Increase of C-Reactive Protein and Decrease of Surfactant Protein A in Surfactant after Lung Transplantation

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In this study, we asked whether the serum acute-phase protein C-reactive protein (CRP) increased in large surfactant aggregates after lung transplantation and analyzed the changes in composition and interfacial adsorption activity of those aggregates. Single left lung transplantation was performed in weight-matched pairs of dogs. A double-lung block from the donor animal was flushed with either modified Euro-Collins solution (EC) (n = 6) or University of Wisconsin solution (UW) (n = 6) at 4°C followed by immersion in cold EC or UW for 22 h. The left donor lung was transplanted. The recipient dog was then reperfused for 4.5 h. Irrespective of the preservation fluid, gas exchanged was impaired in the transplanted lung after 4.5 h of reperfusion. Large surfactant aggregates obtained from this lung showed reduced ability to rapidly adsorb to an air-liquid interface. Phospholipid (PL) content and PL composition of surfactant from lung transplants was similar to that of the control lungs. However, the content of surfactant protein A decreased after reperfusion. In addition, Western blot analyses showed that levels of CRP increased in surfactant from transplanted but not from donor lungs. The addition of human CRP to control surfactant (CRP:PL weight ratio, 0.01:1) caused a decrease of surfactant adsorption. We conclude that the impairment of adsorption facilities of surfactant from transplanted lungs may be correlated with decreased levels of surfactant protein A and increased levels of CRP. The presence of elevated levels of CRP in bronchoalveolar lavage could be a very sensitive marker of lung injury.

Lung transplantation has become an option for selected patients with end-stage obstructive or infective lung disease. However, progress with lung transplantation lagged behind other organs, such as kidney, liver, and heart, in part because of problems unique to the lung (1). The lung is a delicate organ that can develop significant dysfunction in response to minor injury. The severe ischemia-reperfusion injury associated with lung transplantation is similar to the impairment found with acute respiratory distress syndrome (ARDS) (1, 2). In both cases, inflammatory mediators are believed to play a significant role in the cascade of events leading to lung dysfunction (1, 2). The alteration of alveolar surfactant system is an important factor contributing to lung dysfunction in ARDS (2-4) and after lung transplantation during the early reperfusion period (5, 6). In normal lung, alveolar surfactant serves to stabilize the alveoli and distal airways at low lung volumes (7). To fulfill this function, surfactant is composed of a complex mixture consisting of lipids (mainly phospholipids) (8) and three surfactant apolipoproteins (SP-A, SP-B, and SP-C) (9). A iteration in alveolar surfactant leads to a decrease in compliance, ventilation-perfusion mismatch including shunt flow due to altered gas flow distribution, and lung edema formation (2). The possible mechanisms of surfactant alterations in ARDS and maybe in lung transplants include (1) damage or inhibition of surfactant compounds by inflammatory mediators (proteases, oxygen free radicals, phospholipases, cytokines, or lipid mediators) (10, 11), (2) inhibition of the biophysical activity of surfactant by plasma protein leakage (12), and (3) reduced or altered generation of surfactant-active compounds (2, 13, 14).

Little is published on the changes in lung surfactant after lung transplantation. Recent studies (5, 6) indicated that the biophysical activity of alveolar surfactant was impaired during the early reperfusion period in part as a consequence of the inhibition of surfactant by serum proteins that leaked in large amounts into alveolar fluid. The purpose of this study was to

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characterize the changes in the composition and activity of pulmonary surfactant isolated from canine transplanted lungs and to investigate the presence of the acute-phase protein C-reactive protein (CRP) in surfactant from donor and transplanted lungs. CRP is produced predominantly by hepatocytes after infection or inflammation and secreted into the serum. In addition, alveolar macrophages can also produce and secrete CRP to the alveolar space (15). The CRP mRNA levels in isolated macrophages are up-regulated by in vitro lipopolysaccharide stimulation. This protein is a potent inhibitor of surfactant adsorption (16) and has a high calcium-dependent affinity for phosphocholine (17), which is the headgroup of the most abundant component of pulmonary surfactant, phosphatidylcholine.

