Biochimica et Biophysica Acta 1848 (2015) 813–820

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Cholesterol-mediated surfactant dysfunction is mitigated by surfactant protein A

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ARTICLE INFO

Article history:
Received 24 July 2014
Received in revised form 5 December 2014
Accepted 9 December 2014
Available online 16 December 2014

Keywords:
Pulmonary surfactant
Cholesterol
SP-A
Mechanical ventilation

ABSTRACT

The ability of pulmonary surfactant to reduce surface tension at the alveolar surface is impaired in various lung diseases. Recent animal studies indicate that elevated levels of cholesterol within surfactant may contribute to its inhibition. It was hypothesized that elevated cholesterol levels within surfactant inhibit human surfactant biophysical function and that these effects can be reversed by surfactant protein A (SP-A). The initial experiment examined the function of surfactant from mechanically ventilated trauma patients in the presence and absence of a cholesterol sequestering agent, methyl-β-cyclodextrin. The results demonstrated improved surface activity when cholesterol was sequestered *in vitro* using a captive bubble surfactometer (CBS). These results were explored further by reconstitution of surfactant with various concentrations of cholesterol with and without SP-A, and testing of the functionality of these samples *in vitro* with the CBS and *in vivo* using surfactant depleted rats. Overall, the results consistently demonstrated that surfactant function was inhibited by levels of cholesterol of 10% (w/w phospholipid) but this inhibition was mitigated by the presence of SP-A. It is concluded that cholesterol-induced surfactant inhibition can actively contribute to physiological impairment of the lungs in mechanically ventilated patients and that SP-A levels may be important to maintain surfactant function in the presence of high cholesterol within surfactant.

1. Introduction

In the healthy lung, pulmonary surfactant reduces the surface tension at the alveolar surface to reduce the work of breathing and maintain alveolar stability at low lung volumes [1]. This highly surface active material, synthesized by the alveolar type II cells, is composed of a mixture of phospholipids, a large percentage of which is dipalmitoyl-phosphatidylcholine (DPPC), neutral lipids, the majority of which is cholesterol, and surfactant associated proteins [2]. Once secreted into the alveolar space, surfactant adsorbs to the air–liquid interface to form a surface film of surfactant lipids which reduces the surface tension to below 5 mN/m [1]. The hydrophobic surfactant proteins B and C (SP-B and SP-C) are involved in the formation of this lipid film [3]. Surfactant protein A (SP-A) is a large glycoprotein which is part of the collectin family and is the most abundant of the surfactant-associated proteins, representing approximately 5% of surfactant phospholipids by weight [1,4]. It is reported to be involved in host defense mechanisms within the lung, regulation of surfactant metabolism, as well as contributing to surface tension reduction [5,6]. Within the alveolar space, the mixture of lipids and proteins forms highly organized lipid–protein structures known as large aggregate (LA) subtype of surfactant. It is this subtype that is responsible for the surface activity of surfactant [7]. With alveolar surface area changes associated with continuous cycles of inhalation and exhalation, LA is converted to the small aggregate (SA) subfraction, which represents the inactive form of surfactant that is cleared from the airspace [8].

In diseased states, such as the acute respiratory distress syndrome (ARDS), impairment of the surfactant system represents a fundamental pathophysiologic process contributing to the observed changes in lung function [4]. This biophysical impairment is related to changes in surfactant composition as well as the inhibitory effects of substances, such as serum proteins, that have extravasated into the airspace. This inhibition of surfactant by serum proteins has been studied extensively and it has been demonstrated that serum proteins directly interfere with both the adsorption and surface tension-reducing properties of surfactant [9–11]. Further studies have shown that this mechanism of surfactant inhibition can be mitigated by SP-A [12]. Other alterations to the surfactant system that have been linked to surfactant dysfunction in the

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http://dx.doi.org/10.1016/j.bbamem.2014.12.009
0005-2736/

setting of ARDS include i) changes in the concentration of the phospholipid components, specifically those of the active LA subfraction, ii) decreased concentration of surfactant associated proteins, SP-A, SP-B and SP-C, and iii) elevated concentrations of neutral lipids, predominantly cholesterol, relative to phospholipids within the LA subfraction of surfactant [4,13–15].

With respect to the latter observation, the inhibitory effects of elevated cholesterol levels within pulmonary surfactant have been extensively examined *in vitro* [16–19]. Adding high levels of cholesterol to surfactant can inhibit surfactant function through disruption of the lateral organization of the surfactant lipids in the surface film. More recently, a series of *in vivo* studies have demonstrated that the inhibition of pulmonary surfactant observed in an animal model of ventilator-induced lung injury may occur through an elevation in the levels of cholesterol within the surfactant [20,21]. It is unknown whether this phenomenon occurs clinically in mechanically ventilated patients and whether SP-A can mitigate these effects. Therefore, based on these observations we hypothesized that elevated cholesterol levels within surfactant can inhibit the biophysical function of human surfactant and that these effects can be mitigated by SP-A.

2. Methods

2.1. Surfactant samples

The current study utilized three surfactant preparations: 1) LA samples obtained from a cohort of mechanically ventilated trauma patients (described below), 2) surfactant obtained from a patient with known pulmonary alveolar proteinosis and 3) a commercially available exogenous surfactant, bovine lipid extract surfactant, BLES (BLES Biochemicals Inc., London, ON, Canada). Pulmonary surfactant collected from a patient with pulmonary alveolar proteinosis was obtained *via* therapeutic whole lung lavage after obtaining informed consent. Briefly, the patient was anesthetized and intubated using a dual lumen endotracheal tube in order to ventilate each lung independently. Whole lung lavage was then performed on the left lung and the lavage material was processed immediately to obtain surfactant LA, as described [20,22].

