Surfactant-associated Protein A is important for maintaining surfactant large-aggregate forms during surface-area cycling

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Alveolar surfactant can be separated into two major subfractions, the large surfactant aggregates (LAs) and the small surfactant aggregates (SAs). The surface-active LAs are the metabolic precursors of the inactive SAs. This conversion of LAs into SAs can be studied *in vitro* using a technique called surface-area cycling. We have utilized this technique to examine the effect of trypsin on aggregate conversion. Our results show that trypsin increases the conversion of LAs into SAs in a concentration- and time-dependent manner. Immunoblot analysis revealed that surfactant-associated Protein A (SP-A) was the main target of trypsin. To examine further the role of SP-A in aggregate conversion, we tested the effect of Ca^{2+} and mannan on this process. The absence of Ca^{2+} (1 mM EDTA) and the presence of mannan both increased the formation of SAs. Electron microscopy revealed that highly organized multilamellar and tubular myelin structures were present in samples that converted slowly to SAs. We concluded that SP-A is important for maintaining LA forms during surface-area cycling by stabilizing tubular myelin and multilamellar structures.

INTRODUCTION

Pulmonary surfactant isolated from lung lavages consists of two major subfractions: the large surfactant aggregates (LAs) and the small surfactant aggregates (SAs) [1]. These subfractions differ in morphological appearance, buoyant density, surfactant protein composition and surface activity [2–6]. The LA fraction contains multilamellar structures and the unique lattice-like structure called tubular myelin. The LAs also contain the surfactant-associated proteins SP-A, SP-B and SP-C, and represent the surface-active form of alveolar surfactant. The SA subfraction on the other hand consists of small vesicles, contains lower amounts of the surfactant-associated proteins and is not capable of reducing the surface tension when tested either *in vitro* or *in vivo* [3,7].

The metabolic relationship between these two subfractions has been studied *in vivo* by pulse–chase experiments [5,6]. These studies showed that the larger heavier subfractions (LAs) are the metabolic precursors of the smaller lighter surfactant subtypes (SAs). This conversion of LAs into SAs *in vivo* can be reproduced and studied *in vitro* using a technique known as surface-area cycling [8]. The SAs formed during this process are morphologically similar to those obtained from lung lavage [4,8]. Furthermore SAs formed via surface-area cycling, like the SA fraction obtained from lung lavage [4,9], do not reduce the surface tension to low values. Gross and Schultz [10] have reported the requirement for serine protease activity for aggregate conversion *in vitro*. This protease has been tentatively identified as a 76 kDa glycoprotein and has been named convertase [11]. More recent studies have shown that the target for convertase appears to be SP-B [4].

The importance of the surfactant subfractions in lung function has become apparent from studies using animal models of acute lung injury [1,12–14], which have shown that the ratio of SAs to LAs is increased in injured lungs compared with normal lungs. Moreover, this relative increase in the inactive form (i.e. SAs) of surfactant is thought to contribute to surfactant and lung dysfunction associated with acute lung injury. Subsequent surface-area-cycling experiments indicate that an increased rate of LA into SA conversion is, at least partly, responsible for the observed increase in the SA/LA ratio of alveolar surfactant isolated from injured lungs [15].

In the current study we have utilized a bovine natural surfactant preparation to study further the mechanisms of aggregate conversion *in vitro*. Knowledge of these mechanisms might lead to a better understanding of the manner in which conversion is altered in lung injury and ultimately lead to therapeutic interventions aimed at maintaining surfactant in the LA forms.

MATERIALS AND METHODS

Surfactant preparation and reconstitution

Natural bovine surfactant was prepared as described previously [16]. The lyophilized powder was resuspended in 150 mM NaCl/1.5 mM $CaCl_2$ and centrifuged at 40000 ***g*** for 15 min at 4 °C. The pellet, designated natural surfactant, was resuspended in conversion buffer (0.15 M NaCl, 10 mM Tris/HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM EDTA, pH 7.4) and frozen in aliquots at −20 °C.

Surface-area-cycling experiments

Natural surfactant was 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

Abbreviations used: LA, large surfactant aggregate; SA, small surfactant aggregate; SP, surfactant-associated protein.

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to a rotator (Roto-torque rotator, Cole-Parmer Instruments, Laval, Quebec, Canada). 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 during each cycle [4,8]. 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.

