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Research Paper

Differences in Surfactant Lipids Collected from Pleural and Pulmonary Lining Fluids

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Purpose. The type and relative importance of saturated and unsaturated phospholipid components of surfactant within the epithelial lining fluid (ELF) of the inner and outer surfaces of the lung is not known.
Methods. Seven healthy dogs were anesthetized and a bronchoalveolar lavage (BAL) was performed, immediately followed by a pleural lavage (PL). Lipid was extracted from lavage fluid and then analyzed for saturated, primarily dipalmitoylphosphatidylcholine (DPPC), and unsaturated phosphatidylcholine (PC) species using high-performance liquid chromatography (HPLC) with combined fluorescence and ultraviolet detection. Dilution of ELF in lavage fluids was corrected for using the urea method.
Results. DPPC (494.7 ± 213.9 μg/mL) was the predominant PC present in ELF collected from the alveolar surface. In contrast, significantly higher ($p = 0.028$) proportions of unsaturated PC species were measured in PL fluid (~105 μg/mL), particularly stearoyl-linoleoyl-phosphatidylcholine (SLPC), which could not be measured in fluid collected from the alveoli, compared to DPPC (2.6 ± 2.0 μg/mL).
Conclusions. This study indicates that unsaturated PC species seem to be more important than saturated species, particularly DPPC, in the pleural cavity, which has implications for surfactant replenishment following pleural disease or thoracic surgery.

KEY WORDS: bronchoalveolar lavage; lung; phospholipid; pleural lavage; surfactant.

INTRODUCTION

Surface-active phospholipid (SAPL), more commonly known as "surfactant," plays an essential role in pulmonary function by reducing surface tension (1–5), masking irritant receptors (6) and promoting "dewatering" of the lungs (7,8). Surfactant is produced by alveolar type II cells and, although composed also of proteins, approximately 90% of surfactant by weight is phospholipid lipid (5,9), with phosphatidylcholine (PC) comprising approximately 70–80% of the phospholipid, followed by phosphatidylglycerol and phosphatidylinositol (~8–15%) (9–11).

In the lung, there are saturated and unsaturated species of PCs, with saturated PCs being the predominant class and, of these, dipalmitoylphosphatidylcholine (DPPC; PC 16:0/16:0) is the most surface active (9,11). The disaturated nature of DPPC confers high resistance to compression and opposes surface tension forces in the alveolus (to values <5 mN/m), generating a very high surface pressure, thereby preventing alveolar collapse at end expiration (12,13). DPPC is the primary component of synthetic surfactants that have been used as therapy for experimental lung injury (14), adult respiratory distress syndrome (ARDS), and other lung diseases (15). DPPC has also been shown to mask receptors on bronchial epithelium (6,16), which may be a useful application for synthetic and natural surfactants in the treatment of airway hyperreactivity and asthma.

Recent studies have found surfactant in other parts of the body, including pleura (17), articular cartilage (18), tendon (19), gastric wall (12,20), eustachian tube (21), and peritoneum (22). The phospholipid composition at these sites, however, has been found to vary from surfactant collected from alveoli (12,21). Functionally, nonalveolar surfactants display inferior compressibility and achieve lower (~20 mN/m) minimal surface tension (12,17), which seems to be related to PCs being predominantly unsaturated, rather than DPPC (12,21). However, unsaturated PCs, unlike DPPC, have phase transition temperatures far below body temperature that enable them to adsorb to biological membranes, such as the stomach wall (23). More importantly, preliminary *in vitro* studies in our laboratory indicate that the unsaturated PCs, palmitoyl-linoleoyl-phosphatidylcholine (PLPC; PC 16:0/18:2) and palmitoyl-oleoyl-phosphatidylcholine (POPC; PC 16:0/18:1), were able to reduce the coefficient of friction much further than DPPC (24).

The role of unsaturated PCs in the pleural fluid (PF) has not been determined. One early study demonstrated the existence of SAPL in PF that displayed strong surface activity to reduce surface tension by 24–26 mN/m (17). Pleural

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surfaces are constantly interacting and, as such, the SAPL lining could be expected to resemble alveolar surfaces, which are also quite dynamic, compared to gastric wall and eustachian tube. To date, there have been no studies comparing surfactant or surfactant composition between pleural and alveolar surfaces in the same individual. One problem is that PF is not routinely collected from healthy individuals and information concerning PF volume and composition in normal humans has only recently been measured using a technique of pleural lavage (PL) during concurrent surgery for essential hyperhidrosis (25).