Surfactant from mechanically ventilated trauma patients was obtained *via* bronchoalveolar lavage (BAL). Approval to obtain BAL samples from this patient population for research purposes was granted by the Human Subjects Research Ethics Board at the University of Western Ontario and signed consent from substitute decision makers for each patient. Patients were screened for inclusion into the study based upon an admission diagnosis of trauma with an Injury Severity Score (ISS) >12, resulting from a blunt mechanism of injury, on the severity of injury, an age of 16 years or older and intubated with the expectation of a need for mechanical ventilation over 24 h. All aspects of clinical care, including mechanical ventilation strategy were left to the discretion of the attending physician. After obtaining informed consent, BAL samples were collected within 24 h of intubation and repeat samples were collected 48 h and 96 h after the first BAL for patients who remained intubated for that duration in order to assess changes in surfactant over time [23]. BAL was performed in anatomical areas at the discretion of the operator which corresponded to CT or chest X-ray areas demonstrating normal-appearing lung. Patients received intravenous sedation using midazolam and fentanyl and received bag-ventilation *via* an endotracheal tube during the procedure. A bronchoscope was introduced *via* the endotracheal tube and wedged in the airway of the selected segment. 50 mL of sterile saline was injected *via* the bronchoscope and suctioned into a sterile container three times, for a total of 150 mL, targeting a 50% return of lavage fluid. After the BAL procedure, patients were returned to their previous ventilator settings. Immediately following the BAL procedure, the lavage volume was measured and processed as previously reported [14,22]. Total protein analysis, IL-6 measurement, and surfactant isolation and measurements were performed as previously described [20,24]. Ten lavage samples, obtained from 8 patients, were determined to have sufficient quantities of surfactant to be included for the biophysical analysis to evaluate the role of cholesterol.

2.2. Surfactant sample processing

In order to evaluate the role of cholesterol in the surfactant samples, two complementary approaches were utilized. Human trauma patient surfactant was depleted of cholesterol in the chamber of a captive bubble surfactometer. For BLES and alveolar proteinosis LA samples, cholesterol was removed from air-dried chloroform-extracted samples *via* acetone precipitation. Cholesterol depletion was verified using a commercially available free cholesterol assay kit (Wako Chemicals, Richmond, VA, USA) and the cholesterol depleted surfactant was subsequently reconstituted with 0, 5, 10, or 20% (w/w phospholipid) cholesterol in chloroform, dried, and resuspended at a final phospholipid concentration of 5 mg/mL (140 mM NaCl, 10 mM Hepes, and 2.5 mM $CaCl_2$, pH = 6.9) or 10 mg/mL (150 mM NaCl), for *in vitro* and *in vivo* analyses respectively. A separate set of samples was reconstituted in a similar fashion with an additional 5% (w/w phospholipid) of human SP-A. Human SP-A was purified from alveolar proteinosis surfactant as previously described [25]. Briefly, alveolar proteinosis surfactant isolated from a whole lung lavage was mixed with 1-butanol at room temperature followed by centrifugation at 3300 *g* for 30 min. The pellet was resuspended in 1-butanol and centrifuged a second time. The pellet was resuspended in a 20 mM octyl-8-D-glucopyranoside/100 mM NaCl/10 mM Hepes buffer (pH 7.4) and centrifuged at 200,000 *g* for 30 min. The pellet was resuspended in 5 mM TRIS, pH 7.4, and dialysed against this buffer with a MW cut-off 6000–8000. Insoluble material was removed by centrifugation (200,000 *g* for 30 min) and the supernatant containing SP-A stored at −70 °C [25].

2.3. In vitro analyses

The surface activity of various surfactant preparations was assessed using a computer-controlled captive bubble surfactometer (CBS) as described previously [19,20,26]. Briefly, a surfactant sample was introduced at the surface of a small bubble with the shape of the bubble monitored for assessment of surface tension. Samples were first analyzed for adsorption by monitoring the bubble for 5 min under a static condition. To determine the minimum achievable surface tension, the sample bubbles underwent dynamic compression−expansion cycles at a rate of 20 cycles/min. Minimum surface tension was recorded for cycles 1, 2, 5 and 10. For samples from human trauma patients, additional CBS measurements were made to assess the role of cholesterol. For these measurements, the CBS chamber containing buffer with 20 mM methyl-β-cyclodextrin (MβCD) was used to sequester cholesterol from the sample as previously reported [17,20,27,28]. In addition to those published studies, preliminary studies were performed to test the efficacy of the concentration of MβCD. Specifically, it was shown that an alveolar proteinosis sample reconstituted to 20% (w/w) cholesterol reached minimum surface tension values of 17.6 ± 1.6 mN/m, whereas the 20% (w/w) cholesterol analyzed in a buffer containing 20 mM MβCD reached minimum surface tension values of 4.4 ± 1.3 mN/m which was not significantly different than samples with 5% cholesterol.

For surface film structure analysis, Langmuir–Blodgett films were prepared using a Kibron μTrough SE (Helsinki, Finland) as previously described [17,29]. Briefly, films were spread by depositing droplets of surfactant samples uniformly throughout the air–water interface to an initial surface pressure of 30 mN/m (surface tension approximately 42 mN/m). The films were compressed and expanded three times at a rate of 0.10 nm^2 $molecule^{-1}$ min^{-1} to a maximum surface pressure of 50 mN/m and a deposit was taken at the fourth compression at a surface pressure of 50 mN/m (surface tension approximately 22 mN/m). Topographical atomic force microscope (AFM) images were obtained using a Nanoscope III scanning force multimode

Table 1
Patient characteristics.

