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A comparative study of mechanisms of surfactant inhibition

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Abstract

Pulmonary surfactant spreads to the hydrated air–lung interface and reduces the surface tension to a very small value. This function fails in acute respiratory distress syndrome (ARDS) and the surface tension stays high. Dysfunction has been attributed to competition for the air–lung interface between plasma proteins and surfactant or, alternatively, to ARDS-specific alterations of the molecular profile of surfactant. Here, we compared the two mechanisms in vitro, to assess their potential role in causing respiratory distress. Albumin and fibrinogen exposure at or above blood level concentrations served as the models for testing competitive adsorption. An elevated level of cholesterol was chosen as a known adverse change in the molecular profile of surfactant in ARDS. Bovine lipid extract surfactant (BLES) was spread from a small bolus of a concentrated suspension (27 mg/ml) to the air–water interface in a captive bubble surfactometer (CBS) and the bubble volume was cyclically reduced and increased to assess surface activity of the spread material. Concentrations of inhibitors and the concentration and spreading method of pulmonary surfactant were chosen in an attempt to reproduce the exposure of surfactant to inhibitors in the lung. Under these conditions, neither serum albumin nor fibrinogen was persistently inhibitory and normal near-zero minimum surface tension values were obtained after a small number of cycles. In contrast, inhibition by an increased level of cholesterol persisted even after extensive cycling. These results suggest that in ARDS, competitive adsorption may not sufficiently explain high surface tension, and that disruption of the surfactant film needs to be given causal consideration.

Keywords: Captive bubble surfactometer (CBS); Pulmonary surfactant; Fibrinogen; Serum albumin; Cholesterol; Polyethylene glycol (PEG); Bovine lipid extract surfactant (BLES)

1. Introduction

Pulmonary surfactant consists of about 40% w/w of the saturated phospholipid dipalmitoylphosphatidylcholine (DPPC) as well as unsaturated phospholipids, cholesterol (5–10% w/w) and surfactant associated proteins. It is secreted by the alveolar type II epithelial cells as lamellar bodies into the aqueous lining that covers the epithelium to the air [1–4]. Lamellar bodies expand into tubular myelin [5] which spreads to form a tightly packed molecular film at the lung's interface to the air [6–10]. The film counteracts the otherwise high surface tension (γ) of the interface (about 70 mN/m for a free air–water interface) and reduces it almost to 0 mN/m at residual lung capacity [11] when the film is most tightly packed. During inhalation γ rises as the lung expands, but stays below 10 mN/m during tidal breathing [12]. Low surface tension is needed for ease of breathing and to reduce the Laplace pressure preventing collapse of the alveoli. The area reduction associated with reaching near-zero surface tension during exhalation is minimal, indicating that in the healthy lung the compressibility of the surfactant film is very small [12]. Note that to our knowledge, absolute values of film compressibility in the lung have not been published to date. This is likely because the effective change in the interface during breathing cannot be directly measured and so compressibility is not readily obtainable.

Acute respiratory distress syndrome (ARDS) is a common and devastating spectrum of disease that has a high overall mortality rate. The published, population-based incidence of ARDS ranges from 1.5 to $5.3/10^5$ population/year and a mortality rate of 36% to 60% [13]. The strongest clinical evidence of a cause–effect relationship was identified for sepsis, aspiration, trauma, and multiple transfusions [13]. A central component of this condition is pulmonary surfactant inhibition

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and concomitant elevation of surface tension. In ARDS, inflammation gives rise to lipases and proteases, radical oxygen species (ROS) and other inflammatory mediators within lung tissue. Damage to the alveolar wall allows these compounds, together with blood–serum components, access to the alveolar space where they can impair pulmonary surfactant. Since surfactant impairment is a major factor in the morbidity and mortality of ARDS, it has been targeted by replacement surfactant therapy. However, success has been minimal (for a review, see [14]). This is likely because the exogenous surfactant also becomes inactivated in the affected lung. It is now generally believed that successful treatment will depend on an in-depth understanding of the impairment mechanisms.

Current understanding of surfactant inhibition has suggested two alternative mechanisms, one being competition for the interface [14–17]. According to this model, blood proteins, inflammation specific proteins or other surface active substances adsorb to the air–water interface and compete with the pulmonary surfactant for the interface. A high surface tension will then result from an absence of surfactant film over significant portions of this interface. The other model of surfactant inhibition attributes high surface tension to impairment of the surfactant film itself [18–24]. Small amphiphilic molecules such as free fatty acids, cholesterol and lysolipids are thought to associate with the surfactant film and render it dysfunctional [18–20]. The detrimental effect of surfactant lipid and protein oxidation by ROS also falls into this category of surfactant impairment [21–24].

In the current study, we compared the two alternative mechanisms of surfactant inhibition, competition for the interface versus impairment of the surfactant film itself in vitro under conditions which attempt to more adequately reflect those in the lung than the conditions chosen in earlier studies (see "Discussion"). The captive bubble surfactometer (CBS) was used for measuring the surface activity, because it comes closest to mimicking lung function as determined in vivo from pressure–volume studies [25]. The CBS consists of a chamber, filled with buffer, with an air bubble floating against a convex agarose ceiling. Varying the volume in the chamber alters bubble size changing the surface tension. Alterations in γ at the air–liquid interface in the CBS are seen as a flattening of the bubble as γ falls and a rounding as it rises [26]. We investigated films formed from clinically used bovine lipid extract surfactant (BLES) [27]. BLES contains all lipids of natural surfactant with the exception of cholesterol. We used BLES as is to reflect normal surfactant and after the addition of 20% w/w cholesterol to model the elevated amount found in diseased lungs [28,29]. We have found that this level of cholesterol, while not being the only neutral lipid change in diseased lung, produces marked inhibition of surfactant while lower physiological levels do not [19]. BLES also contains the two highly hydrophobic surfactant associated proteins SP-B and SP-C in natural proportions. To study the effect of serum proteins, we added either serum albumin or fibrinogen to the aqueous phase. The protein concentrations (40 mg/ml for albumin, 3 mg/ml for fibrinogen) were chosen to reflect what the alveolus might be exposed to in extreme ARDS. Albumin was also tested at 80 mg/ml. The protein was added either before or after a BLES film had been formed at the bubble air–water interface to assess the competing hypotheses. Surfactants were administered to the air–water interface in a small but concentrated (27 mg/ml phospholipids) aqueous bolus of surfactant [19,30]. We then observed the change in γ to see how serum proteins affected film formation. Next the bubble size was rapidly increased in size and changes in γ were followed to monitor the films' ability to incorporate additional material and then accommodate this addition. Thereafter the bubble size was slowly decreased and increased (quasi-static cycles), followed by rapid cycles (dynamic cycles). A similar experimental protocol was pursued with BLES containing 20% w/w cholesterol. Finally, we also explored whether the addition of PEG to the aqueous phase reversed the inhibition by cholesterol or albumin similar to the published findings for serum protein inhibition.

