Gestational Age-Dependent Changes in Plasma Inositol Levels and Surfactant Composition in the Fetal Rat

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ABSTRACT. To study the possibility that changes in fetal surfactant composition depend on the availability of inositol, we isolated surfactant material from lungs of fetal and neonatal rats and estimated their plasma inositol concentration. During the 18- to 22-day gestational period the amount of surfactant increases from 0.17 to 3.10 μ mol phospholipids/g wet lung. From day 20 onward, 70% or more of the phospholipids is phosphatidylcholine. In this period the relatively high percentage of phosphatidylinositol (8%) in the lung surfactant decreases to 4% whereas the percentage of phosphatidylglycerol increases from 2 to 8% at parturition. During gestation the phospholipid/protein ratio of the surfactant material increase from 3 to 11 and the highest ratio is found immediately after birth. It decreases again 24 h after birth to values characteristic for surfactant from adult rats. The plasma inositol concentration drops during the 18- to 22-day period from 0.81 to 0.26 mmol/liter and a similar decrease in inositol concentration occurs in amniotic fluids. The phosphatidylglycerol/phosphatidylinositol ratio of surfactant correlated negatively with the fetal plasma inositol concentration. It is most likely that the reduction in the level of fetal plasma inositol resulting from a declining production and an increasing metabolism, causes the decrease in phosphatidylinositol and increase in PG content of the surfactant of the fetal rat. (*Pediatr Res* 20: 24-27, 1986)

Abbreviations

PI, phosphatidylinositol
PG, phosphatidylglycerol
DSPC, disaturated phosphatidyl choline

The production of fetal lung surfactant is initiated during the last period of pregnancy. During its maturation changes occur both in the amount produced and in its phospholipid composition (1).

In the human fetus these compositional changes include: 1) enrichment in phosphatidylcholine and especially saturated phosphatidylcholine (2, 3), 2) a temporary increase in the percentage of PI and later on a decrease in its percentage with a concomitant increase in the percentage of PG (4).

In man, PG is an important minor phospholipid of surfactant and its appearance in amniotic fluid is an indication that the lung will function normally at birth (4).

It has been suggested that in the fetus the percentage of PI and PG changes because of a decrease in fetal plasma inositol con-

centration (5). Both PI and PG have the same precursor (CDP-diacylglycerol) and several investigators have shown that in adult rabbits the production of PI and PG depended on changes in the concentration of inositol (6-8). In the fetus, such a relationship has not yet been studied.

The aim of this study was therefore to determine whether compositional changes occur in fetal rat lung surfactant and if these correlate with changes in plasma inositol levels.

MATERIALS AND METHODS

Animals. Young female Wistar rats ($n = 74$) weighting approximately 175 g were obtained from CPB.TNO (Zeist, The Netherlands). They were mated for 4 h once a week and diagnosis of pregnancy was based on increases in body weights and abdominal palpation. The day after mating was called day 1 of gestation.

Material was obtained during the 18- to 22-day period and shortly after birth (1 h and 1 day postpartum). The pregnant rats were anaesthetized with Nembutal and blood was sampled from the vena cava caudalis. Fetuses were delivered by cesarean section and "amniotic fluid" (amniotic fluid contaminated by allantoic fluid) was also collected. Fetal blood was obtained after dissection of the great vessels. The fetal lungs were removed from the thorax and separated from the trachea and the heart. Newborn rats and their does were killed by ether inhalation and blood and lung tissue were obtained as described in the "Results." There were no differences in the amount or composition of surfactant isolated from rats, anaesthetized with Nembutal or does, killed by ether inhalation (Table 1, maternal).

