Role of cholesterol in the biophysical dysfunction of surfactant in ventilator-induced lung injury

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Departments of 1Physiology and Pharmacology, 2Medicine, and 3Obstetrics and Gynecology, and 4Lawson Health Research Institute, University of Western Ontario, London, Ontario; and 4Department of Cell Biology and Anatomy, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada

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Vockeroth D, Gunasekara L, Amrein M, Possmayer F, Lewis JF, Veldhuizen RA. Role of cholesterol in the biophysical dysfunction of surfactant in ventilator-induced lung injury. Am J Physiol Lung Cell Mol Physiol 298:L117–L125, 2010. First published November 6, 2009; doi:10.1152/ajplung.00218.2009.—Mechanical ventilation may lead to an impairment of the endogenous surfactant system, which is one of the mechanisms by which this intervention contributes to the progression of acute lung injury. The most extensively studied mechanism of surfactant dysfunction is serum protein inhibition. However, recent studies indicate that hydrophobic components of surfactant may also contribute. It was hypothesized that elevated levels of cholesterol significantly contribute to surfactant dysfunction in ventilation-induced lung injury. Sprague-Dawley rats (n = 30) were randomized to either high-tidal volume or low-tidal volume ventilation and monitored for 2 h. Subsequently, the lungs were lavaged, surfactant was isolated, and the biophysical properties of this isolated surfactant were analyzed on a captive bubble surfactometer with and without the removal of cholesterol using methyl-β-cyclodextrin. The results showed lower oxygenation values in the high-tidal volume group during the last 30 min of ventilation compared with the low-tidal volume group. Surfactant obtained from the high-tidal volume animals had a significant impairment in function compared with material from the low-tidal volume group. Removal of cholesterol from the high-tidal volume group improved the ability of the surfactant to reduce the surface tension to low values. Subsequent reconstitution of high-cholesterol values led to an impairment in surface activity. It is concluded that increased levels of cholesterol associated with endogenous surfactant represent a major contributor to the inhibition of surfactant function in ventilation-induced lung injury.

Mechanical ventilation (MV) is an essential supportive therapy in all patients with ALI/ARDS to maintain adequate gas exchange (1). Unfortunately, MV can also contribute to the progression of lung injury (9, 25). Two aspects of MV shown to contribute to this progression are the overdistension and repetitive collapse of alveoli (9, 26). The mechanisms by which these aspects contribute to lung injury can be studied in normal animals by using a high-tidal volume (Vt) and no positive end-expiratory pressure (PEEP). The resulting damage to the lung is termed ventilator-induced lung injury (VILI). Alterations to surfactant represent one of the main mechanisms by which overdistension and collapse induce damage during the progression of VILI (2, 23, 36, 37, 38). Changes to the surfactant system such as decreased levels of surfactant proteins, altered lipid composition, and the association of serum proteins leaked in from the capillaries are all believed to contribute to the dysfunction of surfactant, and, consequently, decreased lung function (23, 39).

The most extensively studied mechanism of surfactant dysfunction is serum protein inhibition (6, 17, 32). Despite the strong evidence supporting this role of serum protein inhibition, a recent study by our lab demonstrated that alterations to surfactant composition itself also contributed to the impairment of biophysical function in VILI (23). Quantification of several surfactant components revealed a significant increase in the level of cholesterol in surfactant from animals with VILI. This observation, and the recent studies demonstrating that the addition of supraphysiological levels of cholesterol to exogenous surfactant preparations inhibit surfactant function in vitro (12, 13, 18, 20), led to our hypothesis that cholesterol significantly contributes to surfactant dysfunction in VILI.

To address this hypothesis, surfactant was isolated from adult rats that had VILI induced by high-tidal volume ventilation and from control ventilated animals. The biophysical properties of the isolated surfactants were analyzed on the captive bubble surfactometer (CBS) with and without the removal of cholesterol using methyl-β-cyclodextrin (MβCD).

MATERIALS AND METHODS

Animal experimentation. A total of 30 male Sprague-Dawley rats (Charles River, St. Constant, PQ, Canada), weighing 350–430 g, were included in this study. All procedures were approved by the animal use subcommittee at the University of Western Ontario, under the guidelines of the Canadian Council of Animal Care.