2. Materials and methods

2.1. Surface activity assessment

The surface activity of the surfactant was determined using a laboratory-built, fully computer controlled CBS evolved from the apparatus described earlier [7]. The chamber of the CBS was filled with a buffer solution (140 mM NaCl, 10 mM Hepes, and 2.5 mM CaCl, pH 6.9), with or without protein, as described below and a small (0.035–0.040 ml) bubble was allowed to float up to the chamber's concave agarose ceiling. Then, ~0.05 μl of the surfactant was deposited at the air–buffer interface by means of a transparent capillary. This allowed a precisely defined volume of surfactant to be deposited under visual control. The bubble was imaged by a video camera (Pulnix TM 7 CN) and recorded for later analysis. The chamber was kept at 37 °C during the experiment. A 5 min adsorption (film formation) period followed the introduction of the surfactant into the chamber during which the bubble was not manipulated and the change in γ was monitored. The chamber was then sealed and the bubble was rapidly (1 s) expanded to a volume of 0.13 ml. Five min after the bubble was expanded quasi-static cycling commenced. In the quasi-static portion of the experiment the bubble size was first reduced and then enlarged in a stepwise fashion by altering the internal volume of the chamber. Each step had two components; a 3 s change in volume followed by a 4 s delay when the chamber volume remained unchanged allowing the film to "relax". There was a 1 min inter-cycle delay between each of four quasi-static cycles and a further 1 min delay between the quasi-static and dynamic cycles. In the dynamic cycle portion of the experiment, the bubble volume was smoothly varied over the same range as the last quasi-static cycle for 20 cycles at a rate of 20 cycles/min. Bubble volume, interfacial area, and γ were calculated using height and diameter of the bubble as described [31].

2.2. Surfactant

BLES (a kind gift from the manufacturer BLES Biochemicals Inc, London Ontario see Yu et al. [27] for analysis of BLES composition) in non-buffered normal saline (pH 5–6) with calcium added and at a phospholipid concentration of 27 mg/ml was used as is for the protein-inhibition experiments or after the addition of cholesterol (Sigma Chemicals, St. Louis, MO) for the cholesterol-inhibition experiments. Cholesterol cannot be added reliably and quantitatively to BLES while in aqueous suspension. Therefore, BLES was first taken up in organic solvent, cholesterol added and the mixture returned into an aqueous solution as follows. A 1:1:1 ratio solution of methanol, chloroform, and BLES by volume was first vortexed and then spun at 100 g for 5 min. The chloroform phase contained most of the BLES and was saved. For complete recovery of BLES, the water/methanol phase was extracted a second time with chloroform and the two fractions of BLES in chloroform pooled. To this pool, cholesterol in chloroform was added to a final concentration of 20% w/w with respect to BLES phospholipids. The solution was dried under N_2 and re-suspended in buffer

(140 mM NaCl, 10 mM Hepes and 2.5 mM $CaCl_2$; pH 6.9) to obtain an aqueous suspension of BLES and cholesterol at a phospholipid concentration of 27 mg/ml.

2.3. The aqueous phase of the CBS

Sucrose, 10% w/w, was added to the buffer to increase its density so that the surfactant suspension would float to make and remain in contact with the bubble upon injection (see below). Adding sucrose does not affect surface activity of surfactant [19]. For the protein-inhibition experiments, proteins were added to the buffer either before, or after the surfactant was placed at the air–buffer interface of the CBS. The proteins remained present throughout all tests. Dry powders of bovine serum albumin (catalogue-number: A7906) and fibrinogen from human plasma (catalogue-number F4883) were purchased from Sigma-Aldrich. Dry serum albumin was dissolved in buffer to a final concentration of 40 mg/ml or 80 mg/ml and fibrinogen to a concentration of 3 mg/ml. In two instances, polyethylene glycol (PEG) was introduced into the chamber, after the surfactant had been injected. PEG (MW 6000, Fluka Chemicals Co., Switzerland) was dissolved in buffer and injected into the sample chamber to a final concentration of 25 mg/ml.

2.4. Representation of CBS results

Film formation was plotted as surface tension γ versus time. For quasi-static and dynamic cycles, surface tension (γ)–bubble area (A) isotherms were plotted and the film's two-dimensional compressibility (β) was also calculated, according to the following equation, to further describe the surfactant films' surface activity

$$\beta = (1/A) \cdot (\Delta A/\Delta\gamma).$$

In this equation A is the absolute bubble area at a given surface tension γ, and values for β at $\gamma = 10$ mN/m ($\beta_{\gamma 10}$) are given in the results.

2.5. Statistical analysis of CBS experiments

Nonparametric tests (SPSS 14.0) of differences in surface tension during film formation and in minimum surface tension attained in bubble cycling were performed with $P<0.05$ used as the standard for significance in all cases. The Freidman test for multiple related measures was used first to test for main within treatment effects and the Wilcoxon paired related measures test was used for subsequent orthogonal comparisons after overall significance was determined. The Kruskal–Wallis and the Mann–Whitney U tests for independent samples were similarly used to determine overall between treatment effects and to make subsequent specific orthogonal comparisons.

2.6. Electron microscopy

Five μl each of an aqueous suspension of either BLES of 27 mg/ml, BLES at 5 mg/ml, or BLES at 5 mg/ml plus PEG were put down onto a bare electron microscopy grid, excess of the suspension removed by filter paper and the grid injected into liquid ethane. Grids were transferred onto a cryo-holder and imaged in a Tecnai F-20 (FEI) at −100 °C without further staining.

3. Results

3.1. Film formation

Film formation was studied in two ways. As the first step in the experimental protocol, we injected the aqueous suspension of BLES close to the bubble from a transparent capillary (Fig. 1a). The surfactant then made contact with the bubble surface and the resultant drop in γ was observed over 5 min (Fig. 1b). The drop in surface tension after injection of surfactant as a function of time was indicative of the ability of surfactant to form a functional film at a clean or serum protein-covered interface. In the following sections, results are shown for adsorption of BLES onto a serum protein-free buffer and a buffer containing either serum albumin or fibrinogen. Finally, adsorption of BLES with 20% w/w cholesterol onto a serum-free buffer is described. Results on the adsorption of the films for all conditions are summarized in Table 1.