Lung surfactant. At least 0.5 g of lung tissue from one or a pool of animals from the same litter was minced in 1.1 M sucrose and homogenized in a Potter-Elvehjem tube, equipped with a Teflon pestle (clearance 0.5 mm). A sample of the homogenate was retained for determination of the tissue phospholipid composition. The rest was filtered through one layer of Olympus lens paper and a seven-step discontinuous sucrose density gradient (0.8-0.2 M sucrose) was layered on the top of the filtrate (9). Separation was obtained by centrifugation at $140 \times g$ for 15 minutes followed by $45,000 \times g$ for 5 h in a Beckman J2-21 High Speed Centrifuge with a fixed angle rotor (type JA 20). We collected an opaque band or in case of "18 day" material (if hardly anything was visible) the band corresponding with the 0.4 M sucrose layer and stored the material at -20°C (surfactant fraction). Samples of the surfactant fraction and lung tissue homogenate were extracted with three volumes of chloroform and methanol (2:1). The chloroform layer was collected and the upper layer was extracted again with two volumes of chloroform. The chloroform layers were combined and evaporated to dryness under nitrogen. The extract of lipids was used for: 1) estimation of the total phospholipid phosphorus content according to the method of Bhagwanani *et al.* (10). 2) separation of the individual phospholipids by one dimensional thin layer chromatography

Table 1. Phospholipid composition of surfactant of fetal, newborn, and adult rats (mean $\pm$ SEM)

	Fetal age (days)					Newborn	Maternal
	18	19	20	21	22		
Phospholipid classes (%)							
Lysophosphatidylcholine	2.0 $\pm$ 0.7	3.3 $\pm$ 0.3	1.7 $\pm$ 0.3	1.7 $\pm$ 0.3	2.5 $\pm$ 0.3	3.1 $\pm$ 0.8	3.2 $\pm$ 0.2
Sphingomyelin	4.9 $\pm$ 1.1	7.1 $\pm$ 0.8	4.2 $\pm$ 1.1	1.8 $\pm$ 0.6	1.8 $\pm$ 0.3	1.7 $\pm$ 0.5	2.5 $\pm$ 0.2
Phosphatidylcholine*	59.1 $\pm$ 1.4	64.8 $\pm$ 0.8	70.6 $\pm$ 2.0	75.5 $\pm$ 1.2	77.5 $\pm$ 0.9	76.4 $\pm$ 0.7	77.9 $\pm$ 0.4
Phosphatidylserine	(62. $\pm$ 0.5)†	(4.0 $\pm$ 1.0)	(3.9 $\pm$ 0.3)	(2.9 $\pm$ 0.8)	(2.4 $\pm$ 0.8)	(3.0 $\pm$ 0.5)	ND‡
PI	6.7 $\pm$ 0.5	4.3 $\pm$ 0.6	7.4 $\pm$ 0.5	5.9 $\pm$ 0.4	4.3 $\pm$ 0.2	3.6 $\pm$ 0.3	2.9 $\pm$ 0.1
Phosphatidylethanolamine	17.9 $\pm$ 2.2	15.8 $\pm$ 1.9	11.4 $\pm$ 1.3	6.7 $\pm$ 0.8	3.1 $\pm$ 0.5	4.4 $\pm$ 0.3	3.0 $\pm$ 0.2
PG	4.0 $\pm$ 1.7	1.5 $\pm$ 0.4	2.7 $\pm$ 0.4	5.8 $\pm$ 0.4	8.3 $\pm$ 0.4	8.1 $\pm$ 0.6	8.4 $\pm$ 0.2
Unidentified	5.0 $\pm$ 1.0	3.1 $\pm$ 0.8	2.1 $\pm$ 0.4	2.5 $\pm$ 0.3	2.5 $\pm$ 0.2	2.8 $\pm$ 0.2	2.0 $\pm$ 0.1
No. of samples	5 (5)†	5 (5)	8 (3)	7 (4)	10 (10)	8 (8)	46

* Phosphatidylcholine was not always separated from phosphatidylserine and the results present the sum of the percentages of the (PC + PS) spot.

† Percentages of phosphatidylserine of those cases where separation was achieved.

‡ Not determined.

(HPTLC plate, Merck no. 60 F254), using a solvent of chloroform/ethanol/H₂O/triethylamine (30:34:7:35) as described by Touchstone *et al.* (11).

The phospholipid spots were visualized under UV light after spraying the plate with an aqueous solution of 0.01% Rhodamine 6G. They were scraped directly into tubes for phosphorus determination.

For the determination of phosphorus/protein ratio, proteins were determined in surfactant fractions according to the BioRad Protein Assay modification of the method of Bradford (12). Bovine serum albumin was used as standard.