Patient	Age	M/F	PO_2/FIO_2 (mm Hg)	Ventilation mode	PEEP	Time of BAL after intubation (h)	BAL volume (mL)	Total surfactant (μg PL)	% large aggregates	Protein (mg/mL)	IL-6 (pg/mL)
1	72	F	270	SIMV/PSV	5	117	46	317	79	0.3	52
2	84	M	265	PSV	5	61	12	1480	72	4.5	710
3A	64	M	222	PSV	10	71	18	1860	77	2.3	254
3B			280	AC/PC	8	209	28	1076	93	0.8	44
4	38	M	89	SIMV	10	21	14	1054	71	1.2	1104
5	30	M	130	AC	5	60	27	1939	75	0.6	246
6	82	M	144	PSV	10	65	23	2363	71	3.1	2668
7	20	M	383	AC	5	15	27	5353	70	2.6	2133
8A	22	F	62	AC	8	12	25	2416	70	2.5	4314
8B			71	AC	12	65	27	2677	80	2.3	2131

SIMV synchronized intermittent mandatory ventilation
PSV pressure support ventilation
ACV assist control ventilation
PCV pressure control ventilation
PL phospholipid.

microscope (Digital Instruments, Santa Barbara, CA). Image analysis was performed using the Nanoscope III software (version 5.12r3).

2.4. In vivo analyses

To analyze surfactant function *in vivo*, a lung lavage model of surfactant depletion in rats was utilized as previously reported [24]. All procedures were approved by the animal use subcommittee at the University of Western Ontario in agreement with the guidelines of the Canadian Council of Animal Care. Briefly, rats were anesthetized, connected to a mechanical ventilator (8 mL/kg tidal volume (Vt), respiratory rate (RR) of 54–58 breaths per minute (bpm), 5 cm H_2O positive end-expiratory pressure (PEEP) and a fraction of inspired oxygen (FiO_2) of 1.0.) and depleted of endogenous surfactant pools by repetitive saline lavage. After surfactant depletion, indicated by a reduction in PO_2/FiO_2 values to below 150 mm Hg, rats were randomized to receive either an intratracheal instillation of i) 1 mL/kg bodyweight (BW) bolus of air, ii) 1 mL/kg BW volume of surfactant (10 mg phospholipid/mL) with 5% (w/w) cholesterol (n = 6), iii) surfactant with 5% cholesterol (w/w) + 5% (w/w) SP-A (n = 7), iv) surfactant with 20% (w/w) cholesterol (n = 6) or v) surfactant with 20% (w/w) cholesterol + 5% (w/w) SP-A (n = 6). Rats were then ventilated for 120 min using ventilation parameters as described above. Airway pressure, hemodynamics, and blood gas measurements were recorded every 15 min. Following the 2 h of ventilation, animals were administered with sodium pentobarbital (75 mg/kg BW) and exsanguinated by transection of the descending aorta. A whole-lung lavage was performed, processed and analyzed as previously described [21,22].

2.5. Statistical analysis

Analysis of surfactant function for samples from mechanically ventilated trauma patients was performed using a paired t-test. Remaining statistical analyses utilized a two-way ANOVA followed by Tukey's post-hoc test. Means are reported ± SEM. Values were considered significantly different at a probability value < 0.05.

3. Results

3.1. In vitro analysis of surfactant from mechanically ventilated trauma patients

The first experiment analyzed ten BAL samples obtained from eight mechanically ventilated trauma patients. Clinical, physiological and BAL data from these 8 trauma patients are shown in Table 1. For two of the patients (patients #3 and #8) two separate BAL samples obtained at different time points were analyzed. As shown in Table 1, this patient population ranged in age from 24 to 84 years and had oxygenation and compliance values that varied significantly. In addition, BAL samples were obtained at various times after intubation, ranging from 12 to 209 h. Amounts of surfactant recovered in BAL fluid varied among patients, however, in all patients the majority of surfactant recovered was in the LA form (average 75.8 ± 7.1%). The concentration of total protein and the inflammatory cytokine, interleukin-6, also varied widely among patients.

Aliquots of the LA samples obtained from patients were analyzed for biophysical activity on a CBS with and without the cholesterol sequestering agent, MβCD. The observed surface tension values for two different time points after adsorption (60 and 300 s) are shown in Fig. 1. Surface tension values were significantly lower in samples incubated with MβCD as compared to the controls at both 60 and 300 s. Fig. 2 shows minimum surface tension values from all 10 samples for 4 dynamic compression–expansion cycles. All samples achieved lower minimum surface tensions in the presence of MβCD as compared to without MβCD. This effect was statistically significant at each cycle analyzed.

3.2. In vitro analysis of the effect of SP-A on cholesterol mediated inhibition of human alveolar proteinosis surfactant

Fig. 3 shows the effect of increasing concentrations of cholesterol on the surface tension reducing properties of surfactant following adsorption of human alveolar proteinosis surfactant in the absence (A) or presence of SP-A (B). Surfactant containing 0% and 5% (w/w) cholesterol, without SP-A, demonstrated rapid adsorption reaching equilibrium surface tension values of approximately 23 mN/m in 30 s. Samples containing 10% and 20% (w/w) cholesterol had slower adsorption compared to the 0 and 5% (w/w) cholesterol samples. This difference was statistically significant from 5 to 60 s for 10% (w/w) cholesterol, and from 5 to 30 s for 20% (w/w) cholesterol, as compared to the 0% (w/w) cholesterol sample.