For all samples, upon making contact, the surfactant spread over the entire bubble and lowered the surface tension. However, the time course of film formation differed significantly for different treatments (Fig. 1b). Spreading BLES onto a protein-free buffer resulted in a drop of γ to between 35.0 and 30.0 mN/m within 0.15 s for all but three samples. Thereafter, γ dropped further to reach a value of 23.5 (±0.05) mN/m within 30 s (e.g. Fig. 1b, black curve). The equilibrium surface tension obtained with BLES is ~23.5 mN/m (e.g. [19]). Reaching this surface tension indicates that the process of film formation is complete. Hence, film formation could be seen as two processes with an initial extremely fast drop in γ reflecting surfactant material spreading from the point of contact to cover the interface. The following slower further drop in γ to equilibrium may reflect alterations in the surfactant film architecture after the surface has been covered.

Spreading BLES onto a serum protein containing buffer was chosen to test for the effect of competitive adsorption, by first giving the protein time to adsorb to the interface before injecting surfactant. The initial drop in surface tension was significantly slower under this condition. With serum albumin at the interface, the initial drop in γ to below 35.0 mN/m took longer than 0.15 s irrespective of whether the protein concentration was 40 or 80 mg/ml in the buffer. The results were more varied when the buffer contained fibrinogen. Here one sample dropped below 35.0 mN/m in less than 0.15 s and one took over 7 s to pass this threshold. Despite this initially slower surfactant adsorption, the time for these samples to reach equilibrium was not significantly different from those without protein in the buffer. Hence, surface coverage by surfactant appeared to have been slowed down by the presence of serum proteins, but after an initial coverage, film refinement progressed rapidly enough to obscure the effect by the time equilibrium was reached. Initial film formation was also statistically significantly slower when cholesterol was added to the surfactant, and surface tension took longer than 0.15 s to drop below the 35.0 mN/m criterion in all but one case. This slower initial spreading did not significantly affect the ability to reach equilibrium within 30 s with only one case failing to reach this criterion. The remaining sample then asymptotically approached equilibrium over the allotted 5 min (Fig. 1b, grey curve).

Next, the bubble was rapidly expanded to approximately double the size of the air–water interface and γ observed for another 5 min. Surfactant not only forms a single layer but spreads to the interface with patches of multiple surface associated layers when prepared as in the current study [19,32]. Adsorption of additional surfactant from this surface associated reservoir to the interface was thus observed upon rapid expansion. For films devoid of cholesterol the surface tension peaked

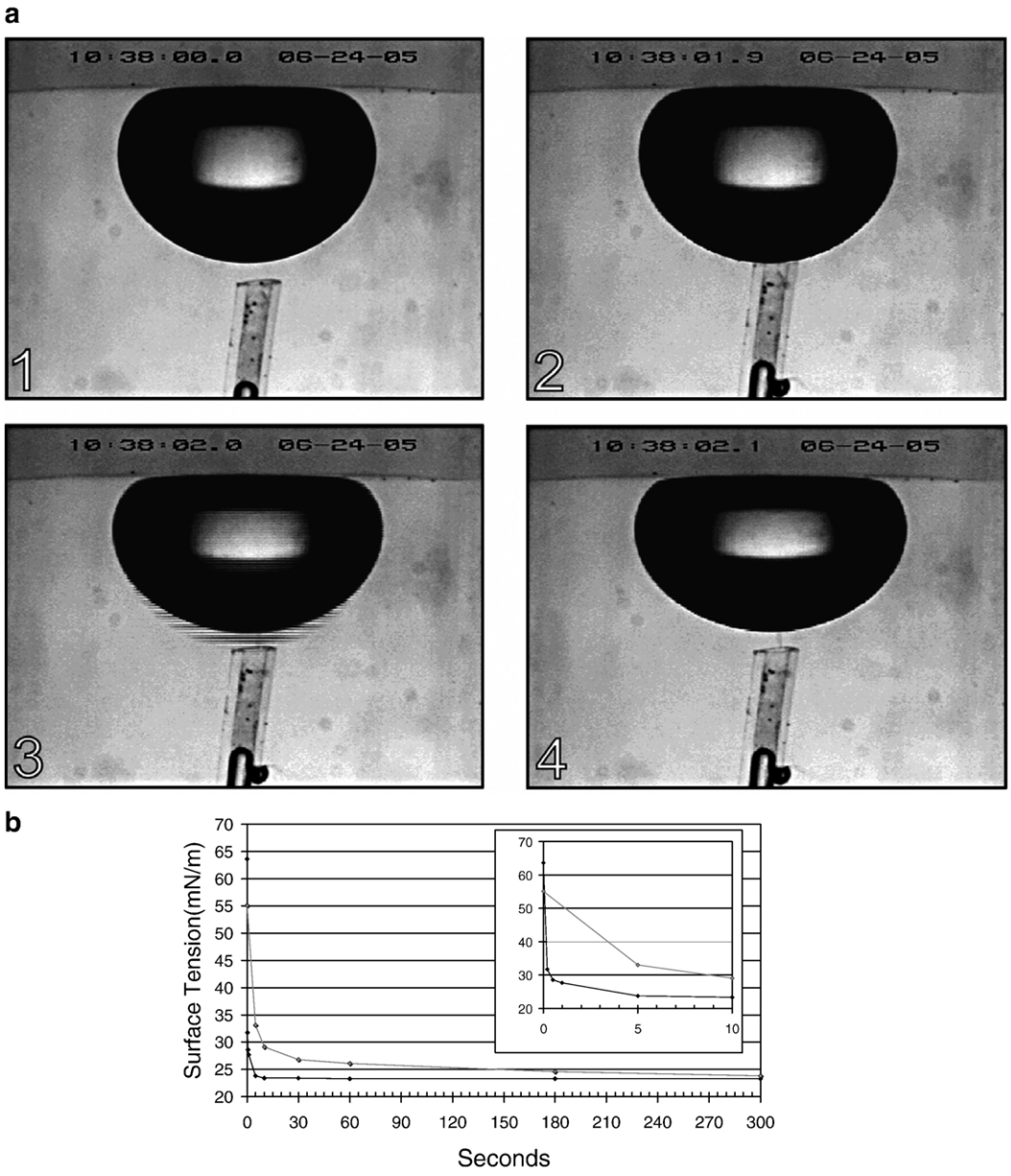

Fig. 1. Adsorption of surfactant. (a) (1) An air bubble in buffer floats against the agar ceiling of the captive bubble surfactometer chamber (image width 6 mm). (2) A transparent capillary is advanced just to the air–water interface from below and a small amount of surfactant ejected (BLES, 27 mg/ml). This material contacts the bubble surface causing it to flatten (3). Due to interlacing of the video image, the old shape of the bubble is evident in frame 3 field 1 as fine horizontal stripes, whereas the new shape appears solid in the second field of this frame. The time display at the top left of each image indicates a time change of 0.1 s between image frames 3 and 4. This change in shape brings the bubble out of contact from the capillary (4). (b) Two plots of the surface tension over time as calculated from video frames such as shown in (a). The inset represents the initial part of the curve at higher time resolution. The dark curve is an example of BLES being adsorbed to the air–water interface of a bubble in buffer, in the absence of a potential inhibitor. Note the very rapid initial film formation. The plot in grey shows the adsorption of BLES containing 20% w/w cholesterol. Initial film formation is slightly slower under this condition.

at similar levels after rapid expansion both in the presence and absence of serum proteins while films containing 20% w/w cholesterol reached significantly higher surface tensions. Hence, insertion of new material from the surface associated reservoir was less efficient for films containing excess cholesterol than for films without cholesterol. However, by 60 s after expansion completed, all films had returned to equilibrium with only one surface tension value remaining above 24.0 mN/m. In our experimental protocol, rapid expansion followed by a 5 min waiting period concluded the assessment of film formation.

