Reconstitution of surfactant activity by using the 6 kDa apoprotein associated with pulmonary surfactant

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Lipid extracts of bovine pulmonary surfactant containing the 6 kDa apoprotein, but lacking the 35 kDa apoprotein, can mimic the essential characteristics of pulmonary surfactant on a pulsating-bubble surfactometer. Reconstituted surfactant can be produced by combining silicic acid fractions containing 6 kDa apoprotein and phosphatidylglycerol with phosphatidylcholine. Treatment of the protein-containing fraction with proteolytic enzymes abolishes its efficacy. These results indicate that the presence of the 6 kDa apoprotein can account for some of the essential physical and biological characteristics of pulmonary surfactant. Immunodiffusion studies indicate that, contrary to earlier suggestions, the 6 kDa apoprotein is not structurally related to the major surfactant apoprotein that has a molecular mass of 35 kDa.

INTRODUCTION

The mammalian lung is stabilized by a specialized material, the pulmonary surfactant, which reduces the surface tension at the air/liquid interface of the alveoli (Goerke, 1974). Pulmonary surfactant contains a number of lipids, including disaturated and unsaturated phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE). Natural surfactant also contains two major apoproteins with nominal molecular masses 35 and 11 kDa. Previous investigations on the role of these proteins in surfactant function have largely emphasized the 35 kDa moiety (King, 1984*a,b*, 1985; Hawgood *et al.*, 1985). Nevertheless, it has become apparent that protein-poor lipid extracts of pulmonary surfactant retain some of the physical and biological properties attributed to natural surfactant (Notter, 1984; Possmayer *et al.*, 1984; Berggren *et al.*, 1985). Treatment of prematurely delivered infants with a heat-sterilized emulsion of 'lipid-extract surfactant' (defined below) resulted in a marked improvement in lung function and a reduction in neonatal mortality (Enhörning *et al.*, 1985). The present investigation reports that the lipid-extract surfactant used in this trial lacked the 35 kDa apoprotein but contained the 6 kDa apoprotein. Such extracts retain their surfactant activity when assayed on a pulsating-bubble surfactometer. The results also demonstrate that active preparations can be reconstituted by combining the 6 kDa apoprotein with the appropriate lipids.

MATERIALS AND METHODS

Preparation of natural surfactant and surfactant components

Bovine lung surfactant and lipid extracts were prepared from freshly slaughtered cattle by a slight modification of methods described in previous publications from this laboratory (Yu *et al.*, 1983; Weber & Possmayer, 1984). 'Natural surfactant' refers to the white material collected at the interface after gradient centrifugation over 0.75 M-sucrose/0.9% NaCl and redispersed in saline

(0.9% NaCl) alone. 'Lipid-extract surfactant' refers to the phospholipid fraction precipitated from chloroform with cold acetone.

Samples of lipid-extract surfactant containing 300 mg of lipid in chloroform were applied to a column containing 5 g of activated silicic acid (200–400 mesh, Bio-Sil A; Bio-Rad). Fractions (50 ml each) were quantitatively analysed by t.l.c. with chloroform/ethanol/triethylamine/water (30:34:35:8, by vol.). Protein was determined by a modification of the Lowry method and phospholipid phosphorus by the Rouser method as previously described (Yu *et al.*, 1983).

Reconstitution of surfactant samples

The fractions from the silicic acid column in chloroform/methanol (9:1, v/v) were mixed as desired, dried under N₂ and dispersed in 0.9% NaCl/1.0 mM-CaCl₂ to yield 1% lipid and 0.01% protein (w/v). The suspensions were dispersed with a wrist-action shaker for 1 h at room temperature and incubated at 37 °C for 1–2 h. Where indicated, protein fractions were treated overnight at 37 °C with trypsin or Pronase (Sigma Chemical Co., St. Louis, MO, U.S.A.) at 2 mg/ml in 50 mM-Hepes/1.0 mM-CaCl₂/0.9% NaCl/0.02% NaN₃, pH 7.5, before reconstitution.

