Degradation of surfactant-associated protein B (SP-B) during *in vitro* conversion of large to small surfactant aggregates

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Pulmonary surfactant obtained from lung lavages can be separated by differential centrifugation into two distinct subfractions known as large surfactant aggregates and small surfactant aggregates. The large-aggregate fraction is the precursor of the small-aggregate fraction. The ratio of the small non-surface-active to large surface-active surfactant aggregates increases after birth and in several types of lung injury. We have utilized an *in vitro* system, surface area cycling, to study the conversion of large into small aggregates. Small aggregates generated by surface area cycling were separated from large aggregates by centrifugation at 40 000 *g* for 15 min rather than by the normal sucrose gradient centrifugation. This new separation method was validated by morphological studies. Surface-tension-reducing activity

of total surfactant extracts, as measured with a pulsating-bubble surfactometer, was impaired after surface area cycling. This impairment was related to the generation of small aggregates. Immunoblot analysis of large and small aggregates separated by sucrose gradient centrifugation revealed the presence of detectable amounts of surfactant-associated protein B (SP-B) in large aggregates but not in small aggregates. SP-A was detectable in both large and small aggregates. PAGE of cycled and non-cycled surfactant showed a reduction in SP-B after surface area cycling. We conclude that SP-B is degraded during the formation of small aggregates *in vitro* and that a change in surface area appears to be necessary for exposing SP-B to protease activity.

INTRODUCTION

Pulmonary surfactant is a mixture of about 90 % lipid and 10 % protein that reduces the surface tension across the air–liquid interphase of the alveoli. Surfactant contains at least three surfactant-associated proteins (SPs), SP-A, SP-B and SP-C [1]. SP-A is a water-soluble glycoprotein while SP-B and SP-C are two small hydrophobic proteins. Another lung-specific glycoprotein has been identified and named SP-D; however, the association of this protein with surfactant is still controversial [2].

Differential centrifugation or sucrose-gradient centrifugation can separate alveolar surfactant, obtained by lung lavages, into different subfractions [3–5]. The larger or denser subtype contains tubular myelin, lamellar bodies and large vesicles [4]. These large aggregates contain all three surfactant-associated proteins and are capable of reducing surface tension to low values. Pulse–chase studies have demonstrated that the large-aggregate subtype is the precursor of smaller, lighter subfractions [3,5]. The small-surfactant-aggregate fraction, which consists mainly of small vesicles, contains less SPs and is functionally inferior to the large aggregates [6,7].

Rapid changes in phospholipid distribution between the different subfractions have been observed at the onset of air breathing in rats [8]. Before birth surfactant consists mainly of the heavy subtype, whereas after birth an increased small-aggregate fraction is observed. Changes in the pool sizes of surfactant subtypes also occur in a number of lung injury models. In *N*-nitroso-*N*-methylurethane (NNMU)-induced lung injury in adult rabbits, the ratio of small to large aggregates was increased several fold over that in control rabbits [9]. Similar findings were reported for an ischaemic/reperfusion injury related to experimental lung transplantation [9a]. On the other hand,

radiation pneumonitis in rats resulted in a decreased ratio of small to large aggregates compared with control animals [10].

Gross and Narine have developed a system *in vitro*, surface area cycling, to study the mechanisms involved in the conversion of large aggregates into small surfactant aggregates [11]. In this procedure a tube containing resuspended large surfactant aggregates is rotated end-over-end thereby changing the surface area, this results in the conversion of large aggregates into small aggregates. The large and small aggregates are separated using sucrose-gradient centrifugation. Gross and Schultz have used this model to demonstrate the involvement of a serine protease, convertase, in the conversion of surfactant aggregates [12]. This protease has recently been identified as a 75 kDa protein closely associated with surfactant phospholipid [13].

Surface area cycling has also been employed to study the altered surfactant subfraction ratios after lung injury. In NNMU-induced lung injury the increased ratio of small to large surfactant aggregates was correlated with an increased conversion of large into small aggregates *in vitro* [14]. The authors suggested that an increase in protease levels may be responsible for the increased conversion rate [14]. The decreased ratio of small to large aggregates in radiation pneumonitis has been explained by an increase in protease inhibitor concentration [15].

In this report we describe the development of a more rapid method of separating large and small aggregates after surface area cycling. In addition, evidence is presented for the degradation of SP-B during cycling.