3.2. Film expansion and compression

After film formation, the bubble volume first was slowly stepwise decreased and increased (quasi-static cycles). Quasi-static cycles reveal whether a surfactant film is able to sustain a low surface tension over time. This is an important parameter of surfactant function. A large proportion of the lung is experiencing minimal or no area change during tidal breathing and these parts of the lung too require stable near-zero surface tension to maintain the alveolar structure. The volume was then cycled dynamically to

Table 1
Summary of results

	Film spread <0.15 sec	1st compression		1st dynamic cycles		2nd quasi-static cycles		3rd quasi-static cycles	
		γmin<2 [mN/m]	β_γ10 [m/N]	γmin<2 [mN/m]	β_γ10 [m/N]	γmin<2 [mN/m]	β_γ10 [m/N]	γmin<2 [mN/m]	β_γ10 [m/N]
Control	100%	93%	11.3	100%	7.2				
80 mg/ml albumin injected after film formation	100%	0%	–	100%	7.6	25%	8.7	100%	6.9
80 mg/ml albumin and PEG injected after film formation	80%	60%	14.2	100%	4.0	100%	4.6	0%	
40 mg/ml albumin injected after film formation	75%	50%	10.0	100%	6.0	100%	6.5	0%	
80 mg/ml albumin in the buffer during film formation	0%	50%	23.9	100%	5.8	100%	7.7		
40 mg/ml albumin in the buffer during film formation	0%	50%	13.7	100%	7.0	100%	5.9		
3 mg/ml fibrinogen in the buffer during film formation	33%	33%	39.2	66%	8.1	100%	8.2		
20% cholesterol added to BLES	0%	0%	–	0%	–	0%	–	–	–
20% cholesterol added to BLES, PEG in the buffer	33%	0%	–	0%	–	0%	–	–	–

The column headed "film spread<0.15 s" indicates for each condition the percentage from all individual tests where the surface tension γ fell below 35 mN/m within 0.15 s. This criterion is indicative of whether film formation was slowed by the presence of inhibitors or not. The second column shows the percentages of films that reached very low surface tension (γ_{min}<2 mN/m) for the first compression after film formation (i.e. the first compression of the first quasi-static compression–expansion cycles), and the following columns show the first series of dynamic cycles, the second, and the third of quasi-static cycles respectively. Another measure of function, the median compressibility at a surface tension of 10 mN/m (median $\beta_{\gamma 10}$ [m/N] shown in the second column of each set) indicates whether a film was sufficiently incompressible. In the lung, a low compressibility of the surfactant film is found, as the very low surface tension required at end expiration occurs with a small area change. In the table, "–" denotes incidences where a surface tension of 10 mN/m was not achieved, and, hence, compressibility not measured. Cells with a grey background denote significantly impaired function.

simulate lung inflation and deflation during normal breathing. Quasi-static and dynamic cycles were repeated to see whether impaired surfactant regained function or functional surfactant became impaired upon further cycling. All results on film expansion and compression are summarized in Table 1.

Fig. 2 shows the compression component of the isotherms for BLES, spread on buffer containing no protein (control, upper row), 80 mg/ml albumin (middle row) or 3 mg/ml fibrinogen (lower row). The individual compression curves for the repeats under each condition are denoted by different shades of grey. The main results are also summarized in Table 1. During the first compression, all films exposed to protein were at least partially dysfunctional in that the surface tension dropped less rapidly than control during size reduction. At γ=10 mN/m, median compressibility was 20 m/N as compared to 11 m/N for the control. Note that at γ =10 mN/m a compressibility on the order of 10 m/N is consistent with molecules becoming more tightly packed at the interface. Much larger values indicate that some film collapse occurs and molecules are removed from the interface. Some films reached a surface tension near zero (γ_{min}<2mN/m), others did not. Interestingly, there was not a tight association between compressibility and low γ_{min} during the first compression of the films.

However, inhibition was not persistent for all films formed with protein present in the buffer during further cycling. All signs of inhibition had virtually disappeared by the forth quasi-static cycle (median $\beta_{\gamma 10}$ 6–7 m/N, similar to control). It is notable that this full recovery of function took place even though the films were continuously exposed to the protein.

Surprisingly, exposure to higher levels of protein *after* film formation resulted in films that failed to show meaningful reductions in surface tension and rarely displayed a surface tension below 15 mN/m from the start to the end of quasi-static testing (Fig. 3). In dynamic cycling, surfactant inhibition was all but gone (median $\beta_{\gamma 10}$ 8 m/N) but dysfunction showed up again in the following set of quasi-static cycles (median $\beta_{\gamma 10}$ 13 m/N). We allowed one of these latter films to rest overnight after at which time it was no longer impaired. In contrast, for films exposed to a lower amount of serum albumin (40 mg/ml) in the buffer after film formation signs of inhibition had all but disappeared by the forth quasi-static cycle (median $\beta_{\gamma 10}$ 8 m/N). In summary, exposure to albumin and fibrinogen had no lasting effect on function under the conditions tested. An effect, when present, was only observed in quasi-static cycling but not in dynamic cycles. Interestingly, 80 mg/ml albumin had only limited effect when present at the time of film formation and the