For experiments involving surface-area cycling with trypsin, trypsin (Gibco, Toronto, Ont., Canada) was dissolved in conversion buffer and added to the surfactant samples at a final concentration of 0.2 mg/ml (unless otherwise specified in the text).

To investigate the effect of Ca^{2+} on aggregate conversion, natural surfactant samples were surface-area-cycled in a Ca^{2+}-free conversion buffer which contained either 1 mM EDTA or 1 mM EGTA. In addition, conversion characteristics of surfactant samples in conversion buffer containing 20 mM $CaCl_2$ were also studied.

The importance of the carbohydrate-binding site of SP-A was studied using mannan (Sigma, St. Louis, MO, U.S.A.). Mannan was dissolved in conversion buffer, the pH was adjusted to 7.4 and the mannan was added to the surfactant samples to the concentrations specified in the text.

Separation of LAs and SAs

After the cycling or incubation period, LAs and SAs were separated by centrifugation at 40000 ***g*** for 15 min at 4 °C [4]. The total phospholipid of the LA fraction (pellet) and the SA fraction (supernatant) were determined by lipid extraction [17] and subsequent phosphorus analysis [18].

Biophysical assays

Surfactant samples were resuspended in 0.15 M NaCl/1.5 mM $CaCl_2$ to a final concentration of 2.5 mg of phospholipid/ml. The surface-tension-reducing activity of the samples was assessed using a pulsating bubble surfactometer (Electronetics Corporation) as described by Enhorning [19]. 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 minimum bubble radius ($R_{min.}$) of 0.4 mm at 36 °C. The pressure across the air/liquid interface is monitored by a pressure transducer. Surface tension was calculated using 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 $R_{min.}$ and $R_{max.}$ were expressed.

Electrophoresis and Western-blot analysis

For SP-A, SDS/12 % polyacrylamide gels were developed by the method of Laemmli [20]. For SP-B, electrophoresis was performed using 16 % Tricine/SDS/PAGE as described by Schägger and Gebhard [21]. After electrophoresis the proteins were transferred to nitrocellulose using a Bio-Rad semi-dry transfer apparatus (Bio-Rad Laboratories, Mississauga, Ont., Canada). Transfer was carried out at 13 V for 30 min. The gels, nitrocellulose and filter paper were prewetted with 25 mM Tris/192 mM glycine. For the Western blotting, the nitrocellulose was blocked for 30 min with 3 % gelatin in Tris-buffered saline (20 mM Tris/500 mM NaCl, pH 7.5), washed with 0.05 % Tween 20 in buffer and incubated with the primary antibody in 1 % gelatin/0.05 Tween 20 in buffer for 1 h. The primary antibodies were a polyclonal anti-(bovine SP-A) antibody (R362) and a monoclonal anti-(human SP-B) antibody (1B9). 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 IgG (1:3000 dilution). After washing, the colour was developed with buffer containing 20 mM Tris/HCl, pH 9.5, 20 mM NaCl, 1 mM $MgCl_2$, 0.04 μM *p*-Nitroblue Tetrazolium chloride and 3.8 μM 5-bromo-4-chloro-3-indolyl phosphate, for 5–10 min. The reaction was stopped by washing with water.

Morphological studies

Natural surfactant suspensions were fixed in glutaraldehyde (2.5 % final concentration) in the conversion buffer [22]. 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 then 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. Transmission electron micrographs were made of representative areas.

RESULTS

The effects of the addition of trypsin to natural surfactant on aggregate conversion is shown in Figure 1. The effects of adding different concentrations of trypsin to natural surfactant on SA formation is shown in Figure 1(a). Whereas only 21.7 % of this surfactant preparation was converted into SAs during 3 h of surface-area cycling, the addition of trypsin to the natural surfactant led to a dose-dependent increase in SA formation. Incubation of natural surfactant at 37 °C with the highest concentration of trypsin (0.25 mg/ml) but without surface-area

