Fetal Lung Maturation IV: The Release of Phosphatidic Acid Phosphohydrolase and Phospholipids into the Human Amniotic Fluid

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Extract

The relationship between surface active material and phosphatidic acid phosphohydrolase (PAPase) activity was examined in human amniotic fluid after centrifugation at $105,000 \times g$ for one hour. The distribution of PAPase activity between the pellet and supernatant fractions was similar to that of phospholipids in the amniotic fluid. The majority of the PAPase activity was found to be associated with the pellet fraction. Similarly, 82% of the phospholipid was also present in this fraction. Of the total palmitate present in the original sample, approximately 90% was found in the pellet after centrifugation. Palmitic acid constituted 75% of all fatty acids present in the phosphatidylcholine identified in the pellet. By sharp contrast only 4% of the total protein was pelleted by centrifugation. The pelleted fraction isolated from amniotic fluid after $105,000 \times g$ centrifugation closely resembles the lamellar bodies in: (1) its phospholipid pattern; (2) palmitate concentration in phosphatidylcholine; and, (3) the presence of PAPase activity with a high specific activity. Collectively, these findings lend strong support to the concept that a lamellar-like body is released from fetal lung into the amniotic fluid.

Speculation

Inadequate production of surface active material by the lung plays an important role in the pathogenesis of respiratory distress syndrome of the newborn. The measurement of key enzymes responsible for the regulation of phosphatidylcholine synthesis would be useful as another index of fetal lung maturity. The response to prenatal therapy to induce surfactant synthesis or release could be monitored by the assay of these enzymes in the amniotic fluid and help to define the normal intrauterine stimuli for the surge of phosphatidylcholine synthesis.

Several investigators have found that the maturation process of the fetal lung is associated with an increased production of a lipoprotein possessing surface-active properties. The major phospholipid of this lipoprotein is dipalmitoyl-phosphatidylcholine (DPPC); moreover this phospholipid is thought to be responsible for most of the surface-active properties of the complex. The several enzymes that catalyze the biosynthesis of DPPC have been studied in both adult and developing fetal lung (3). We have found that phosphatidic acid phosphohydrolase activity [phosphatidate phosphohydrolase (EC. 3.1.3.4)] (PAPase), a key enzyme in the regulation of phospholipid biosynthesis (8, 17), begins to increase in fetal rabbit lung on day 26 of gestation and its activity continues to increase and reaches adult levels just prior to parturition (18). In contrast to the observed increase in PAPase activity, the activity of the enzyme CDP-choline: diglyceride choline phosphotransferase [EC. 2.7.8.2 (CPTase)] in lung of fetal rabbits did not change during the course of gestation (5, 18). Tissue specificity for the increase in PAPase activity in the lung was suggested by the finding in liver PAPase activity did not increase during fetal development (18). We have also demonstrated PAPase activity in human amniotic fluid (9). Furthermore, 4 to 5-fold higher activity of PAPase was found in the amniotic fluid obtained from pregnancies of more than 35 weeks than in amniotic fluid from pregnancies before the 32nd week of gestation. In a study of the sequential changes in amniotic fluid PAPase activity in 21 gravidas in whom repeated amniocentesis were performed beginning at 30 weeks of gestation a continuing increase in activity was observed that reached peak activity by the 37th week. The observed increase in amniotic fluid PAPase activity preceded the "surge" of acetone precipitable phosphatidylcholine by approximately one and one half weeks. Also, the specific activity of PAPase in the nasopharyngeal fluid was found to be 3 to 4 times greater than that of the amniotic fluid (10). These findings support the concept that PAPase originates in the fetal lung. The purpose of the present study was to ascertain the relationship between the surface-active material and PAPase activity in human amniotic fluid.

METHODS AND MATERIALS

Amniotic Fluid Fractionation

Twenty-five ml of amniotic fluid were collected at the time of cesarean section from gravidas at term. To remove intact cells and cell debris, the samples were chilled immediately and centrifuged at 4°C at 700 x g for 5 minutes. The supernatant fraction of this centrifugation was removed and is referred to subsequently as the original amniotic fluid sample. An aliquot of the original sample was centrifuged at 105,000 x g for 60 minutes at 4°C. The supernatant fraction and the pellet were analyzed separately.

Gas Liquid Chromatography

The lipids were extracted from amniotic fluid by the method described by Folch *et al.*, (4). The chloroform was removed by evaporation under nitrogen at 25°C. The fatty acid composition of this lipid fraction was determined by procedures we have described previously (19).

