Surface-area cycling of different surfactant preparations: SP-A and SP-B are essential for large-aggregate integrity

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Surface-area cycling is an *in vitro* procedure for the conversion of large into small surfactant aggregates. In this procedure a tube containing a surfactant suspension is rotated end-over-end at 37 °C so that the surface area of the suspension changes twice each cycle. We have utilized this method to study the mechanisms involved in aggregate conversion. Several different surfactant preparations were analysed: (1) bovine natural surfactant, a sucrose-gradient-purified material containing surfactant phospholipid and surfactant-associated proteins (SP-) SP-A, SP-B and SP-C; (2) bovine lipid-extract surfactant, which contains the surfactant phospholipids and SP-B and SP-C; (3) mixtures of dipalmitoyl phosphatidylcholine and phosphatidylglycerol (7:3, w/w) reconstituted with one or more surfactant proteins. Aggregate conversion was measured by phosphorus analysis of a 40000 *g* supernatant (small aggregate) and pellet (large aggregates) before and after surface-area cycling. Surface-area cycling of lipid extract surfactant or lipids plus SP-B or SP-C resulted in rapid aggregate conversion. Lipids alone were not converted. Only a small percentage of purified natural surfactant was converted into small aggregates. Addition of SP-A to lipid extract surfactant could inhibit aggregate conversion of this material, but this was only observed when an additional 1% (w/w) of SP-B was added to the lipid extract. It is concluded that SP-A is important for large-aggregate integrity. It appears that SP-A acts in conjunction with SP-B. The presence of SP-B and/or SP-C is required for aggregate conversion; it is proposed that this reflects the necessity for lipid adsorption in aggregate conversion.

INTRODUCTION

Pulmonary surfactant prevents alveolar collapse by reducing the surface tension across the air/lipid interface of the alveoli. Surfactant is a mixture of approx. 90% lipid and 10% protein [1]. The protein component of surfactant consists of four proteins: surfactant-associated protein A (SP-A), SP-B, SP-C and SP-D [2]. SP-A is a collagen-like glycoprotein involved in the formation of tubular myelin [3], the enhancement of lipid adsorption [4,5], the counteraction of blood protein inhibition [6], the regulation of secretion and re-uptake of surfactant [7] and an involvement in host defence mechanisms [8]. SP-B and SP-C are two small hydrophobic proteins important for the formation and maintenance of the surface-active monolayer [9,10]. The bulk of the fourth surfactant protein, SP-D, is not associated with the surface-active surfactant fraction [11,12]. It has been suggested that SP-D has a role in lung defence [11].

Pulmonary surfactant isolated from lung lavages consists of different subfractions. These subfractions differ in morphological appearance, buoyant density and protein composition [13,14] The relationship between the different subfractions has been studied *in vivo* by pulse–chase experiments. These studies showed that larger, heavier, subfractions are the metabolic precursors of the smaller, lighter, surfactant subtype [13,14].

The conversion of large into small surfactant aggregates *in vivo* can be reproduced and studied *in vitro* by a technique known as 'surface-area cycling' [15]. Results obtained with surface-area cycling have correlated well with *in vivo* findings. For example, in *N*-nitroso-*N*-methylurethane-induced lung injury in rabbits an increased ratio of small to large surfactant aggregates in lung lavage was associated with an increased rate of conversion of large into small aggregates *in vitro* [16]. Similarly, the decreased ratio of small to large surfactant aggregates in lung lavage observed in radiation pneumonitis in rats was associated with a decreased conversion rate of large into small aggregates [17,18]. Furthermore, the small aggregates obtained from either alveolar lavage or after surface-area cycling do not contain SP-B and are impaired in their biophysical activity [19].

To investigate the mechanisms involved and the roles of individual surfactant components in aggregate conversion, experiments described here were performed with different surfactant preparations. After surface-area cycling of these preparations, which differ mostly in surfactant-associated-protein content, the samples were analysed for large and small aggregates, as determined by centrifugation at 40000 *g*, and surface-tension-reducing activity as determined using the pulsating bubble surfactometer. The effect of cycling on the morphology of the surfactant preparations was also examined.

