

# Phosphatidylglycerol in lung surfactant. III. Possible modifier of surfactant function

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**Abstract** Lamellar bodies and alveolar lavage from adult mammalian lung contain unusually high concentrations of phosphatidylglycerol that could serve as a sensitive indicator of surfactant. Phosphatidylglycerol was absent and phosphatidylinositol was correspondingly prominent in surfactant from the preterm rabbit fetus. Phosphatidylglycerol rapidly appeared and phosphatidylinositol decreased following the delivery. Surfactant isolated from the prematurely born rabbit or from humans with respiratory distress syndrome never contained phosphatidylglycerol.

Comparison between lamellar bodies from fetal and postnatal rabbits revealed remarkably similar composition except for the acidic phospholipids; however, the physicochemical properties were different. The compressibility of the surface film (i.e. the ratio of the fractional decrease in surface area and the corresponding decrease in surface tension) at low surface tensions was higher with fetal than with postnatal surfactant, whereas the difference in minimum surface tensions was small. These data suggest that phosphatidylglycerol is not an essential component required for the formation of the complex, but it improves the properties of surfactant in stabilizing the alveoli.

**Supplementary key words** lamellar inclusion body · dipalmitoyl lecithin · lung mechanics

Lung surfactant lines the air-liquid interface of the terminal airways. By reducing the surface tension on expiration, it contributes to the stability of these tiny air spaces (1, 2). Surfactant is synthesized in type II alveolar cells and is stored in intracellular lamellar inclusion bodies prior to the release into alveoli. Surfactant lining of interfaces is deficient in some life-threatening conditions. Perhaps the most significant of these is the respiratory distress syndrome (RDS) of the newborn, a developmental disease characterized by progressive atelectasis of lungs (3, 4). This condition can be predicted by phospholipid analysis in the amniotic fluid prior to birth (5). Disaturated lecithin is the major component of surfactant. In addition, proteins and certain other lipid components are present (see ref. 6 for review).

Phosphatidylglycerol is the second most abundant of the surfactant lipids (7-10) and like disaturated lecithin, is present in uniquely high concentration. We

have speculated that its possible role is as (1) an intermediate or an end product of some other surfactant component, (2) a structural component of lamellar bodies necessary for their assembly, or (3) a component contributing to surfactant function (9).

Studies on biosynthesis of phospholipids in lung fractions has yielded no evidence in favor of the first alternative (11). CDP-diglyceride serves as a common precursor for both phosphatidylinositol and phosphatidylglycerol, and the latter compound seems to be an end product rather than a precursor. The present approach to investigate surfactant phosphatidylglycerol—its ontogeny in mammals and distribution in some vertebrate species—enabled us to further evaluate its function.

## METHODS

New Zealand albino rabbits, adult chickens, and adult male bullfrogs (*Rana catesbiana*) were used for isolation of surfactant.

Lamellar bodies were isolated from lung homogenate as described earlier (11). The mitochondrial fraction containing lamellar bodies was isolated by differential centrifugation. Lamellar bodies were then isolated using density gradient and differential centrifugation. The extracellular surfactant was washed from the airways using endobronchial saline lavage (12) and was purified by a modification of the method of King and Clements (13). The wash medium was layered over 5 ml of 1.64 M NaBr, 0.15 M NaCl, 10 mM MgCl<sub>2</sub>, 5 mM Tris-HCl, 0.1 mM EDTA, and 0.05 mM NH<sub>4</sub>HCO<sub>3</sub> (pH 7.4), and centrifuged for one hour at 27,000 rpm using a SW-27 rotor at 4°C. The interphase was collected and suspended in the same solution as above except that 1.40 M NaBr was used. The resulting mixture was centrifuged at 27,000 rpm for 12 hr. The material floating on the top

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was collected and suspended in a small volume of the 1.64 M NaBr–NaCl–MgCl<sub>2</sub>–Tris–EDTA–NH<sub>4</sub>HCO<sub>3</sub> solution. A continuous density gradient (from 1.124 g/cm<sup>3</sup> to 1.057 g/cm<sup>3</sup>) was layered on the mixture. After centrifuging for 3 hr at 27,000 rpm, a white surfactant band at about 1.080–1.090 g/ml was collected. It was suspended in 0.1 mM EDTA, 0.05 mM NH<sub>4</sub>HCO<sub>3</sub> and centrifuged at 27,000 rpm for 1 hr. The pellet represented the purified surfactant. Mitochondria, microsomes, and plasma membranes were isolated as previously described (11).

