PHOSPHOLIPID BIOSYNTHESIS IN LUNG LAMELLAR BODIES

Hugh L. Spitzer, Janice M. Rice, Paul C. MacDonald, and John M. Johnston

Departments of Biochemistry and Obstetrics
and Gynecology and the
Cecil H. and Ida Green Center
For Reproductive Biological Sciences
Southwestern Medical School
The University of Texas Health Science Center at Dallas
Dallas, Texas 75235

Received July 9,1975

SUMMARY

Lung Type II epithelial cells contain lamellar bodies, the storage form of surfactant, which is predominately dipalmitoyl-phosphatidylcholine. Isolated lamellar bodies, free of microsomal and mitochondrial contamination have significant phosphatidic acid phosphohydrolase activity. Calculations based on enzyme distribution indicate that as much as 40% of the phosphatidic acid phosphohydrolase activity within the Type II cell is associated with the lamellar bodies. This high concentration of phosphatidic acid phosphohydrolase, a key enzyme in phospholipid biosynthesis, suggests that the phosphatidylcholine stored within the lamellar bodies may be synthesized at the perilamellar surface.

The PC concentration in pulmonary tissue of fetal rabbits begins to increase markedly four days prior to birth (1). Similar findings have been obtained in studies of other species. This increase in PC is attributable to the accumulation of dipalmitoyl-PC which is stored in lamellar bodies within the Type II epithelial cells of the lung. The dramatic increase in fetal lung PC suggests an alteration in the activity of the enzymes involved in its bioysnthesis. The activity of choline kinase (ATP:choline phosphotransferase, EC 2.7.1.32), choline phosphate cytidyltransferase (CTP: cholinephosphate cytidyltransferase, EC 2.7.7.15), and CDP-choline:diglyceride transferase (CDP-choline;1,2-diglyceride choline phosphotransferase, EC 2.7.8.1) has been measured in fetal lung tissue at various gestational ages. No increase in enzymatic activity was found in rat (2), rabbit (1,3,4) or human (5) fetal

Abbreviations:PC = phosphatidylcholine, DPC = disaturated phosphatidylcholine, PAPase = phosphatidic acid phosphohydrolase

lung throughout gestation except for one report by Farrell, et al. (6) showing an increase in CDP-choline diglyceride transferase in fetal rat lung 3 days prior to birth.

In order to determine what factors may account for the accumulation of PC in pulmonary tissue we turned our attention to phosphatidic acid phosphohydrolase (EC 3.1.3.4) an enzyme which occupies a central position in the biosynthesis of lipids. It has been suggested that PAPase is an important regulatory enzyme in lipid biosynthesis in regenerating liver (7), in liver triglyceride synthesis (8), in lipid synthesis following fasting (9) and in phospholipid biosynthesis in brain following neural stimulation (10). Recently we reported that PAPase activity increased 4-8 fold in fetal rabbit lung beginning at 26 days gestation (3) one day before the first detectable accumulation of DPC. PAPase activity was also demonstrated in human amniotic fluid (11). Moreover, the activity of this enzyme was shown to increase during the latter weeks of human gestation prior to the observed rise in the lecithin/sphingomyelin ratio (12). Based on these findings it was suggested that PAPase may occupy a central role in providing substrate for the synthesis of DPC at a critical time in gestation. From autoradiographic studies of murine and avian lung in organ culture Adamson and Bowden (13) suggested that the perilamellar membrane of the Type II lung cell is the site of surfactant synthesis. This conclusion is consistent with the histochemical findings of Meban (14) who found PAPase activity present in the Type II cell and demonstrated that this activity was associated with the perilamellar surface of the lamellar bodies. Therefore, an investigation was undertaken to determine if the perilamellar membrane contained the essential enzymatic activities compatible with the continuous synthesis and secretion of dipalmitoyl-PC by the lung. Our initial efforts, reported here, were directed toward the assessment of PAPase activity in isolated lamellar bodies.

MATERIALS AND METHODS

Lamellar bodies were isolated from 60 grams of fresh adult pig lung by a technique that employed certain features of published methods (15-17). The tissue was cut into small pieces and all visible bronchi and blood vessels