METHODS

Surfactant preparation and reconstitution

Bovine natural surfactant was prepared as described previously [10]. The freeze-dried powder was resuspended in saline/1.5 mM $CaCl_2$ and centrifuged at 40000 *g* for 15 min at 4 °C. The pellet was resuspended in conversion buffer (0.15 M NaCl/10 mM Tris/1 mM $CaCl_2$/1 mM $MgCl_2$/0.1 mM EDTA, pH 7.4) and frozen at −20 °C. Lipid extract surfactant (bLES) was prepared by chloroform extraction and subsequent acetone precipitation of natural surfactant as described by Cockshutt et al. [6]. Bovine SP-A was purified using a mannose-affinity column [6]. Bovine SP-B and SP-C were isolated as previously described [20].

Abbreviations used: DPPC, dipalmitoyl phosphatidylcholine; PG, phosphatidylglycerol; bLES, bovine lipid extract surfactant; SP, surfactant-associated protein; $r_{max.}$, maximum bubble radius; $r_{min.}$, minimum bubble radius; ARDS, adult respiratory distress syndrome.

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For the reconstitution of SP-B and SP-C with lipid, the proteins were combined with dipalmitoyl phosphatidylcholine/phosphatidylglycerol (DPPC/PG, 7:3, w/w) and extracted by the method of Bligh and Dyer [21]. The lipid/protein mixtures in the chloroform layer were dried under nitrogen. The samples were resuspended in conversion buffer by vortex-mixing for 15 min at room temperature. An identical approach was used for the reconstitution of SP-B with bLES. BLES reconstituted with SP-A was prepared by resuspending nitrogen-dried bLES with buffer containing SP-A. Ca^{2+} was added to a final concentration of 5 mM, and the suspension was incubated at 37 °C overnight.

Surface-area cycling

Different surfactant preparations were resuspended in conversion buffer at a concentration of 0.25 mg of phospholipid/ml. Aliquots (2 ml) were placed in plastic tubes (Falcon 2058), capped, and attached to a rotator (Roto-torque rotator; Cole–Parmer Instruments). The tubes were cycled at 40 rev./min at 37 °C so that the surface area changed from 1.1 cm^2 to 9.0 cm^2 twice each cycle [15,16]. Unless otherwise specified, samples were cycled for 180 min. Identical, non-cycled, control samples were kept at 37 °C for the same duration as the cycled samples.

Separation of large and small aggregates

Large and small aggregates were separated by centrifugation at 40000 *g* for 15 min at 4 °C [19]. Total phospholipid in the large aggregate pellet, and in the small surfactant aggregates remaining in the supernatant, were determined by lipid extraction [21] and subsequent phosphorus analysis [22].

Sucrose-density-gradient centrifugation

Density centrifugation was performed as described previously [15,16,19]. Samples were loaded on a linear sucrose gradient from 0.1 M to 0.75 M sucrose in conversion buffer. The gradients were centrifuged for 60 h at 74000 $g_{max.}$ at 6 °C in an SW-28 swinging-bucket rotor in a Beckman L-70 ultracentrifuge (Beckman Instruments). Each gradient was fractionated into 1 ml fractions. Aliquots were taken for refractive-index determination and phospholipid analysis. The refractive index was measured at room temperature and used to calculate the sucrose density of the fractions. The lipids were extracted into chloroform [21] and phospholipid phosphorus measurements were performed [23].

Morphological studies

Surfactant suspensions were fixed in glutaraldehyde (2.5% final concn.) in the conversion buffer. After the addition of fixative, samples were incubated at 37 °C for 3 h, and then centrifuged for 10 min at 7000 *g*. After this primary fixation the pellets were incubated in 1% OsO_4/1.5% $K_4Fe(CN)_6$ for 1 h. Samples were dehydrated in a graded series of ethanol concentrations and rinsed in two changes of 100% acetone. Polybed 812 (Polysciences, Warrington, PA, U.S.A.) was used to embed the samples. Thin sections were counterstained with uranyl acetate and lead citrate. Transmission electron micrographs were made of representive areas.

Biophysical assays

Large and small aggregate samples obtained before and after surface-area cycling were extracted by the method of Bligh and Dyer [21]. Dried extracts were resuspended in 0.15 M NaCl/1.5 mM $CaCl_2$ to a final concentration of 5 mg of phospholipid/ml. Samples were incubated for at least 90 min at 37 °C before being analysed with a pulsating bubble surfactometer (Electronics Corporation) as described by Enhorning [24], using the chambers supplied by the manufacturer. With this technique a bubble is created in a surfactant suspension. After 10 s the bubble is pulsated at 20 pulsations/min between the maximum bubble radius ($r_{max.}$) of 0.55 mm and a the minimum bubble radius ($r_{min.}$) of 0.4 mm at 37 °C. The pressure across the air/liquid interface is monitored by a pressure transducer. Surface tension was calculated by the law of Young and Laplace, which states that the pressure across a sphere is directly proportional to twice the surface tension and indirectly proportional to the radius. Surface tensions at $r_{min.}$ were expressed.