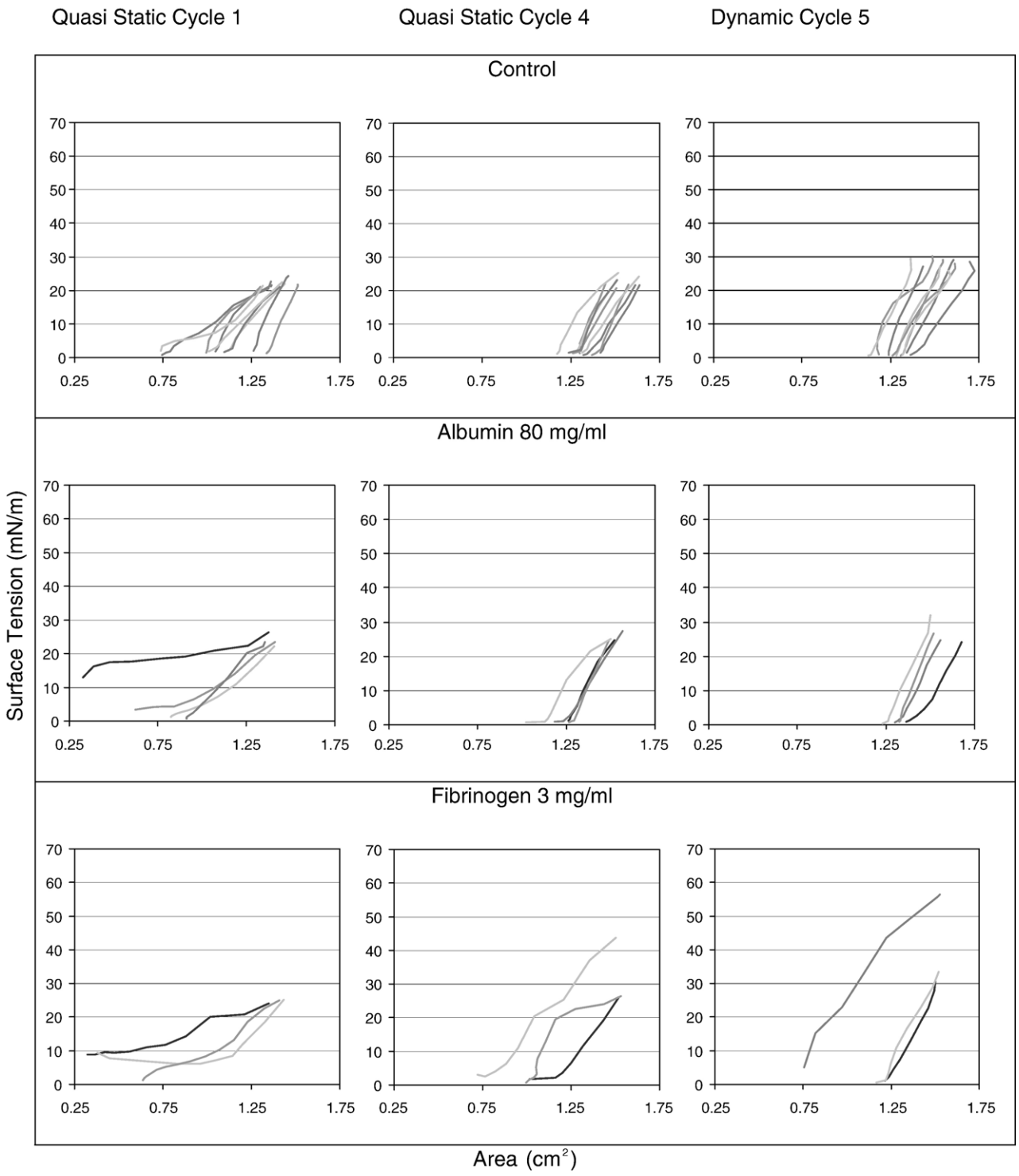

Fig. 2. Area–surface tension isotherms of BLES films exposed to plasma proteins. The isotherms were acquired during film compression. The films were adsorbed to the air–water interface on unadulterated buffer (upper row) or buffer already containing either albumin (80 mg/ml, middle row) or fibrinogen (3 mg/ml, lower row). For the first, control, condition 14 tests were carried out but only eight representative cases are shown for clarity and for the two experimental conditions, three or four individual tests were carried out, indicated by different shades of grey. Note that under these two conditions, all films showed inhibition in the first compression, but regained full function thereafter.

most pronounced inhibitory effect when brought into contact with BLES films after film formation if PEG was not added after the protein. However, even this latter inhibition disappeared over time and area cycling.

In contrast, 20% cholesterol in surfactant abrogated function with no recovery (Fig. 4). γ stayed above 15 mN/m throughout testing. Even a waiting period of 12 h did not lead to recovery of function of BLES containing 20% cholesterol (Table 1).

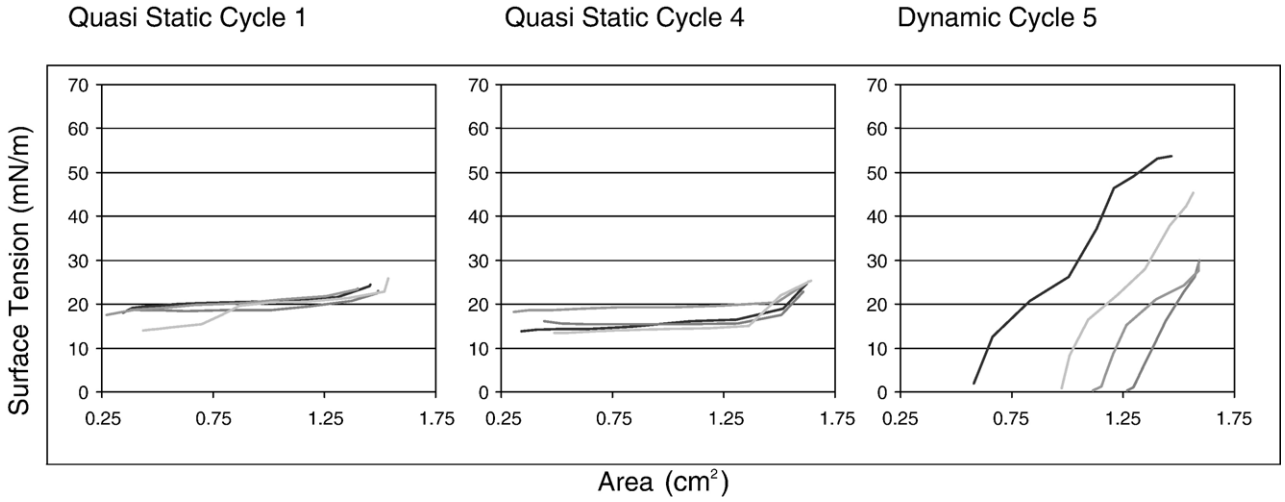

Fig. 3. Area–surface tension isotherms of BLES films exposed to albumin (80 mg/ml). Unlike in Fig. 2, the films were adsorbed to clean buffer–air interface first and the albumin injected afterward. Four individual tests were carried out, indicated by different shades of grey. Under this condition, all films showed severe inhibition during the course of four quasi-static cycles, but were functional upon dynamic compressions. These films regained full function upon later series of quasi-static compressions (not shown).