In the current study, we used a canine model of bronchoalveolar lavage (BAL) followed by PL to compare saturated (DPPC) and unsaturated phospholipid [PLPC, POPC, stearoyl-linoleoyl-phosphatidylcholine (SLPC; PC 18:0/18:2), and dioleoylphosphatidylcholine (DOPC; PC 18:1/18:2)] in the epithelial lining fluid (ELF) from the pulmonary and pleural surfaces of the lung within the same healthy individual.

MATERIALS AND METHODS

Animals

Seven mixed breed dogs (three female and four male), weighing 18–34 kg (mean 24.3 kg), were used in this study. These animals had been presented to the School of Veterinary Science for euthanasia and this study was approved by the University of Queensland Animal Ethics Committee (approval number: SVS/597/03). All dogs were healthy based on clinical examination and there was no evidence of respiratory disease on thoracic auscultation.

BAL and PL Technique

Each dog was anesthetized with an intravenous injection of sodium thiopentone (22 mg/kg; Merial Ltd., Parramatta, NSW, Australia) and placed in sternal recumbency. A 9.2-mm fiber-optic endoscope (Pentax EPM-3000 Video Endoscopy System; Pentax Corp, Englewood, CO, USA) was then immediately inserted via the trachea and wedged into the left caudal lung lobe. Three 20-mL aliquots of warmed (25°C) sterile saline were flushed down the catheter port of the endoscope, with immediate suction used to withdraw each flush before the subsequent aliquot was instilled. The lavage samples were combined in a sterile plastic vial and gently mixed before storage on ice. The abdominal cavity was opened and a blood sample withdrawn from the caudal vena cava. A 6-cm incision was then made in the skin over the ninth intercostal space at the level of the costo–chondral junction and the parietal pleura approached by blunt dissection. A small incision was made in the pleura and warm (25°C) sterile saline (20 mL) was syringed into the interpleural space and the dog was rocked gently for 20 s. The lavage fluid was then withdrawn by gentle suction and placed on ice. The lung was examined immediately after PL and the samples were discarded if there was any evidence of gross pathological changes. Dwell time of lavage fluid in pulmonary and pleural spaces was restricted to 30 s.

BAL and Pleural Fluid Analysis

BAL and PL fluid samples were analyzed within 1 h of collection. Cell counts were performed on well-mixed undiluted fluid using a hemocytometer (Improved Neubauer Haemocytometer; Assistant, Germany). Fluid samples (200 μL) then underwent cytocentrifugation (950 rpm for 6 min; Shandon Cytospin 4, Theamo Shandon, Cheshire, UK) and 500-cell differential cell counts were prepared from smears after staining with Wright's stain (Hema-Tek 1000 Automatic Stainer, Ames, IL, USA). Urea in BAL and pleural fluids and in plasma were also measured (Olympus AU400 automatic analyzer; Olympus, Tokyo, Japan). The sensitivity of this technique was maximized for the low concentration of urea in BAL and pleural fluids by doubling the volume (20 μL instead of 10 μL) used for analysis, which gave linear results, using standard curves of urea dissolved in saline for lavage fluids over a concentration range of 0.05–2.5 mg/dL ($r^2 = 0.94$).

Samples of BAL (2 mL) and PL (4 mL) fluid were placed in 10 mL of chloroform on ice within 1 min of collection, and stored at −20°C until extraction according to the method of Folch *et al.* (26). Total phospholipid concentration was measured using thin layer chromatography (27), whereas individual saturated and unsaturated PCs were quantified using high-performance liquid chromatography (HPLC) with combined fluorescence and ultraviolet detection (28).

Statistical Analysis

The proportion of ELF in BAL and PL fluid samples was calculated as

$$\text{BAL(or PL) fluid area concentration} \times 100 \;/\text{plasma urea concentration (29).}$$

The concentrations of each PC species in BAL and PL fluids, after correction for dilution using the urea method, were not normally distributed as we were comparing paired sites within each animal. Wilcoxon's matched pairs rank sum test was used to determine statistical significance. To calculate the relative predominance of saturated *vs.* unsaturated PCs, the ratio of saturated (DPPC) to unsaturated (PLPC + POPC + DOPC + SLPC) was calculated for each dog, resulting in a mean ratio for each site; then the distribution of ratios for each dog was compared using Wilcoxon's matched pairs rank sum test.