For the adsorption measurements, samples were analysed at 5 mg/ml in conversion buffer. Samples were incubated at 37 °C for at least 90 min before being analysed. Adsorption measurements were conducted on the pulsating bubble surfactometer by monitoring the change in pressure across the air/liquid interface during the first 10 s after the formation of the bubble. Surface tension was calculated as described above.

RESULTS

The formation of small aggregates (defined as the phospholipid fraction remaining in the supernatant after centrifugation at 40000 *g*) during surface-area cycling was investigated with three different surfactant preparations: (1) bovine natural surfactant, a preparation containing SP-A, SP-B, SP-C and a small amount of other proteins [6]; (2) bLES, containing the surfactant phospholipids and the two hydrophobic proteins SP-B and SP-C; and (3) lipids, a preparation containing only DPPC/PG (7:3, w/w). Figure 1 shows that a small percentage of natural surfactant was converted into small aggregates during 3 h of cycling. Surface area cycling of bLES led to the rapid formation of small aggregates, whereas lipids alone were not converted into small aggregates (Figure 1).

The effects of surface-area cycling on the morphology of the surfactant preparations is shown in Figure 2. Non-cycled natural surfactant (Figure 2a) contained a large number of the lattice-like tubular myelin structures. After cycling of natural surfactant (Figure 2b), the amount of tubular myelin is significantly reduced; however, highly organized multilayers and dense lipid structures were still observed. Electron micrographs of non-cycled bLES

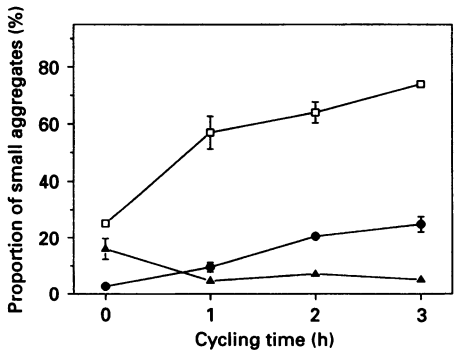

Figure 1 Formation of small aggregates by three different surfactant preparations

The following samples were analysed: purified natural surfactant (●), bLES (□) and lipids (DPPC/PG, 7:3, w/w; ▲).