The lipids were extracted and the phospholipids subsequently isolated using two-dimensional thin-layer chromatography as described earlier (11). The phosphorus contents were measured directly from the silica gel. The weights of the phospholipids were calculated by assuming that all the fatty acid components were palmitate. Individual “neutral lipids” were isolated on 5% ammonium sulfate silica gel H plates developed first in hexane–ethyl ether–acetic acid 50:50:1 (v/v), dried for 5 min at 70°C and redeveloped in hexane–ether 19:1(v/v). Thereafter the plates were charred and “neutral lipids” were quantified with reflectance densitometry (14). The fatty acids of phospholipids were analyzed after phospholipase A<sub>2</sub> digestion using gas–liquid chromatography (15). Disaturated lecithins were quantified essentially according to Mason, Huber, and Vaughan (16).

The proteins of lamellar bodies were assayed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Lamellar bodies were dialyzed for 24 hr against 0.1 mM EDTA, 0.05 mM NH<sub>4</sub>HCO<sub>3</sub> and the dialyzate was lyophilized. The lipids were extracted according to King et al. (17). Polyacrylamide gel electrophoresis was performed as described by Talbot and Yphantis (18); a phosphate buffer was used. After electrophoresis the gels were fixed and stained for protein according to Diezel, Kopperschaler, and Hofman (19).

Surface tension measurements were performed

with a Langmuir–Wilhelmy surface balance, essentially as described by King and Clements (20). The maximum surface area was 63 cm<sup>2</sup> and the minimum was 14 cm<sup>2</sup>. The compression and expansion of the surface took place in 50 sec. The temperature was 36°C.

Adsorption of surfactants from the aqueous suspensions to the air–liquid interphase was studied by measuring the decrease in surface tension as described by King and Clements (20). Lamellar bodies (20 nmoles phospholipid-phosphorus/ml) were suspended in 0.14 M NaCl, 4 mM KCl, 2 mM MgCl<sub>2</sub>, 5 mM Tris-HCl (pH 7.4), and sonicated. Twenty-five ml of the mixture was incubated for 5 min at 37°C with magnetic stirring (120 rpm) in a round Teflon dish, 5 cm in diameter. Thereafter the liquid surface was aspirated and the surface tension was continuously recorded.

Succinate:cytochrome c reductase (EC 1.3.99.1) and NADPH:cytochrome c reductase (EC 1.6.2.3) were measured as described by Sottocasa et al. (21), and 5′-nucleotidase (EC 2.7.8.2) as described by Emmelot and Bos (22). Protein was measured according to Lowry et al. (23). Linear density gradients were prepared using a gradient mixer connected to a polystaltic pump.

## RESULTS

Lamellar inclusion bodies of the lung were isolated from pre- and postnatal rabbits. The purity of the organelles was defined by mitochondrial (succinate:cytochrome c reductase), microsomal (NADPH:cytochrome c reductase) and plasma membrane (5′-nucleotidase) markers, as well as by electron microscopy. According to the marker enzymes, both fetal and postnatal lamellar bodies were relatively pure (**Table 1**). Electron microscopy revealed some smooth membranes in addition to lamellar bodies, but there was no detectable difference between the preparations from the fetus and the newborn.

TABLE 1. Distribution of “marker” enzymes in subcellular fractions of 30-day-old rabbit fetus and 3- to 8-day-old newborns

	Succinate-cytochrome c Reductase		NADPH-cytochrome c Reductase		5′-Nucleotidase	
	Fetus	Newborn	Fetus	Newborn	Fetus	Newborn
	<i>nmoles/min/mg protein</i>					
Mitochondria	112	161				
Microsomes			41	139		
Plasma membranes					189	225
Lamellar bodies	ND <sup>a</sup>	ND	ND	12	12	24

<sup>a</sup> Not detectable.

