Indicates values that were significantly different ($P < 0.05$) between ethanol-fed animals at 40 and 68 days gestation.

thesis. In perinatal guinea pig liver, PC16:0/18:2, 18:0/18:2, PC16:0/16:0, and PC16:0/18:1 species are synthesized almost entirely by the *de novo* pathway, whereas PE *N*-methylation contributes significantly to the synthesis of 22:6(n-3)-containing PC species and is the sole source of 20:4(n-6) species.[39] Thus, it is possible that ethanol may induce a differential decrease in PC16:0/18:2, PC18:0/18:2, 16:0/16:0, and PC16:0/18:1 concentrations by reducing flux through the *de novo* pathway, but not PE *N*-methylation. This suggestion is also supported partly by the relative resistance of PE to the effects of ethanol exposure compared with PC. The absence of significant effects of ethanol on hepatic PE composition may reflect differences between mechanisms that regulate PE molecular species content and those that define the specificity of PC synthesis.[25,39]

In contrast to late pregnancy in women and in the rat, pregnancy in the guinea pig is characterized by a marked hypolipidaemia in which plasma PC concentration decreased to below 50% of that found in non-pregnant animals by 25 days gestation and remained at this level until term.[24] This decrease in plasma PC concentration is consistent with rapid hydrolysis of serum lipoproteins by placenta lipoprotein lipase, which is about 50 fold/g tissue greater in the guinea pig compared with the human.[40] Feeding ethanol to pregnant guinea pigs resulted in a lower plasma PC concentration at day 40 compared with controls, due to decreased contents of all plasma PC species. Plasma PC concentration represents a balance between the rate of lipoprotein secretion by the liver and hydrolysis by lipoprotein and hepatic lipases. Thus the decrease in plasma PC at 40 days in ethanol-fed animals may either represent increased lipoprotein hydrolysis or decreased lipoprotein secretion, although in pregnancy increased degradation of lipoproteins at the placenta to facilitate rapid supply of fatty acids to the growing fetus is probably the more likely explanation. Conversely, at term the ethanol-induced increase in plasma PC concentration, principally due to increased PC16:0/18:2, PC16:0/18:1, PC18:0/18:2, and PC16:0/18:0 contents, may represent increased lipoprotein secretion or decreased hydrolysis. In addition, because there were no significant changes in the concentration of hepatic saturated, and mon- and di-unsaturated PC species at term, the increased contents of plasma PC16:0/18:2, PC16:0/18:1, PC18:0/18:2, and PC16:0/18:0 at 68 days gestation suggest either selective increases in secretion of these species on lipoproteins or differential decreases in turnover.

The results of this study show that chronic ethanol consumption in the pregnant guinea pig results in complex differential modifications to the molecular species compositions of both maternal hepatic and plasma phospholipids. However, these data do not support the suggestion that maternal ethanol consumption during pregnancy leads to reduced supply of 22:6(n-3) from mother to fetus. Ethanol has been shown to reduce the expression of the growth cone-associated protein GAP-43 in neuroblastoma cells *in vitro*.[41] Such impairment of processes that lead to neurite formation in developing brain would result in reduced requirement for 22:6(n-3) for incorporation into newly synthesized plasma membrane and may thus provide one possible mechanism by which ethanol exposure may directly affect 22:6(n-3) accumulation into fetal brain PE.

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