Effects of Prenatal Treatment with Betamethasone, *L*-Carnitine, or Betamethasone-*L*-Carnitine Combinations on the Phosphatidylcholine Content and Composition of the Foetal and Maternal Rat Lung[1]

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Dedicated to Professor Dr. Erich Kaiser on the occasion of his 70th birthday

Pregnant rats received 0.10 or 0.20 mg/kg body weight betamethasone, or 100 mg/kg body weight *L*-carnitine, or *L*-carnitine 100 mg/kg plus betamethasone 0.05 or 0.10 mg/kg body weight, or saline (controls) for three days before delivery of foetuses at day 19 of gestation. Dose-related effects on the dipalmitoyl phosphatidylcholine content and the phosphatidylcholine species composition of foetal and maternal lungs were determined. Betamethasone (0.10 and 0.20 mg/kg) or *L*-carnitine (100 mg/kg) significantly increased ($p < 0.05$) the dipalmitoyl phosphatidylcholine content in the foetal lungs, while only small changes were found in relative terms. Combinations of betamethasone (0.05 or 0.10 mg/kg) with *L*-carnitine (100 mg/kg) also significantly increased the dipalmitoyl phosphatidylcholine content of the foetal lungs above control values ($p < 0.01$) and above the values achieved with betamethasone alone ($p < 0.05$). In the maternal lungs a significant increase of the dipalmitoyl phosphatidylcholine content above the control values was only found after treatment with betamethasone-carnitine combinations, whereas compared with the foetal lung the relative increase of dipalmitoyl phosphatidylcholine as a fraction of total phosphatidylcholine was more pronounced after betamethasone treatment. The gas chromatographic method used separates two monoenoic phosphatidylcholine species with 32 carbon atoms in the acyl residues. These two phosphatidylcholine species showed striking differences between adult and foetal lungs. Palmitoleyl palmitoyl phosphatidylcholine predominates in the maternal lung, whereas palmitoyl palmitoleyl phosphatidylcholine is the major monoenoic phosphatidylcholine species with 32 carbon atoms in the foetal lung. These two species were not affected in maternal or foetal lung by betamethasone or *L*-carnitine treatment. In contrast, after treatment with betamethasone-carnitine combinations, a significant increase of the fraction of palmitoyl palmitoleyl phosphatidylcholine was found in foetal but not in the maternal lung. The results of the present study demonstrate that maternal glucocorticoid and carnitine treatment affects the maternal as well as the foetal lung but with different effects on the dipalmitoyl phosphatidylcholine content and phosphatidylcholine species composition.

Introduction

The problem associated with a preterm birth are due to the immaturity of one or more organ systems. Because the neonatal respiratory distress syndrome is the most severe complication, its prevention is a matter of major concern.

Pulmonary surfactant is a complex aggregation of phospholipids, cholesterol, and lung-specific apolipoproteins which line the alveolar surface. Phosphatidylcholine species are by far the most abundant component of the phospholipid fraction. Dipalmitoyl phosphatidylcholine (1,2-dipalmitoyl-*sn*-glycero-3-phospho-choline) is mainly responsible for surface activity (1, 2).

A lack of surfactant causes a disturbance of alveolar gas exchange. This is seen in immature infants suffering from respiratory distress syndrome (3). Pulmonary instillation of exogenous surfactant is known to be effective in reducing the severity of the disorder (4). Altered chemical composition and functional activity of surfactant has also been demonstrated in adult respiratory distress syndrome, and it has been suggested that these abnormalities occur early in the disease process (5). The main causes of adult respiratory distress syndrome in pregnancy were found to be infection, preeclampsia or eclampsia (6).

Even in normal pregnancies plasma carnitine levels at delivery are decreased to about half of the concentrations seen in non-pregnant women (7−10). As shown for rats, maternal carnitine levels are also significantly lower in tissues (11). Maternal administration of *L*-carnitine during pregnancy increased the content of dipalmitoyl phosphatidylcholine in the foetal rat lung (12, 13). Maternal administration of carnitine in pregnancies with the risk of imminent premature delivery reduced both the incidence of respiratory distress syndrome and foetal morbidity (14). Carnitine is essential for the trans-

port of long chain fatty acids into the mitochondrial matrix. Furthermore carnitine is important as a reversible sink for acyl residues and the generation of free coenzyme A (15).