were removed. The tissue was homogenized in 120 ml of buffer, 0.05 M Tris-HCl, 0.15 M NaCl, 0.0001 M EGTA, pH 7.4, for 2 min in a Servall Omini Mixer and filtered through cheese cloth twice. The volume of the filtrate was adjusted to 240 ml by the addition of buffer. A 30 ml aliquot was layered over 10 ml of 0.95 M sucrose (all sucrose solutions were made up in the original buffer) and centrifuged at 18,000 x g for 10 min. Three Fractions were obtained: a pink supernatant (Fraction I), a white layer at the gradient interface (Fract-II) and a large pellet (Fraction III). Fraction II which was enriched with lamellar bodies was diluted to 200 ml buffer and centrifuged at 23,000 x g for 20 min. The resulting supernatant fraction was combined with Fraction I and the pellet was resuspended in 100 ml of buffer and 18 ml layered over a 2-step gradient of 10 ml each of 0.41 M and 0.65 M sucrose. After centrifugation at 100,000 x g for 30 min a clear upper layer (Fraction IIa), a dense white band (Fraction IIb) and a pellet (Fraction IIc) were obtained. Fractions IIa and IIc were combined for analysis. Microsomes were prepared both from Fraction I after adjusting it to 0.25 M sucrose and from an original sample of tissue which was homogenized in 0.25 M sucrose. Cell debris and nuclei were removed by centrifugation at 700 x g for 10 min mitochondria at 13,000 x g for 20 min following by pelletization of microsomes at 100,000 x g for 60 min.

Lipid extraction and analyses were by standard procedures (18). The amount of disaturated PC was calculated after determining the fatty acid composition of the total PC by gas chromatographic analysis. For this calculation it was assumed that 95% of the unsaturated fatty acids were esterified at the 2 position of PC. Protein was measured by the method of Lowry, et al. (19). PAPase activity was determined by the release of ^{32}Pi from [^{32}P]-phosphatidic acid (3). NADPH-cytochrome c reductase, a microsomal marker enzyme, and cytochrome c oxidase, a mitochondrial marker enzyme were measured by methods previously reported (20).

RESULTS

In our preliminary efforts to obtain lamellar bodies free of microsomal and mitochondrial contamination, several published procedures were attempted with varying degrees of success. The results indicated that microsomes or mitochondria once bound to lamellar bodies could be removed only with great difficulty, a problem which has been encountered by others (21). For these reasons the method described was employed. The first density gradient centrifugation results in Fraction I containing 80-85% of the microsomes as evidenced by the activity of NADPH-cytochrome c reductase (Table I). Fraction II was enriched with lamellar bodies contaminated with small amounts of microsomes and mitochondria. Fraction III, the pellet, contained >97% of the mitochondria as evidenced by the activity of cytochrome c oxidase.

The second density gradient centrifugation yielded a lamellar body preparation (Fraction IIb) essentially free of microsomal contamination (Table I). In four experiments the lamellar bodies had an NADPH-cytochrome c reductase specific activity that averaged 0.9 (range 0-2.2), and a trace

Table 1. STRUCTURAL AND ENZYMATIC ANALYSIS OF PIG LUNG SUBCELLULAR FRACTIONS

	Protein	PAPase		Reductase	
	% of Total	S. A.	% of Total	S. A.	% of Total
Whole Homogenate	100	16.6*	100	3.2	100
Fraction I	89.5	8.7	46.9	2.9	81.1
IIb	0.1	51.2	0.3	<1.3	<0.1
IIa & c	1.8	126.0	13.6	13.8	7.7
III	18.3	40.6	44.8	3.0	17.2

S. A. = specific activity in nmoles min^{-1} mg $protein^{-1}$. Values are from a representative experiment. *PAPase per 100 gm wet wt is 176,623 units of activity.

amount of cytochrome c oxidase was observed in one study. The specific activity of PAPase in isolated lamellar bodies was higher than that in microsomes or mitochondria. This suggests that the PAPase activity in lamellar bodies is not the consequence of contamination by other subcellular organelles. The PC associated with the lamellar bodies contained 69 mole % palmitate. Further confirmation of the purity of lamellar bodies in this fraction is provided by its high DPC concentration, the phospholipid/protein ratio and the electron microscopic identification which are consistent with previously published data (16,22).

The significance of the initial gradient centrifugation of this procedure is emphasized by comparing the enzymatic and structural composition of microsomes prepared from Fraction I to microsomes prepared without prior removal of the lamellar bodies (Table 2). If DPC is employed as a marker for the presence of lamellar bodies it is apparent that microsomes from Fraction I contain

Table 2. STRUCTURAL AND ENZYMATIC ANALYSIS OF PIG LUNG MICROSOMES AND LAMELLAR BODIES

	mgPL / mg protein	DPC	PAPase nmoles min^{-1}	Reductase mg $protein^{-1}$
LB	8.9	50	51.2	<1.3
Mic_I	0.9	24	12.4	18.5
Mic	1.2	46	32.0	23.1

LB = lamellar bodies = Fraction IIb; Mic_I = microsomes prepared from Fraction 1: Mic = microsomes prepared from whole homogenate without prior removal of lamellar bodies. PL = phospholipid. DPC = mole % of total phosphatidylcholine in fraction which has two saturated fatty acids.

significantly less lamellar bodies than those prepared by conventional methods.

The high PAPase specific activity associated with Fractions IIa&c which contains microsomes and lamellar bodies has been a consistent and at present unexplained finding. Experiments are now being carried out to determine if there is a synergistic effect between lamellar bodies and microsomes which would explain the high PAPase specific activity.