Finally, we tested whether inhibition by cholesterol or inhibition brought about by 80 mg/ml albumin after film formation could be reversed by the addition of PEG to the buffer. PEG has been shown to be effective in reversing surfactant inhibition by proteins (e.g. [17] and references therein). This observation was explained by the action of PEG to drive surfactant to the air–water interface by a mechanism called depletion attraction [33–38]. PEG was thought to specifically counter surfactant inhibition by competitive adsorption by driving the protein off the interface and allowing surfactant to adsorb. As expected, PEG had no effect on cholesterol inhibited surfactant (Table 1). However, inhibition by 80 mg/ml albumin on previously formed surfactant films was effectively reversed by adding PEG even with our current experimental protocol. This was surprising as the surfactant film resided at the interface prior to both, exposure to albumin and exposure to PEG. Hence, the inhibition is not likely to have been by competitive adsorption in the first place and PEG improved surfactant function in an as yet unknown way.

3.3. Cryo-electron microscopy

Current results on surfactant inhibition by serum proteins differ from results reported earlier on that subject in that our results were not consistent with surfactant inhibition by competitive adsorption. The one difference in experimental procedures that might best account for these divergent results may be the current use of highly concentrated surfactant over the more dilute suspensions used in the earlier studies. We therefore studied the influence of concentration on the structures

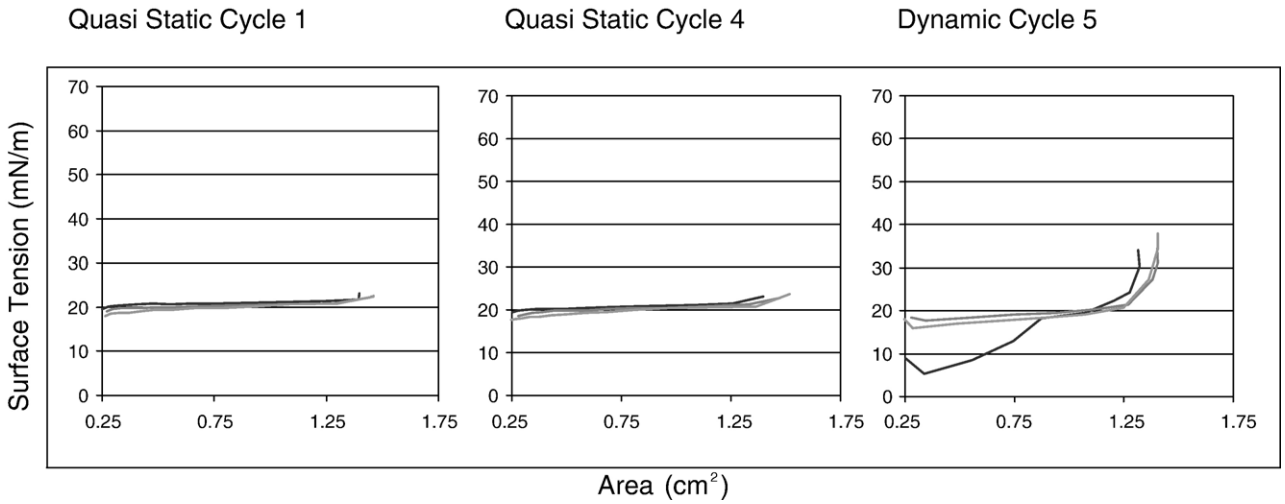

Fig. 4. Area–surface tension isotherms of BLES films containing 20% w/w cholesterol. Three individual tests were carried out, indicated by different shades of grey. Under this condition, all films showed severe inhibition during the course of all quasi-static and dynamic compression–expansion cycles. Function was also not regained upon later series of compression–expansion cycles (not shown).

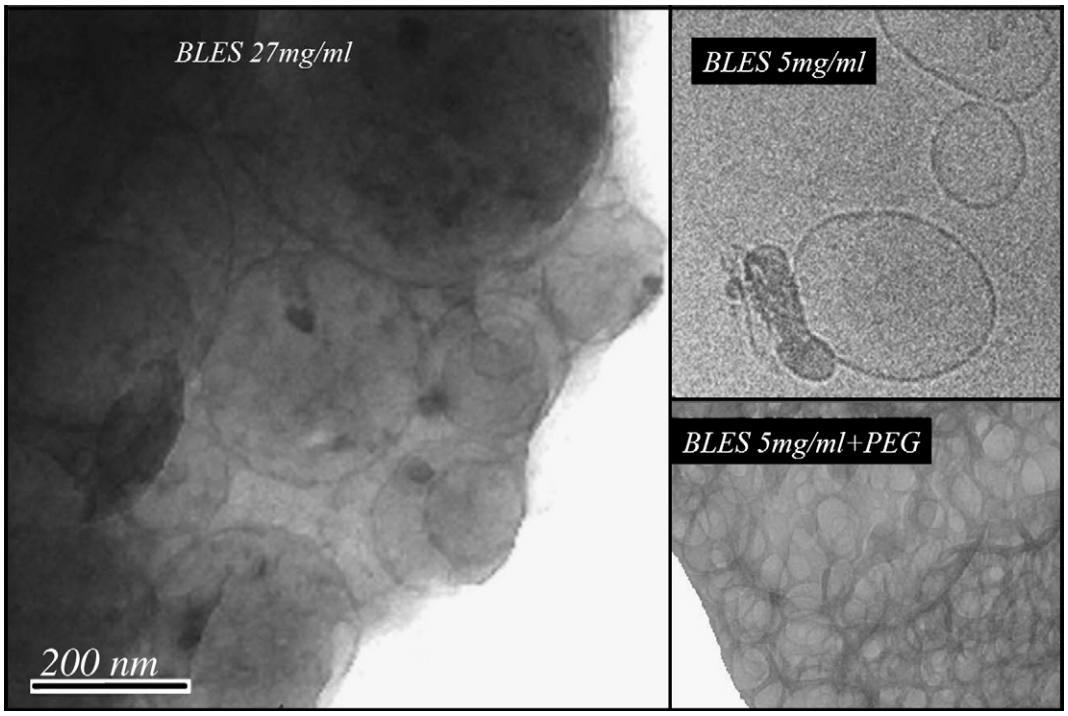

Fig. 5. (left) Cryo-electron micrographs of an aqueous suspension of BLES of 27 mg/ml such as used in the current study, BLES at 5 mg/ml (top right) and BLES at 5 mg/ml plus PEG (bottom right). At high concentration or at low concentration in the presence of PEG, BLES forms dense aggregates of vesicular structures. At low concentration, BLES forms unilamellar vesicles. For each sample, 5 μl of the suspension was put down onto a bare electron microscopy grid, excess of the suspension removed by filter paper and the grid injected into liquid ethane. Grids were transferred onto a cryo-holder and imaged in a Tecnai F-20 (FEI) at −100 °C.

formed by surfactant in suspension by cryo-electron microscopy of unstained aqueous suspensions (Fig. 5). At high concentration (27 mg/ml) such as used for the CBS studies, BLES formed dense aggregates of vesicular structures (Fig. 5, left). Large vesicles were packed with smaller vesicles or other spherical lipid particles. At a lower concentration (5 mg/ml), BLES formed unilamellar vesicles (Fig. 5, top right). This or a lower concentration reflects the condition used in earlier studies of surfactant inhibition by serum proteins. Adding PEG to 5 mg/ml of surfactant lead to condensed surfactant aggregates in the buffer (Fig. 5, bottom right), similar to those observed with 27 mg/ml of surfactant.