TABLE 2. Lipid composition of lamellar bodies from fetal and newborn rabbit

	Fetus 30 days	Newborn 2-8 days
<b>Phospholipids</b>	81.0 ± 8.9 <sup>a</sup>	81.1 ± 8.0
<b>Acidic:</b>		
Phosphatidylglycerols	0.4 ± 0.2 <sup>a</sup>	7.9 ± 1.2
Phosphatidylinositols	10.7 ± 1.0	3.9 ± 0.6
Phosphatidylserines	1.8 ± 0.5	1.4 ± 0.5
Bis-(monoacylglycerol) phosphates	0.1 ± 0.1	0.7 ± 0.1
Phosphatidic acids	0.4 ± 0.1	0.2 ± 0.1
	13.4 ± 1.1	14.1 ± 1.1
<b>"Neutral"</b>		
Lecithins	79.6 ± 9.0	79.5 ± 7.3
Phosphatidylethanolamines	5.1 ± 0.4	4.5 ± 0.5
Sphingomyelins	0.9 ± 0.5	0.6 ± 0.4
Lysolecithins	0.4 ± 0.4	0.3 ± 0.1
	86.0 ± 8.8	84.9 ± 7.5
<b>"Neutral lipids"</b>	6.5 ± 1.5	6.1 ± 1.4
Cholesterol	49.2 ± 1.2	45.9 ± 1.5
Free fatty acids	18.5 ± 6.1	21.3 ± 10.0
Diglycerides	15.4 ± 3.1	18.0 ± 4.9
Triglycerides	7.7 ± 3.0	6.6 ± 3.2
Monoglycerides	4.6 ± 1.5	3.3 ± 3.2
Cholesterol esters	3.0 ± 3.0	2.0 ± 0.8
	98.4 ± 23.0	97.1 ± 18.0
<b>Proteins</b>	12.5 ± 2.9	12.8 ± 3.4

<sup>a</sup> The results are expressed as weight percent ± SD of five independent measurements. Neutral glycolipids were not detected (<0.5%).

Three days prior to term, significant amounts of lamellar bodies were recovered from the lung. However, phosphatidylglycerol first appeared at term (1.6% of phospholipid-phosphorus), and its content increased 3.3-fold during the first two hours after birth. A sharp burst in surfactant phosphatidylglycerol was triggered by premature delivery up to two days prior to term. Animals that were more premature (28 days gestation or younger) did not develop phosphatidylglycerol after birth and respiratory distress

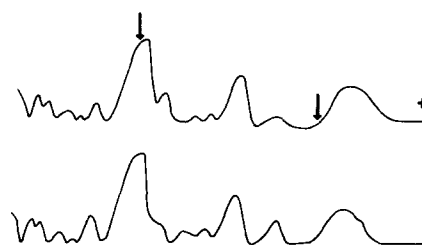


Fig. 1. Polyacrylamide gel electrophoresis of proteins from lamellar bodies. The upper densitometric tracing shows the pattern from 30-day-old rabbit fetus, and the lower one from 2-day-old newborn. The locations of protein standards run on separate gels are shown by arrows: bovine serum albumin on the left, chymotrypsin on the right.

ensued. The content of acidic phospholipids in lamellar bodies and alveolar lavage did not significantly differ during development (cf. ref. 24).

Table 2 shows the lipid composition of pre- and postnatal lamellar bodies. In the fetus phosphatidylinositol was essentially the only acidic phospholipid, whereas postnatal lamellar bodies had mainly phosphatidylglycerol and less phosphatidylinositol. The other lipids did not differ significantly. There were no differences in either percentage of disaturated lecithins in total lecithin or the fatty acid composition of the total lecithin from pre- and postnatal lamellar bodies (Table 3).

Proteins from the lamellar bodies were studied using sodium dodecyl sulfate gel electrophoresis. After lipid extraction and subsequent electrophoresis, three major bands with approximate molecular weights of 60,000, 35,000 and 18,000 daltons were detected in the gel. The protein patterns of fetal and postnatal lamellar bodies were similar (Fig. 1).

Surfactant recovered from a variety of healthy adult mammals always contained a large phosphatidylglycerol fraction. Reptiles possessed a similar surfactant phospholipid pattern, whereas bird surfactant did not contain phosphatidylglycerol. The healthy newborn humans always had phosphatidylglycerol.