Animal surgery and ventilation were performed as previously reported (23). Briefly, the mass of the rat was recorded, the animals were anesthetized, and both jugular veins and the right carotid artery were exposed and cannulated to deliver anesthetic/analgesic (0.5–2.0 mg·100 g−1·h−1 propofol), a neuromuscular inhibitor (0.2 ml·h−1 2.2
was extracted by the method of Bligh and Dyer (4). The amount of
the LA pellet was resuspended in 2 ml of 0.15 M sterile saline and
containing the large aggregate (LA) subfraction of surfactant (21).
A whole lung lavage procedure was performed as previously
described, computer-controlled CBS (12, 31). Briefly, the
ventilator that was calibrated to deliver a Vt of 30 ml/kg was used for
animals randomized to the high Vt strategy. After meeting inclusion
criteria, all animals were disconnected from the ventilator for 5 s
and reconnected to the ventilator of the appropriate strategy. PIP was
monitored throughout ventilation, and arterial blood gas measure-
ments were taken at baseline and every 15 min thereafter.

Isolation of surfactant. Following 2 h of either low or high Vt
ventilation, animals were killed with an overdose of pentobarbital
sodium (0.2–0.6 ml at 65 mg/ml) administered intravenously to lower
blood pressure and exsanguinated via transection of the descending
aorta. A midline sternectomy was performed to expose and visualize
the lungs followed by determination of the pressure-volume curve
(23). A whole lung lavage procedure was performed as previously
described, and differential centrifugation was used to isolate the pellet
containing the large aggregate (LA) subfraction of surfactant (21).
The LA pellet was resuspended in 2 ml of 0.15 M sterile saline and
was extracted by the method of Bligh and Dyer (4). The amount of
phospholipids in the chloroform extract was quantified using a Duck-
Cheng phosphorus assay (10). The remainder of the LA fraction for
each animal was lipid extracted, dried under nitrogen (N2), and stored
at −20°C for further analysis.

Assessment of surface tension. Before surface tension assessment,
each LA surfactant sample was resuspended to a concentration of 27
mg·ml⁻¹ in buffer (140 mM NaCl, 10 mM HEPES, and 2.5 mM CaCl₂,
PH 6.9). The surface activity of the surfactants was assessed using a
previously described, computer-controlled CBS (12, 31). Briefly, the
chamber of the CBS was filled with a buffer solution (140 mM NaCl,
10 mM HEPES, and 2.5 mM CaCl₂, PH 6.9) containing 10% sucrose
(by mass) to increase the buffer density above that of surfactant.
A small bubble (0.035–0.045 ml) was introduced into the chamber and
allowed to float up and rest against the concave agarose plug at the top
of the chamber. Approximately 0.1–0.15 μl of LA sample was
deposited just below the air-liquid interface by a transparent capillary
attached to a micromanipulator used for the precise approach of the
capillary to the bubble (12). The bubble was imaged using a video
camera (Pulnix TM-7CN, S/N: 017505) and recorded for future
analysis. Throughout the experiment, the chamber was kept at 37°C.
Upon injection of surfactant, a 5-min adsorption period (initial ad-
sorption) was allowed, during which time there was no manipulation
of the bubble, and the change in the shape of the bubble was
monitored. The chamber was then sealed and the bubble was rapidly
expanded to a volume of 0.13 ml over a 1-s interval. Again, a 5-min
period was allowed to monitor the change in bubble shape (rapid
expansion). Following this 5-min period, a quasistatic cycle was
performed by compressing and then expanding in a stepwise manner in increments of 20% of the total volume to set the
minimum (0.0090 ml) and maximum bubble volume (0.13 ml) for
subsequent assessment (12). Dynamic cycling, in which the bubble
volume was smoothly changed to the defined volumes for 20 cycles at
a rate of 20 cycles/min, was then performed to determine the mini-
num achievable surface tension during compression (minimum sur-
face tension, MST). The surface tensions of the bubbles were calcu-
lated based on the shape of the bubble at various stages throughout the
CBS technique. Briefly, during initial adsorption and rapid expansion,
images at precisely 0, 1, 5, 10, 30, and 300 s were analyzed for surface
tension. During dynamic cycling, every fifth image was analyzed, and
the lowest minimum surface tensions that were achieved for cycles 1,
2, 5, 10, and 20 were determined.