4. Discussion

The current findings indicate that under the experimental conditions chosen here, surfactant inhibition is likely caused by a dysfunctional film rather than by inhibition via competitive adsorption of plasma proteins to the air–lung interface. This is in contrast to earlier studies that demonstrated competition for the interface by serum proteins such as used in the current study [14–17]. The following sections will first address the experimental differences between the current and the earlier studies that could account for this discrepancy. We will then make the argument why studying surfactant inhibition with highly concentrated surfactant rather than dilute surfactant solutions may better approximate the conditions in the alveolar hypophase and produce results that could be more immediately relevant to the diseased lung.

According to the inhibition via competitive adsorption model, serum proteins form a film at the air–water interface. The layer of serum proteins prevents, or greatly delays, surfactant adsorption by repelling surfactant aggregates approaching the interface from the buffer because of its hydration shell as well as through electrical double layer repulsion [39,40]. In agreement with this proposed mechanism of inhibition, the addition of polymers such as polyethylene glycol (PEG) or hyaluronic acid to the buffer reversed this type of surfactant inactivation in these earlier studies. The polymers cause surfactant to flocculate, but also drive it to the interface by a mechanism termed depletion attraction [33–38].

In contrast, in the current study film formation was only minimally delayed by the presence of a film of proteins. Despite an initially slower drop in surface tension, the total time required to reach the equilibrium surface tension of 23.5 mN/m was not increased by plasma proteins in the buffer. In testing after film formation only limited inhibition by protein was observed in the first quasi-static cycle when present at the time of film formation. A high concentration of protein introduced after film formation produced significant and persistent inhibition but if PEG was added after the protein or a lower concentration of protein was used then the observed inhibition was modest and transient like that seen with protein present at the interface at the time of film formation. Hence, while there clearly was an effect of proteins at the interface in our experiments, the effect was transient and so any protein film at the interfaces seems to have been efficiently replaced by a film of surfactant.

The disagreement between earlier studies supporting inhibition by competitive adsorption and the current study is best explained by difference in the concentration of the surfactant applied; 27 mg/ml for the current study as compared to <2 mg/ml for most of the earlier studies [14–17] and is consistent with the findings by Holm et al. [18], where serum albumin abolished surfactant function at a surfactant concentration of 1 mg/ml but had no effect at only three times this concentration. The results by Holm and our current findings also show that surfactant inhibition by competitive adsorption is not linearly dependent on surfactant concentration and, hence, not merely subject to mass action. Rather, it appears to be related to the critical concentration, above which surfactant condenses from a dispersed suspension of unilamellar vesicles into extended mesostructures, depending on surfactant and buffer composition. Such transition has been studied experimentally and theoretically for lipids [41] and is evident for BLES in the electron micrographs shown in Fig. 5. According to this view, surfactant will be susceptible to inhibition by competitive adsorption if in the dispersed form and not be affected by competing serum proteins if present in condensed form. BLES forms unilamellar vesicles (Fig. 5, top right) at 5 mg/ml and dense aggregates at 27 mg/ml (Fig. 5 left). Diffusion of the vesicles to the interface and insertion into the interface are slow even in the absence of plasma proteins (e.g. [7]) and so films form over a time period measured in minutes. Under these conditions, the vesicles are apparently unable to effectively overcome a barrier posed by a film of protein. In contrast film formation from dense aggregates is effective even in the presence of a protein film at the interface. Once an aggregate has come into contact with the interface, it might spread over the interface, unaffected by the energy barrier encountered by vesicles. Because each densely packed particle delivers a much larger amount of surfactant to the interface than its unilamellar vesicular counterpart, film formation will be accordingly faster. Interestingly, BLES also forms dense aggregates at low concentration when in the presence of PEG. This would suggest that the earlier described effect of PEG in overcoming surfactant inhibition by serum proteins may not only have been the effect of driving the (dilute) surfactant to the interface [14–17], [33–38] but also due to its effect on flocculating the surfactant and turning it into this physical form which spreads to an air–water interface so much more effectively.

When evaluating the validity of the current over earlier findings for explaining surfactant dysfunction in ARDS, one needs to assess, which of the surfactant concentrations, 27 mg/ml chosen here or the more typical concentrations of <5 mg/ml better approximate the alveolar hypophase in the diseased lungs. The concentration of surfactant has not been directly measured to date, neither in the healthy nor the diseased lung. The best available estimate of the alveolar hypophase surfactant concentration to our knowledge is that of Clements et al. [42] which they roughly calculate to be 120 mg/ml phospholipids. These authors later, referring to the work of Bray, note that their estimate likely is a conservative one [34,43]. Bray [44] proposed that local concentrations of surfactant at the interface could be enhanced by hyaluronic acid or other organizing factor in the surfactant hypophase. Another line of evidence indicating that the surfactant hypophase is an organized "structure" with locally enhanced concentration is that found in EM studies of the lung. None of these studies present any evidence for the existence of a uniform suspension of unilamellar vesicles of surfactant, capped at the air–fluid interface by a phospholipid monolayer. Rather from the earliest studies [45] the surfactant hypophase is seen as containing consistent highly organized osmophilic structures including lamellar bodies, tubular myelin, and a multilamellar superficial layer. Several authors have also noted a tight association between tubular myelin and the superficial lamellar structures [45]. It should be remembered that the osmium used to produce the contrast in these images is specifically localized to phospholipids the most abundant constituent of surfactant and so reveal the structured non-homogeneous distribution of surfactant in the hypophase. Subsequent investigations have largely ruled out artifact as a significant factor in these studies and revealed more subtle details of this organization [5,7,46,47]. This leads us to conclude that the complex organization in the lung is functionally important for the surface tension altering effects of pulmonary surfactant and that the "self organization" occurring with more concentrated material would be qualitatively different from that formed in more dilute surfactant solutions. In a recent comparative study of BLES and other hydrophobic animal extract surfactants with natural surfactant in vitro showed comparable surface activities in a CBS but also quite similar structures (with the exception of the lack of tubular myelin for the animal extract surfactants) at the air–water interface as obtained by electron microscopy [8]. This is irrespective of the deficiency in the water soluble surfactant associated proteins A and D (SP-A, SP-D) for the hydrophobic extract surfactants.