An aliquot of the lipid fraction was placed on a Silica gel HR plate, previously activated at 100°C for 30 minutes. The developing solvent was chloroform:methanol:acetic acid:0.9% saline (75:25:10:6). The separated lipids were visualized after exposure to I_2 vapors. The area migrating parallel to an authentic phosphatidylcholine was scraped from the TLC plate. The fatty acid composition of the various phospholipids was determined by gas liquid chromatography of the methyl esters of the fatty acids (19). Each analysis was performed in duplicate.

PAPase activity was measured by a modification of the method of Coleman and Hübscher (9). Inorganic phosphate was quantified by the method described by Bartlett (11). The amniotic fluid pellet fraction was further characterized by sucrose gradient centrifugation separation. In this study, PAPase was measured by the rate of release of P_3^{32} utilizing a recently developed radioactive substrate assay procedure (20). Protein content was measured by the method of Lowry *et al.*, (11). Total phospholipids and the phosphatidylcholine fractions were quantified by the procedure described by Parker and Peterson (14).

RESULTS

The distribution of the activity of PAPase as well as that of the quantity of phospholipid, palmitate and protein are presented in Table 1. The results obtained from the analyses of the supernatant fraction, following centrifugation at 105,000 x g for 60 minutes, and for the pellet of this centrifugation are expressed as percentages of that found in the original sample. Amniotic fluids from six gravidas were analyzed and the tabulated results are the average of these six samples. From these data it is apparent that most of the PAPase activity was found primarily in the 105,000 x g pellet. In some samples, as much as 90% of the PAPase activity of the original sample was found in the pellet. Similarly, the phospholipid was also found principally in the pellet fraction. Of the total palmitate present in the original sample (700 x g supernatant fraction), approximately 90% was found in the pellet after 105,000 x g centrifugation. While the PAPase, phospholipids, and palmitate content was found in the pellet, by sharp contrast, only a small amount of the total protein was pelleted by 60 minutes of centrifugation at 105,000 x g.

The phospholipid present in the 105,000 x g pellet fraction was further characterized by an analysis of its phospholipids and their fatty acid content.

The results of these studies are presented in Table 2. Of the phospholipid in the pellet, 78% was phosphatidylcholine. The phosphatidylcholine was further analyzed to ascertain the fatty acid distribution. Of the total fatty acids associated with the phosphatidylcholine 80% was palmitic acid. The finding of this disproportionately high concentration of phosphatidylcholine together with the marked enrichment of this fraction with palmitate strongly suggests that the material being sedimented from amniotic fluid by high speed centrifugation was similar if not identical to lung surface-active material.

By a sucrose gradient centrifugation system, the resuspended pelleted material obtained from amniotic fluid layered as a band at a density similar to that reported for both lamellar bodies (20) and surfactant (15). Furthermore, the activity of PAPase was predominately localized in this band and the specific activity of PAPase in this fraction was similar to that found in lamellar bodies (21). These results also strongly support the view that the material pelleted by high speed centrifugation of amniotic fluid was surface-active material.

Several lines of evidence gathered from this study give strong support to the concept that the release of the surface-active material by the fetal lung is associated with a parallel release into the amniotic fluid of one of the major enzymes involved in the biosynthesis of phosphatidylcholine, PAPase. Moreover, the PAPase activity and the surface-active material were found in the same fractions. In other studies we have also shown that lamellar bodies isolated from lung tissue also contain a significant amount of PAPase (20) and CPTase (21). In these studies it was observed that the specific activity of PAPase in lamellar bodies was the highest of any of the various subcellular organelles isolated from lung tissue (20). The pelleted fraction isolated from the amniotic fluid after 105,000 x g centrifugation closely resembles the lamellar bodies in (1) its phospholipid pattern, (2) palmitate concentration in phosphatidylcholine; and, (3) the presence of PAPase activity of high specific activity. The density of the pellet obtained from amniotic fluid of term gestations was also found to be similar to that of lamellar bodies isolated from lung tissue and similar also to that of the surface-active materials isolated by lung lavage. Each of these components, i.e. isolated lamellar bodies, surface-active material isolated by lung lavage and the 105,000 x g pellet of human amniotic fluid were found to contain PAPase activity of high specific activity. Collectively, these findings provide strong support for the concept that a lamellar-like body is released from the fetal lung into the amniotic fluid. The results of previous studies by Novy, *et al.*, (13) of the pellets obtained following centrifugation of amniotic fluid from term gestations monkeys also indicate that a lamellar-like structure to be present in the amniotic fluid of this species. The chemical and enzymatic studies of human amniotic fluid reported herein, together with the findings of Novy and colleagues, that include electron micrographs, constitute convincing evidence that the fraction obtained by centrifugation is of lung origin. This obtains since the physical and chemical data as well as the morphologic studies strongly resemble those characteristic of lamellar bodies isolated from lung tissue. The finding of such a close association of the activity of PAPase with both the surface-active material in the lamellar bodies and the surface-active material found in amniotic fluid is also consistent with the view that these materials enter the amniotic fluid as an associated particle. Previously we have reported studies in which it was demonstrated that human amniotic fluid PAPase was of fetal lung origin (10). Novy, *et al.*, (13) found that the lamellar bodies present in monkey amniotic fluid were of fetal lung origin. Together these findings negate the possibility that PAPase and lamellar bodies were released into amniotic fluid from tissue sites other than the lung.