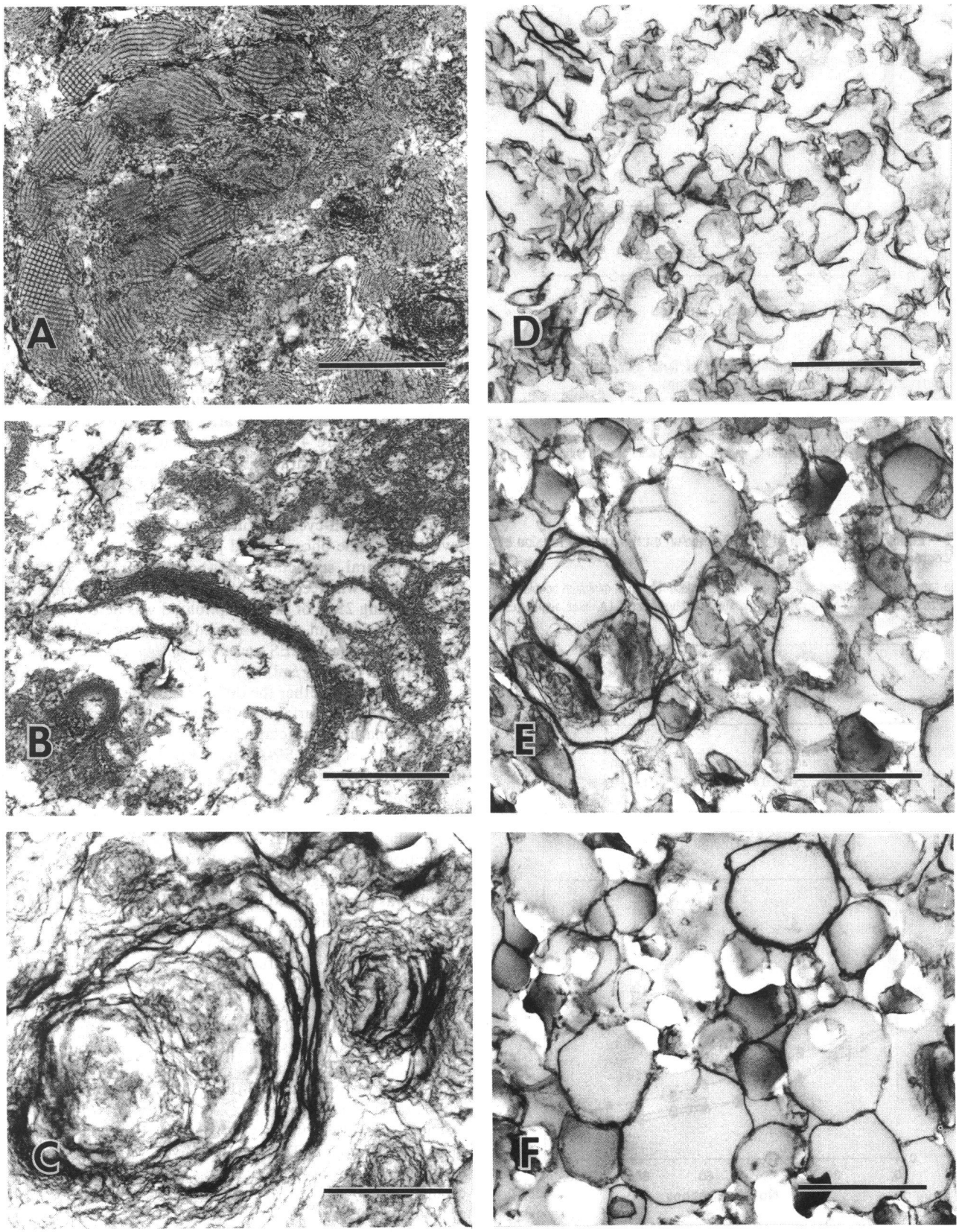

Figure 2 Electron micrographs of non-cycled and cycled surfactant preparations

Panels A and B, natural surfactant (A, non-cycled; B, cycled); panels C and D, bLES (C, non-cycled; D, cycled); panels E and F, lipids (E, non-cycled; F, cycled). Representative areas were photographed and are printed at a final magnification of 22769 × (the scale bar represents 1.0 μm).

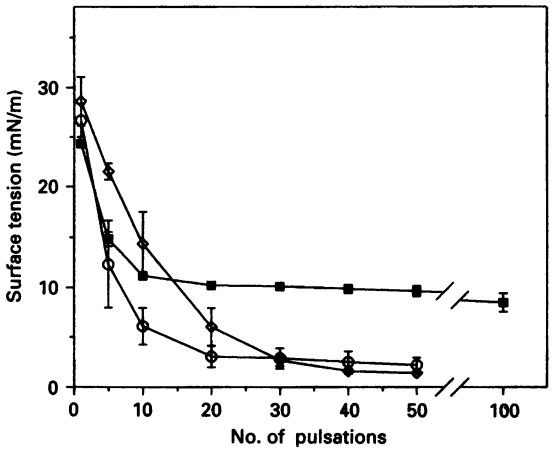

Figure 3 Surface-tension-reducing ability of lipid extracts of large and small aggregates isolated from natural surfactant

Large aggregates were obtained as a 40000 *g* pellet from cycled (◇) and non-cycled (○) large natural surfactant. Small aggregates (■) were obtained from the supernatant of a 40000 *g* centrifugation of cycled natural surfactant (values at minimum bubble size are expressed).

Table 1 Effect of the addition of 1% SP-B (w/w) on the surface tension of small aggregates

Extracted samples were analysed at 5 mg/ml, and the surface tension at minimum bubble size after 10 s adsorption and after 50 pulsations is shown. Abbreviations: LA, large aggregates; SA, small aggregates.