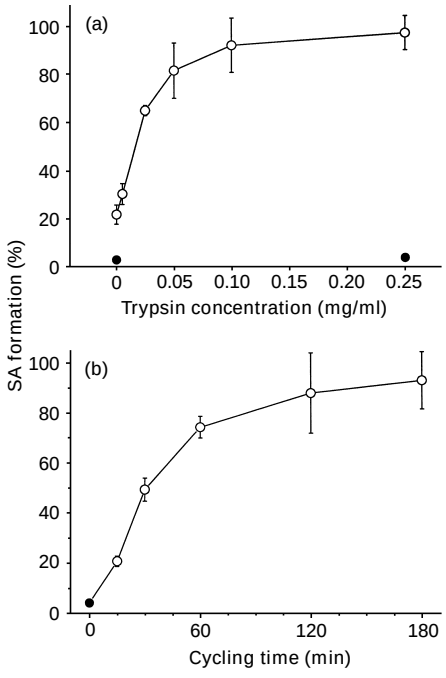

Figure 1 Effect of trypsin on aggregate conversion of natural surfactant

(**a**) Effect of different concentrations of trypsin on SA formation by surface-area cycling; (**b**) effect of surface-area-cycling time on SA formation by natural surfactant plus trypsin (0.2 mg/ml). ○, Cycled; ●, non-cycled. Values are means ± S.E.M. of three experiments.

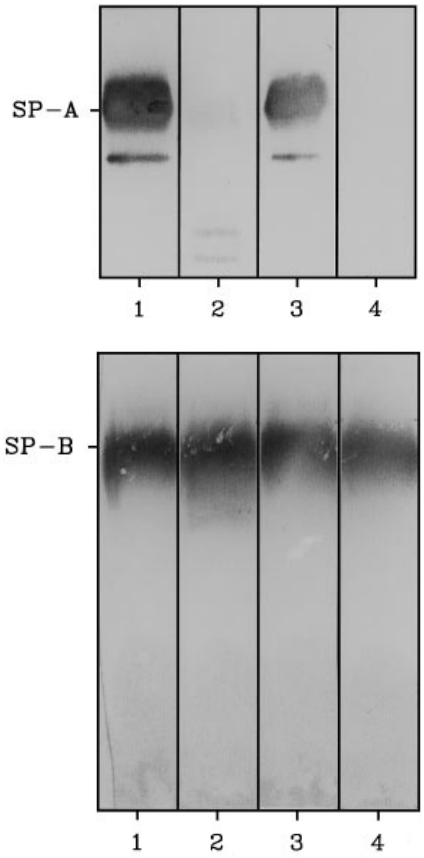

Figure 2 Effect of trypsin on the levels of immunoreactive SP-A and SP-B

Natural surfactants with and without incubation with trypsin (0.2 mg/ml) for 3 h at 37 °C were analysed. Proteins were separated by gel electrophoresis, transferred to nitrocellulose and blotted with antibodies against SP-A (top) or SP-B (bottom). Equal amounts (50 μg of phospholipid) were loaded on the gels. Lane 1, natural surfactant sample not incubated with trypsin; lane 2, natural surfactant sample incubated with trypsin; lane 3, natural surfactant sample after surface-area cycling (3 h, 37 °C); lane 4, natural surfactant sample incubated with trypsin after surface-area cycling.

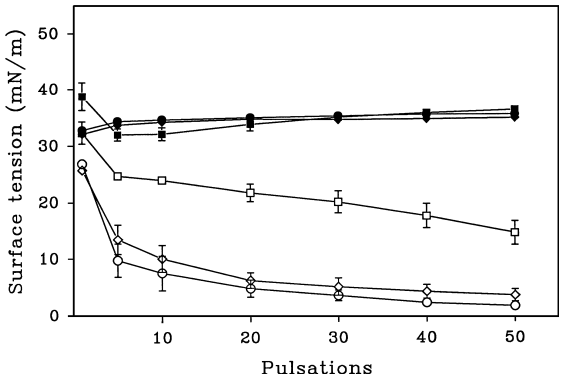

Figure 3 Effect of trypsin on the surface activity of natural surfactant

Surface activity of natural surfactant (○, ●), natural surfactant incubated for 3 h at 37 °C with trypsin (□, ■) and natural surfactant incubated for 3 h at 4 °C with trypsin (◇, ◆) (0.2 mg/ml) was analysed on a pulsating bubble surfactometer. Samples were analysed at 5 mg/ml. Means ± S.E.M. of three separate analyses are shown. Open symbols represent surface-tension values at minimum bubble size and filled symbols represent the values at maximum bubble size.

Table 1 Effect of Ca^{2+} on aggregate conversion

Natural surfactant samples were surface-area-cycled for 3 h at 37 °C in buffer containing 1 mM Ca^{2+}, 1 mM EDTA/no Ca^{2+}, 1 mM EGTA/no Ca^{2+} or 20 mM Ca^{2+}. Values are means ± S.E.M. of three experiments.