Surface-tension determinations

Surfactant activities were measured with a pulsating-bubble surfactometer (Enhörning, 1977; Yu *et al.*, 1983; Possmayer *et al.*, 1984). A bubble communicating with ambient air is created by withdrawing a small quantity of surfactant suspension from the test chamber (Fig. 1). The bubble is pulsed between a maximum radius (r_{max}) of 0.55 mm and a minimum radius (r_{min}) of 0.40 mm at 37 °C. Surface tensions are calculated from the pressure tracings after 1 min pulsation using the Laplace equation (Possmayer *et al.*, 1984).

Other methods

SDS/polyacrylamide-gel electrophoresis was conducted as described by Laemmli (1970). The 6 kDa

Abbreviations used: DPPC, dipalmitoyl phosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

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Fig. 1. Diagrammatic representation of the pulsating-bubble surfactometer

apoprotein was delipidated by spotting aliquots on a t.l.c. plate and chromatographing with chloroform/ethanol/triethylamine/water (30:34:35:8, by vol.). The protein was then eluted with chloroform/methanol/acetic acid/water (45:20:6:1, by vol.). Antisera were raised in rabbits by using standard procedures (MacPherson *et al.*, 1981). Immunodiffusion studies were conducted by the method of Ouchterlony (1953).

RESULTS AND DISCUSSION

Isolation of surfactant components

Natural surfactant prepared from bovine lung has a protein pattern (Fig. 2, lane 2) similar to that previously reported (Yu *et al.*, 1983). Repeated extraction of natural surfactant with chloroform/methanol by the method of Bligh & Dyer (1959) results in lipid extracts containing approx. 1% protein (measured by the Lowry method) with respect to phospholipid. These extracts appear devoid of any 35 kDa apoprotein, but contain the 6 kDa apoprotein (Fig. 2, lane 3). Owing to the large amounts of phospholipid, this protein migrates as a broad, blurred band. After delipidation by t.l.c. the protein migrates as a single discrete band of 5–6 kDa (Fig. 2, lane 4). The lipid extracts contained approx. 80% PC, 12% PG, and 3% PE. Chromatography of lipid extracts on silicic acid (Fig. 3) resolved a small fraction (F_I) of protein plus PG, followed by a fraction (F_{II}) containing protein, PG and PE. The combined fractions (F_I plus F_{II}) contained about 5% protein (w/w). Fraction III (F_{III}) contained pure PC in which protein could not be detected.

Reconstitution of protein and phospholipids

It is generally accepted that dipalmitoyl phosphatidylcholine (DPPC), which accounts for approx. 50% of the PC in lung surfactant, is primarily responsible for lowering the surface tension in the alveoli. Nevertheless, neither DPPC (results not shown) nor the PC fraction F_{III} from the silicic acid column was effective in reducing the surface tension of a pulsating bubble to the same extent

Fig. 2. Polyacrylamide-gel electrophoresis of bovine surfactant and its lipid extract

SDS/polyacrylamide-gel electrophoresis was conducted with 3% and 12% discontinuous gels. Proteins were stained with Coomassie Blue. (1) Standards; (2) natural surfactant; (3) lipid extract; (4) delipidated protein from lipid extract.

as unfractionated lipid-extract surfactant (Table 1). Reconstitution of PC, PG and protein resulted in surface-tension characteristics comparable with whole surfactant or lipid extracts. Although lipid extracts contain 3% PE, the activity of recombinants was not affected by the presence of this lipid.