MATERIALS AND METHODS

Preparation of large aggregates

Normal dog lungs were lavaged twice via the trachea using approx. 1 litre of 0.15 M NaCl/1.5 mM CaCl₂. The lung lavage

was centrifuged at 150 *g* for 10 min at 4 °C to remove cells and cellular debris. A large-surfactant-aggregate pellet was obtained by centrifugation at 40000 *g* for 15 min at 4 °C. The large aggregates were resuspended in conversion buffer (0.15 M NaCl, 10 mM Tris, 1 mM CaCl₂, 1 mM MgCl₂, 0.1 mM EDTA, pH 7.4) and immediately frozen at -20 °C.

Surface area cycling

Large surfactant aggregates were resuspended in conversion buffer at a concentration of 0.25 mg of phospholipid/ml. Samples (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 cm² to 9.0 cm² twice each cycle [11,14]. 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.

Some experiments utilized the protease inhibitor phenylmethanesulphonyl fluoride (PMSF). PMSF was dissolved in ethanol (500 mM) and added to the samples to a final concentration of 10 mM. An equal amount of ethanol was added to samples not receiving PMSF.

Separation of large and small aggregates

Sucrose-density-gradient centrifugation was used to separate the large and small surfactant aggregates [11,14]. Samples were loaded on to a linear sucrose gradient ranging 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. Each gradient was fractionated into 1 ml fractions. Aliquots were taken for refractive index determination (50 µl), phospholipid measurement (0.8 ml) and immunoblot analysis for SP-A and SP-B (50 µl). The refractive index was measured at room temperature and used to calculate the sucrose density of the fractions. The lipids were extracted into chloroform [16] and phospholipid phosphorus measurements were performed [17].

As an alternative to sucrose-density-gradient centrifugation, large and small aggregates were separated by centrifugation at 40000 *g* for 15 min at 4 °C. Total phospholipid in the large-aggregate pellet and in the small surfactant aggregates remaining in the supernatant were determined by lipid extraction [16] and phosphorus analysis [18]. A validation of this procedure is given in the Results and discussion section of this paper.

Morphological studies

Surfactant suspensions were fixed in glutaraldehyde (2.5 % final concentration) in the conversion buffer. After the addition of fixative, samples were incubated at 37 °C for 4 h, and then centrifuged for 10 min at 7000 *g*. After this primary fixation the pellets were incubated in 1 % (w/v) OsO₄/1.5 % (w/v) K₄Fe(CN)₆ for 1 h. Samples were dehydrated in a graded series of alcohol and rinsed in two changes of 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. Representative areas were photographed and printed at a final magnification of 3545 ×.

Dot-blot and Western-blot analyses

Samples were spotted on nitrocellulose using a Bio-Dot micro-filtration apparatus. The nitrocellulose was blocked for 30 min with 3 % (w/v) gelatin in Tris-buffered saline (20 mM Tris/500 mM NaCl, pH 7.5), washed with 0.05 % Tween 20 in

Tris-buffered saline, and incubated with the primary antibody in 1 % (w/v) gelatin/0.05 % Tween 20 in buffer for 1 h. The blot was washed twice with 0.05 % Tween 20 in buffer and incubated for 1 h with the secondary antibody, alkaline phosphatase-conjugated goat anti-(mouse Ig) (Bio-Rad, 1 in 3000 dilution). After washing, the colour was developed with 20 mM Tris/20 mM NaCl/1 mM MgCl₂/0.04 µM *p*-Nitroblue tetrazolium chloride/3.8 µM 5-bromo-4-chloro-3-indoylphosphate (pH 9.5) for 5 to 10 min. The reaction was stopped by washing with water. For SP-B the primary antibody was a monoclonal anti-SP-B antibody, raised against natural human SP-B, which recognizes the SP-B dimer. For SP-A a monoclonal anti-(canine SP-A) antibody was used.

For Western blots, SDS/12 % (w/v) polyacrylamide gels were developed according to Laemmli [19]. After electrophoresis the proteins were transferred to nitrocellulose using a Bio-Rad Trans-Blot Cell. Transfer was carried out at 4 °C, 100 V for 4 h using 25 mM Tris/192 mM glycine/20 % (v/v) methanol as a transfer buffer. SP-A was detected on the nitrocellulose as described for the dot-blot analysis using a polyclonal rabbit anti-(bovine SP-A) antibody as a primary antibody and alkaline phosphatase-conjugated goat anti-(rabbit IgG) as the secondary antibody.

