PHOSPHATIDYL GLYCEROL IN LUNG SURFACTANT: 1. SYNTHESIS IN RAT LUNG MICROSOMES

Mikko Hallman and Louis Gluck

Department of Pediatrics, University of California, San Diego
La Jolla, California

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Summary - Phosphatidyl glycerol is present in lamellar bodies and in the material obtained by alveolar wash representing 12.3 and 11.5%, respectively, of the total phospholipid phosphorus. Lung microsomes catalyze the formation of phosphatidyl glycerol from the known precursors, L-glycerol 3-phosphate and CDP-diglyceride. The rate of [^{14}C]L-glycerol 3-phosphate incorporation into phosphatidyl glycerol was 30% higher in microsomes as compared to mitochondria. The addition of mercuric chloride inhibited the synthesis of phosphatidyl glycerol, and stimulated the incorporation into another as yet incompletely identified lipid. After pulse labeling of microsomal phosphatidyl glycerol in vitro, further incubation of microsomes with lamellar bodies or alveolar wash resulted in nearly quantitative appearance of label in surfactant.

According to Morgan, lung surfactant contains significant amounts of phosphatidyl N,N-dimethyl ethanolamine (PDME) (1). His presumptive findings apparently were verified. In addition to its surface tension reducing properties PDME also was investigated as the intermediate in the N-methylation pathway for disaturated lecithin, the major component of surfactant (2-3). However, others were unable to identify PDME in the lung but instead found phosphatidyl glycerol (PG) (4-5).

The present report supports the latter alternative. Although there is close thin layer chromatographic similarity between the two compounds, they may be differentiated by other studies. No PDME but significant amounts of PG was found in the two components of the surfactant system: lamellar bodies and the material lining lung alveoli. The synthesis of PG in the microsomal fraction and its subsequent transfer to lamellar bodies was demonstrated.

MATERIALS AND METHODS

Adult male rats of Sprague-Dawley strain were used. Lamellar bodies were isolated by modifying the method of Page-Roberts (6) as follows: 30% homogenate of alveolar tissue in 0.3M sucrose, 0.01 M Tris-Cl, 0.1mM EDTA (pH 7.4) was layered on a discontinuous density gradient containing 10ml

Abbreviations: PDME, phosphatidyl N,N-dimethyl ethanolamine; PG, phosphatidyl glycerol; TLC, thin layer chromatography.

of 0.8M sucrose-Tris-EDTA, and 2.5ml of 0.45M sucrose-Tris-EDTA, and spun at 35,000 rpm for 40 minutes using a Ti 60 rotor. One part of the interphase was added to ten parts of 0.58M sucrose-Tris-EDTA and centrifuged as described by Page-Roberts (6). The material floating on the top was diluted with 0.4M sucrose-Tris-EDTA, and spun at 56,000 xg for 20 minutes.

Nuclei (7), mitochondria, and microsomes (8) were derived from the first pellet. Each fraction was washed once by resuspension. Cell sap was obtained by centrifuging the tissue homogenate at 105000g for 60 minutes. The alveolar washings were performed as described earlier (9). Cell sap and alveolar wash were concentrated using Diaflo UM 2 ultrafilters (Amicon, Lexington, Mass.).

Lipids were extracted according to the method of Folch et al. (10). The phospholipids were isolated by means of two-dimensional thin layer chromatography (TLC) using washed silicagel H (Merck, Darmstadt). The first dimension was developed using tetrahydrofuran: methylal: methanol: $2NH_4$ (10:8:2:1.1 v/v), and the second in chloroform: methanol: water (65:25:4 v/v). Between the runs the plates were heated for five minutes at 70°C. Carrier phosphatidyl glycerol was used when the compound was not detectable by means of iodine vapor.

CDP-diglyceride was prepared using rat liver microsomes in the presence of phosphatidic acid (derived from egg lecithin by phospholipase-D (11) treatment), and [^{3}H]-CTP (19). The compound was isolated using two-dimensional TLC, and the label was identified by means of Varian Aerograph Model 6000 thin layer scanner (Walnut Creek, Calif.). R_f values of 0.25 and 0.15 were obtained for the first and second dimension, respectively. CDP-diglyceride had an absorption maximum at 278 nm (pH 7.5).

The radioactivity in the scraped gel was measured with a Beckman LS-250 liquid scintillation system using the technique described by Webb et al. (13).

Phosphorus was measured by modification of Bartlett method (14). Free glycerol, released after phospholipase-D treatment, was assayed according to Eggstein et al. (15), using reagents obtained from Boehringer GmbH (Mannheim, Germany). Total phospholipid-glycerol was analyzed as described by Renkonen (16). Quantitative periodate oxidation was performed according to Kiyasu et al. (17). Succinate dehydrogenase activity was measured by the method of Lee and Lardy (18). The protein content was assayed according to Lowry (19).