However, SP-A and SP-D might play an important role in the injured lung, when surfactant in the alveolar fluid becomes diluted and dispersed by edema. SP-D and SP-A both have been shown to keep surfactant from dispersing in a more dilute suspension [48] and prevent it from becoming more susceptible to inhibition by competitive adsorption. The naturally occurring hyaluronic acid in the alveolar fluid is another substance that keeps surfactant condensed and close to the air–alveolar interface [44]. Together, the above in vitro and in vivo findings lead us to conclude that the approach chosen in the current study over earlier experiments is more likely to reflect the situation in the lung and the choice of BLES at 27 mg/ml is appropriate when assessing surfactant inhibition. As a consequence, competitive adsorption is not likely occurring in the lung as the initial inhibitory mechanism. However, we would not rule out the possibility that when its constituents become degraded by mechanisms discussed below and other disease related-mechanisms, surfactant may no longer maintain a condensed structure and may eventually become susceptible to inhibition by competitive adsorption as well.

There are more reasons for surfactant inhibition by competitive adsorption not to occur as the primary inhibition mechanism. Firstly, serum albumin is in the alveolar space even in the healthy lung at relatively high concentrations where it does no damage to the surfactant system. Ishizaka et al. reported

8 mg/ml albumin in the alveolar fluid of a lung in healthy individuals [49]. This is above a buffer concentration beyond which the equilibrium surface tension of serum albumin becomes largely independent of the buffer concentration and competitive adsorption should have reached its maximum level (~0.1 mg/ml; [50]). Furthermore, for competitive adsorption to occur, a protein film would need to be in place before surfactant had time to form a film on its own and lower the surface tension to below the equilibrium surface tension value for the respective protein (~43 mN/m for albumin and ~37 mN/m for fibrinogen, according to the current study). At an equilibrium surface tension of surfactant below 24 mN/m, protein molecules are unlikely to insert into the interface and displace surfactant. On the other hand, surfactant with its higher surface activity, evident from a lower equilibrium surface tension, can drive a protein film from the interface.

While our findings do not support surfactant inhibition by competitive adsorption as the primary inhibitory mechanism, serum proteins still caused functional inhibition of surfactant. The first quasi-static compression of the surfactant films revealed elevated compressibility. The interfacial area needed to be reduced at least by 50% to lower the surface tension from equilibrium to near zero as compared to about 15% in the absence of proteins. For all conditions tested, some films never reached a low surface tension in the presence of proteins in Q-Stat 1. Remarkably, the inhibition by proteins was most pronounced when the exposure occurred after the surfactant films had been formed and a high concentration of surfactant was used. This inhibition was transient and all cases, except those where a high concentration of albumin was added after film formation, showed quick and sustained recovery. The latter had slower and somewhat transient recovery.

We have no detailed explanation how serum proteins interfere with surfactant. However, comparison with films inhibited by cholesterol is illustrative. Film adsorption to the equilibrium value was not affected by an excess of cholesterol or exposure to proteins in the buffer but the films could not withstand a film pressure associated with a surface tension below equilibrium. This resulted in an extended plateau in the surface tension–area isotherm for the cholesterol inhibited films which was greater than the most extreme cases of protein inhibition. Below equilibrium surface tension, an interfacial film is metastable by definition (i.e. once matter has left the film, it can no longer return). It is a unique mechanical property of a pulmonary surfactant to not collapse under this condition. This property has been associated with specific film architecture of a monolayer and dispersed multilayer patches [51,52] and excess cholesterol has been shown to disrupt this structure [20]. By analogy, serum protein might also interfere with this film structure. Nag et. al [53] showed that albumin interacts intimately with the lipids of surfactant causing them to redistribute into a phase pattern not present in the absence of the protein.

In dynamic cycling, all protein exposed films achieved near-zero surface tension even when they showed inhibition during quasi-static cycling before and after the dynamic cycles. Film collapse is time dependent and films may remain intact under pressure for a short while but collapse over longer time periods. Dynamic cycling is therefore a less rigorous stability test and reveals the impairment only for the most severely inhibited surfactants. To judge whether surfactant maintains near-zero surface tension in areas of the lung that are not undergoing area change during tidal breathing, quasi-static cycling or other near static surfactant evaluations are therefore required.

A surprising finding of this study was that films allowed to form in absence of serum protein became (transiently) inhibited after exposure to proteins while films formed in the presence of serum protein were already functional after a single compression–expansion cycle. This effect was most pronounced for 80 mg/ml albumin, which resulted in minimal inhibition when present during film formation but was most inhibitory if added to the aqueous phase after film formation and then showed recovery over with successive cycles with proteins present. Hence, pulmonary surfactant appears to possess a mechanism that makes it tolerant to exposure to serum proteins.

5. Conclusion

The current study contradicts surfactant inhibition by competitive adsorption by serum albumin and fibrinogen as the primary mechanism of surfactant inhibition. It appears unlikely to us that this mechanism would hold true for any other plasma or inflammatory protein present in the injured lung as long as the surfactant itself is undamaged. Instead, we found that inhibition by proteins appears to occur via interference with the surfactant film itself. Inhibition was transient for the two proteins tested and the surfactant films became insensitive to their continued presence. This is likely important for normal lung function where at least serum albumin is always present in the alveolar space and is just elevated in ARDS. There is good evidence that other serum or inflammatory proteins may cause more lasting surfactant impairment than albumin and fibrinogen (e.g. [54]). We expect this to occur at least initially via interference with the film structure and not through competitive adsorption. However, we note that surfactant inhibition by competitive adsorption might occur, once the surfactant has become deficient in its composition and become unable to maintain a condensed structure.

Identification of the relevant mechanism of surfactant impairment in ARDS is important for devising new strategies to counter the inhibition and overcome the current lack of success with exogenous surfactant treatment. The current study does not support the rationale for a treatment that primarily addresses competitive adsorption by introducing hydrophilic polymers such as PEG into the alveolar space as proposed (e.g. [17,40]). Our results for cholesterol inhibition show that addition of PEG has no positive effect on surfactant inhibited by cholesterol. An effective treatment, may have to specifically counter the effect of cholesterol and other small hydrophobic molecules and also target other mechanisms, including oxidation of surfactant through the reactive oxygen species present in the injured lung [21–24,55] and interference with serum and inflammatory proteins.

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