It has been reported that addition of PG can improve the quality of surfactant reconstituted either with (King & MacBeth, 1981; Hawgood *et al.*, 1985) or without (Morley *et al.*, 1979; Bangham *et al.*, 1979; Obladen *et al.*, 1979; Yu *et al.*, 1984) 35 kDa apoprotein. Consequently it was deemed important to obtain additional evidence implicating the 6 kDa apoprotein present in F_I and F_{II} in surfactant activity. In agreement with previous results (Metcalf *et al.*, 1980; Possmayer *et al.*, 1984), the surface-tension-reducing ability of lipid-extract surfactant is resistant to trypsin but susceptible to Pronase (Table 2). For reconstitution studies, the protein-containing fraction was treated with proteolytic enzymes before lipid addition. Table 2 reveals that recombinants employing protein subjected to trypsin are inactive. These results not only support the view that the 6 kDa apoprotein is required for reconstitution, but also indicate that this protein becomes trypsin-sensitive once PC is removed. It appears likely that Pronase can degrade the 6 kDa apoprotein in the presence or absence of lipids. However,

Fig. 3. Silicic acid chromatography of lipid-extract surfactant

The inset shows a gel electrophoretogram of F_I .

because of the extensive streaking, samples treated with either proteinase still overlapped with controls on SDS/polyacrylamide gels. Consequently, it was not possible to demonstrate directly that degradation had occurred. Therefore it is conceivable that the effects noted with Pronase could be due to the physical presence of

exogenous protein in the manner previously reported for a 110 kDa serum protein by Ikegami *et al.* (1984).

Surfactant apoproteins apparently function by promoting the formation of a surface-active monolayer from bilayer lipids. The precise mechanism(s) by which apoproteins affect lipid structure and/or mobility is not understood. Possmayer *et al.* (1984) recently proposed that the surface-active monolayer might be formed via two processes termed the 'protein-facilitated' and the 'protein-mediated' mechanisms. Both processes promote the adsorption and spreading of DPPC to yield an equilibrium surface tension of 27 mN (27 dynes)/m. The protein-facilitated process requires only low concentrations (10–15 $\mu\text{g/ml}$) of lipid, but does require native undenatured 35 kDa apoprotein. This process is sensitive to boiling and exposure to organic solvents, trypsin and 5 mM-EDTA. The same treatments do not affect the protein-mediated process. This latter process requires relatively high concentrations of lipid (2500–5000 $\mu\text{g/ml}$) and protein. The results presented here clearly implicate the 6 kDa apoprotein in the latter process.

Although the suggestion that pulmonary surfactant can form a monolayer of DPPC by two processes requires further clarification, this proposal serves to clarify many apparently conflicting observations (Notter, 1984; Possmayer *et al.*, 1984). The reason why two apparently separate processes requiring different proteins have evolved to stabilize the mammalian lung is not clear. A number of independent investigations, including several human trials, demonstrate that lipid-extract surfactants possess biological activity (Fujiwara, 1984; Notter, 1984; Possmayer *et al.*, 1984; Enhorning *et al.*, 1985). The results presented in the present paper strongly suggest that this activity is related to the 6 kDa apoprotein. Whitsett and his co-workers (J. A. Whitsett, B. L. Ohning, J. Ross, T. Meuth, B. A. Weaver, D. L. Holm,

Table 1. Surface activities of reconstituted phospholipids and protein from silicic acid column chromatography (means $\pm$ S.E.M., $n = 4$)

'Control' refers to unfractionated lipid extract (1%, w/v).

Condition	Surface tension (mN/m) at 1 min of pulsation	
	$r_{\text{max.}}$	$r_{\text{min.}}$
Control	27.0 ± 1.0	0 ± 0
F_{III} (PC)	45.0 ± 1.2	20.0 ± 0.8
$F_{III} + F_I$ (PC + PG + protein)	27.0 ± 1.0	0.5 ± 0.5
$F_{II} + F_I$ (PC + PG + PE + protein)	27.0 ± 1.0	0.5 ± 0.5

Table 2. Effect of proteolytic digestion on surface activities of lipid extract and reconstituted surfactants (means $\pm$ S.E.M., $n = 4$)

Condition	Surface tension (mN/m) at 1 min of pulsation			
	Lipid-extract surfactant		Reconstituted surfactant	
	$r_{\text{max.}}$	$r_{\text{min.}}$	$r_{\text{max.}}$	$r_{\text{min.}}$
Control	27.0 ± 0	0 ± 0	28.0 ± 1.0	1.0 ± 1.0
Trypsin	28.0 ± 1.0	0 ± 0	37.0 ± 1.4	14.0 ± 0.9
Pronase	47.0 ± 2.0	17.0 ± 1.5	45.0 ± 2.5	18.0 ± 1.6