RESULTS

BAL fluid cell numbers from the dogs in the current study (Table I) were similar to that reported as normal for clinically healthy dogs (31). The recovery of saline from BAL and PL was 75.7 ± 6.8 and 89.2 ± 3.4%, respectively. The proportion of ELF in BAL fluid and PL fluid was 3.4 ± 1.5 and 32.4 ± 12.1%, respectively. The concentration of saturated (DPPC) and unsaturated (PLPC, POPC, DOPC, and SLPC) PCs in BAL and PL fluids are listed in Table II, before and after correction for dilution by the urea method.

There was significantly more DPPC ($p = 0.0180$) and POPC ($p = 0.0280$) in BAL fluid compared to PL fluid, whereas there was significantly more SLPC ($p = 0.0180$) in PL fluid compared to BAL fluid. The ratio of saturated to unsaturated PCs was −1.38 in BAL fluid and 0.03 in PL fluid, with DPPC being the predominant species in BAL fluid, whereas unsaturated PCs predominated in PL fluid ($p = 0.028$).

DISCUSSION

This is the first report comparing PCs species between the inner (alveolar) and outer (pleural) surface of the normal lung. The results conclusively demonstrate that unsaturated PCs are the predominant species in the pleural cavity, whereas DPPC, a disaturated PC, seems integral to alveolar function.

A canine model was used in the current study because of the ethical and practical difficulties of collecting both pleural and alveolar lining fluids in healthy human subjects. There is extensive literature available concerning human alveolar constituents during health or disease, whereas measurement of the volume and cellular content of "normal" pleural fluid has only recently become possible in man during surgery to correct essential hyperhidrosis (25). The canine respiratory system is a useful model for human asthma and airway smooth muscle function (32,33), and investigation on interpleural fluid dynamics has relied on canine models (34,35). The canine model used in the current study permits virtually simultaneous comparison of two adjoining, but functionally different, surfaces in the healthy respiratory system.

Use of the urea model to estimate dilution was first proposed in 1986 (29), but has since proven controversial. The theory relies on an assumption that urea concentrations between plasma and epithelial lining fluid (ELF) are similar and constant, with no movement between the vascular and extravascular components during lavage (29). A problem occurs with long dwell time (>2 min) of lavage fluid as urea may flow out of the vasculature and therefore overestimate ELF, particularly during disease conditions where the permeability of the barrier separating plasma from ELF often increases (36). It has since been proposed that urea may still be a suitable endogenous marker, particularly in PL fluid, if dwell time is limited to <1 min, the ELF is collected from healthy subjects, and care is taken to prevent contamination of lavage fluid with red blood cells (25,37). It should also be noted that exogenous markers of dilution were not practical in the current study because of possible contamination of PL fluid following BAL.

Table I. BAL Fluid Cell Populations (Mean ± SD) from Dogs in the Current Study ($n = 7$) Compared to Normal Dogs ($n = 6$)

	Current study	Normal dogs[a]
Total nucleated cell/μL	233 ± 53	200 ± 86
Differential (%)		
Macrophages	64 ± 14	70 ± 11
Lymphocytes	8 ± 4	7 ± 5
Neutrophils	2 ± 3	5 ± 5
Eosinophils	11 ± 7	6 ± 5
Basophils	2 ± 1	r1 ± 1

[a] Clinically, radiographically, and histologically normal dogs (31).