DISCUSSION

There is general agreement that the Type II epithelial cell of the lung is the source of surfactant; however, the precise intracellular site of its synthesis has not been established. Numerous enzymes, including PAPase have been demonstrated by histochemical studies to be associated with the perilamellar membrane of the lamellar bodies. The presence of PAPase, a key enzyme in lipid biosynthesis suggests that the lamellar body perilamellar membrane may be directly involved in the synthesis of surfactant DPC. In order to determine the importance of the lamellar body PAPase within the Type II cells it is necessary to make a number of extrapolations. Type II cells com-

prise approximately 5-10% of the total cell population of the lung. In our calculations we made the following assumptions: All the DPC in the lung (402 μmole DPC/100 gr wet wt) is associated with the lamellar bodies, the PAPase: DPC ratio in Fraction IIb (14.5 units of PAPase activity/μmole DPC) is representative of all lamellar bodies and the remaining PAPase activity is evenly distributed throughout the entire population of lung cells. From the data given and from the assumptions it can be calculated that greater than 40% of the intracellular PAPase present in the Type II cell is associated with the lamellar bodies.

Using the assumptions noted above it would appear that there is significant PAPase activity associated with the lamellar bodies compared to other subcellular organelles within the Type II cell. These data taken in conjunction with reports (7-10) that PAPase is a key regulatory enzyme in lipid biosynthesis (7-10) support the concept that the perilamellar membrane may be metabolically responsible for the synthesis of DPC. Furthermore, the high PAPase specific activity associated with the lamellar bodies add further support to our concepts that this enzyme can be specifically related to lung maturation (3) and that the PAPase found in amniotic fluid denotes surfactant secretion (11,12). Thus it would appear that PAPase activity in amniotic fluid may offer a highly specific reflection of lung maturation.

ACKNOWLEDGEMENTS

The authors wish to thank Ms. Diana Watson and Mary Bob Wylie for expert technical assistance. Supported, in part, by grants from USPHS AM-3108, The Robert A. Welch Foundation, and NIH 1 PO1 HD08672.

REFERENCES

1. Gluck, L., Sribney, M., and Kulovich, M. V. (1967) Pediat. Res. 1, 247-265.
2. Weinhold, P. A., Sanders, R., and Stern, W. (1973) Respiratory Distress Syndrome pp 29-45, Academic Press, N. Y.
3. Schultz, F. M., Jimenez, J. M., MacDonald, P. C., and Johnston, J. M. (1974) Gynecol. Invest. 5, 222-229.
4. Farrell, P. M. and Zackman, R. D. (1973) Science 179, 297-298.
5. Zackman, R. D. (1973) Pediat. Res. 7, 632-637.
6. Farrell, P. M., Lundgren, D. W., and Adams, A. J. (1974) Biochem. Biophys. Res. Commun. 57, 696-700.

7. Magiapane, K. A., Lloyd-Davies, K. A., and Brindley, D. N. (1973) Biochem. J. 134, 103-112.
8. Lamb, R. G., and Fallon, H. J. (1974) Biochim. Biophys. Acta 348, 116-178.
9. Vavrecka, M., Mitchell, M. P., and Hübscher, G. (1969) Biochem. J. 115, 139-145.
10. Schact, J. and Agranoff, B. W. (1973) Biochem. Biophys. Res. Commun. 50, 934-941.
11. Jimenez, J. M., and Schultz, F. M., MacDonald, P. C., and Johnston, J. M. (1974) Gynecol. Invest. 5, 245-251.
12. Jimenez, J. M., Schultz, F. M. and Johnston, J. M. Obstet. Gynecol. In Press.
13. Adamson, I. Y. R., and Bowden, D. H. (1973) Exp. Mol. Path. 18, 112-124.
14. Meban, C. (1972) J. Cell. Biol. 53, 249-252.
15. Frosolono, M. F., Charmo, B. L., Pawlowski, R., and Slivka, S. (1970) J. Lipid Res. 11, 439-457.
16. Pruitt, K. M., Cherng, M. J., and Spitzer, H. L. (1971) Arch. Intern. Med. 127, 390-394.
17. DiAugustine, R. P. (1974) J. Biol. Chem. 249, 493-534.
18. Balint, J. A., Beeler, D. A., Treble, D. H., and Spitzer, H. L. (1967) J. Lipid Res. 8, 486-493.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
20. Polheim D., David, J.S.K., Schultz, F. M., Wylie, M. B., and Johnston, J. M. (1973) J. Lipid Res. 14, 415-421.
21. Page-Roberts, B. A. (1972) Biochim. Biophys. Acta, 260, 334-338.
22. Gil, J. and Reiss, O, K. (1973) J. Cell Biol. 58, 152-171.