	Surface tension (mN/m)	
	10 s adsorption	50 pulsations
LA	28.8±1.3	3.5±0.9
SA	40.2±3.4	18.9±0.5
SA+1% SP-B	25.9±0.4	4.6±0.6

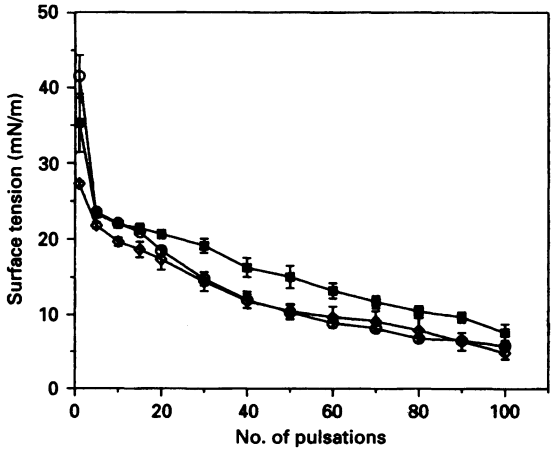

Figure 4 Surface-tension-reducing ability of lipid extracts of large and small aggregates isolated from bLES

Large aggregates were obtained as a 40000 *g* pellet from cycled (◇) and non-cycled (○) bLES. Small aggregates (■) were obtained from the supernatant of a 40000 *g* centrifugation of cycled natural surfactant (values at minimum bubble size are expressed).

(Figure 2c) showed many multivesicular-like structures, but, after cycling, bLES mainly consisted of small vesicles (Figure 2d). Lipids alone consisted mainly of large unilamellar vesicles, and this structure was not altered by surface area cycling (Figures 2e and 2f).

We have previously observed that small aggregates recovered after surface-area cycling of canine large surfactant aggregates have poor surface-tension-reducing properties [19]. The biophysical activities of large and small aggregates obtained after cycling of bovine natural surfactant are shown in Figure 3. Lipid extracts of large surfactant aggregates from non-cycled and cycled natural surfactant reduced surface tension to near zero values within 50 pulsations. Non-cycled large aggregates reduced the surface tension slightly faster than the large aggregates obtained after cycling. Lipid extracts of the small aggregates formed during cycling did not reach near zero values even after 100 pulsations (Figure 3). However, addition of 1% SP-B to these lipid extracts of small aggregates gave a surface-tension-reducing activity similar to that of large aggregates (Table 1).

The surface-tension-reducing activity of lipid extract of small and large aggregates obtained after surface-area cycling of bLES is shown in Figure 4. Although somewhat less active than the large-aggregate preparations of natural surfactant, both large and small aggregates obtained from bLES reduced surface tension to low values within 100 pulsations.

Analysis of non-cycled natural surfactant on a sucrose gradient revealed a single phospholipid peak at a density of 1.083 g/ml. Cycled natural surfactant showed two peaks: 75% of the phospholipid was recovered at a density of 1.085 g/ml, whereas the remaining 25% of the phospholipid was detected as a broad peak at 1.051 g/ml. Sucrose-gradient centrifugation of non-cycled and cycled bLES revealed the same density for both preparations (1.058 g/ml).

To determine whether the difference in the conversion rates of bLES and natural surfactant (Figure 1) was due to the presence of SP-A in natural surfactant, SP-A was added to bLES. Addition of 5 or 10% (w/w) SP-A to bLES did not affect the rapid aggregate conversion of bLES (results not shown). Neither incubation at 37 °C overnight nor increasing the Ca^{2+} concentration to 5 mM had any effect (results not shown). Surface-area cycling of bLES plus 20% (w/w) SP-A in 5 mM Ca^{2+} after incubation at 37 °C overnight resulted in a slight decrease in the amount of small aggregates generated. However, when the bLES sample was supplemented with an additional 1% (w/w) SP-B, a marked decrease in small aggregate formation was observed in the presence of 20% (w/w) SP-A.

To examine this effect further, different concentrations of SP-A were added to bLES or to bLES plus 1% (w/w) SP-B. As stated above, the addition of SP-A to bLES does not result in a significant inhibition of surfactant aggregate conversion. However, bLES plus SP-B shows a reduced conversion, even when only 1% (w/w) SP-A is added (Table 2). At 5 or 10% (w/w) SP-A added to bLES plus SP-B the small-aggregate formation was at its lowest level (Table 2).