Biophysical assay

Lipid extracts of cycled or non-cycled, large and small surfactant aggregates were obtained by chloroform extraction using the method of Bligh and Dyer and subsequent acetone precipitation [16,20]. Dried extracts were resuspended in 0.15 M NaCl/1.5 mM CaCl₂ 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 (Electronetics Corporation) as described by Enhörning [21]. With this technique a bubble is created in a surfactant suspension at 37 °C. After 10 s the bubble is pulsated between the maximum bubble radius (*R*_{max}) of 0.55 mm and the minimum bubble radius (*R*_{min}) of 0.4 mm at a rate of 20 pulsations/min. The pressure across the air-liquid interface is measured 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*_{max} and *R*_{min} were expressed.

Electrophoresis

Tricine/SDS/PAGE was used as described by Schägger and Von Jagow [22]. Equal amounts of a lipid extract of the surfactant preparations were electrophoresed under non-reducing conditions on a tricine/16 % (w/v) polyacrylamide gel. The gels were stained using silver stain (Bio-Rad).

RESULTS

The small and large aggregates in lung lavages are frequently separated using high-speed centrifugation [3,6,9]. However, for surface area cycling, aggregate separation has normally been performed by time consuming density centrifugation [11,14]. To test whether centrifugation at 40000 *g* could be used to separate the small and large aggregates after surface area cycling, samples of large surfactant aggregates were cycled for various periods of time. After cycling, the surfactant suspensions were centrifuged for 15 min at 40000 *g* and the phospholipid content of the supernatants and pellets were determined by phosphorus analysis. The results (Table 1) show a steady increase in the amount of phosphorus in phospholipid in the supernatant with increasing

Table 1 The effect of surface area cycling on the amounts of phosphorus recovered in the 40 000 *g* supernatant and pellet

Samples were surface-area-cycled for various amount of time. All samples were kept at 37 °C for the entire 180 min period. The centrifugation and phosphorus analysis were performed as described in the Materials and methods section. Values are given as means $\pm$ S.E.M.

Time (min)	Phosphorus content (μ g)	
	Small aggregates	Large aggregates
0	2.05 $\pm$ 0.14	12.92 $\pm$ 2.8
15	2.73 $\pm$ 0.21	10.78 $\pm$ 0.14
30	3.95 $\pm$ 0.17	10.34 $\pm$ 0.88
60	5.61 $\pm$ 0.41	6.95 $\pm$ 0.59
120	9.86 $\pm$ 0.57	5.30 $\pm$ 0.16
180	11.20 $\pm$ 0.43	3.73 $\pm$ 0.30

time of cycling and a corresponding steady decrease in phospholipid in the pellet.

To validate the centrifugation method for aggregate separation after surface area cycling, the results obtained by this method were compared with those of the density centrifugation method used in previous studies. Large surfactant aggregates were cycled for 180 min. Non-cycled and cycled samples, as well as the supernatant obtained after a 40 000 *g* spin of cycled surfactant, were centrifuged on sucrose gradients. Figure 1 shows the phosphorus content in the different fractions of these gradients. Surfactant that has not been cycled consists mainly of large aggregates (density 1.07–1.06), while after cycling there is a marked increase in small aggregates (density 1.064–1.04). The supernatant obtained by 40 000 *g* centrifugation after cycling contains lipid phosphorus with the same density range as the

Figure 1 Sucrose density gradient centrifugation of 40 000 *g* supernatant, non-cycled and cycled large aggregates

The 40 000 *g* supernatant analysed by sucrose gradient was obtained from the centrifugation of a surface area cycled large aggregate sample. The supernatant analysed was derived from a cycled sample with the same amount of phosphorus as the cycled and the non-cycled preparations.

small aggregates peak of cycled surfactant. The percentage of phosphorus in small aggregates after cycling was found to be similar with the two methods, 59 % for the gradient method and 58 % for the centrifugation method.