METHODS

Experimental Groups

Single left lung transplantation procedures were performed in weight-matched pairs of dogs (20 to 25 kg) as described previously (18). A total of 24 dogs were randomly divided into two groups (n = 12 per group; 6 donors and 6 recipients): Group 1, lung flushing via the pulmonary artery clamp was removed first to allow for backflow and dealing with vascular anastomosis was done with a single 5-0 suture. The pulmonary artery anastomosis was performed with a single 4-0 prolene suture, and each atrial appendage was isolated with a side-biting clamp. The left lung and the left main bronchus proximal to the last branch. Lastly, the left pulmonary artery was cross-clamped and cardioplegic (cysteamine plus 20% dextrose in water) solution (C.C. no. 85-23, revised in 1985). The heart was removed, and the double-lung block excised. Both lungs were immersed in E.C. or UW solution at 4°C for 22 h until ischemia time and cross-clamping of the aorta followed, and cardioplegic (cysteamine plus 20% dextrose in water) solution (C.C. no. 85-23, revised in 1985). The donor chest was opened by median-sternotomy, and ligatures were placed around both venae cavae. A fiter heparin (3 mg/kg body wt) was administered intravenously, a single bolus of 1 ml (500 μg) prostaglandin E1, diluted in 10 ml of saline was injected into the right ventricle outflow tract. When blood pressure dropped, inflow occlusion and cross-clamping of the aorta followed, and cardioplegic (cysteamine plus 20% dextrose in water) solution (C.C. no. 85-23, revised in 1985). The lung preservation solution (60 ml/kg body wt) was administered from a bag raised to 30 cm above table level, and continuous ventilation was maintained while both pleural cavities were opened and copiously irrigated with cold saline.

Donor Operation

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Recipient Operation

Weight-matched recipients underwent a left posterolateral thoracotomy through the fifth intercostal space. The hemiazygous vein was ligated and divided, and the pericardium was opened anterior to the pulmonary veins. The three anastomotic stumps were prepared after clamping of the left pulmonary artery at the point of its bifurcation and the left main bronchus proximal to the last branch. Last, the left atrial appendage was isolated with a side-biting clamp. The left lung removed from recipient dogs was used as control lung to obtain control surfactant.

The donor left lung was anastomosed to the recipient. The airway anastomosis was performed with a single 4-0 prolene suture, and each vascular anastomosis was done with a single 5-0 suture. The pulmonary artery clamp was removed first to allow for backflow and dealing with vascular anastomosis, then the donor left lung was transplanted into the recipient animal.

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Results

Four hours after the implantation procedure was complete, the right main bronchus and right pulmonary artery were cross-clamped for 30 min for the assessment of graft function on the basis of arterial blood gases. The right main bronchus and right pulmonary artery branch were previously dissected from the left pleural cavity. This was done prior to the left pneumonectomy.

BAL Processing and Isolation of Pulmonary Surfactant

A fiter 4.5 h of reperfusion period, the transplanted lung was lavaged twice with 4°C saline (50 ml/kg body wt). BALs were pooled and immediately centrifuged at 1000 × g for 2 h at 4°C to remove cells and cell debris. The volume of the remaining cell-free BAL was recorded, and an aliquot was taken for protein determination. BAL L was stored at −20°C until used for surfactant isolation.

Large surfactant aggregates (heavy subtype surfactant) were obtained as previously described (13). Briefly, BAL L was centrifuged at 100,000 × g for 2 h at 4°C to obtain the large surfactant aggregates in the resulting pellet. B because of the amount of leaked serum protein in BAL L of transplanted lungs, measured by the Lowry method, large surfactant aggregates obtained by sedimentation were contaminated by lipids and proteins from blood transudated into the air spaces. T o test the surfactant aggregates obtained by sedimentation were contaminated by lipids and proteins from blood transudated into the air spaces, T o test the surfactant aggregates obtained by sedimentation were contaminated by lipids and proteins from blood transudated into the air spaces, T o test the surfactant aggregates obtained by sedimentation were contaminated by lipids and proteins from blood transudated into the air spaces, T o test the surfactant aggregates obtained by sedimentation were contaminated by lipids and proteins from blood transudated into the air spaces.

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calculated for each of them in the composition of SP-B. The same procedure was performed for SP-B quantitation from the mentioned amino acids.