The effect of the SPs (1% w/w) on the conversion of DPPC/PG mixtures is shown in Table 3. Both hydrophobic proteins, SP-B and SP-C, can promote the formation of small aggregates during cycling, with SP-C being slightly more effective than SP-B. Lipids alone or in the presence of SP-A do not form small aggregates.

Table 4 shows the adsorption of lipid/protein mixtures as assayed on the pulsating bubble surfactometer. High surface tensions of approx. 70 mM/m were observed with either lipids alone or with lipids to which SP-A had been added. The surface tension of these samples was not significantly different from the surface tension of conversion buffer alone. This indicates that, in

Table 2 Effects of adding different concentrations of SP-A to bLES or bLES plus 1% SP-B before surface-area cycling

SP-A added (% of lipid)	Small aggregates (%)			
	Non-cycled		Cycled	
	bLES	bLES + 1% SP-B	bLES	bLES + 1% SP-B
0	7.1 ± 1.0	10.8 ± 0.1	49.2 ± 8.8	48.7 ± 2.2
1	5.0 ± 0.3	5.0 ± 0.3	54.0 ± 5.7	30.0 ± 3.5
5	2.9 ± 0.2	4.3 ± 0.5	55.8 ± 10.8	16.4 ± 1.3
10	3.1 ± 0.3	2.8 ± 0.2	42.8 ± 4.0	13.2 ± 1.3
20	4.5 ± 0.5	2.9 ± 0.2	32.7 ± 9.2	20.1 ± 4.6

Table 3 Effects of the addition of 1% SP-A, SP-B or SP-C to lipid (DPPC/PG, 7:3, w/w) on the formation of small aggregates during surface-area cycling

	Small aggregates (%)	
	Non-cycled	Cycled
Lipids	11.9 ± 1.1	10.0 ± 0.7
+ 1% SP-A	7.2 ± 0.7	11.1 ± 1.9
+ 1% SP-B	4.3 ± 0.8	29.8 ± 4.5
+ 1% SP-C	3.3 ± 0.3	42.5 ± 2.8

Table 4 Effects of the addition of 1% SP-A, SP-B or SP-C to lipid (DPPC/PG, 7:3, w/w) on lipid adsorption

Extracted samples were analysed at 5 mg/ml, and the surface tensions after 10 s adsorption are shown.

	Surface tension after 10 s adsorption (mN/m)
Buffer	73.0 ± 1.5
Lipids	71.0 ± 0.5
+ SP-A	68.6 ± 0.8
+ SP-B	40.4 ± 2.6
+ SP-C	29.6 ± 0.8

the absence of protein, the lipids in these samples did not rapidly adsorb at the air/liquid interface. When SP-B or SP-C was added to DPPC/PG there was a rapid adsorption of lipids to the interface (Table 4); this effect was more pronounced with SP-C than with SP-B.

DISCUSSION

Large- to small-aggregate conversion constitutes part of the extracellular metabolic pathway of pulmonary surfactant [7,13,14]. To avoid the complexity of *in vivo* experiments, Gross and Narine [15] developed an *in vitro* technique, surface-area cycling, to study the conversion of surfactant aggregates. Using this technique it has been found that conversion of large to small surfactant aggregates is dependent on repeated changes in surface area and proteinase activity [15,19,25]. Degradation of SP-B has been observed during the conversion of normal large aggregates [19]. This had led to the following hypothesis: an increase in surface area leads to the adsorption of lipid to the air/liquid interface resulting in the exposure of SP-B to proteinase activity [19]. The present studies were conducted to obtain further support for this working hypothesis.

The results presented here suggest that only those samples that adsorb rapidly to an air/liquid interface, such as bLES and lipids plus SP-B or SP-C, can be converted into small aggregates during surface-area cycling. Conversion of these reconstituted systems is independent of proteinase activity. When bLES samples were cycled in tubes filled to a level where an air/liquid interface was still present, but where rotation did not cause a change in surface area, there was no detectable formation of small aggregates (results not shown). These results are consistent with the view that adsorption is necessary for aggregate conversion. Furthermore, it suggests that phospholipids at the air/liquid interface can form small vesicles upon a rapid decrease in surface area.

Highly purified natural surfactant rapidly absorbs to the air/liquid interface [4,5,6]. However, this preparation is not rapidly converted into small aggregates. This indicates that, although adsorption is required, it is not necessarily sufficient for aggregate conversion. The small aggregates formed during surface-area cycling of this purified natural surfactant are not surface-active, consistent with the degradation of SP-B by a serine proteinase [19].