Glucocorticoids, especially betamethasone, are frequently used to accelerate foetal pulmonary maturity and to decrease the risk of respiratory distress syndrome in preterm infants (16, 17). However, a considerable number of infants fail to respond to this therapy (16). The concept that glucocorticoids only trigger receptors on foetal lung fibroblasts and/or type II cells to induce synthesis of surfactant lipid and protein components has been judged much too simplistic, especially since glucocorticoids cause foetal growth retardation in rabbit (18) and rat foetuses (19).

The initial purpose of this study was to evaluate whether the glucocorticoid dosage which is known to be effective on foetal lung development also induces changes of the dipalmitoyl phosphatidylcholine content and the phosphatidylcholine molecular species composition in maternal rat lungs. The second aim was to compare the effects of different betamethasone-L-carnitine combinations on the phosphatidylcholine species composition of foetal and maternal lungs, since this drug combination is under investigation in clinical trials.

Materials and Methods

Study design

Sixty pregnant Wistar rats with an average weight of 300 g and an expected gestation period of 22 days were prospectively randomised and divided into 6 subgroups. The day after mating was considered day 1 of gestation. The rats received intraperitoneal injections of different doses of betamethasone, L-carnitine, and L-carnitine-betamethasone combinations, or saline (controls) from day 16 to day 18 of gestation. In all groups the foetuses were delivered by Cesarean section on day 19 of gestation.

Animal procedures

The rats were intubated and anaesthetised with piritramide (Dipidolor®, 15 µg/kg) and an additional injection of D-glucochloralose (5%) as necessary. The animals were ventilated with a tidal volume of 15 ml/kg. Immediately after delivery the foetal trachea was clamped before spontaneous inspiration could occur. The foetuses were thoracotomised by two parasternal incisions and the lungs were removed. The lungs of the foetuses of each litter were pooled and homogenised. A hypodermic syringe was inserted in the maternal vena cava, and the lung was rinsed by passing physiological saline via the right ventricle. Thereafter the lung was removed and homogenised.

Lipid extraction

Lipids were extracted and washed using the method of *Folch* et al. (20). The main phospholipid classes were separated by one-dimensional thin-layer chromatography using the solvent system chloroform/methanol/10 g/l aqueous potassium chloride (43 + 47 + 4, by vol.) (12).

Determination of phosphatidylcholine molecular species

Dipalmitoyl phosphatidylcholine and other phosphatidylcholine species were determined as the corresponding diacylglycerol tri-

methylsilylether derivatives by gas-liquid chromatography (21). A 10 m (0.32 mm I.D.) fused silica capillary column with chemically bonded DB-5 (0.15 µm coating thickness) was used for all analyses. Hydrogen was used as the carrier gas at 40 kPa (8—10 ml/min flow rate) and nitrogen as the make-up gas. The oven temperature was programmed from 260 °C to 320 °C, at a rate of 3 °C/min. The analyses were carried out on a Dani Model 86.10HT and a Dani Model 8521 gas chromatograph (Dani SpA., Monza, Italy) each equipped with a programmed temperature vaporiser injector. Recording, converting, peak area calculation, and data processing were carried out by personal computer using Chrom-Card software (Fisons Instrument SpA, Milan, Italy). The results of quantitative determinations are expressed as amounts per g dry weight of the lungs, since this measurement tends to underestimate rather than overestimate changes in dipalmitoyl phosphatidylcholine content in the different treatment groups (22).

Chemicals

Chloroform, methanol, ethanol, pyridine, hexamethyldisilazane, and thin-layer chromatography plates (silica gel 60) were obtained from E. Merck (Darmstadt, Germany). *Bacillus cereus*-derived phospholipase C was obtained from Boehringer-Mannheim (Germany). Dimyristoyl-sn-glycero-3-phosphocholine was supplied by Sigma Chemical Company (St. Louis, MO) and L-Carnitine-Leopold® by Leopold Ltd. (Graz, Austria). Betamethasone was purchased from Boehringer Ingelheim (Germany).

Statistical analysis

Statistical comparisons between groups were made using analysis of variance followed by *Dunnet*'s t-test for multiple comparison (23). All values are given as mean ± SD.