For samples containing 5% (w/w) SP-A (Fig. 3B) there were no significant differences among the samples reconstituted with different levels of cholesterol. Comparisons between surfactant samples containing similar levels of cholesterol with or without SP-A showed that samples containing 10% (w/w) cholesterol + 5% (w/w) SP-A had a significantly reduced surface tension from 0 to 60 s as compared to samples with 10% (w/w) cholesterol without SP-A. Similarly, surfactant containing 20% (w/w) cholesterol with 5% (w/w) SP-A showed a significantly lower surface tension from time points 10 to 60 s as compared to surfactant containing 20% (w/w) cholesterol without SP-A.

The minimum achievable surface tension of the alveolar proteinosis surfactant samples over the course of ten dynamic expansion–compression cycles is shown in Fig. 4. Within each surfactant group, there were

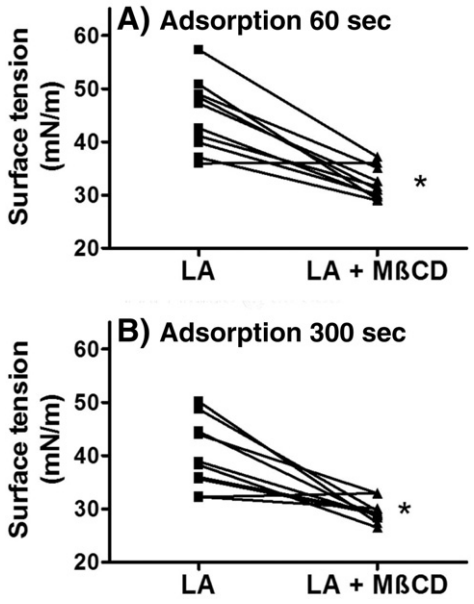

Fig. 1. Effect of MβCD on *in vitro* adsorption of patient surfactant samples. Surface tension after adsorption of surfactant isolated from individual mechanically ventilated trauma patients with and without incubation with MβCD. * = $p < 0.05$ LA + MβCD *versus* LA.

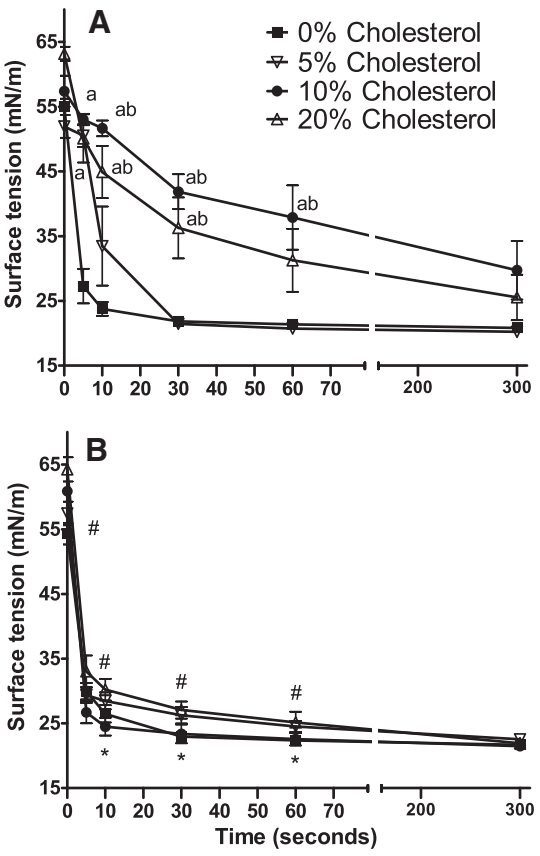

Fig. 3. Effect of SP-A and cholesterol on surfactant adsorption *in vitro*. Surface tension (mN/m) during 300 s of initial adsorption of human alveolar proteinosis surfactant reconstituted with different levels of cholesterol without (A) and with (B) 5% (w/w) SP-A, as indicated in the legend. Values are reported as mean ± standard error, a = $p < 0.05$ *versus* 0% (w/w) cholesterol, b = $p < 0.05$ *vs.* 5% (w/w) cholesterol, # = $p < 0.05$ *versus* 10% (w/w) cholesterol without SP-A, and * = $p < 0.05$ *vs.* 20% (w/w) cholesterol without SP-A, n = 3/group.

no significant differences in minimum surface tension throughout all expansion–compression cycles. Samples with 10% (w/w) cholesterol and 20% (w/w) cholesterol, without SP-A (Fig. 4A), had significantly higher surface tensions than the 0% (w/w) cholesterol and 5% (w/w) cholesterol groups throughout all cycles. Among alveolar proteinosis surfactant samples reconstituted with SP-A (Fig. 4B), there were no significant differences in minimum surface tension between groups containing 0, 5 or 10% (w/w) cholesterol throughout all compression–expansion cycles. Surfactant containing 20% (w/w) cholesterol + 5% (w/w) SP-A had a significantly higher minimum surface tension than all other surfactant groups reconstituted with 5% (w/w) SP-A. Additionally, there were significant differences between groups treated with SP-A and those without. Specifically, surfactant with 5% (w/w) cholesterol + 5% (w/w) SP-A had