The quantitation of SP-B and SP-C from LES by this method is reproducible. This method was validated by determining known amounts of SP-B and SP-C from mixtures prepared from these proteins previously isolated by chromatographic separation on Sephadex LH-60 and quantified by amino acid analysis (21). On the other hand, the amino acid composition of surfactant hydrophobic proteins analyzed in LES from transplanted lungs (EC or UW) was similar to that found in control lungs. This suggests that LES from transplanted lung is free of possible hydrophobic fragments derived upon enzymatic cleavage of plasma proteins.

Isolation of Canine SP-A

Canine SP-A was isolated from large surfactant aggregates of control lungs using sequential butanol and octyl glucoside extractions (24). SP-A concentrations were estimated by quantitative amino acid analysis as described above. The protein was stored in small aliquots in 5 mM Tris/HCl buffer, pH 7.4 at −20°C. Electrophoretic analysis of SP-A was performed under reducing conditions (50 mM dithiothreitol) by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using stacking and running gels of 4 and 12% acrylamide, respectively. Gels were stained with Coomassie Brilliant Blue R-250.

Measurement of SP-A and CRP in Surfactant

Electrophoretic analysis of surfactant from control and transplanted lungs (EC and UW) were performed as described above. A plot was either the same protein amount (15 μg protein) or the same phospholipid amount (200 nmol phospholipid) from all of the surfactant samples. In addition, 8 μg of canine SP-A was always applied in each gel. A fer electrophoresis, samples were transferred to nitrocellulose using a Bio-Rad Trans-Blot Cell. Transfer was carried out at 200 V constant voltage and 100 mA total increment of intensity, using 25 mM Tris (pH 8.3), 192 mM glycine, 20% (vol/vol) methanol as a transfer buffer. After drying, the nitrocellulose was blocked for 15 min with 10% dried skim milk in PBS-T (137 mM NaCl, 10 mM Na₂HPO₄, 0.18 mM KH₂PO₄, 2.7 mM KCl [pH 7.4], and 0.1% [vol/vol] Tween 20) and washed five times with PBS-T. Then, the primary antibody (anti-SP-A or anti-CRP) was added and incubated for 1 h in PBS-T. The blot was washed five times with PBS-T and incubated for 15 min with the secondary antibody, peroxidase-labeled anti-rabbit IgG antibody (Amersham International, Buckinghamshire, U.K.). A fer washing five times, detection reagents were added and SP-A or CRP observed on a Hyperfilm™ ECL. Quantification was finally achieved by densitometric evaluation in an Ultrascan 2002 densitometer (LKB, Uppsala, Sweden). For SP-A, an anti-(human-SP-A) polyclonal antibody, kindly supplied by Dr. J. A. Whitsett (University of Cincinnati, Cincinnati, Ohio), was used. For CRP, an anti-(rat-CRP) polyclonal antibody was generously gifted by Dr. S. Mookerjea (Memorial University, Cincinnati, OH), was used. For CRP, an anti-(rat-CRP) polyclonal antibody was generously gifted by Dr. S. Mookerjea (Memorial University, Cincinnati, OH).

Adsorption Assay

The ability of surfactant to adsorb and spread at an air-water interface could be detected under the experimental conditions. The amount of surfactant phospholipids in surfactant from transplanted lungs was not significantly different from that of control lungs. However, a significant increase of the protein/phospholipid ratio was found in transplanted lungs preserved with either EC or UW solution despite the purification procedure used to separate surfactant from blood components transudated into the air spaces. The protein/phospholipid ratio of surfactant from transplanted lungs preserved with EC solution was not significantly different from those with UW solution. Further phospholipid analysis of LES revealed a very similar phospholipid composition in control and transplanted (EC or UW) lungs (Figure 2).