Results and Discussion

The present study is the first to systematically examine the effects of treating the mother animal with betamethasone, L-carnitine, and betamethasone-L-carnitine combinations. A rat model was used, because rabbits are not suitable: prenatal carnitine levels are low in man, rats, piglets, and sheep, but not in rabbits and guinea pigs (24). A disadvantage of the rat model is, however, that for technical reasons it is impossible to lavage or determine the mechanical properties of the lungs in animals on the 19th gestational day (25), and that survival analysis cannot be performed, since rat foetuses delivered on day 19 of gestation do not survive (26). Thus we evaluated the effects of the different drugs on the dipalmitoyl phosphatidylcholine content and the phosphatidylcholine species composition of foetal and maternal lungs.

Dipalmitoyl phosphatidylcholine content.

Injection of the mother with 0.10 and 0.20 mg/kg body weight betamethasone or 100 mg/kg body weight L-carnitine resulted in a significant ($p < 0.05$) increase of the dipalmitoyl phosphatidylcholine content in the foetal lungs, whereas in the maternal lungs only treatment with 0.20 mg/kg betamethasone caused a significant increase of the dipalmitoyl phosphatidylcholine content, compared with control values (tab. 1). Combinations of both 0.05 or 0.10 mg/kg betamethasone with 100 mg/kg

Tab. 1   Phosphatidylcholine species in foetal and maternal rat lung containing two C_{16}-fatty acids

Treatment	n	16:0/16:0-PC* (g/kg dry weight)	16:0/16:0-PC*	16:1/16:0-PC**	16:0/16:1-PC***
			(Fraction of total phosphatidylcholines, %)		
Foetal rat lung					
NaCl (Controls)	8	5.8 ± 0.7	18.4 ± 2.3	4.4 ± 0.4	6.7 ± 0.7
Betamethasone (0.1 mg/kg)	8	7.9[a] ± 2.5	21.1 ± 2.4	4.0 ± 1.0	7.0 ± 1.9
Betamethasone (0.2 mg/kg)	8	8.2[a] ± 1.9	18.4 ± 3.5	3.9 ± 0.4	6.0 ± 1.7
Carnitine (100 mg/kg)	8	7.6[a] ± 0.6	18.2 ± 1.1	4.2 ± 0.3	5.9 ± 1.1
Carnitine (100 mg/kg) + Betamethasone (0.05 mg/kg)	8	8.4[b] ± 0.8	22.4 ± 2.2	3.3 ± 0.3	8.4[a] ± 0.9
Carnitine (100 mg/kg) + Betamethasone (0.1 mg/kg)	8	9.4[b] ± 1.2	20.7 ± 2.6	3.0 ± 0.2	8.3[a] ± 1.3
Maternal rat lung					
NaCl (Controls)	8	16.0 ± 2.9	33.2 ± 4.9	6.7 ± 1.3	4.7 ± 0.7
Betamethasone (0.1 mg/kg)	8	15.8 ± 3.4	41.1[a] ± 3.0	7.3 ± 0.7	4.4 ± 0.5
Betamethasone (0.2 mg/kg)	8	21.2 ± 7.8	41.9[a] ± 5.8	7.3 ± 0.4	4.7 ± 0.4
Carnitine (100 mg/kg)	8	18.9 ± 3.4	36.7 ± 3.7	6.5 ± 0.8	4.5 ± 0.7
Carnitine (100 mg/kg) + Betamethasone (0.05 mg/kg)	8	19.6[a] ± 1.5	35.1 ± 2.1	7.2 ± 0.2	5.4[a] ± 0.3
Carnitine (100 mg/kg) + Betamethasone (0.1 mg/kg)	8	20.0[a] ± 1.7	38.9[a] ± 2.5	7.4 ± 0.4	5.3[a] ± 0.3

The values are given as mean ± SEM.
n indicates the number of experiments.
[a] $p < 0.05$,   [b] $p < 0.01$.

* 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine
** 1-Palmitoleyl-2-palmitoyl-*sn*-glycero-3-phosphocholine
*** 1-Palmitoyl-2-palmitoleyl-*sn*-glycero-3-phosphocholine

L-carnitine also increased the dipalmitoyl phosphatidylcholine content of the foetal and maternal lungs significantly above control levels ($p < 0.05$). Doubling of the betamethasone dosage from 0.10 to 0.20 mg/kg caused only a minor further increase of the dipalmitoyl phosphatidylcholine content of the foetal lungs, whereas the adult lungs showed a significant increase (tab. 1).