Aquasol, 5-[^{3}H]CTP, and [glycerol-^{14}C(U)]L-glycerol-3-P, were purchased from New England Nuclear (Boston, Mass.). L-glycerol-3-P and phospholipase-D (cabbage), were obtained from Calbiochem (San Diego, Calif.). PG, and phosphatidic acid were products of Supelco, Inc., (Bellefonte, Pa.), and PDME was obtained from Sigma Chemical Co. (St. Louis, Mo.). All solvents were of analytical grade.

RESULTS

Identification of phosphatidyl glycerol - Authentic PG, authentic PDME, and the component present in fairly large quantity in surfactant, had virtually identical R_f's on TLC using the following solvents: Chloroform: methanol: NH_4 H_2O (130:60:5:4 v/v), chloroform: methanol: H_2O (65:25:4 v/v) and chloroform: methanol: acetic acid: H_2O (160:50:12:4 v/v). However, using tetrahydrofuran: methylal: methanol: 2M NH_4 (10:8:2:1.1 v/v) authentic PG and the surfactant component had R_f of 0.3 whereas authentic PDME did not migrate (R_f0.0).

The compound was recovered from surfactant using two-dimensional TLC. It contained 2.1 moles glycerol per 1.0 mole phosphate. Quantitative periodate oxidation yielded 0.48 µmoles per 0.40 µmoles of compound-P added. Upon phospholipase-D treatment 0.36 µmoles glycerol in the water phase and 0.40 µmoles phosphate in the lipid phase were recovered per 0.40 µmoles of compound-P added. When the lipid component was developed in TLC both in acidic and basic solvents, 84 and 90% of the phosphorus, respectively, was recovered in the spot where phosphatidic acid should migrate. This evidence, together with the strong evidence presented earlier (4) leads to the conclusion that PG is a major component of surfactant, whereas no phospholipid was recovered in the spot where PDME should migrate.

Localization of phosphatidyl glycerol - After lecithin, PG was the most abundant phospholipid in lamellar bodies and alveolar wash of the rat and it was found in moderate amounts in the cell sap fraction also (Table I). This also may represent the surfactant system, since the quantity of phosphatidyl glycerol in cell sap was decreased by 60% when alveolar wash was performed prior to the isolation of the fraction.

Incorporation of L-glycerol-3-P into phosphatidyl glycerol - Table II shows

Table I. The concentrations of phosphatidyl glycerol in the lung

	nmoles/mg. prot.	% of phospholipid-P
Whole tissue	9	3.5
Nuclei	0	0.4
Mitochondria	5	1.2
Microsomes	11	1.8
Lamellar bodies	650	12.3
Alveolar wash	274	11.5
Cell sap	4	6.1

Table II. Incorporation of [^{14}C]L-glycerol 3-P into phosphatidyl glycerol in various cell fractions

	CDP - diglyceride	cpm x 10^{-3}/mg prot. -	+
Lung			
Nuclei	0.4 mg. prot.	0.4	0.9
Mitochondria	0.4 mg. prot.	8.8	20.1
Microsomes	0.4 mg. prot.	9.4	26.3
Lamellar bodies	0.1 mg. prot.	5.8	8.5
Cell sap	0.4 mg. prot.	0.8	1.0
Liver			
Mitochondria	0.4 mg. prot.	5.5	20.1
Microsomes	0.4 mg. prot.	1.7	2.0

Reaction mixture contained 0.2mM L-glycerol-3-P (0.5 µCi of ^{14}C), 70 mM Tris-Cl (pH 7.4), 2.5 mM reduced glutathione, and 0.15 mM CDP-diglyceride, when indicated, in a total volume of 100 µl. Incubation took place at 37°C for 60 minutes.

the incorporation of [^{14}C]L-glycerol-3-P into PG in various fractions obtained from lung and liver. Liver microsomes had only trace activity as compared to mitochondria (cf. ref. 17). On the other hand, the microsomal fraction obtained from the lung showed significant activity of L-glycerol-3-P incorporation into PG. This cannot be due to mitochondrial contamination since the corresponding activity in lung mitochondria was 76% of that found in microsomes. On the basis of the measurements of succinate dehydrogenase activity in mitochondria and microsomes, the microsomal fraction contained less than 5% of mitochondrial contamination.

The activity found in lamellar bodies was considerably lower than that of microsomes and mitochondria, but was not entirely absent. Due to the low protein content of lamellar bodies, even a small contamination may be a source of error. Indeed, trace amounts of microsomal type membranes were present in this fraction as detected by electron microscopy. This seems to explain the activity found in lamellar bodies.

Additions of CDP-diglyceride and mercuric chloride - The addition of CDP-diglyceride into mitochondria and lung microsomes profoundly increased the incorporation. However, some activity was found even in the absence of added CDP-diglyceride, suggesting the presence of trace amounts of CDP-diglyceride in the membranes obtained from the rat.