In vitro surfactant function was determined by measuring the ability of surfactant to adsorb and spread at an air-water interface in a diffusion-independent system (Figure 3). A stable interfacial adsorption rate is dependent on the concentration of surfactant material in the hypophase, the concentration of surfactant phospholipids was deliberately chosen so as to obtain a measurable rate of change of surface pressure (σ) which was not instantaneous, to be sure that any change on the rate of surfactant adsorption could be detected under the experimental conditions. The amount of surfactant phospholipids injected into the hypophase was the same for all the samples from control and transplanted groups. Figure 3 shows that the adsorption rate decreased in surfactant from transplanted lungs. The results obtained with EC solution were not significantly different from those with UW solution. Figure 4A shows electrophoretic analysis of pulmonary surfactant from control and transplanted lungs and Western blot analysis of SP-A. Serum protein contamination is easily visible by SDS-PAGE in partly purified large surfactant aggregates from lung transplants. The content of SP-A decreased by 57% in surfactant from transplanted lungs (Figure 4B). No significant difference was observed between the EC and UW groups. That marked decrease in the SP-A content

<table>
<thead>
<tr>
<th>Step</th>
<th>Preservation Solution</th>
<th>P_{O_2} (mm Hg)</th>
<th>P_{CO_2} (mm Hg)</th>
</tr>
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<tbody>
<tr>
<td>Baseline</td>
<td>EC</td>
<td>504 ± 36</td>
<td>44 ± 11</td>
</tr>
<tr>
<td></td>
<td>UW</td>
<td>412 ± 55</td>
<td>40 ± 6</td>
</tr>
<tr>
<td>Post-surgery</td>
<td>EC</td>
<td>436 ± 47</td>
<td>50 ± 15</td>
</tr>
<tr>
<td></td>
<td>UW</td>
<td>452 ± 40</td>
<td>42 ± 6</td>
</tr>
<tr>
<td>After 4 h of repuffusion</td>
<td>EC</td>
<td>56 ± 24*</td>
<td>75 ± 21</td>
</tr>
<tr>
<td></td>
<td>UW</td>
<td>76 ± 40*</td>
<td>49 ± 13</td>
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Definition of abbreviations: EC = Euro-Collins solution; UW = University of Wisconsin solution.

RESULTS

Graft function after 4.5 h of repuffusion was assessed by measuring P_{O_2} later than the right main bronchus and right pulmonary artery were cross-clamped for 30-min (Table 1). A significant decrease of P_{O_2} was found. No significant difference was observed between EC and UW solutions.

Figure 1 shows an analysis of phospholipid content and protein/phospholipid ratio in large surfactant aggregates and in LES (hydrophobic protein/phospholipid). The content of phospholipids in surfactant from transplanted lungs was not significantly different from that of control lungs. However, a significant increase of the protein/phospholipid ratio was found in transplanted lungs preserved with either EC or UW solution despite the purification procedure used to separate surfactant from blood components transudated into the air spaces. The protein/phospholipid ratio of surfactant from transplanted lungs versus control lungs was 1.47 ± 0.5 for the EC group and 1.48 ± 0.6 for the UW group. On the other hand, the hydrophobic protein/phospholipid ratio significantly decreased in transplanted lungs preserved with EC solution but not in those preserved with UW solution. Further phospholipid analysis of LES revealed a very similar phospholipid composition in control and transplanted (EC or UW) lungs (Figure 2).

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occurred after reperfusion because SP-A content did not decrease in lungs preserved with either EC or UW solution (data not shown). The content of SP-B and SP-C in a known amount of phospholipids was estimated by amino acid analysis of LES (Table 2). Irrespective of the preservation fluid, SP-B but not SP-C slightly decreased in surfactant from transplanted lungs.

Immunoblot analysis for CRP is shown in Figure 5. The content of CRP significantly increased in surfactant from transplanted lungs preserved with either EC or UW solution. Differences between the EC and UW group did not achieve statistical significance. The content of CRP did not change in surfactant from donor lungs flushed with EC or UW solution (data not shown). Thus, the increase of CRP content in surfactant from transplanted lungs occurred after reperfusion.

To find out if CRP might in part be responsible for the observed decrease in surfactant adsorption activity, we studied

Figure 1. Phospholipid content and protein/phospholipid ratio in surfactant from control and transplanted lungs. Total protein was measured by the Lowry method. Hydrophobic protein was measured from lipid extract of surfactant (LES) by quantitative amino acid analysis. Phospholipid content was determined both in surfactant and in LES. EC = Euro-Collins solution; UW = University of Wisconsin solution.