Fig. 4. Characterization of 35 kDa and 6 kDa surfactant apoproteins by immunodiffusion

1, Lung surfactant (0.25 mg/ml); 2, 35 kDa protein (0.1 mg/ml); 3, bovine serum (undiluted); 4, 6 kDa protein (0.1 mg/ml); 5, 6 kDa protein (0.5 mg/ml); 6, 6 kDa protein (1 mg/ml); 7, 6 kDa protein (0.5 mg/ml); 8, bovine serum (undiluted); 9, 35 kDa protein (1 mg/ml); 10, 35 kDa protein (0.5 mg/ml). Antisera were raised in rabbits. 35 kDa apoprotein was eluted from SDS/polyacrylamide gels (Hunkapiller *et al.*, 1983). Lipid extract was used as the source of 6 kDa apoprotein for raising antiserum. Delipidated 6 kDa apoprotein was used as antigen. Approx. 20 μ l of undiluted antisera were applied to the centre wells and 10 μ l of potential antigen were used in the peripheral wells. Abbreviations used: anti-35k and anti-6k, anti-(35 kDa protein) and anti-(6 kDa protein) antisera.

D. L. Shapiro & R. H. Notter, unpublished work) have recently shown that the organic-solvent extracts of calf lung surfactant used to prepare surfactant for the clinical trials conducted by Kwong *et al.* (1985) and Shapiro *et al.* (1985) contain only 5–6 kDa apoprotein, yet have the full biophysical activity of whole lung surfactant. However, the relative importance of the protein-facilitated (35 kDa apoprotein) process and the protein-mediated (6 kDa apoprotein) process in the lung under normal conditions must still be determined.

Possibly owing to its hydrophobic nature, the 6 kDa apoprotein has been studied less extensively than its 35 kDa counterpart. The isolation of a low-molecular-mass apoprotein was first reported for dog surfactant by King *et al.* (1973). This apoprotein was assigned a molecular mass of 10–12 kDa. Although early results (see King, 1984b) indicated that the major surfactant apoproteins share common epitopes, antisera raised against the 6 kDa and the 35 kDa apoproteins from bovine surfactant exhibit no cross-reactivity (Fig. 4). Phizackerley *et al.* (1979) reported that pig lung surfactant contained several hydrophobic proteins. Although further studies are required, N-terminal analysis indicates the 6 kDa apoprotein reported here is more than 90% pure. Suzuki *et al.* (1982, 1984) observed that partially purified protein fractions containing the hydrophobic apoproteins of less than 15 kDa from pig surfactant were more effective in promoting DPPC adsorption than fractions containing the 35 kDa apoprotein.

However, the possibility that the 6 kDa apoprotein

described here corresponds to a separate entity cannot be discounted. Fisher and co-workers have reported that rat surfactant contains a 6.2 kDa hydrophobic protein which promotes uptake of surfactant lipids by type II cells in culture (Claypool *et al.*, 1985; Fisher *et al.*, 1985). Depending on the mode of preparation, various amounts of other proteins can be isolated with surfactant (King, 1984a), but their relevance to surfactant function is vague. The present studies clearly implicate the 6 kDa apoprotein in the physicochemical and biological properties associated with pulmonary surfactant. Further investigations are required to confirm these observations and to determine the precise mechanism by which surfactant apoproteins influence lipid–lipid interaction and monolayer formation.

Note added in proof

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Since this manuscript was submitted, we have received permission to quote studies by Y. Suzuki, T. Curstedt, G. Grossman, T. Kobayashi, R. Nilsson, K. Nohara & B. Robertson (personal communication). Their investigations demonstrated not only that surfactant reconstituted from the low-molecular-mass (15 kDa or less) apoprotein is active with the pulsating-bubble apparatus, but have also demonstrated that these recombinants exhibited biological activity with prematurely delivered rabbits.

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