Evaluating the effect of cholesterol. Two separate protocols were
utilized to remove cholesterol from the extracted LA samples by
MβCD (Sigma-Aldrich, cat. no. M7439-1G). In experiment 1, LA
samples were injected into the chamber of the CBS, which contained
either buffer alone, as described above, or a buffer containing 20 mM
MβCD to remove cholesterol from the injected sample (28, 31, 42).
Samples were then incubated within the bubble chamber for 1 h
following the initial adsorption step as described above. Thus, this
experiment tested four experimental conditions for the isolated
LA: 1) LA from low Vt injected in a control buffer, 2) LA from low
Vt injected in MβCD-containing buffer, 3) LA from high Vt injected
in a control buffer, and 4) LA from high Vt injected in MβCD-
containing buffer.

In experiment 2, cholesterol was removed from the extracted samples
in a test tube before injection into the CBS. Based on preliminary
experiments, aliquots of the extracted LA were resus-
pended in 2 ml of 5 mM MβCD solution in saline, whereas another
aliquot was resuspended in saline as a control. The samples were
incubated overnight at 37°C. After incubation, the volumes were
centrifuged at 40,000 g to remove the MβCD and isolate the LA
pellets, which were resuspended in a 2-ml aliquot of saline. The
resuspended LA were then centrifuged again at 40,000 g, and the
remaining LA pellet was resuspended in saline again, and an aliquot
was taken to determine PL phosphorus content (10). Of note, this
technique for cholesterol removal was characterized using radioactive
cholesterol and several different surfactant preparations varying in their
cholesterol content. The results demonstrated the removal of
80–85% of the cholesterol from all preparations. In addition, a
separate experiment showed that MβCD treatment of LA from either
normal rats or rats exposed to either low Vt or high Vt ventilation did
not result in changes in the molecular species of phosphatidylcholine
and phosphatidylglycerol, as assessed by mass spectrometry analysis.

To validate that the potential differences in biophysical function in
this experiment were specifically due to cholesterol removal, choles-
terol was added back to values corresponding to those observed in
either low Vt or high Vt samples. MβCD-incubated samples obtained
from animals that were ventilated using low Vt MV were supple-
mented back to a final concentration of 8% cholesterol, and the high
Vt MβCD group was supplemented to 13% cholesterol. Finally,
extracted samples that were not incubated with MβCD from the low
Vt group were supplemented with an additional 5% cholesterol, to
increase the final concentration of cholesterol to the 13% that was
observed in the high Vt group (23).

Thus, experiment 2 involved the surface tension assessments of
seven different conditions: 1 and 2) saline incubated samples from
both low Vt and high Vt groups, 3 and 4) MβCD-incubated samples
from both low Vt and high Vt groups, 5 and 6) cholesterol-supple-
mented, MβCD-incubated samples from both low Vt and high Vt
groups, and 7) cholesterol-supplemented sample from the low Vt

group.

Statistical analysis. All values are reported as means ± SE.
Statistical analysis was performed using a two-way repeated measures
ANOVA to investigate differences over time, with a Tukey post hoc
test to investigate one-way interactions between groups. Surface
tension assessments in experiment 1 were also analyzed using a
three-way ANOVA followed by a two-way ANOVA to investigate
any interactions of effects. Two-way repeated measures ANOVAs were performed using SigmaStat for Windows, version 3.0 (Systat Software, San Jose, CA). Three-way ANOVAs were performed using SPSS version 16.0 for Windows (SPSS, Chicago, IL). Values are considered significantly different at $P < 0.05$.

RESULTS

Animal experimentation. The mean mass of animals in this study was $391 \pm 3$ g, with no significant differences between groups. Physiological parameters during the 2-h ventilation period for animals in both experiments are shown in Table 1. Animals ventilated using low Vt maintained consistent $P_{aO_2}/F_{I_O_2}$ values above 400 mmHg over the entire time course of ventilation. Animals receiving high Vt had $P_{aO_2}/F_{I_O_2}$ values that decreased over time such that at 90 and 120 min of ventilation, these values were significantly lower than both those measured at baseline and those measured in the low Vt MV group at the same time point. Animals receiving low Vt MV maintained $P_{aCO_2}$ values between 40 and 43 mmHg throughout the 2 h of ventilation, whereas animals in the high Vt MV group exhibited significantly lower $P_{aCO_2}$ values compared with baseline values and compared with the low Vt group. Blood pressure was not significantly different among the low Vt and high Vt group at up to 90 min of ventilation. At 120 min, the blood pressure in the high Vt animals was significantly lower than those in the low Vt animals. Heart rate was not significantly affected by either low Vt or high Vt ventilation.