The differences in the extent of aggregate conversion of bLES and natural surfactant suggest that SP-A is important for the integrity of large surfactant aggregates. This hypothesis was supported by the observation that the addition of SP-A to bLES can block the conversion into small aggregates. Since these SP-A-containing surfactant samples rapidly adsorb at the air/liquid interface, SP-A might be involved in the reformation of large aggregates during the decrease in surface area.

Unexpectedly, the inhibition of conversion with SP-A was only observed after an additional 1% (w/w) SP-B was added to the lipid extract surfactant. This requirement for an additional 1% SP-B to block the conversion of bLES by SP-A is an interesting observation. This result could imply a stoichiometric relationship between SP-A and SP-B. Since SP-A forms different oligomeric structures (dimers, trimers and 18-mers), and bLES already contains approx. 0.5–1.0% SP-B [26], the molar ratio of SP-A/SP-B is difficult to estimate. Another explanation for the requirement of additional SP-B is that a considerable proportion of the SP-B is inaccessibly packed in the lipid vesicles and cannot interact readily with exogenously added SP-A. This suggestion is supported by observations made in this laboratory during the development of an e.l.i.s.a. assay for measurements of SP-B. When identical amounts of SP-B were analysed in the presence and absence of surfactant lipids, it was found that presence of lipid markedly reduced the amount of detectable SP-B (K. Inchley, R. A. W. Veldhuizen and F. Possmayer, unpublished work). Regardless of the mechanism involved, the cycling results support a possible interaction between SP-A and SP-B in large aggregates. Since both SP-A and SP-B bind lipids [3], the interaction between SP-A and SP-B may be mediated through lipid binding or altered lipid structures. Alternatively, there may be a direct protein–protein interaction between the two.

Both SP-A and SP-B are required for the formation of tubular myelin *in vitro* [3,27]. Immunogold labelling of the SP-A in tubular myelin has suggested that SP-A is located in the corners of this lattice-like structure [28]. It has been proposed that SP-A binds lipid and/or SP-B in the lattice corners, while self-aggregation of the headgroups of SP-A oligomers stabilizes the structure [29]. It has been suggested that tubular myelin can rapidly adsorb to the air/liquid interface [30].

On the basis of this proposed molecular structure of tubular myelin, the following revised hypothesis for the mechanisms involved in surface-area cycling of natural surfactant can be

made. Tubular myelin or a tubular-myelin-like structure adsorbs rapidly to the air/liquid interface during an increase in surface area. This rapid adsorption might be accomplished by the detachment of the connections that stabilize the tubular-myelin structure. During the decrease in surface area, reattachments of those connections lead to the re-formation of tubular myelin or tubular-myelin-like structures which behave as large aggregates. SP-A in conjunction with SP-B is involved in this reformation process. Cleavage of SP-B during the adsorption process, as suggested previously [19], would prevent the reassembly of large aggregates. Similarly, in the absence of SP-A, large aggregates adsorb to the air/liquid interface, but cannot reform into large aggregates, thus leading to the formation of small aggregates.

In several different animal models of lung injury the ratio of small (inactive) to large (active) surfactant aggregates is increased [31–33]. Understanding the mechanisms involved in aggregate conversion might lead to treatment strategies for lung injury focused on maintaining large-aggregate integrity.

The results described here suggest that the loss of SP-A or a reduction of the amount of this protein can contribute to the formation of small aggregates. Increases in proteolytic activity have been observed in lung lavages from adult-respiratory-distress-syndrome (ARDS) patients [34,35]. A recent report by Gregory et al. [36] showed a decreased level of SP-A in large aggregates from ARDS patients. Unfortunately, the small/large aggregate ratio was not determined in that study [36]. SP-A was also decreased in large aggregates isolated after experimental lung transplantation [31]. In that study, the small/large surfactant aggregate ratios were significantly increased. Similarly, analysis of surfactant aggregates in septic sheep revealed an increased ratio of small to large aggregates associated with a decreased level of SP-A [33].

In summary, we have shown that SP-A is important for maintaining large-aggregate integrity in vitro. SP-A appears to interact in conjunction with SP-B. These observations suggest that decreased levels of SP-A [31,33,36] in lung injury might, in part, be responsible for the increased small/large surfactant aggregate ratio in these lung injuries [31–33].

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