The 40 000 *g* centrifugation procedure for separating large and small aggregates was further validated by electron microscopy. Figure 2 shows the morphological difference between large and small surfactant aggregates. Dense lipid structures, tubular myelin and large vesicles are observed in the large aggregates (Figure 2a), while small aggregate fractions obtained after cycling

Figure 2 Electron micrographs of large and small surfactant aggregates

(a) Large aggregates, obtained from a non-cycled sample. (b) Small aggregates, obtained from a 40 000 *g* supernatant after surface area cycling of large surfactant aggregates. Representative areas were photographed and printed at a final magnification of 3545 $\times$.

Table 2 The effect of incubation at 37 °C and subsequent surface area cycling in the presence (+) and absence (—) of 10 mM PMSF on the formation of small aggregates as determined by centrifugation at 40 000 *g*.

Time (h)		Presence of PMSF		40 000 <i>g</i>	Phospholipid in small aggregates (% of total)
Incubation	Cycling	Incubation	Cycling		
6	0	—	—	—	7
3	3	—	—	—	63
3	3	—	—	+	64
3	3	—	+	+	15
3	3	+	+	+	12

Figure 3 The effect of surface area cycling on the biophysical activity of lipid extracts of canine surfactant

(a) Percentages of total phospholipid in small (closed bars) and large (open bars) aggregates before and after surface area cycling. (b) Biophysical activities of lipid extracts of non-cycled (circles) and cycled (squares) surfactant on the pulsating-bubble surfactometer. Samples were analysed at a concentration of 5 mg of phospholipid/ml at 20 pulsations/min. The surface tension at R_{min} is expressed by the open symbols and the surface tension at R_{max} is expressed by the closed symbols.

contain only small vesicles and appear relatively uniform (Figure 2b).

The effect of incubation and cycling with the protease inhibitor PMSF on surfactant conversion is shown in Table 2. Large aggregates were incubated for 3 h at 37 °C, and then cycled for 3 h at 37 °C. PMSF was added either before the incubation, before the cycling or after cycling. Small and large aggregates were separated by centrifugation at 40 000 *g* and measured by

Figure 4 Biophysical activities of lipid extracts of large and small surfactant aggregates

Large aggregates were obtained as a 40 000 *g* pellet from non-cycled (circles) and cycled (squares) samples, small aggregates (triangles) were obtained from the 40 000 *g* supernatant of the cycled sample. Samples were analysed at a concentration of 5 mg of phospholipid/ml at 20 pulsations/min. (R_{min} = open symbols, R_{max} = closed symbols).

phosphorus analysis. The results (Table 2) show inhibition of conversion by PMSF when the protease inhibitor is present during cycling. Preincubation in the presence or absence of PMSF does not affect the results. Addition of the protease inhibitor after cycling does not influence the amount of small aggregates generated.

The biophysical activities of lipid extracts of cycled and control surfactant examined with the pulsating-bubble surfactometer are shown in Figure 3. Non-cycled surfactant contains about 98 % large surfactant aggregates, whereas cycled surfactant contains 50 % large and 50 % small aggregates (Figure 3a). Low surface tension values were obtained within 50 pulsations with lipid extracts of non-cycled surfactant (Figure 3b), whereas lipid extracts of cycled surfactant do not reach near zero values within 50 pulsations.

To observe whether the difference between the biophysical activity of non-cycled and cycled surfactant is related to the generation of small aggregates, small and large aggregates were assayed with the pulsating-bubble surfactometer. Large and small aggregates were isolated by 40 000 *g* centrifugation. Lipid extracts of large aggregates from both non-cycled and cycled surfactant rapidly reduce the surface tension to near zero values (Figure 4). Small aggregates exhibit very poor surface-tension-reducing activity.

Lipid extracts of surfactant contain the surfactant lipids and SP-B and SP-C, but do not contain SP-A. The lipid profile of non-cycled and cycled, large aggregates and small aggregates appeared to be similar by t.l.c. (results not shown). Immunoblots detected the presence of SP-A in both large and small aggregates isolated by 40 000 *g* centrifugation. However, SP-B was only detected in large aggregates (results not shown).