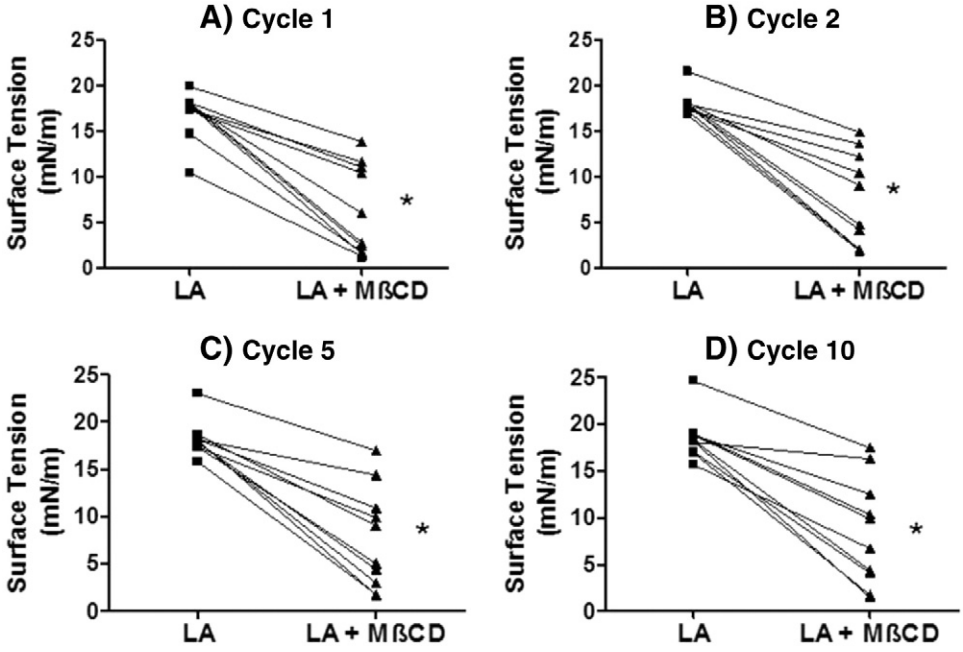

Fig. 2. Effect of MβCD on minimum surface tension during compression–expansion cycles of patient surfactant. Minimum surface tensions of surfactant isolated from individual mechanically ventilated trauma patients with and without incubation with MβCD, during various dynamic compression–expansion cycles. * = $p < 0.05$ LA + MβCD *versus* LA.

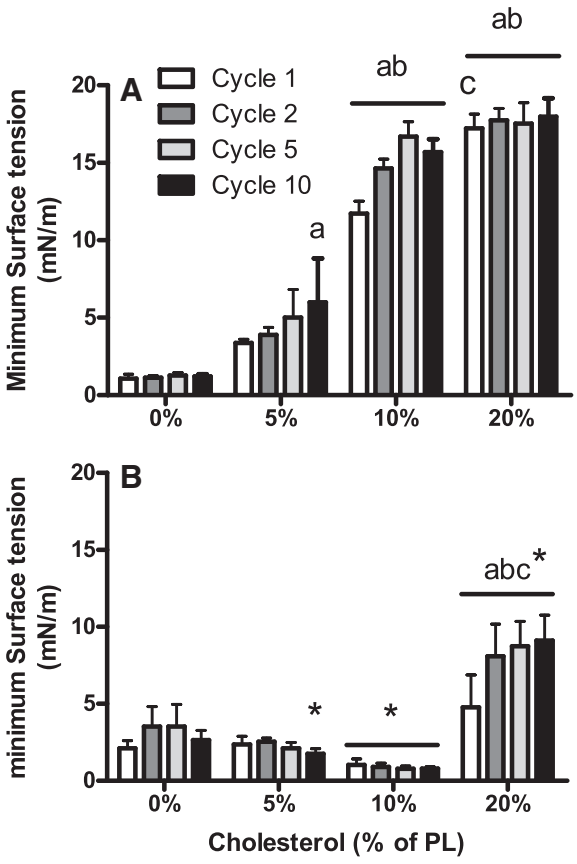

Fig. 4. Effect of SP-A and cholesterol on minimum surface tension during repeated compression–expansion cycles *in vitro*. Minimum surface tension (mN/m) of human alveolar proteinosis surfactant reconstituted with different levels of cholesterol without (A) and with (B) 5% (w/w) SP-A during various dynamic compression–expansion cycles. Values are reported as mean ± standard error, a = $p < 0.05$ *vs.* 0% (w/w) cholesterol, b = $p < 0.05$ *vs.* 5% (w/w) cholesterol, c = $p < 0.05$ *vs.* 10% (w/w) cholesterol and * = $p < 0.05$ *versus* same sample without SP-A, n = 3/group.

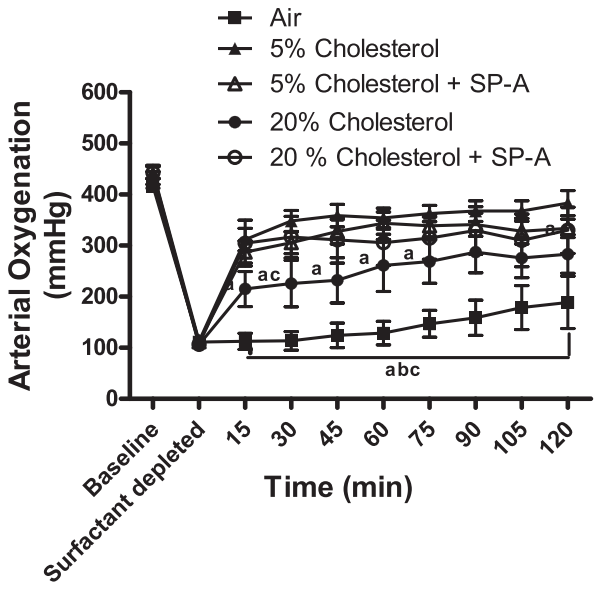

Fig. 5. *In vivo* activity of surfactants with various amounts of cholesterol and SP-A. Arterial oxygenation content (mm Hg) after endogenous surfactant depletion and administration of BLES with various amounts of cholesterol ± 5% (w/w) SP-A and 120 min of mechanical ventilation. Values are means ± SE, n = 6–7/group, a = $p < 0.05$ *vs.* 5% (w/w) cholesterol, b = $p < 0.05$ *vs.* 5% (w/w) cholesterol + 5% (w/w) SP-A, and c = $p < 0.05$ *vs.* 20% (w/w) cholesterol + 5% (w/w) SP-A.

a significantly lower minimum surface tension at the end of cycle 10 as compared to surfactant with 5% (w/w) cholesterol without SP-A. Similarly, surfactant with 10 and 20% (w/w) cholesterol + 5% (w/w) SP-A had significantly lower minimum surface tensions when compared to similar samples lacking SP-A throughout all expansion–compression cycles.