Several mechanisms may be responsible for the different glucocorticoid dose effects in the foetal and adult lung. In response to glucocorticoids a polypeptide, the fibroblast-pneumocyte-factor, is produced and secreted by foetal lung fibroblasts (27, 28). It seems plausible that the effects of the lower betamethasone dose on the foetal lung is mediated by this factor, since higher concentrations of glucocorticoid are required to produce an effect on type II cells (27, 28). This is supported by previous findings that in glucocorticoid-treated animals differentiation of foetal lung tissue was advanced in regions with broad epithelial-mesenchymal contact, i. e. in the terminal branches of the pseudoglandular outgrowths in the subpleural regions (29).

In foetal lung the effect of hormones on the CTP : choline-phosphate-cytidylyl-transferase[2]) is due to activation of existing enzyme rather than stimulation of its synthesis (1). This is in accordance with evidence that the foetal lung contains a large amount of this enzyme in the active form; in contrast, more of the enzyme in

the adult lung is in the active form (30). Consequently in the adult lung glucocorticoid stimulation of CTP : choline-phosphate-cytidylyltransferase activity occurs via the classical mechanism, mediated by the glucocorticoid receptor and dependent on mRNA and protein synthesis (31), for which higher doses of the hormone are necessary.

Furthermore there is ample evidence that monoenoic fatty acids induce a shift from the inactive form of CTP: choline-phosphate-cytidylyltransferase to the active species and thus play a key role in the developmental regulation of this enzyme. Late pregnancy is characterised by increased levels of cholesterol esters, triacylglycerols, and unesterified fatty acids (32). This implies that the enzyme activity is not only linked to developmental and drug-induced changes, but also to pregnancy-related changes of lipid metabolism.

Foetuses of several species, including humans, accumulate carnitine in the last trimester of pregnancy (24). The corresponding maternal carnitine levels are significantly lower not only in blood but also in tissues (11). It is well known that carnitine promotes phospholipid synthesis in different tissues (12, 13, 33, 34). This effect may be even more pronounced in carnitine-treated animals with insufficient tissue carnitine levels.

Phosphatidylcholine molecular species containing palmitic- and palmitoleic acid

As previously described (21) the gas chromatographic method employed distinguishes between two isomeric

[2]) Enzyme:
CTP : choline-phosphate-citidylyltransferase (EC 2.7.7.15)

Tab. 2 Relative phosphatidylcholine species composition in foetal and maternal rat lungs

Treatment	n	PC-30*	PC-32*	PC-34*	PC-36*	PC-38*
		(Fraction of total phosphatidylcholines, %)				
Foetal rat lung						
NaCl (Controls)	8	4.6 ± 0.8	30.6 ± 1.8	37.8 ± 2.7	19.8 ± 2.3	7.4 ± 2.3
Betamethasone (0.1 mg/kg)	8	4.6 ± 0.9	32.0 ± 3.4	35.5 ± 3.3	19.7 ± 1.3	7.9 ± 1.8
Betamethasone (0.2 mg/kg)	8	4.0 ± 0.9	27.9 ± 3.6	36.1 ± 2.2	21.3 ± 2.6	10.0 ± 2.8
Carnitine (100 mg/kg)	8	3.5[a] ± 0.4	27.8 ± 2.3	37.1 ± 1.4	21.2 ± 2.4	10.3 ± 1.8
Carnitine (100 mg/kg) + Betamethasone (0.05 mg/kg)	8	4.6 ± 0.6	33.0 ± 2.9	32.6[a] ± 1.5	18.9 ± 1.6	8.8 ± 0.9
Carnitine (100 mg/kg) + Betamethasone (0.1 mg/kg)	8	4.6 ± 0.7	34.6[a] ± 3.9	32.7[a] ± 1.3	18.8 ± 2.0	7.7 ± 3.4
Maternal rat lung						
NaCl (Controls)	8	4.8 ± 0.9	46.0 ± 5.9	29.2 ± 1.8	15.1 ± 3.1	4.3 ± 3.1
Betamethasone (0.1 mg/kg)	8	4.7 ± 0.4	53.6[a] ± 3.0	27.2 ± 1.2	11.1[a] ± 1.8	1.7[a] ± 0.7
Betamethasone (0.2 mg/kg)	8	4.7 ± 0.6	54.3[a] ± 6.1	27.6 ± 2.1	10.4[a] ± 2.6	2.2[a] ± 0.6
Carnitine (100 mg/kg)	8	4.4 ± 0.4	49.2 ± 4.9	28.8 ± 0.9	13.5 ± 2.7	3.2 ± 2.4
Carnitine (100 mg/kg) + Betamethasone (0.05 mg/kg)	8	5.3 ± 0.8	47.6 ± 1.7	27.0 ± 1.2	13.4 ± 1.2	4.2 ± 1.5
Carnitine (100 mg/kg) + Betamethasone (0.1 mg/kg)	8	5.3 ± 0.4	50.8 ± 2.8	27.7 ± 0.8	12.3 ± 2.9	3.3 ± 1.9