**p < 0.01 and *p < 0.05 versus control.

Figure 2. Phospholipid composition of surfactant from control and transplanted lungs. PC = phosphatidylcholine; PG = phosphatidylglycerol; PI = phosphatidylinositol; PS = phosphatidylserine; PE = phosphatidylethanolamine; SM = sphingomyelin; LPC = lysophosphatidylcholine; X = bismonoaoylglycerol phosphate; EC = Euro-Collins solution; UW = University of Wisconsin solution.

Figure 3. Interfacial adsorption kinetics of pulmonary surfactant isolated from control and transplanted lungs. \( \pi \) = surface pressure; EC = Euro-Collins solution; UW = University of Wisconsin solution. The final concentration of phospholipids in the hypophase was 59.8 nmol/ml for all surfactant preparations obtained from control \((n = 6)\) and transplanted lungs \((EC, n = 6; UW, n = 6)\). Values are expressed as mean ± SD.
the influence of human CRP (Calbiochem, La Jolla, CA) on the adsorption rate of control surfactant at various CRP/phospholipid weight ratios (Figure 6A). At a very low CRP/phospholipid weight ratio (0.01:1) (0.45 \( \mu \)g CRP/ml), surfactant adsorption rate was impaired. Higher CRP/phospholipid ratios caused higher inhibition of surfactant adsorption. The inhibition of surfactant adsorption by CRP was completely reversed by addition of phosphocholine. However, the reduced adsorption rate of surfactant from lung transplants was not improved by addition of excess phosphocholine (Figure 6B), indicating that other factors, such as the decrease of SP-A and SP-B levels and the presence of other serum proteins, must be also involved in the impairment of surfactant adsorption activity. Interestingly, addition of SP-A, in the absence or the presence of phosphocholine, was unable to reverse inhibition of the adsorption activity of surfactant from transplanted lungs.

DISCUSSION

Pulmonary edema associated with pulmonary hypertension and hypoxemia often occurs in early post-transplant period.

Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>nmol SP-B/( \mu )mol PL</th>
<th>nmol SP-C/( \mu )mol PL</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.06 ± 0.2 (n = 10)</td>
<td>2.27 ± 0.4 (n = 10)</td>
</tr>
<tr>
<td>Transplanted (EC)</td>
<td>0.75 ± 0.3 (n = 6)*</td>
<td>1.63 ± 0.5 (n = 6)</td>
</tr>
<tr>
<td>Transplanted (UW)</td>
<td>0.73 ± 0.2 (n = 6)†</td>
<td>1.95 ± 0.6 (n = 6)</td>
</tr>
</tbody>
</table>

Definition of abbreviations: SP-B = surfactant protein B; SP-C = surfactant protein C; PL = phospholipid; EC = Euro-Collins solution; UW = University of Wisconsin solution. *SP-B and SP-C content was estimated by quantitative amino acid analysis as described in Methods. †Monomeric SP-B.

Figure 5. Levels of immunoreactive CRP in pulmonary surfactant isolated from control and transplanted lungs. Fifteen micrograms of surfactant proteins from control (n = 6) and transplanted lungs (EC, n = 6; UW, n = 6) were separated by gel electrophoresis, transferred to nitrocellulose, and blotted with polyclonal antibodies against CRP. Eight micrograms of canine SP-A were also loaded on the gels (left lane). Quantitation of CRP was achieved by densitometric evaluation of the immunoreactive bands. **p < 0.01 and *p < 0.05 versus control.
This is usually termed ischemia-reperfusion injury (1). It is believed that microvascular injury associated to ischemia-reperfusion is due to massive pulmonary polymorphonuclear leukocyte (PM N) adhesion to endothelial cells. A dhesion of PM Ns is mediated by CD18 leukocyte integrin during reperfusion and is further increased by the release of pro-inflammatory cytokines by alveolar macrophages (such as tumor necrosis factor-α or interleukin-1β) (26). A ctitiation of adherent neutrophils worsens microvascular injury by causing the release of active oxygen species, proteolytic enzymes, and additional cytokines (27). Once the cascade of events resulting in reperfusion injury is initiated, secondary effects such as alteration of the alveolar surfactant system (5, 6) may complicate the clinical picture. The present study analyzes the composition and activity of the surfactant system after lung transplantation and investigates whether the acute-phase protein CRP increases in surfactant from transplanted lungs.