3.3. In vivo analysis of SP-A on cholesterol mediated inhibition of exogenous surfactant

Surfactant depleted rats were treated with exogenous surfactant, BLES, containing 5 or 20% (w/w) cholesterol, with and without 5% (w/w) SP-A to determine the physiological effects of excess cholesterol in the presence and absence of SP-A in surfactant. Fig. 5 depicts arterial oxygenation (PaO_2) over the course of 120 min of mechanical ventilation. In animals receiving BLES plus 5% (w/w) cholesterol, PaO_2 values were significantly increased 15 min after surfactant administration compared to animals receiving an air bolus control and remained significantly higher throughout the remaining 120 min of mechanical ventilation. Rats administered exogenous surfactant containing 20% (w/w) cholesterol had oxygenation values that were not significantly different than animals treated with an air bolus alone over the entire course of mechanical ventilation. In the two groups receiving exogenous surfactant containing either 5% or 20% (w/w) cholesterol with SP-A, oxygenation was significantly increased 15 min after administration and remained significantly higher oxygenation throughout ventilation as compared to rats treated with an air bolus alone. Analysis of collected surfactant from lavage after exogenous surfactant administration and mechanical ventilation showed that there were no significant differences in the pool sizes of either the total amount or the LA and SA subfractions of surfactant, regardless of cholesterol or SP-A content of the surfactant administered (Fig. 6).

3.4. Surface film analysis of cholesterol containing surfactant with and without SP-A

In order to further investigate the effects of cholesterol and SP-A on surfactant function, samples of BLES similar to those used in the above *in vivo* experiments were examined by AFM. Based on previous studies demonstrating that cholesterol impairs functional surfactant multilayer formation, samples were analyzed at a surface tension of approximately 22 mN/m at which, in functional surfactant, multilayers are observed [29]. The results of samples containing 5% (w/w) cholesterol (Fig. 7A) show higher (lighter) areas surrounding the darker "wells" with height analysis demonstrating that these higher areas are 1 to 3 bilayers in thickness. The addition of 5% (w/w) SP-A led to a similar pattern, with bi- and multi-layers (Fig. 7B) surrounding the lower, darker, areas. The pattern of samples with 20% (w/w) cholesterol was noticeably different, with higher, multiple layer film areas being limited to a small number of sites. Although 5% (w/w) SP-A added to the 20% (w/w) cholesterol sample did not completely restore surface film morphology towards the structures observed for 5% (w/w) cholesterol containing sample, more widely distributed areas of multilayers were observed.

4. Discussion

The current study tested the hypothesis that elevated cholesterol levels within surfactant can inhibit the surface tension reducing function of human surfactant and that these effects can be mitigated by SP-A. Our initial experiment focused on mechanically ventilated trauma patients. Despite the heterogeneous nature of this cohort of patients, including age, ventilation parameters, oxygenation parameters and timing of lavage procedure subsequent to the onset of mechanical ventilation, a consistent improvement in surfactant function was observed across all 10 samples with the addition of MβCD. This information was

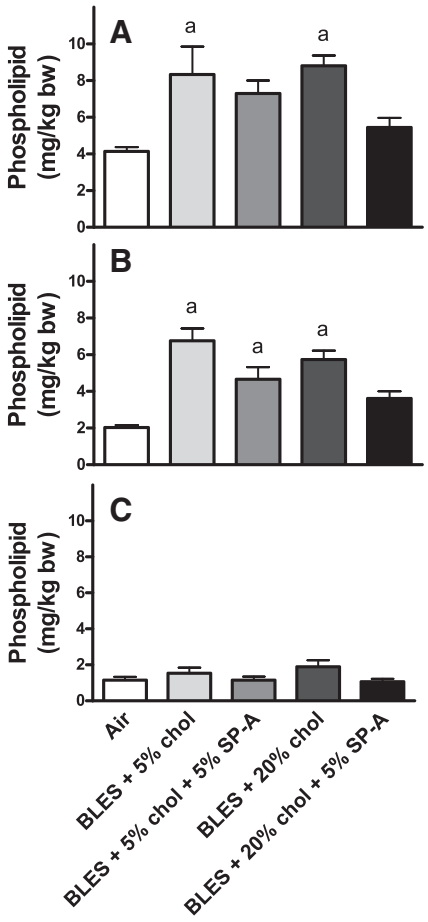

Fig. 6. Surfactant pool sizes following 120 min of mechanical ventilation. Surfactant pool sizes (mg phospholipid/kg BW) of rats depleted of endogenous surfactant followed by the administration of BLES with various amounts of cholesterol ± 5% (w/w) SP-A and 120 min of mechanical ventilation. Measurement of (A) total surfactant (B) large and (C) small aggregate pool sizes. Values are means ± SE, n = 6–7/group, a = $p < 0.05$ *vs.* air group.

extended by demonstrating that human alveolar proteinosis surfactant function was inhibited when reconstituted with high levels of cholesterol *in vitro*. Furthermore, it was also demonstrated that a clinically relevant exogenous surfactant preparation, BLES, had impaired function in the presence of excess cholesterol content when administered to surfactant deficient animals. Together with published evidence of cholesterol-mediated inhibition of surfactant in mechanically ventilated animals [20,21], these results support the conclusion that cholesterol-induced surfactant inhibition can actively contribute to physiological impairment of the lungs in mechanically ventilated patients.