The values are given as fraction (%) of total of phosphatidylcholine species ± SEM.
n indicates the number of experiments.

* PC-30 etc., total carbon atoms in acyl residues is 30 etc.
[a] $p < 0.05$.

monoenoic species with 16 carbon atoms in each acyl residue. With regard to these phosphatidylcholine molecular species, there are striking differences between adult and foetal lungs (tab. 1). 1-Palmitoleyl-2-palmitoyl-*sn*-glycero-3-phosphocholine predominates in the maternal lung, whereas in the foetal lung the major phosphatidylcholine monoenoic species with two C_{16}-acyl residues is 1-palmitoyl-2-palmitoleyl-*sn*-glycero-3-choline. In the foetal and maternal lungs the fraction of lung palmitoyl palmitoleyl phosphatidylcholine species was significantly increased by treatment with betamethasone-*L*-carnitine combinations but not in those groups administered either *L*-carnitine or betamethasone alone.

Palmitoyl palmitoleyl phosphatidylcholine may be a precursor of dipalmitoyl phsophatidylcholine synthesis by the acyl residue remodelling pathway (35). Increased dipalmitoyl phosphatidylcholine synthesis by this pathway is not necessarily accompanied by a reduction of phosphatidylcholine species with 34 carbon atoms in the acyl residues as shown for the developing lung (tab. 2).

Composition of the phosphatidylcholine molecular species

The higher dipalmitoyl phosphatidylcholine content in *L*-carnitine treated animals, resulting from an increase in the rate of the de novo synthesis of phosphatidylcholine, is dependent on the composition of fatty acids available and not necessarily associated with changes in the composition of phosphatidylcholine molecular species (tab. 2).

Late pregnancy in the rat (gestational ages 16–21 days) has been reported to be accompanied by a specific increase in hepatic phosphatidylcholine molecular species containing palmitic acid at the *sn*-1 position and polyunsaturated essential fatty acids at the *sn*-2 position (36). Similar metabolic changes may occur in the lung. Prenatal betamethasone treatment increased the fraction of phosphatidylcholine with 16 carbon atoms in each acyl residue (PC-32) in maternal but not in foetal lungs. This increase was compensated by a reduction of other phosphatidylcholine species in the higher mass range.

It is well established, that the palmitoyl linoleyl molecular species undergoes a remodelling mechanism in the adult lung, thereby serving as the main source for the surfactant dipalmitoyl phosphatidylcholine (2). Consequently, if betamethasone treatment induced a higher rate of dipalmitoyl phosphatidylcholine synthesis via the phosphatidylcholine species remodelling pathway, a reduced amount of phosphatidylcholine with a sum of 34 carbon atoms in the acyl residues (PC-34) will result. This has been described previously (2) and is confirmed by our data for foetal lung tissue (tab. 2). However, surprisingly a different picture emerges for adult lung tissue. Here a rise in the PC-32 fraction is accompanied by a reduction of the contribution of the PC-36 and PC-38, but not the PC-34 species. This suggests that glucocorticoids stimulate fatty acid synthesis with the main product palmitic acid converted to PC-32 or that they activate a remodelling mechanism converting PC-36 and PC-38 species to dipalmitoyl phosphatidylcholine.

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