Inositol. The inositol concentration in amniotic fluid and in plasma of fetal, newborn and adult (female) rats was determined using the enzymatic procedure as described by Garcia Buñuel and Garcia Buñuel (13). We dialyzed the sample by centrifugation (800 $\times$ *g* for 90 min) to remove excess of proteins and cleared the dialysate of intrinsic NADH, glucose, and glucose-6-phosphate with HCl and hydroxylamine. The excess of hydroxylamine was destroyed by boiling the sample for 10 min.

Inositol dehydrogenase (Sigma), in a stock solution of Tris buffer containing bovine serum albumin, barbital, and NAD (Sigma) is added to the cooled sample and the mixture is incubated at 25° C for 30 min. The amount of reaction product: NADH, is measured by fluorometry. We used the difference in fluorescence of samples with and without inositol dehydrogenase to calculate the inositol concentration. All determinations were done in duplicate. Results are presented as mean $\pm$ SEM, and correlation coefficients and regression functions were estimated by using General Statistics of the Hewlett Packard 9825 A software.

RESULTS

The amount of material, isolated by density gradient centrifugation from fetal, newborn, and adult rats is shown in Figure 1. On day 18 very low amounts of phospholipid material were found in the 0.4 M sucrose layer (0.17 μ mol/g wet lung) and its composition (Table 1) resembles that of fetal lung tissue homogenate (results not shown).

From day 19 onward, the amount of surfactant in the fetal lung increased significantly (Fig. 1) its composition still resembles that of 18-day material. Thereafter the composition of the so-called surfactant fraction changes significantly and lamellar bodies could be found easily. The percentage of phosphatidylcholine increased and the percentages of sphingomyelin and of phosphatidylethanolamine decreased and the highest percentage of PI (7.4%) was found in the 20-day surfactant material while the percentages of PG were very low.

Both the amount and the percentage of PG increased from

Fig. 1. Changes in the amount of fetal surfactant phospholipids during gestation and first postnatal day compared to the amount of surfactant in the adult rat. The results are the mean $\pm$ SE of five to 10 assays of fetal material and of 46 assays of adult surfactant. Significant increase in the amount of surfactant was seen from day 19 onward ($p < 0.005$).

day 20 onward while the percentage of PI decreased. During that period the phospholipid/protein ratios increased also until after birth (Fig. 2). However, during the first postnatal day the phospholipid/protein ratio decreased again quickly; the values of the 1-h-old animals ($n = 4$) were twice as high as those of the adult rats but those of the 1-day-old newborns ($n = 3$) did not differ from the values of the adult rats.

The plasma inositol concentration of the fetal, newborn, and adult (female) rats is shown in Figure 3. There was a significant decrease in the fetal inositol levels during gestation ($r = 0.878$, $p < 0.001$, $n = 46$) and after birth, the inositol concentration was the same as in the adult rats in which the inositol levels did not change during pregnancy.

A significant relationship was found between the fetal plasma inositol levels and the PI/PG ratio of lung tissue ($r = 0.863$, $p < 0.001$, $n = 42$) and PI/PG ratio of surfactant fraction from day 19 onward ($r = 0.785$, $p < 0.001$, $n = 36$) (Fig. 4).

In amniotic fluid there were similar gestational age-dependent changes in inositol concentrations ($r = 0.688$, $p < 0.001$, $n = 45$) as observed in fetal plasma although at day 18 the standard error was large (Fig. 3).

Fig. 2. Changes in the phospholipid/protein ratio of fetal surfactant (O) and after birth (☆) and the phospholipid/protein ratio of surfactant of the adult rat (*). The ratio increases significantly from day 21 onward ($p < 0.01$) until birth and decreases significantly postnatally ($p < 0.01$). The results are the mean $\pm$ SE of five to 10 assays of fetal material, four and three assays of newborn surfactant and of 41 assays of adult rat surfactant.

Fig. 3. The decrease of inositol concentration in plasma and amniotic fluid during gestation (●, O) and the level of plasma inositol in the adult rat during pregnancy (*). Inositol was estimated indirectly by fluorometry as described in "Materials and methods." The results are the mean $\pm$ SE of three to nine assays of fetal plasma, five to nine assays of maternal plasma, and six to nine assays of amniotic fluid.