Table II. Saturated (DPPC) and Unsaturated (PLPC, POPC, DOPC, and SLPC) Constituents of Phospholipid (μg/mL of Sample Fluid) in BAL and PL Fluids (Mean ± SD) from Clinically Healthy Dogs (n = 7) After Correction for Dilution in Lavage Fluid Using Urea as an Endogenous Marker

	BAL fluid	PL fluid
DPPC	494.7 ± 213.9	2.6 ± 2.0
	(16.8 ± 11.6)	(0.84 ± 0.82)
PLPC	61.8 ± 35.2	18.1 ± 3.8
	(1.3 ± 1.1)	(5.6 ± 1.9)
POPC	237.0 ± 166.6	17.8 ± 8.3
	(6.6 ± 4.3)	(6.4 ± 4.4)
DOPC	42.1 ± 44.2	6.5 ± 7.0
	(0.7 ± 0.8)	(2.3 ± 2.3)
SLPC	0.0 ± 0.0	62.7 ± 27.8
	(0.0 ± 0.0)	(19.6 ± 10.9)

Values enclosed in parentheses represent concentrations in BAL and PL fluid without correction for dilution.
There was significantly more DPPC ($P = 0.0180$) and POPC ($P = 0.0280$) in BAL fluid compared to PL fluid, whereas there was significantly more SLPC ($P = 0.0180$) in PL fluid compared to BAL fluid. The saturated/unsaturated PCs ratio was −1.38 in BAL fluid and 0.03 in PL fluid, with DPPC the predominant species in BAL fluid, whereas unsaturated PCs predominated in PL fluid ($P = 0.028$).

Several factors may account for the difference in surfactant species on two sides of the same organ. Obviously, the propensity of DPPC to reduce minimum surface tension to a value below 5 mN/m is essential for normal alveolar function (12,13). However, unsaturated PCs are the predominant species in all other biological surfaces tested to date, including gastric mucosa (12,20), tendon (19), eustachian tube (21), and articular surfaces (24). Importantly, preliminary *in vitro* studies in our laboratory have found *only* unsaturated PCs in human peritoneum (24). It would seem that properties of PCs other than reduction in surface tension are required in nonalveolar biological surfaces (4,38), particularly boundary lubrication (39), release "anti-stick" effects (40), and physical barrier formation (20). It has already been shown that SAPL within PL fluid reduced friction by up to 70% within pleural surfaces, whereas degradation of PCs leads to the condition of "pleural rub" (17).

An alternative explanation for limited saturated PCs in nonalveolar surfaces is that DPPC, in particular, is energetically very expensive to produce (12). It would seem reasonable to argue that if the surface-tension reducing properties are not required, substitution with unsaturated PCs at nonalveolar surfaces would be biologically favorable. However, it must also be considered that the solid nature of DPPC at body temperature requires a complex system of unsaturated acidic phospholipids and specific apoproteins to encourage adherence to epithelial surfaces (4,12,38). Conversely, the plasma transition temperatures of unsaturated PCs are much lower than body temperature and they readily adsorb to epithelial surfaces (12). It has already been demonstrated that surface adsorption is essential for any surface-active agent (12,38).

Results of the current study suggest that unsaturated rather than saturated PCs are essential to the normal function of nonalveolar biological surfaces, particularly the pleura. This leads to the potential therapeutic applications of unsaturated PCs in pleural diseases, particularly under conditions (41)

where pleural fluid, and subsequent lubricating properties, are diminished. Replacement of surfactant has been shown to be beneficial in the treatment of acute lung injury (15) or to reduce bronchial hyperreactivity in asthmatics (6,16). However, purified natural surfactant or commercially available synthetic surfactants contain primarily DPPC as the active ingredient (15). Several studies have already shown that SAPL applied to biological surfaces *in vivo* significantly improves boundary lubrication (39,42,43). Importantly, SAPL has also been shown to significantly reduce peritoneal adhesions following surgery (22,44). There may be a role for SAPL therapy, containing predominantly unsaturated PCs, following pleural disease or intrathoracic surgery.

In conclusion, further studies are required to specifically determine the advantages of unsaturated PCs over saturated PCs in terms of lubrication, although it is already known that unsaturated PCs more readily adsorb to biological surfaces, such as the pleura, an essential property for boundary lubrication. It is already known that unsaturated PCs are the predominant species at many other nonalveolar sites, including, now, the pleura, which seems logical given their superior physical and physiological advantages over DPPC in terms of boundary lubrication and adherence to biological surfaces. Synthetic surfactant preparation containing predominantly unsaturated PCs may therefore be preferable at nonalveolar sites, such as the pleura, to promote normal function during or following pleural disease, plus as an adjunct to limiting postoperative adhesions following intrathoracic surgery.

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