TABLE 3. Fatty acid composition of lecithins in lamellar bodies from 30 day-old rabbit fetus and 2- to 8-day-old newborns

	1-position		2-position	
	Fetus	Newborn	Fetus	Newborn
14:0	0.4 ± 0.4 <sup>a</sup>	0.4 ± 0.2	1.2 ± 0.5	0.8 ± 0.7
16:0	85.2 ± 4.9	87.4 ± 5.7	59.3 ± 3.9	58.6 ± 4.8
16:1	2.0 ± 0.5	1.4 ± 0.2	10.0 ± 1.4	12.3 ± 1.7
18:0	4.9 ± 0.7	3.2 ± 0.7	3.4 ± 1.2	3.0 ± 0.6
18:1	5.6 ± 0.4	5.1 ± 0.5	24.0 ± 6.1	23.0 ± 5.0
18:2	1.2 ± 0.7	1.5 ± 0.2	1.3 ± 0.7	1.4 ± 0.8
18:3 + 20:1	0.7 ± 0.1	1.0 ± 0.4	0.8 ± 0.2	0.9 ± 0.1
% saturated	90.5 ± 5.1	91.0 ± 5.7	63.9 ± 4.0	62.4 ± 4.7

<sup>a</sup> The results are expressed as the mean ± SE of the duplicate measurements.

TABLE 4. Representative phospholipid composition of surfactants from various sources.<sup>a</sup>

	Bullfrog	Chicken	Newborn Human	
			Healthy	Respiratory Distress Syndrome
Lecithins	76.3	79.2	77.9	76.2
Phosphatidylglycerols	9.1	0.0	8.9	0.0
Phosphatidylinositols	4.5	8.9	4.2	11.9
Phosphatidylethanolamines	6.4	5.2	5.1	6.0
Bis-(monoacylglycerol) phosphates	1.0	0.0	1.1	0.0
Phosphatidylserines	1.5	3.0	1.4	2.1
Sphingomyelins	0.8	2.9	1.0	3.0

<sup>a</sup> % of lipid-phosphorus.

Babies with respiratory distress syndrome sometimes had surfactant in the airways, but it never contained phosphatidylglycerol. The lecithins from preparations shown in **Table 4** always had more than 50% disaturated species.

We further studied whether the specific differences in the acidic phospholipids possibly would be reflected as a difference in certain physicochemical characteristics. The adsorption of lamellar bodies from an aqueous suspension at the air-liquid interphase was studied by measuring the decrease in surface tension with a Wilhelmy dipping plate. Fetal and postnatal lamellar bodies decreased the surface tension in 8 min at 37°C from 71 dynes/cm to 49 and 48 dynes/cm, respectively. The time course was similar in both cases (data not shown), indicating that both surfactants were able to adsorb to the interphases. Secondly, the minimum surface tension was measured upon compression on a modified Wilhelmy balance. Lamellar bodies from both fetuses and newborns decreased the surface tension to below 10 dynes/cm (fetus 30 days:  $7.8 \pm 0.9$  ( $n = 7$ ) dynes/cm, newborn

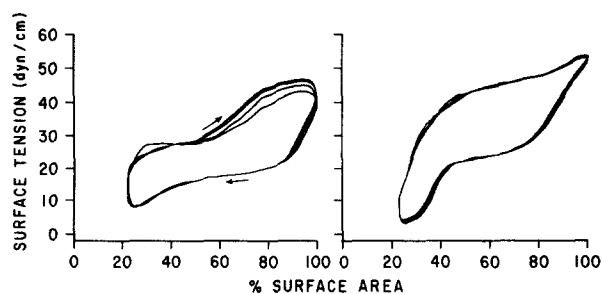
1-7 days:  $5.2 \pm 1.2$  ( $n = 6$ ) dynes/cm), indicating high surface activity. The amount of material required for less than 10 dynes/cm was similar with both surfactants. Thirdly, the effect of surface compression on surface activity was studied (**Fig. 2**). During compression of postnatal surfactant the surface tension fell in a characteristic manner (cf. ref. 13). However, fetal surfactant behaved differently; below 20 dynes/cm the surface tension decreased gradually. Therefore, these two surfactant layers had different compressibility ( $C = 1/A \text{ dA/d}\gamma$ ) at low surface tensions. Between 15 and 10 dynes/cm the compressibility of surfactant without phosphatidylglycerol (0.05-0.17 cm/dyne) was higher than that with phosphatidylglycerol (0.01-0.02 cm/dyne) at 36°C. The difference in compressibility was evident regardless of the amount of surfactant (15-40 nmoles) applied to the surface.