$PIP$ values during ventilation were recorded at the same time intervals as the blood gas analysis. Animals receiving low Vt MV maintained $PIP$ values between 12 and 13 cmH$_2$O throughout ventilation. High Vt ventilation resulted in significantly higher $PIP$ values (28–38 cmH$_2$O) at every time point compared with the low Vt group. Analysis over time showed that within the high Vt group, measurements of $PIP$ at 90, 105, and 120 min were significantly higher than the 0-min value within this group (results not shown). Measurements of the pressure-volume curves postventilation indicated that animals receiving high Vt MV had significantly lower compliance than low Vt animals, as indicated by a shift downward and to the right in the pressure-volume curve (results not shown). The maximum sustainable volume (Vmax) at 26 cmH$_2$O pressure was significantly lower in animals from the high Vt group compared with animals receiving low Vt ventilation (29.8 ± 1.5 ml/kg vs. 39.4 ± 0.9 ml/kg). In general, these physiological parameters are consistent with VILI and with previous studies utilizing this specific experimental model (23, 40). Isolated surfactant LA samples from the low Vt animals have 5–7% cholesterol, which is similar to that found in normal, nonventilated rats. Isolated surfactant LA samples from the high Vt animals had 12–14% cholesterol.

Surface activity measurements: experiment 1. Following collection of the surfactant LA fraction from the two ventilation groups, experiment 1 evaluated the effect of cholesterol on the surface activity via the inclusion of MßCD within the buffer of bubble chamber. Surface tension measurements during the initial adsorption of surfactant to the air-liquid interface for the four experimental groups are shown in Fig. 1. In the presence of the control buffer, low Vt samples reached equilibrium surface tension (20–23 mN/m) within 10 s and maintained that value over the entire 5-min period. There was no significant difference at 0 s between high Vt and low Vt samples; however, at every time point thereafter, high Vt samples exhibited significantly higher surface tension values compared with the low Vt samples (Fig. 1A). Similar results were obtained in the presence of MßCD in the chamber of the CBS (Fig. 1B) with high Vt samples exhibiting significantly higher surface tension values compared with low Vt samples. Surface tension of MßCD-containing buffer at 0 s, before surfactant injection, was significantly lower compared with control buffer since MßCD possesses slight surface active properties (Fig. 1, B vs. A). At every time point analyzed thereafter, MßCD exhibited no effect on surface tension reduction within each ventilation strategy, as low Vt with the MßCD buffer was not different from low Vt with control buffer, and high Vt with MßCD buffer was not different from high Vt with control buffer (Fig. 1, B vs. A).

Following adsorption measurements and the subsequent incubation with either control or MßCD buffer, the bubble inside the CBS chamber was rapidly expanded with a further measurement of surface tensions over a 5-min period. No significant differences between low Vt and high Vt samples were observed when analyzed in the control buffer. In addition, there were no significant differences between low Vt in MßCD buffer and in control buffer or between high Vt in MßCD buffer and in control buffer. High Vt in MßCD buffer was also

<table>
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<th>PaO$_2$/FIO$_2$, mmHg</th>
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<th>60 min</th>
<th>90 min</th>
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<td>430±5.9</td>
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<td>434±7.3</td>
<td>439±5.4</td>
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<td>440±4.4</td>
<td>438±6.1</td>
<td>361±29.8*†</td>
<td>173±29.3*†</td>
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<th>PaCO$_2$, mmHg</th>
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<td>Low Vt</td>
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<td>Low Vt</td>
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<td>71.5±3.2</td>
<td>72.0±3.1</td>
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<tr>
<td>High Vt</td>
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<td>72.2±2.2</td>
<td>71.8±2.5</td>
<td>70.0±2.9</td>
<td>49.7±3.0*†</td>
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<th>Heart rate, beats/min</th>
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<th>60 min</th>
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<tr>
<td>Low Vt</td>
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<td>311±6</td>
<td>298±5</td>
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<td>High Vt</td>
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<td>296±4</td>
<td>297±7</td>
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Table 1. Physiological parameters of animals measured at baseline and throughout mechanical ventilation

Values are means ± SE, n = 15–16/group. Statistical significance is indicated by *high tidal volume (Vt) vs. low Vt, †vs. 0 min within group, where $P < 0.05$.\n
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Comparison of high Vt in control buffer achieved the lowest MST in the first cycle, which increased significantly over subsequent cycles (Fig. 3A). Surfactant samples in the other three conditions maintained their respective MST values over all cycles analyzed.