DISCUSSION

The composition of fetal lung surfactant is better documented for man (3, 14), rabbits (15, 16), and sheep (17, 18) than for the rat (19).

Fariday *et al.* (20) described the disaturated DSPC content of fetal rat lung lavages, whereas Adamson and King (21) estimated the change in total DSPC of fetal lung tissue. However, Young *et al.* (22) found that surfactant DSPC accounts for only 30% of the total amount of lung DSPC. In our study we determined both the change in composition and the increase in amount of fetal rat lung phospholipids and surfactant from the 18th day onward. The significant amounts of PG in the so-called "surfactant" fraction of 18-day-old fetal rats (approximately 7 nmol/g lung) seems remarkable because the isolated fraction resembled otherwise that of total lung tissue in which PG is less than 1%

Fig. 4. Relationship between the fetal plasma inositol level and the PI/PG ratio in lung tissue (●) or in surfactant (O). Inositol was estimated by fluorometry as described in "Materials and methods." The PI/PG ratio was calculated after thin-layer chromatography and phosphorus estimation as described. The results of the PI/PG ratio and inositol concentration (classes 0.1 mM/liter) are given as mean $\pm$ SE of four to 11 combinations. The PI/PG ratios in lung tissue are significantly higher than those in surfactant material ($p < 0.01$, Sign test for paired samples).

before day 20. The surfactant fraction might be contaminated by mitochondrial PG or cardiolipin which runs in our system almost as fast as PG (23). Furthermore, because of the extremely small amounts of material, relatively large errors are introduced.

After two-dimensional thin-layer chromatography, we found that less than 15% of the PG of the 20-day surfactant and less than 3% of the PG of 22-day surfactant did not cochromatograph with a PG standard. These percentages of contamination of one-dimensionally isolated PG agree with the results of the calculation assuming contamination of the surfactant fraction by "non-surfactant" material as much as the 18-day material. Furthermore, electronmicroscopy of 21-day material showed that the pellet material of the surfactant fraction was not contaminated by other organelles. High speed centrifugation ($40,000 \times g$) was, however, not effective enough to obtain a surfactant pellet of the 19-day material. If surfactant is present then, it might be present as vesicles which need higher g -forces for sedimentation (24).

The phospholipid/protein ratio of fetal rat surfactant appears to change and is at the end of gestation approximately the same as found for the surfactant of the fetal lamb (17) and the surfactant isolated from human amniotic fluids (14). The fetal surfactant phospholipid/protein ratios are higher than those found in the adults of various species (25, 30). The gestational age-dependent increase in this ratio in the fetal rat is probably the result of an increase in the phospholipid content of the lamellar body and could then be a measure of lamellar body maturation. The results of Magoon *et al.* (27) support this suggestion. They estimated the turnover-time of saturated phosphatidylcholine of two lamellar body fractions different in density and phospholipid/protein ratio and they suggest that the longer turnover-time that was found for lamellar bodies with the highest phospholipid/protein ratio results from their intracellular growth.

A high phospholipid/protein ratio of fetal surfactant might be important because larger amounts of surfactant phospholipids than in adults may be needed to overcome the postulated effects of a so-called "inhibitory protein" (28) which might pass the alveolar blood barrier during the immediate postnatal period.

In man, the changes in phospholipid profile, especially the decrease in percentage of PI and the increase in the percentage of PG, correlates with a decreased risk for developing neonatal

respiratory distress (4, 29). However, in diabetic patients fetal surfactant PG appear later in gestation than normally (29, 30). This may indicate that in such fetuses the gestational age-dependent decrease in the plasma inositol levels is delayed.

The changes in the composition of rat surfactant are similar to those in man whereas such changes do not occur in the surfactant of fetal rabbits or lambs. Our results also suggest that the PI/PG ratio in the surfactant decrease with the gestational-age-related decrease in fetal rat plasma inositol concentration. The possibility of a causal relationship is supported by the findings of Quirk *et al.* (32), who noticed that elevating the fetal plasma inositol levels in the rat resulted in higher PI/PG ratio of their lung tissue phospholipids. The fetal rat may therefore be a good model for studying factors which affect fetal lung maturation especially with respect to the synthesis of PI and PG.

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