## DISCUSSION

According to the present results phosphatidylglycerol is not always present in surfactant and consequently is not an integral component required for formation of surfactant. However, the following evidence suggests that it is highly important for surfactant function.

Phosphatidylglycerol was absent in surfactant from the fetal rabbit. It appeared at term and increased after birth (24). This phospholipid was rapidly induced following premature delivery up to two days prior to term. On the other hand, in the fetal rabbit phosphatidylinositol was the most prominent acidic surfactant phospholipid, but it fell after birth. Thus phosphatidylinositol and phosphatidylglycerol appeared stepwise in surfactant, the latter heralding the postnatal stability of the lung (24).

Secondly, in respiratory distress syndrome of the newborn the babies sometimes had copious secretions in the airways. Most of the aspirate sedimented at



**Fig. 2.** Surface tension-area relationship of lamellar bodies isolated from 30-day-old fetus (left) and 7-day-old postnatal rabbit (right). The compression and the expansion of the surface are indicated by arrows. Prior to the measurement, the organelles were frozen and thawed, and an equal volume of absolute ethanol was added. The surface tension was measured using a modified Wilhelmy balance. A suspension of lamellar bodies containing 20 nmoles of phospholipid-phosphorus was applied to the surface of a solution containing 0.14 M NaCl, 2 mM MgCl<sub>2</sub>, 4 mM KCl, 5 mM Tris-HCl (pH 7.4), at 36°C. After two complete cycles the tracings shown above were obtained.

1.084–1.087 g/cm<sup>3</sup> in NaBr density gradient and 60% of the lecithins were disaturated. Phosphatidylglycerol was never present whereas phosphatidylinositol was always the predominant acidic phospholipid. However, the healthy newborn never lacked phosphatidylglycerol (Table 4, ref. 25). Therefore this phospholipid could serve as an indicator of the maturity of the surfactant system.

Thirdly, the surfactant lining the compliant lungs of adult mammals (7–10) and of a representative amphibian (Table 4) contained phosphatidylglycerol in uniquely high concentration. However, phosphatidylglycerol was absent from bird lung, although the complex isolated from the airways contained a high percentage of surface-active disaturated lecithins (58% of total lecithin). This suggests that the rigid bird lung contains surfactant (cf. ref. 2), although its composition is different from that in alveolar or saccular airways of other vertebrates.

Finally, low surface compressibility at low surface tension is an important characteristic of lung surfactant that contributes to alveolar stability during expiration (1). In the present study the comparison between fetal and postnatal surfactants from rabbits revealed little difference in minimum surface tension whereas the compressibility was quite different. It was high in postnatal surfactant at moderately high surface tensions (28 to 20 dynes/cm), but the compressibility characteristically decreased when surface tension fell (cf. ref. 26). On the other hand fetal surfactant had high surface compressibility whenever the surface tension was below 20 dynes/cm, suggesting inferior properties in stabilizing the alveoli (2).

The calculated area per phospholipid at minimum surface tension was about 17 Å<sup>2</sup>/molecule with both surfactants, whereas the limiting area of the stable dipalmitoyllecithin is 36 Å<sup>2</sup>/molecule (6). This suggests significant loss of material from the surface.

The region of high surface compressibility apparently represents a "close-packed" state, where further compression of the film forces molecules out of the surface while enriching the surface in disaturated phospholipids (26). Therefore the difference between the two surfactants may depend on factor(s) controlling the loss of material from the surface film. With the "mature" surfactant the loss of components from the surface during compression (before the minimum surface tension is reached) seems to be mainly during the phase when surface tension exceeds 20 dynes/cm. On the other hand, with the "immature" surfactant, the loss of components starts relatively late but continues close to the minimum surface tension.

The specific role of phosphatidylglycerol and phosphatidylinositol in surfactant function remains to be studied further. The present findings further indicate the importance of studying the biosynthesis and regulation of surfactant components other than solely lecithin. ■

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