To further explore the concept of cholesterol-mediated surfactant inhibition, we investigated the role of SP-A on this process. Using *in vitro* and *in vivo* approaches, the results provided evidence for a beneficial effect of 5% (w/w) SP-A in mitigating the inhibitory effects of cholesterol on surfactant function. Beneficial effects of SP-A in the context of surfactant dysfunction have also been demonstrated in other studies evaluating mechanisms of surfactant inhibition. For example, Cockshutt et al. demonstrated that reconstitution of SP-A in surfactant mitigated the inhibitory effect of serum-proteins on surfactant dysfunction [12]. Similarly, SP-A mitigated surfactant inhibition mediated by oxidized phospholipids and meconium in the context of neonatal aspiration [24,30,31]. Interestingly, naive mice deficient in SP-A possess normal lung and surfactant function under basal conditions [32,33]. Together, these findings suggest that although SP-A may not have an essential role in baseline surfactant function, it may serve an important role in improving or maintaining biophysical function of surfactant in the setting of compromised host surfactant function.

The specific biophysical mechanism(s) of cholesterol-mediated surfactant inhibition have been previously studied *in vitro* [16–19]. Although various molecular models of surfactant function have been proposed, the lateral organization of the surfactant lipids at high surface tensions coupled with the formation of lipid multilayers during compression represent the basis of the majority of proposed mechanisms [11,34]. Ultimately, a film of lipids remaining in a "solid" state capable of reaching low surface tensions appears to be important. Cholesterol can form a complex with some of the saturated phospholipids, mainly DPPC in the more solid area of the film. The inhibitory effect of elevated cholesterol on surfactant function appears to be through increasing the film fluidity, which occurs when the excess cholesterol interacts with some of the more "fluid" unsaturated phospholipids within the film. This was demonstrated by Gunasekara and colleagues [35] who showed that adding 20% cholesterol to BLES inhibited the function of this surfactant preparation, but that this inhibitory effect could be counteracted by adding 40% DPPC, thereby restoring the DPPC-cholesterol ratio. More direct evidence of a role for film fluidity has been obtained using AFM. Using BLES with approximately 10% (w/w) cholesterol it was demonstrated that at high surface tension the lateral structure of the film exhibited altered formation of liquid expanded (fluid) and liquid condensed (solid) domains [17]. We have confirmed these observations with the alveolar proteinosis samples utilized in the current study (data not shown). Leonenko and colleagues further investigated the effects of film structure by AFM by examining the structure at low surface tensions [18]. These studies demonstrated that elevated cholesterol led to impairment in the formation of multilayers during compression. Together these studies suggest that the major impact of cholesterol is on increasing the fluidity of the film, thereby resulting in collapse rather than multilayer formation during lateral compression.

In view of this mechanism of cholesterol inhibition, it is interesting to speculate on the mechanism by which SP-A can counteract this inhibition. Considering that evidence in the literature has demonstrated that SP-A can also counteract protein inhibition of surfactant by various serum proteins and inhibition of surfactant due to oxidation [12,30,31], we propose that this activity of SP-A likely reflects a common property of this protein, rather than unique mechanisms for each different inhibitors. The suggested mechanisms for counteracting these latter forms of inhibition have been through enhance adsorption, formation of tubular myelin and stabilizing multilayer formation [29,36,37]. Our results for cholesterol mediated inhibition support this latter mechanism as AFM analysis showed that SP-A may stabilize multilayer formation even in the presence of a more fluid film due to high cholesterol, although this effect was not a complete reversal (Fig. 7). Further studies are required to specifically investigate the process by which SP-A enhances surfactant function in the presence of various inhibitors.

The clinical relevance of our study was shown by the analysis of samples obtained from mechanically ventilated patients. The majority of previous studies investigating the mechanisms leading to the inhibition of pulmonary surfactant function in humans have focused on well-defined patient populations, such as those with mild or severe ARDS [13,14,38]. Those patients have, by definition, lung dysfunction including low blood oxygenation and reduced lung compliance. It is notable that despite our broad inclusion of trauma patients undergoing prolonged ventilation, cholesterol-mediated surfactant inhibition was consistently observed. This result suggests that prolonged ventilation itself may be a contributing factor to the cholesterol mediated surfactant dysfunction. Although not studied in a heterogeneous population of mechanically ventilated patients, there is strong evidence from mechanically ventilated patients with ARDS that SP-A levels decrease in this setting [4,13,39]. For example, Schmidt and colleagues showed levels of SP-A changing from 4.1% (w/w) in healthy volunteers to 2.6% in patients with ARDS within the first 24 h of diagnosis [4]. In general, considering the widespread use of mechanical ventilation among a broad population of patients in intensive care units, these findings offer further insight into