	SA formation (%)	
	Non-cycled	Cycled
1 mM Ca^{2+}	1.2 ± 0.4	38.1 ± 2.6
1 mM EDTA/no Ca^{2+}	15.2 ± 2.7	64.3 ± 6.3
1 mM EGTA/no Ca^{2+}	8.3 ± 4.2	69.4 ± 8.8
20 mM Ca^{2+}	0.3 ± 0.2	20.6 ± 4.0

cycling did not lead to SA formation. Figure 1(b) shows the percentage of SA formed from natural surfactant plus trypsin (0.1 mg/ml) after different cycling times. The formation of SAs increased steadily over time so that after 3 h of cycling almost all the LAs were converted into SAs.

The effects of adding trypsin to natural surfactant with or without surface-area cycling for 3 h at 37 °C on the levels of immunoreactive SP-A and SP-B are shown in Figure 2. Natural surfactant not incubated with trypsin contains both glycosylated and a small amount of unglycosylated SP-A (Figure 2, top, lane 1). SP-A dimer is also detected. Incubation with trypsin resulted in a profound decrease in detectable SP-A (Figure 2, top, lane 2). After surface-area cycling, natural surfactant without trypsin still contained significant levels of SP-A (lane 3), whereas the sample cycled in the presence of trypsin contained no detectable levels of SP-A (lane 4). On the other hand, it appeared that the level of SP-B was not significantly affected by the incubation with trypsin or by surface-area cycling (Figure 2, bottom).

Incubation of natural surfactant with trypsin also affected the surface activity of the LAs. Figure 3 shows the surface-tension-reducing abilities of natural surfactant, natural surfactant incubated with trypsin at 37 °C for 3 h and natural surfactant incubated with trypsin at 4 °C. Natural surfactant reduced the surface tension to values less than 10 mN/m within 30 pulsations. Surfactant incubated with trypsin at 37 °C, on the other hand, did not produce low surface tension even after 50 pulsations. This impaired activity of the trypsin-incubated samples at 37 °C was not due to direct inhibition by the trypsin protein itself, as surfactant samples incubated with trypsin at 4 °C were capable of reducing the surface tension to low values. The surface activity of surface-area-cycled material was not investigated because of difficulties of obtaining the high concentrations of material necessary for analysis on the pulsating bubble surfactometer.

Table 1 shows the effect of EDTA, EGTA and Ca^{2+} on the conversion of LAs into SAs during surface-area cycling. In the absence of Ca^{2+} (EDTA and EGTA samples) there was a significant ($P < 0.05$) increase in the formation of SAs during surface-area cycling compared with samples at the standard (1 mM) Ca^{2+} concentration. When the Ca^{2+} concentration was increased to 20 mM, on the other hand, there was a decrease in conversion rate compared with that observed with the standard Ca^{2+} concentration, although this difference did not reach statistical significance.

The morphologies of the natural surfactant (1 mM Ca^{2+}), natural surfactant samples incubated with trypsin (3 h, 37 °C), surfactant incubated with 20 mM Ca^{2+} and surfactant incubated