Surface activity measurements: experiment 2. To further elucidate the role of cholesterol in surface tension impairment due to high Vt ventilation, samples were incubated with MβCD before analysis. Surface tension during the initial adsorption of LA to the air-liquid interface is shown in Fig. 4 for the untreated samples, the MβCD-treated samples, and for the three samples in which cholesterol was added. As shown in Fig. 4A, comparison of the two untreated groups revealed that high Vt exhibited a significantly higher surface tension over all time points than the low Vt group. After MβCD treatment (Fig. 4B), the adsorption characteristics of samples obtained from the low Vt and high Vt groups were similar. Comparison of the untreated low Vt with the MβCD-treated low Vt group (Fig. 4, A vs. B) revealed that the MβCD group had significantly higher surface tension values at 1, 5, and 10 s; however, no differences existed at 30, 60, or 300 s. Statistical comparison of the two groups of samples from animals receiving high Vt MV revealed no significant differences at time points up to 60 s; however, at 300 s, surface tension values for the MβCD-treated high Vt group were significantly lower compared with the untreated high Vt samples.

As shown in Fig. 4C, samples from the MβCD-treated low Vt and high Vt groups in which cholesterol was added back to its original values revealed rapid adsorption for the low Vt group and slower adsorption for the high Vt group. These values were similar to those observed in the untreated samples at all time points analyzed (Fig. 4, A vs. C). Finally, untreated samples from the low Vt group, to which cholesterol was supplemented to levels similar as those observed in the high Vt group, had adsorption values that were similar to the untreated high Vt group (Fig. 4, A and C).

Surface tension during rapid expansion of the samples shown in Fig. 4 revealed no significant differences at any time points between any of the groups. The exception to this was a significantly higher surface tension at 1 and 5 s only for the sample from the untreated low Vt group supplemented with cholesterol compared with the samples from untreated low Vt group (results not shown).

The MST values during dynamic compression-expansion cycling of the bubble, for the samples in experiment 2, are shown in Fig. 5. Comparison of the untreated low Vt and high Vt groups revealed higher MST over all cycles in high Vt samples (Fig. 5A). In MβCD-treated samples (Fig. 5B), comparison of high Vt MβCD-treated to low Vt MβCD-treated revealed a higher MST in high Vt samples over cycles 2, 5, 10, and 20, but no difference at cycle 1. Comparison between untreated low Vt and the MβCD-treated low Vt samples revealed no significant differences over cycles 1, 2, 5, or 10; however, cycle 20 was significantly lower in MβCD-treated surfactant samples (Fig. 5, A and B). The effect of MβCD in the high Vt groups was more marked than that in the low Vt
groups. Specifically, comparison of the untreated high Vt samples with the MβCD-treated high Vt samples revealed a lower MST over cycles 1, 2, and 5 in MβCD-treated samples, with no significant difference at cycles 10 and 20 (Fig. 5, A and B). As shown in Fig. 4C, addition of cholesterol to MβCD-treated low Vt samples compared with MβCD-treated high Vt samples revealed a higher MST over all cycles analyzed in samples from animals receiving high Vt. Comparison of these samples to their respective untreated samples (Fig. 5, A vs. C) revealed similar MST over all cycles analyzed. Finally, statistical analysis of low Vt samples with added cholesterol revealed higher MST over cycles 1, 2, 5, and 10 compared with untreated low Vt.

DISCUSSION

The purpose of this study was to investigate the contribution of cholesterol to the biophysical dysfunction of surfactant observed in VILI. The rationale was that 1) MV is known to contribute to the progression of lung injury in patients with ALI/ARDS (5), 2) the neutral lipid component of surfactant, which includes cholesterol, was increased in patients with ALI/ARDS (22) and in VILI (23), and 3) adding cholesterol to exogenous surfactant preparations inhibited surfactant function (12, 13, 18, 20, 33). Our study expanded on these studies by demonstrating, for the first time, that cholesterol-mediated inhibition of surfactant function occurred with samples from an in vivo model. We conclude that increased levels of cholesterol represent a major contributor to the inhibition of surfactant function in VILI. It is speculated that this inhibition may play a role in surfactant dysfunction during MV in the setting of ALI and as such contribute to the progression of lung injury due to MV.