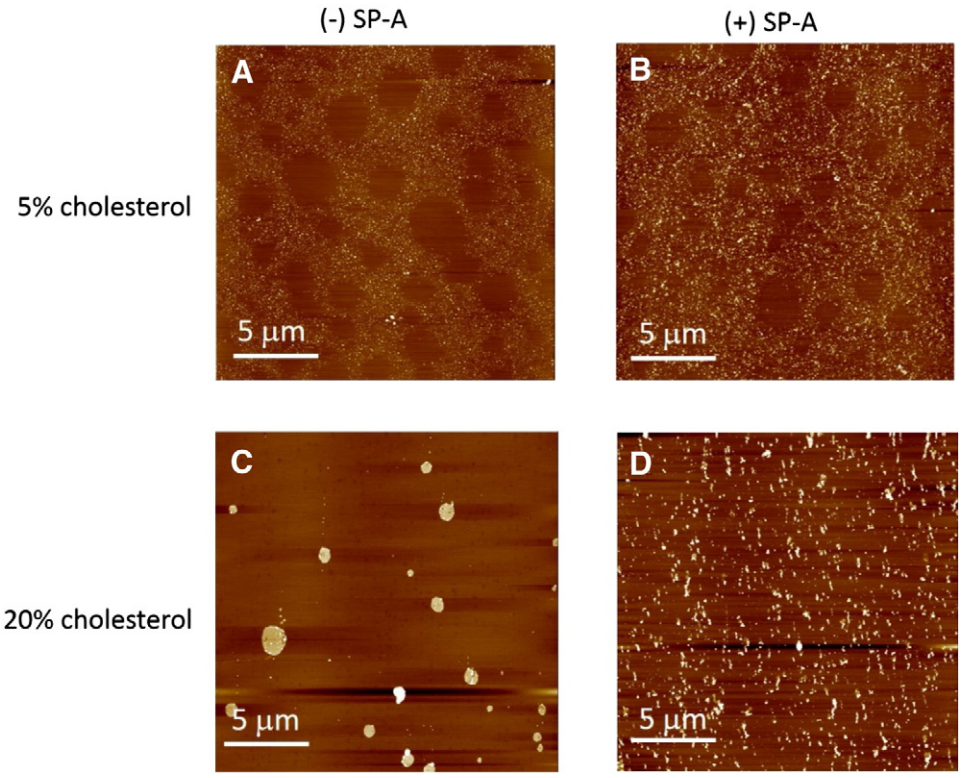

Fig. 7. AFM topographic images of surfactant with various amounts of cholesterol and SP-A. Typical AFM topographic images of BLES films + 5% (w/w) cholesterol (A) (−) SP-A, (B) (+) SP-A or 20% (w/w) cholesterol, (C) (−) SP-A, and (D) (+) SP-A deposited by the Langmuir–Blodgett technique. Each film was subjected to 3 full cycles of compression to surface tension approx. 22 mN/m and expansion to maximum area and deposits were taken on the fourth compression. All scanned areas are 20 × 20 μm^2.

the potential risks of this intervention and may have widespread implications for an even broader population of patients.

Although not the primary focus of this study, the surfactant subfractions from samples obtained from ventilated and surfactant depleted rats were also examined. This analysis allowed a preliminary assessment to determine if elevated levels of cholesterol with surfactant, with and without SP-A, were metabolized differently within the airspace. Previous *in vivo* experiments have suggested that SP-A promotes the re-uptake of surfactant into type-II alveolar cells and may modulate the secretion of surfactant [40,41]. We observed a trend of lower recovered surfactant pool sizes from animals administered surfactant reconstituted with 5% (w/w) SP-A, as compared to animals treated with surfactant lacking the protein, indicating that SP-A may have a regulatory role on surfactant metabolism within the lungs. Interestingly, to our knowledge, there is no information available on the effect of cholesterol on the alveolar metabolism of surfactant pool sizes or subfractions. Based on the lack of differences in pool sizes recovered in our experiment between the animals receiving surfactant with either 5 or 20% (w/w) cholesterol, we propose that cholesterol does not impact alveolar metabolism directly.

Overall, although our study provides strong evidence for the role of surfactant inhibition by cholesterol and mitigation by SP-A, several limitations should be noted. With regard to the data from ventilated patients (Fig. 1, 2), the small amount of surfactant recovered from BAL samples precluded direct measurements of cholesterol concentrations within individual samples or analysis of these samples in the presence of SP-A. These are considered future studies. Additionally, the MβCD utilized to sequester cholesterol may be relatively non-specific and binding of other inhibitory components cannot be excluded. However, three lines of evidence support that the major effect of MβCD is through cholesterol sequestration. First, in our control experiments with alveolar proteinosis sample containing 20% (w/w) cholesterol, we demonstrated that MβCD could restore the function of the surfactant. Second, in published studies with rat surfactant containing 13% (w/w) cholesterol, we measured reduced cholesterol values (to approximately 2%) following incubation with MβCD which was associated with improved activity; furthermore, restoring the cholesterol levels to its original 13% (w/w) value resulted in inhibition similar to the unaltered sample [20]. Third, it was demonstrated that the addition of cholesterol to BLES leads to a change in film structure by AFM which can be reversed with treatment with MβCD [17]. Finally, our study focused only on SP-A in the context of cholesterol inhibition. The role of the hydrophobic surfactant proteins, SP-B and SP-C, in the activity of surfactant with high cholesterol also warrants further investigation.

In conclusion, the current study built upon previous *in vitro* and *in vivo* studies by demonstrating that cholesterol mediated surfactant dysfunction occurs in a cohort of mechanically ventilated patients. Moreover, we show that SP-A can mitigate these effects in both *in vivo* and *in vitro* assays. Further studies are required to determine whether cholesterol inhibition of surfactant may be used clinically to guide the prognosis or treatment in individuals requiring prolonged mechanical ventilation.

Acknowledgements

The authors like to thank BLES Biochemicals Inc. for generously providing BLES. The authors also acknowledge Fred Possmayer for the helpful discussions, Dr Matthias Amrein for the help in establishing the captive bubble surfactometer technique, Lynda McCaig for the help with the animal experimentation, Valeria Puntorieri, Brandon Banaschewski, Julianna Decuzzi and Scott Milos for proofreading the manuscript, and all the clinical staff involved in obtaining BAL samples from trauma patients. This research was funded by operating grants from the Canadian Institutes of Health Research and the Ontario Thoracic Society, as well as local funding from the program of Experimental Medicine and Western University.

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