The first evidence of lung injury in transplanted lungs after long-term preservation was a decrease in arterial PO2 after 4.5 h of reperfusion and increased levels of proteins recovered from BAL, in agreement with other investigators (5, 6). In this study, we partly purified large surfactant aggregates (heavy subtype surfactant) from blood components that leaked into the alveoli. Large surfactant aggregates from lung transplants showed a phospholipid content and composition similar to that of the control group. These results differ from those of Veldhuizen and coworkers (5). They showed that SP-A can prevent but not reverse inhibition of surfactant function. The present study revealed that the decreased content of SP-A in surfactant from transplanted lungs could partly explain the decreased ability of these surfactants to adsorb very rapidly from an aqueous subphase into the air–water interface. Increased levels of CRP were also found in BAL from patients with sepsis-induced ARDS and decreased surfactant adsorption was reported (28).

The precise in vivo function of CRP remains unclear. Several lines of evidence indicate that CRP might participate in pulmonary host defense (29, 30). Interestingly, a recent study (31) indicated that CRP might inhibit lung inflammation by down-regulating alveolar macrophage production of interleukin-1β in response to endotoxin. CRP seems to suppress inflammation in the alveolar space, but excess CRP might be harmful for the lung function because part of CRP present in the alveolar space would interact with large surfactant aggregates. We show here that CRP decreased the adsorption activity of control surfactant at a low CRP-to-surfactant phospholipid weight ratio (0.01:1). The effect of CRP on surfactant adsorption was effectively reversed by addition of phosphocholine, indicating that CRP binds to phosphocholine headgroups of surfactant lipids as previously suggested (16, 17). A ddition of excess phosphocholine to surfactant from lung transplants had no effect on its reduced adsorption activity. The decreased content of SP-A in surfactant from transplanted lungs as well as the contamination with other plasma proteins could also contribute to the diminished adsorption rate of those surfactants. Decreased levels of SP-A were also reported in large surfactant aggregates (5) and in BAL (6) obtained from lung transplants in ARDS (3, 4). We found that the decrease of SP-A content occurred after reperfusion because the content of SP-A did not decrease in donor lungs after long-term storage with either EC or UW solution. Decreased levels of SP-A and SP-B are likely due to degradation by neutrophil proteases (10) or by increased leakage into the bloodstream (32). SP-B is essential for the surface activity of surfactant. SP-A accelerates the adsorption process, modulates surfactant homeostasis, and participates in host defense. In addition, SP-A performs an essential function when surfactant activity is compromised by the presence of inhibitory agents such as serum proteins. In vitro (12) and in vivo (33) studies demonstrated that SP-A counteracted serum protein inhibition of surfactant function. The present study revealed that addition of SP-A to surfactant from transplanted lungs did not reverse inhibition of surfactant adsorption. It is likely that SP-A can prevent but not reverse serum protein inhibition. We speculate that instillation of surfactant containing SP-A in the donor just before anastomosis and reperfusion could prevent some of the surfactant changes that occur as a
consequence of ischemia-reperfusion injury and, therefore, improve transplant function.

In conclusion, this study shows that, irrespective of the preservation fluid: (1) adsorption facilities of surfactant from transplanted lungs are impaired as well as graft function and (2) CRP levels increase in surfactant upon reperfusion whereas the relative content of SP-A significantly decreases. The presence of elevated levels of CRP in BAL could be a very sensitive marker of lung injury. Because most of the changes in the surfactant system seems to occur as a consequence of ischemia-reperfusion injury, we suggest that instillation of exogenous surfactant just before reperfusion might (1) protect endogenous SP-A from degradation by increasing the percentage of SP-A bound to lipids, which is less susceptible to proteolytic degradation (34) and (2) diminish the CRP/surfactant phospholipid ratio, which would decrease the ability of CRP to inhibit surfactant absorption. Alternatively, new strategies of surfactant treatment could include the presence of SP-A or the water-soluble CRP ligand phosphocholine, which diminishes the inhibition of surfactant adsorption by CRP.

References