A more detailed analysis of the effect of surface area cycling on SP-A and SP-B using a sucrose gradient is shown in Figure 5. Small and large aggregates from non-cycled and cycled surfactant were separated by centrifugation on a sucrose gradient. After fractionation, aliquots of each fraction were used for phosphorus analysis. Immunoblot analysis for SP-A and SP-B were performed on every second fraction. As shown in Figure 5(a), non-cycled surfactant consists mainly of large aggregates in which

Figure 5 Sucrose density gradient separation and dot-blot analysis before and after surface area cycling

Surfactant small and large aggregates were separated on a sucrose gradient. The gradient was fractionated and analysed for phosphorus and the presence of SP-A and SP-B. (a) Non-cycled surfactant (20 μ g of phosphorus); (b) cycled surfactant (20 μ g of phosphorus).

Figure 6 Western-blot analysis for SP-A before and after surface area cycling

Lane 1, purified bovine SP-A; lane 2, non-cycled surfactant; lane 3, cycled surfactant; lane 4, large aggregates from non-cycled surfactant; lane 5, large aggregates from cycled surfactant; and lane 6, small aggregates from cycled surfactant.

both SP-A and SP-B are detected. After cycling the surfactant mainly consists of small aggregates (Figure 5b). Immunoblots detected little or no SP-B in these fractions, SP-A on the other hand was detected in the small-aggregate fractions (Figure 5b).

Figure 6 shows a Western-blot analysis of SP-A before and after cycling, as well as from large and small aggregates. In all fractions the apparent molecular mass of the immunoreactive products is similar to purified SP-A.

Figure 7 Tricine gel electrophoresis of lipid extracts (100 μ g of phospholipid) before and after surface area cycling

Lipid extracts were dried under nitrogen, resuspended in non-reducing sample buffer and electrophoresed on a 16% (w/v) tricine gel. Canine SP-B migrates with an apparent molecular mass of 17 kDa. (a) Total lipid extract of non-cycled (NC) and cycled (C) large aggregates. (b) Lipid extracts of small aggregates (SA) and large aggregates (LA) obtained after surface area cycling.

PAGE of equal amounts of lipid-extract surfactant of non-cycled (14% small aggregates) and cycled (67% small aggregates) samples revealed a marked reduction in the amount of SP-B present in the cycled surfactant sample (Figure 7a). This decrease in SP-B relative to phospholipid can be attributed to the formation of small aggregates, as these do not contain any visibly detectable SP-B (Figure 7b). Control experiments using lipid extracts of large aggregates detected SP-B bands of equal intensity before and after cycling. This indicates that the loss of SP-B is not the result of SP-B binding to the plastic tube.

DISCUSSION

Pulmonary alveolar surfactant has several different physical forms, such as lamellar bodies, tubular myelin and vesicles [3,4]. Some of these different forms of surfactant can be separated according to their buoyant densities. Surface area cycling represents an *in vitro* system with which the conversion from large to small surfactant sub-types can be studied [11].

We have changed the time-consuming sucrose gradient separation for a single 40000 *g* centrifugation for separating large and small aggregates. This method has been validated by morphological analysis and by examining the density of 40000 *g*-isolated small aggregates on a sucrose gradient. The density observed for small aggregates in this study is slightly higher than in previous reports [11,12,14]. Whether the difference between our observations and those in other studies arises from species specificity or procedural differences is not known. However, the morphology of the small-aggregate fraction clearly shows the presence of small vesicles and a lack of tubular myelin or myelin-like structures.

Advantages of the 40000 *g* centrifugation method include a dramatic decrease in centrifugation time as well as the ability to analyse a greater number of samples, as a single phosphorus analysis can be used to determine the amount of small aggregates generated by cycling. However, in contrast with sucrose-gradient centrifugation, this method of separation does not differentiate between the heavy and ultra-heavy surfactant subtypes. Therefore, although 40000 *g* centrifugation can be used for rapid analysis of large to small surfactant aggregate conversion, sucrose-density centrifugation remains the method of choice for

a more detailed analysis and for studies on ultra-heavy surfactant sub-types.

A serine protease, named convertase, has been reported to be involved in the conversion of large to small surfactant aggregates *in vitro* [12]. We have tested whether or not convertase activity is dependent on the change in surface area. If convertase cleaves its target protein only during surface area cycling, cycling in the presence of inhibitor would result in inhibition of conversion regardless of the preincubation conditions. Alternatively, if convertase cleaves its target without cycling and in doing so produces a surfactant that can convert into small aggregates, incubation in the absence of inhibitor followed by cycling in the presence of inhibitor would still lead to the formation of small aggregates. Preincubation in the presence or absence of PMSF did not affect the extent of inhibition of aggregate conversion by PMSF during cycling. This suggested that for aggregate conversion to occur the protease activity was necessary during surface area cycling.