In addition to PG, another as yet incompletely identified lipid component was significantly labeled in the presence of [^{14}C]glycerol-3-P and CDP-diglyceride. The addition of mercuric chloride markedly inhibited the formation of PG (cf. refs. 17, 20), whereas the labeling of the unidentified lipid increased (Table III, compound X). The possible identity of this compound as phosphatidyl

Table III. Effect of 5mM mercuric chloride on [^{14}C]L-glycerol-3-P incorporation into lung lipids

	phosphatidyl glycerol (cpm)		compound X (cpm)	
	$-HgCl_2$	$+HgCl_2$	$-HgCl_2$	$+HgCl_2$
Mitochondria	1724	227	1060	4446
Microsomes	1741	297	1570	2730

The conditions were the same as in Table II (CDP-diglyceride added) except 1.2 mM glutathione was used. The unidentified lipid had R_f 0.38, and 0.36 in the first and second dimension of TLC, respectively.

glycerophosphate, the intermediate in the synthesis of PG, remains to be determined.

Pulse-labeling studies - The observed difference in the subcellular localizations of PG and the activity of PG synthesis was the basis of the experiment shown in Table IV. Mitochondrial and microsomal PG was pulse-labeled in the presence of [^{14}C]L-glycerol 3-phosphate and CDP-diglyceride, and the incubation was continued in the presence of fractions containing surfactant. Subsequently, the organelles added first were separated from the material added at 30 minutes by centrifugation in 0.8 M sucrose, and PG radioactivity was assayed in each fraction.

The incubation of microsomal preparation together with alveolar wash or lamellar bodies resulted in nearly quantitative removal of labeled PG from microsomes. On the other hand, the addition of surfactant to lung mitochondria did not affect the distribution of label between intra- and extramitochondrial spaces. Despite the use of fresh mitochondria and in some cases the washing of the organelles by centrifugation through 0.8 M sucrose prior to the experiment, only about half of the labeled PG was found in the mitochondrial pellet. The role of PG released from liver and lung mitochondria *in vitro* is as yet unknown.

DISCUSSION

The presence of substantial quantities of PG in lamellar bodies and in the

Table IV. Effect of surfactant on the subcellular distribution of phosphatidyl glycerol

Add 0 min.	Add 30 min.	Pellet		Supernatant
		cpm $\times 10^{-2}$	protein recovery (%)	cpm $\times 10^{-2}$
Lung				
Microsomes	Medium	108	84	59
Microsomes	Lamellar bodies	6	79	194
Microsomes	Cell sap	26		188
Microsomes	Alveolar wash	3		169
Mitochondria	Medium	56	89	75
Mitochondria	Lamellar bodies	63		68
Liver				
Mitochondria	Medium	50	82	87

Organelles (0.8 mg protein) were incubated under the same conditions as shown in Table II. CDP-diglyceride was included. At 30 min. 10 µl of 14mM L-glycerol-3-P, and 50 µl of 0.3M sucrose-Tris-EDTA, lamellar bodies (0.1 mg protein), cell sap concentrate (0.4 mg protein), or alveolar wash concentrate (0.24 mg protein) was added and the incubation was continued for another 30 min. Subsequently, the incubation mixture was layered on 0.8M sucrose-Tris-EDTA and spun at 105000g for twenty min.

material apparently derived from them, is unique in animal sources (17,21). On the other hand, plant chloroplasts that bear superficial morphological resemblance to lamellar bodies, and some bacteria contain large amounts of PG (22,23).

The present experiments indicate that the microsomal fraction of the lung incorporates L-glycerol-3-P into PG. The observed activity is markedly enhanced by the addition of CDP-diglyceride, suggesting the mechanism of the formation of PG as shown by Kiyasu et al.:

CDP-diglyceride + L-glycerol 3-phosphate → phosphatidyl glycerol + r_i + CMP

Lung mitochondria contain PG synthesizing activity, too, in agreement with the earlier evidence obtained with liver, and brain mitochondria (17,20).

According to the present experiments PG once formed in the microsomal fraction is subsequently associated with the surfactant system (Table IV). It is postulated that lamellar bodies contain a (protein) component with high affinity binding to PG, thus explaining the difference between the site of synthesis and the location

of this phospholipid. The assumed origin of the lamellar body component from microsomal membranes is in agreement with morphological evidence (24,25). On the other hand, the present experiments do not exclude the possibility that the synthesis of PG in mitochondria contributes to the formation of surfactant. However, it is likely that at least part of phosphatidyl glycerol in lung mitochondria serves as a precursor for cardiolipin synthesis (26).

The function of phosphatidyl glycerol still is speculative. Possibly it may:

1.) Contribute directly to reducing the surface tension in the air-liquid interface of alveoli.

2.) Be a structural component of lamellar bodies necessary for the assembly and/or the release of surfactant.

3.) Be an intermediate or end product of some other surfactant component.

These possibilities currently are being tested.

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