The experimental model utilized to obtain the various LA fractions studied was an established model of MV known to
induce VILI in rats (23). Lung dysfunction was verified by demonstrating a severe reduction in PaO2/FIO2 and compliance over 2 h and an increase in PIP values. To specifically study the contribution of cholesterol, the large surfactant aggregates obtained from these animals were extracted with organic solvent to remove nonsurfactant proteins as well as SP-A and SP-D. The extracted samples, containing the hydrophobic components of surfactant, were subsequently analyzed for their ability to reduce surface tension using two complementary approaches. In experiment 1, MβCD was added to the buffer within the chamber of the CBS where it would complex with, and remove, cholesterol from the surfactant film during the 1-h incubation period (28, 42). This method is suitable for the analysis of the role of cholesterol in surfactant using only a very small amount of material and can therefore be utilized in future studies with samples from clinical studies, in which the surfactant yield is often limited. In experiment 2, cholesterol was removed from the samples before surface activity measurements. This method allowed us to verify the effect of cholesterol via the addition of exogenous cholesterol to these samples before the introduction of this material into the CBS. It should be noted that the efficacy of cholesterol removal by MβCD was based on preliminary studies using radioactive cholesterol, and cholesterol levels were not measured in the actual samples used. Using the CBS, we examined surface tension during adsorption, expansion, and compression in LA samples obtained from the various groups.

The main result from the two experiments was that incubation of surfactant samples obtained from animals with VILI (i.e., the high Vt group) with MβCD resulted in an improve-
Lesterol observed with high Vt LA was responsible for deterioration of biophysical activity. In brief, the experiments showed that samples with restored cholesterol levels had similar activities as the original samples. In addition, elevating cholesterol levels in the samples from the low Vt group (8% cholesterol) to levels similar to those observed in the high Vt group (13% cholesterol) resulted in an impaired activity similar to the nonsupplemented high Vt samples. Together, these results provide strong evidence that elevated cholesterol is a major contributor to the dysfunction of surfactant in VILI. It should be noted, however, that this evidence for the inhibitory activity of cholesterol does not preclude the possibility of a role for other inhibitory components that may have been removed during the purification and extraction procedures of the surfactant in this study.

Whereas the overall interpretation of the two experiments was similar, there were also two specific differences between the results from the different approaches that warrant discussion. First, the observed effects of MβCD on the adsorption step of the activity measurement differed between experiment 1 and experiment 2. In experiment 1, MβCD did not have an effect on the adsorption values, whereas in experiment 2, MβCD treatment affected adsorption of samples obtained from both the low Vt and high Vt groups. This difference likely relates to the fact that adsorption in experiment 1 occurred by injection of the cholesterol-containing material into the CBS, with subsequent removal of cholesterol from the adsorbed film by incubation with MβCD. In contrast, experiment 2 measured the adsorption of samples that had cholesterol removed before injection into the CBS. Thus, interpreting the data regarding the role of cholesterol on adsorption specifically can only be based on experiment 2. The results showed a reduced adsorption in samples from the high Vt group compared with the low Vt group that improved upon treatment with MβCD, which is consistent with an inhibitory role of elevated cholesterol. However, treatment of the low Vt samples with MβCD resulted in a reduced adsorption compared with the nontreated low Vt sample. Thus, it appears that elevated cholesterol can inhibit surfactant adsorption, but also that physiological levels of cholesterol are required for normal rapid adsorption.
observations are consistent with previous suggestions that physiological levels of cholesterol enhance the ability of pulmonary surfactant to adsorb to equilibrium surface tension at the air-liquid interface (3, 41).

A second difference between the two approaches relates to the MST values obtained during compression. Whereas the MST of untreated samples from low Vt and high Vt groups were similar between the two experiments, the MST obtained over consecutive cycles was different between the two experiments after MβCD exposure. Specifically, in experiment 1, the MST remained low, whereas in experiment 2, the MST increased over the different cycles measured. It is speculated that this difference in results could be related to difference in the manner or the extent of cholesterol removal between the two approaches. In experiment 1, cholesterol was directly removed from the adsorbed film, which was subsequently analyzed for the MST. The amount of cholesterol removed by this method could not be assessed. In contrast, in experiment 2, ~80–85% of the cholesterol was removed by the MβCD treatment, and the material was depleted before the adsorption step. It is also possible that the differences in adsorption due to cholesterol, as described above, may have affected the subsequent MST measurements. This will require further study.