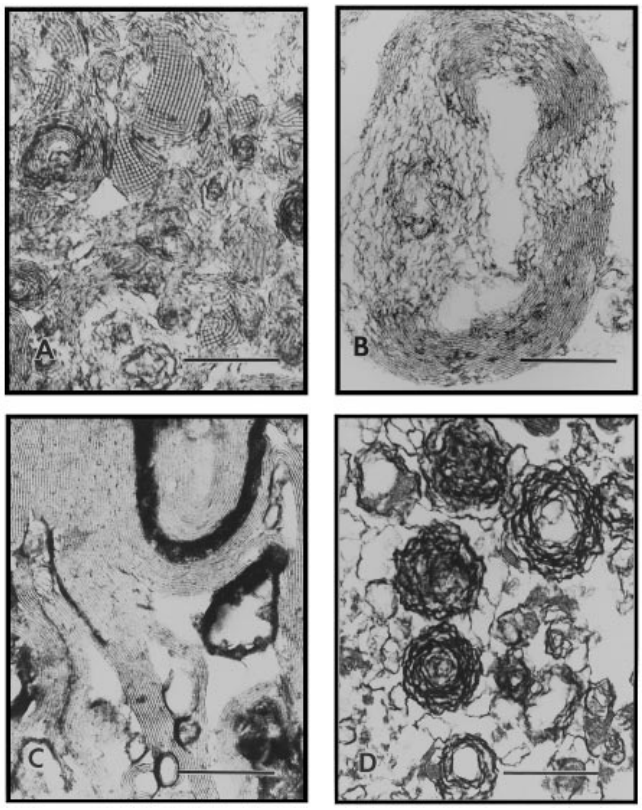

Figure 4 Effects of Ca^{2+} and trypsin on surfactant morphology

Representative electron micrographs of natural surfactant after incubation for 3 h at 37 °C under different conditions are shown: (**A**) standard conditions, 1 mM Ca^{2+}; (**B**) incubation with trypsin (0.2 mg/ml); (**C**) incubation with 20 mM Ca^{2+}; (**D**) incubation with 1 mM EDTA/no Ca^{2+}. The scale bar represents 1 μm.

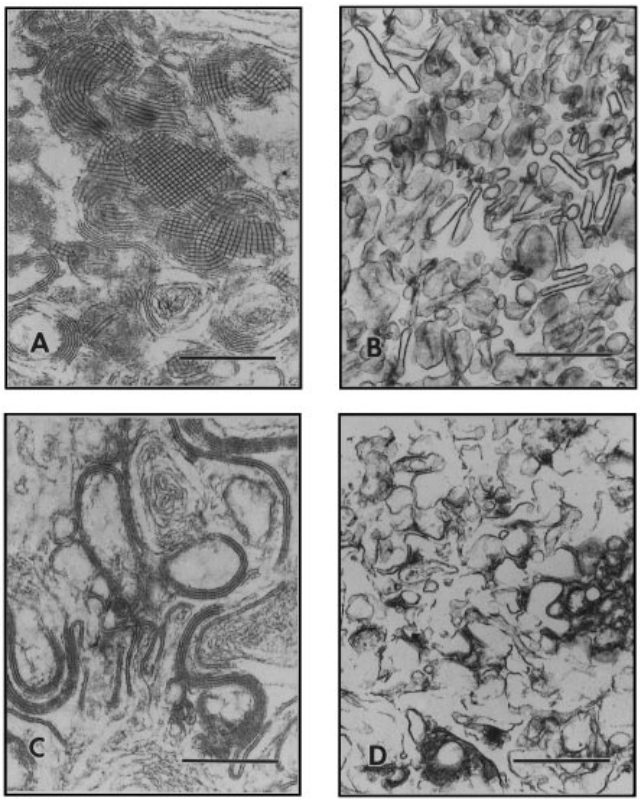

Figure 5 Effects of Ca^{2+} and trypsin on surfactant morphology after surface-area cycling

Representative electron micrographs of natural surfactant after surface-area cycling for 3 h at 37 °C under different conditions are shown: (**A**) standard conditions, 1 mM Ca^{2+}; (**B**) incubation with trypsin (0.2 mg/ml); (**C**) incubation with 20 mM Ca^{2+}; (**D**) incubation with 1 mM EDTA/no Ca^{2+}. The scale bar represents 1 μm.

with 1 mM EDTA (no Ca^{2+}) were examined by electron microscopy. Representative electron micrographs are shown in Figure 4. Natural surfactant (1 mM Ca^{2+}) contained abundant tubular myelin and multilamellar structures (Figure 4a). In contrast, these structures were markedly reduced in samples incubated with trypsin (Figure 4b) and completely absent in the presence of EDTA (Figure 4d) even though these samples were not cycled. Interestingly, the surfactant samples with the high Ca^{2+} concentration had less tubular myelin structure than the samples with 1 mM Ca^{2+} and consisted mostly of highly organized elongated multilamellar structures (Figure 4c).

Representative electron micrographs of samples obtained after surface-area cycling for 3 h at 37 °C are shown in Figure 5. Natural surfactant (1 mM Ca^{2+}) still contained mostly organized multilamellar and tubular myelin structures. It also contained a small amount of poorly organized lipid structures (Figure 5a). Surface-area cycling of natural surfactant in the presence of trypsin led to an abundance of small vesicular structures (Figure 5b). Natural surfactant in buffer containing a high Ca^{2+} concentration (20 mM) resulted in multilamellar lipid structures as well as poorly organized aggregated material after surface-area cycling (Figure 5c). The absence of Ca^{2+} from the surface-area-cycling buffer resulted in small vesicular structures after surface-area cycling.