These results together with studies reported previously from our laboratory and others provide the foundation for a unified hypothesis. This concept is illustrated graphically in Figure 1, in which the Type II lung cell, in association with the Type I pneumocyte, are depicted. The characteristic lamellar bodies containing the surface-active material are shown in the Type II cell, and are considered to be biosynthetic units for the synthesis of the surface-active material (20). The presence of phosphatidylglycerol and its biosynthesis has also been demonstrated in this subcellular fraction (6, 16). Previously it was considered that the membrane surrounding the lamellar bodies in the Type II cell was the site of enriched amounts of PAPase (12). However, from the results of this study we suggest that PAPase may be an integral part of the lamellar body, possibly one of the several proteins known to be associated with the surface-active material. This view is based on the observation that the lamellar bodies isolated from the human amniotic fluid contain a significant quantity of PAPase, yet from the electron microscopic studies of Novy, *et al.*, (13) a distinct perilamellar membrane was not discernible.

The surface-active material synthesized in the lamellar body can migrate to the alveolar surface, is extruded into the alveolar space and thence by fetal respiration reaches the amniotic fluid. The migration of the lamellar body to the cell surface occurs via the microtubule system. Failure of extrusion of surfactant was demonstrated after use of inhibitors of microtubule formation (2). The development of respiratory distress in an adult woman, followed by death after the ingestion of a large dose of colchicine to commit suicide suggests that a similar mechanism for the release of surface-active material may occur in the adult human (7). The surface-active material released could provide the necessary coating of the alveolar surface to prevent collapse.

This unified concept envisions that the lamellar body of the Type II lung cell is an organelle of biosynthetic capability. During the course of human gestation there is a marked increase in the biosynthetic activity of key enzymes in phosphatidylcholine synthesis, e.g., PAPase. This "surge" in activity precedes the augmented synthesis of lecithin characteristic of fetal lung maturation. PAPase, which is intimately associated with the lamellar body, replete with surface-active material reaches the alveolar surface by the microtubule system. The perilamellar membrane fuses with the plasma membrane of the Type II cell, a release of surface-active material to the alveolar space occurs and it subsequently reaches the amniotic fluid by water movement by fetal respiration *in utero*. This would account for the higher specific activity of the enzyme PAPase found in the nasopharyngeal fluid than in amniotic fluid.

SUMMARY

The distribution of PAPase, phosphatidylcholine, and palmitate has been measured in human amniotic fluid following centrifugation at 105,000 x g for 60 minutes. Of the total PAPase activity in the original sample (700 x g supernatant fraction), 67% was found in the pellet. Similarly, the phospholipid was also found principally in the pellet fraction. Of the total palmitate present in the original sample, approximately 90% was precipitated in the pellet. The finding of such a close association of the activity of PAPase with the surface-active material found in the amniotic fluid is consistent with the view that these materials are secreted into the amniotic fluid as a closely associated particle.

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TABLE I

THE DISTRIBUTION OF COMPONENTS IN HUMAN
AMNIOTIC FLUID FOLLOWING CENTRIFUGATION
AT 105,000 x g FOR 1 HOUR

	PAPase	Phospholipid	Palmitate	Protein
Supernatant	33	18	11	96
Pellet	67	82	89	4

TABLE II
CHARACTERIZATION OF THE PHOSPHOLIPID AND FATTY ACID OF
PHOSPHATIDYLCHOLINE IN THE PELLET FRACTION OBTAINED
AFTER CENTRIFUGATION OF HUMAN AMNIOTIC FLUID
AT 105,000 x g FOR 1 HOUR

Phospholipid	Percent
Phosphatidylcholine	78
Fatty acids present in phosphatidylcholine	
14:0	4
16:0	75
18:0	4
18:1	11
18:2	3
20:4	3

Figure 1. Schematic representation of an alveolus with type I and type II cells. The lamellar bodies formed in the type II cell, after being devoided of their perilamellar membrane are extruded into the alveolar space, where they may coat the alveolar surface or are carried as a lamellar-like body into the amniotic fluid via the respiratory tract.