It has been reported that small surfactant sub-types, isolated from lung lavages of normal lungs, possess poor surface activity both *in vitro* and *in vivo* [7,9a]. Our biophysical studies using lipid extracts show that large aggregates subjected to surface area cycling lose some of their surface-tension-reducing ability. This effect was presumably related to the generation of small aggregates, as both cycled and non-cycled large aggregates reduce surface tension rapidly. Similar to small aggregates isolated from lung lavages, small aggregates generated *in vitro* do not reduce surface tension to low values. Lipid extracts contain surfactant lipids and the two hydrophobic surfactant-associated proteins B and C. These hydrophobic proteins appear to be important for the surface-tension reduction by increasing the adsorption and lipid squeeze-out of the monolayers [23,24]. Since lipid-extract surfactant was used in the biophysical assays, the differences observed cannot be attributed to serum protein inhibition or to changes in SP-A content. Since no changes in lipid composition were observed after cycling and a serine protease has been implicated in conversion, we hypothesized that the change in biophysical activity could be due to degradation of one of the hydrophobic proteins. This was investigated by dot-blot analysis after sucrose-gradient separation and gel electrophoresis.

Both the water-soluble SP-A and hydrophobic protein SP-B were analysed. Whereas SP-A was detectable in both large and small aggregates, immunoreactive SP-B was only present in large aggregates. This suggested that SP-B was degraded during surface area cycling. SP-B was not degraded during incubation at 37 °C without surface area cycling, suggesting that the change in surface area was necessary for exposing SP-B to convertase activity. The antibody used for the detection of SP-B was a monoclonal antibody which recognizes the SP-B dimer. Therefore, the loss of immunoreactive SP-B after surface area cycling observed with this antibody could be due to a single cleavage of SP-B.

PAGE confirmed the immunological observations. The intensity of the 17 kDa SP-B band was reduced after surface area cycling. The decrease in SP-B levels appeared to be correlated to the formation of small aggregates which do not contain detectable SP-B. Breakdown products of SP-B were not observed with this system.

Interestingly, SP-A remains associated with small aggregates after surface area cycling. This SP-A could be important for the re-uptake of these lipids by type-II cells [25]. Studies on the relative rates of re-uptake of small aggregates compared with other forms of surfactant such as large aggregates or large vesicles could be informative.

The observation that SP-B was degraded was somewhat

surprising in view of the studies by Higuchi et al. who were able to block aggregate conversion by adding SP-A to large aggregates [14]. However, this blocked conversion could be accounted for by the formation of ultra-heavy surfactant aggregates. SP-A degradation was also suggested by earlier studies that showed small aggregates obtained from lung lavages contain relatively less SP-A than large surfactant aggregates [6], in these studies SP-B was not measured.

From the results reported here we hypothesize that during the increase in surface area surfactant adsorbs to the air-liquid interface. The process of adsorption exposes SP-B to convertase, however, in large aggregates SP-B is inaccessibly packed in the lipid structures. During the subsequent decrease in surface area the SP-B-depleted surfactant would form small aggregates. When convertase is either absent or inhibited, the surfactant adsorbed to the air-liquid interface can reform into large aggregates upon the decrease of surface area. Conversely, when convertase is increased, or its inhibitors decreased, the formation of small aggregates might occur faster. This hypothesis could be extended to the situation *in vivo* in which the respiration would cause the change in surface area. This would explain the results by Spain et al. who observed mainly large aggregates before, and the generation of small aggregates after, the onset of air-breathing in rats [8].

In several lung-injury models the ratio of small to large surfactant aggregates is increased [9,9a]. Although not measured in those studies an increase in neutrophil elastase has been observed in adult respiratory distress syndrome [26,27]. Pison et al. reported that incubation of surfactant preparations with neutrophil elastase can affect the biophysical activity of surfactant, presumably by cleavage of SP-A and one or both of the hydrophobic proteins [28]. This raises the possibility that in lung injury other proteases, such as neutrophil elastase, can contribute to an increased conversion of large aggregates into small aggregates. Reconstitution of purified surfactant with pure proteases in combination with surface area cycling could be used to investigate this possibility further.

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