Regardless of the specific differences between our two approaches, the overall interpretation of both experiments clearly implicates elevated cholesterol as an important inhibitor of surfactant function. The potential molecular mechanism by which increased levels of cholesterol affect the surface tension reducing ability of surfactant during compression stems mostly from reconstitution studies with high amounts of cholesterol added to exogenous surfactant preparations (8, 12, 13, 18, 19). Consistent with our results, addition of 20% cholesterol (by weight) to an exogenous surfactant preparation resulted in an inability to achieve low MST values during the compression phase of dynamic cycling. In addition, atomic force microscopy of the surface films demonstrated that high cholesterol alters the lateral organization of surfactant (18, 19). Specifically, these studies, as well as other experiments with pure lipid films, demonstrated that supraphysiological levels of cholesterol increase surfactant rigidity due to the interaction of cholesterol with more fluid-like lipids (such as the unsaturated lipids of surfactant) (29, 43). This effect is not observed with normal physiological levels of cholesterol, where an interaction occurs between cholesterol and the rigid lipids, which enhances the fluidity (20, 29, 43). Furthermore, it has also been observed that surface films of surfactant with high cholesterol had a tendency to collapse at higher surface tension (13, 20, 30). For example, using atomic force microscopy, it was determined that the lack of film stability in the presence of elevated levels of cholesterol was correlated to an inhibition of a monolayer-to-bilayer conversion (20). Functional surfactant films, containing physiological amounts of cholesterol, underwent a monolayer-to-bilayer conversion upon film compression and resulted in a film with lipid bilayer stacks scattered over a lipid monolayer, whereas the films formed from surfactant with elevated cholesterol formed only a few stacks (20). Overall, these biophysical studies suggest that the increased cholesterol in our samples obtained from animals with VILI decreased the surfactant fluidity and that this may lead to the impaired ability to reach low surface tension during compression due to collapse of the surface film. However, more detailed investigation of the mechanism by which increased levels of cholesterol inhibit surfactant function is warranted.

The current finding that an increased level of cholesterol in surfactant contributes significantly to surfactant impairment may have important clinical significance. Although most of the research on surfactant dysfunction in VILI and ALI/ARDS has focused on serum protein inhibition (17, 27, 43), it has recently been reported that surfactant from patients with ALI/ARDS had increased levels of neutral lipid, and this was thought to contribute to surfactant dysfunction (22). Thus, inhibition by elevated cholesterol levels represents an additional mechanism that could be targeted in studies to limit surfactant dysfunction. It should be noted that the mechanism by which serum proteins inhibit pulmonary surfactant differs from that observed with cholesterol. With proteins, inhibition can normally be relieved through repeated compression-expansion cycling (43). With cholesterol-mediated inhibition, compression-expansion cycling does not relieve inhibition and may even result in higher MST, as observed in our experiments. In this context, it would also be informative to elucidate the source of the elevated cholesterol in surfactant obtained in our model of VILI and in patients with ALI/ARDS. In the healthy lung, it is widely accepted that most (99%) surfactant-associated cholesterol is derived from serum lipoproteins (15, 16). Cholesterol from serum lipoproteins is taken up by the type II cell and incorporated into the surfactant before secretion (15). Furthermore, it has recently been demonstrated that mice fed a high cholesterol diet exhibit an increased level of serum cholesterol and that the surfactant obtained from these mice had increased cholesterol values with impaired biophysical function (24). However, Davidson et al. (7) demonstrated that lowering levels of serum cholesterol by inhibiting the release of hepatic lipoproteins did not affect surfactant cholesterol levels, due to the ability of type II cells to synthesize cholesterol. These findings provide evidence that, at least in the unjured lung, surfactant cholesterol arises from the serum and/or de novo synthesis. In contrast, the source of cholesterol in VILI has yet to be determined. Further investigation into the source of the increased levels of cholesterol is warranted if treatment strategies are to be aimed at mitigating the increase in surfactant cholesterol observed in VILI.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