Table 2 shows the effect of different concentrations of the complex carbohydrate, mannan, on the conversion of LAs into SAs. There was a concentration-dependent increase in the formation of SAs during surface-area cycling in the presence of mannan. The increase was statistically significant for samples cycled in the presence of 5 mg of mannan/ml ($P < 0.05$) and 12.5 mg of mannan/ml ($P < 0.01$). The presence of 12.5 mg of mannan/ml without surface-area cycling did not lead to the formation of SAs. The morphological appearance of natural surfactant in the presence of 12.5 mg/ml mannan (non-cycled) was similar to that of natural surfactant alone (results not shown). After surface-area cycling of natural surfactant samples

Table 2 Effect of mannan on aggregate conversion of natural surfactant

Different concentrations of mannan were added to natural surfactant before cycling. The percentage of SAs formed without surface-area cycling or during surface-area cycling for 3 h is shown. Values are means ± S.E.M. of three separate experiments. n.d., Not determined).

Mannan concentration (mg/ml)	SA formation (%)	
	0 h	3 h
0	2.5 ± 0.9	35.4 ± 2.0
0.5	n.d.	38.2 ± 1.4
2.5	n.d.	41.9 ± 3.0
5	n.d.	49.6 ± 2.0
12.5	1.71 ± 0.4	54.0 ± 2.1

in the presence of mannan, both organized multilamellar structures and unorganized lipid vesicles could be observed (results not shown).

DISCUSSION

Surfactant in the alveolar space exists in different subfractions. The two major subfractions, LAs and SAs, can be separated by differential centrifugation [1]. LAs represent the surface-active form of surfactant and are precursors of the non-surface-active SAs [3]. The relative proportions of LAs and SAs within the airspace changes at birth [23], with exercise [24] and in acute lung injury [1,12–14].

Surface-area cycling is an *in vitro* technique used to study the conversion of LAs into SAs [8]. The effects of temperature, changes in surface area, serine protease activity, serum proteins and surfactant proteins on aggregate conversion have all been studied using the surface-area-cycling technique [4,8–10,22]. For example, Gross and Schultz [10] reported that serine protease activity is required for the conversion of LAs into SAs. In subsequent experiments, this protease was tentatively identified as a 75 kDa glycoprotein which was named convertase. The target for this protease appeared to be SP-B [4]. In the present study we utilized a highly purified surfactant preparation. The low amount of aggregate conversion in this preparation *in vitro* indicated a low level of convertase activity. We used this preparation to study the effects of factors other than convertase on the conversion of LAs into SAs.

Initially, we investigated whether proteases other than convertase could induce the formation of SAs. Preliminary experiments included incubating and surface-area cycling of natural surfactant with a variety of commercially available proteases (results not shown). On the basis of these results, trypsin was selected for further study. Trypsin induced the formation of SAs from natural surfactant in a time- and concentration-dependent manner (Figure 1). Immunoblot analysis and surface-activity studies suggested that the mechanism by which trypsin increased SA formation was via the degradation of SP-A (Figures 2 and 3). The importance of SP-A for the integrity of LA structures was also suggested in two previous studies. First, Higuchi et al. [15] showed that addition of SP-A to LAs isolated from rabbit lungs decreased their conversion rate with subsequent cycling. Second, reconstitution studies using purified SP-A and SP-B indicated that both these proteins are important for maintaining LA forms during surface-area cycling [22].

One of the reported properties of SP-A is its ability to aggregate lipids in a Ca^{2+} dependent manner [25]. Furthermore Haagsman et al. [25] showed that addition of the complex carbohydrate, mannan, could reverse the lipid aggregation induced by SP-A via competition for the carbohydrate-binding sites on SP-A. It was concluded that SP-A–SP-A interactions via the carbohydrate/lectin-binding sites were important for this lipid-aggregation process. In addition, it was suggested that this interaction might be involved in tubular myelin formation. Other evidence for the importance of SP-A in the tubular myelin structures has come from reconstitution studies using purified lipids and surfactant-associated proteins. In these studies SP-A, together with SP-B, phospholipids and Ca^{2+} were essential for the formation of tubular myelin [26,27]. In addition, immunocytochemical studies have demonstrated that SP-A is located in the corners of the tubular myelin structure [28].

To test whether SP-A-dependent lipid aggregation is an important factor in maintaining LA integrity we investigated the effects of removing Ca^{2+} and of adding mannan on SA formation. SA formation was increased in the presence of EDTA and in the presence of mannan. In addition, morphological analysis of the trypsin-incubated and EDTA-incubated samples revealed a significant reduction in tubular myelin and organized multilamellar structures. Together these results suggest that SP-A-dependent lipid aggregation is indeed important for the integrity of LAs, presumably by SP-A stabilizing the tubular myelin and multilamellar structures. We hypothesize that, in normal lungs, these structures provide a mechanism by which surfactant can adsorb to an expanding air/liquid interface during inspiration and reform into LA structures with the decrease in surface area during expiration.

In reviewing the data obtained from the various surface-area-cycling experiments reported to date, it would appear that several different mechanisms for aggregate conversion occur *in vitro*. For example, the conversion of LAs obtained from normal lungs is associated with degradation of SP-B by convertase. It is also apparent that the conversion of LAs into SAs occurs as the result of degradation of SP-A or decreased lipid aggregation, as reported in the present study. Furthermore Ueda and co-workers [9] recently reported an increased rate of SA formation after the addition of serum proteins to rabbit LAs before surface-area cycling. Although several different mechanisms may lead to SA formation *in vitro*, a change in surface area was found consistently to be necessary for the formation of SAs. For example, whereas incubation alone with a high concentration of trypsin did not lead to SA formation, surface-area cycling in the presence of trypsin caused the rapid formation of SAs. A change in surface area was also found to be necessary for aggregate conversion when serum proteins were added to rabbit LAs [9]. *In vivo*, a change in surface area would be accomplished via respiration. Indeed, three *in vivo* studies have demonstrated the effects of breathing on surfactant aggregate pool sizes. Whereas before birth most of the surfactant consists of LA forms, after the onset of respiration, i.e. a change in surface area, significant increases in the amounts of SAs are observed [23,29,30].

The physiological importance of surfactant aggregate forms present in the airspace has become apparent from observations of changed surfactant aggregates in several animal models of acute lung injury. In these studies a significant increase in the SA/LA ratio of surfactant isolated from injured compared with normal lungs was observed. These changes were proposed to contribute to surfactant and lung dysfunction in these animals [1,12–14]. Using the surface-area-cycling technique, it was observed that LAs obtained from injured lungs were converted more rapidly into SAs than those from normal lungs [15]. Based on the current study, it is possible that this increased conversion rate was due to decreased levels of SP-A. Although SP-A levels were not measured in the study of Higuchi et al. [15], there have been several reports of decreased levels of SP-A in the lavage material obtained from injured lungs [12,13]. If decreased levels of SP-A result in increased conversion of LAs into SAs in the airspace of the injured lungs, this could explain the increased SA/LA ratio that has also been observed in lung injury. This hypothesis is supported by previous observations. Decreased SP-A levels were associated with increased SA/LA ratios in the lung injury induced by experimental lung transplantation in dogs [13] and caecal-ligation-and-puncture-induced sepsis in adult sheep [12] and in humans with acute respiratory distress syndrome [31]. An alternative mechanism by which aggregate conversion might be increased in lung injury was suggested by Ueda et al. [9], who showed increased aggregate conversion *in vitro* after the addition of serum proteins to their LA fraction.

In addition to its role in maintaining LA integrity discussed in the current study, SP-A has also been implicated in a variety of other surfactant processes. These functions of SP-A include

enhancement of lipid adsorption [32], counteraction of blood protein inhibition of surfactant [33], formation of tubular myelin [26,27], a role in surfactant uptake and secretion from Type II cells [29] and a role in host defence mechanisms [34]. The variety of functions described for SP-A indicates that decreased concentrations of this protein in acute lung injury could significantly affect surfactant metabolism and function at several levels. It is interesting to note that current exogenous surfactant preparations used for surfactant supplementation in acute lung injury do not contain SP-A. This may partially explain the necessity to use high dosages of exogenous surfactant in lung injury since, in the absence of SP-A, the exogenous material might be more susceptible to protein inhibition and convert more rapidly to poorly functioning SAs.

In conclusion, several factors can influence the formation of SAs *in vitro*. The present studies provide some insight into the mechanisms responsible for the accelerated conversion of LAs into SAs in lung injury.

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