Pulmonary surfactant and lung transplantation
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Pulmonary Surfactant after Cold Lung Storage for Transplantation.

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Supported by the Netherlands Asthma Foundation, Leusden, and the "Fokkens-Kerkhoff Stichting", Groningen, the Netherlands. H.P.H. and J.J.B. were supported in part by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization of Scientific Research (NWO)

Submitted
Abstract

The function of pulmonary surfactant is impaired after cold storage and subsequent reperfusion of lung transplants. It is unclear, however, to what extent pulmonary surfactant changes already during the cold storage. We investigated pulmonary surfactant from rat lungs after cold deflated storage for 0h, 6h, and 20h, and in an additional experiment after cold inflated (50% oxygen) storage for 20h. Pulmonary surfactant was obtained by bronchoalveolar lavage. mRNA of the surfactant-associated protein A (SP-A) was measured in lung tissue. We found that the phospholipid composition of surfactant and the percentage of heavy subtype surfactant were not affected by lung storage for up to 20h. The SP-A/PL ratio was slightly decreased (by approximately 25 % ) after 20h lung storage. SP-A mRNA was not decreased. This study shows that pulmonary surfactant does hardly change during cold lung storage. The decrease in SP-A/PL ratio is relatively small and may be of little clinical relevance. Significant changes in pulmonary surfactant occur only after reperfusion of lung transplants.

The composition of pulmonary surfactant has been found to be changed and its function deteriorated immediately after transplantation of lungs (1, 2). The main cause of the detrimental changes of surfactant is thought to be ischemia-reperfusion injury (2). It is unclear, however, to what extent components of pulmonary surfactant change already during the period of cold ischemic storage. In some studies the phospholipid composition of surfactant changed early during storage (3). In other studies the phospholipid composition remained unchanged during a period of 12 hours of cold storage, but the surfactant-associated protein A (SP-A) decreased by half (1). To address the question whether pulmonary surfactant changes during storage, we assessed the composition (phospholipids, SP-A, inhibiting serum proteins) and in vitro function of surfactant lavaged from explanted rat lungs that had been stored for 0, 6, or 20 hours. In addition, the amount of SP-A mRNA was measured in lung tissue. In the experiments with explanted lungs that had been stored deflated for up to 20 hours we hardly found changes in pulmonary surfactant. Therefore, we added an experiment where lungs were kept inflated during 20 hours of ischemic storage.
Methods

Changes in pulmonary surfactant during storage were investigated in explanted right lungs from male, specific-pathogen free LEW rats (Charles River). Lungs that had been stored for 0, 6, or 20 hours were compared with normal control lungs (n= 4, 4, 5, and 5, respectively). During explantation the lungs were ventilated with room air (PEEP 3 cm H\(_2\)O, PIP 17 cm H\(_2\)O). After dissection, the lungs were flushed with saline through the pulmonary artery (80 ml/ kg body weight at 6-8 °C, maximum pressure 40 cm H\(_2\)O) and subsequently submersed in saline at 6-8 °C as described previously (4). The main bronchus was left open, so that the lung collapsed after explantation. Lungs for 0 hour storage were investigated immediately after the explantation procedure. The normal lungs were not flushed or stored.

In an additional experiment, a group of lungs was kept inflated during storage for 20 hours; again, surfactant changes in the lungs were compared with normal lungs (n= 7 in each group). Differences with the previous experiments are that the lungs were ventilated with 50% O\(_2\) during explantation, that they were inflated once till 25 cm H\(_2\)O to expand all atelectatic spots, and that subsequently, at an airway pressure of 10 cm H\(_2\)O, the bronchus was ligated.

After the storage period, the lungs were lavaged 5 times with ice cold saline (2) for assessment of the surfactant components in bronchoalveolar lavage fluid (BALF). Immediately thereafter lungs were cut into small pieces, snap frozen in liquid nitrogen and stored at –70 °C for later measurement of SP-A mRNA.

The components of surfactant were all measured using methods previously described (4) . In an aliquot of BALF the amount of phospholipids (PL) was measured. Heavy-subtype surfactant was isolated in 1 ml of BALF by centrifugation at 40,000 g for 15 minutes in a fixed angle rotor (TLA 100, Beckman). The amount of phospholipids measured in this fraction was calculated as a percentage of PL in total BALF. The percentage of surfactant phosphatidylcholine (PC) was measured after separation of PL by thin layer chromatography. SP-A was measured by sandwich ELISA. Values of surfactant components in BALF are expressed relative to the measured PL because smaller BALF volumes were recovered after inflated storage than after deflated storage (table 1). The in vitro function of surfactant was measured in a pulsating bubble surfactometer (PBS, Electronetics). Briefly, the surface tension at minimal bubble size after 20 pulsation's in an organic solvent extracted surfactant suspension ( 2 mg PL/ml in 0.9 % NaCl, 1.5 mM
CaCl₂, 37 °C) was used as measure of surfactant function (4) To measure the amounts of 1.7 kb and 1 kb SP-A mRNA in lung tissue total RNAs were isolated with RNAzol-B according to the instructions of the manufacturers (Cinna/Biotecx, Houston, TX) and quantified by measuring the absorbance at 260 nm. Aliquots of 25 µg RNA were subjected to Northern blot hybridization as described before (5), using a 32P-labeled 662-bp rat SP-A cDNA fragment (kindly provided by Dr. T. Lacaze-Masmonteil) as a probe. The intensities of the hybridization signals of both SP-A mRNAs were quantified by measurement in a Fujix BAS 1000 Bio-imaging Analyzer System (Fuji). When making multiple comparisons, statistical analysis was carried out by one-way analysis of variance. In those cases where the F-test indicated that there was a significant difference (p < 0.05) among groups, comparisons with the control condition were made using Dunnett’s test. When 2 means were compared, analysis was performed with Student’s t-test (2-tailed).

### Table 1. Surfactant variables in BALF after cold lung storage

<table>
<thead>
<tr>
<th></th>
<th>Recovered BAL (m/kg BW)</th>
<th>Heavy subtype (% of PL)</th>
<th>PC (% of PL)</th>
<th>SP-A/PL µg/mg</th>
<th>Protein/PL (mg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Deflated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal (n=5)</td>
<td>103.3 ± 6.1</td>
<td>66.1 ± 6.8</td>
<td>77.6 ± 3.3</td>
<td>84.4 ± 23.9</td>
<td>5.9 ± 1.5</td>
</tr>
<tr>
<td>0h storage (n=4)</td>
<td>107.2 ± 6.9</td>
<td>67.5 ± 7.9</td>
<td>78.6 ± 2.9</td>
<td>74.5 ± 16.4</td>
<td>3.9 ± 1.2</td>
</tr>
<tr>
<td>6h storage (n=4)</td>
<td>106.6 ± 5.8</td>
<td>76.2 ± 10.4</td>
<td>71.7 ± 7.7</td>
<td>76.9 ± 26.0</td>
<td>4.0 ± 1.1</td>
</tr>
<tr>
<td>20h storage (n=5)</td>
<td>107.7 ± 6.3</td>
<td>74.1 ± 6.4</td>
<td>72.8 ± 5.0</td>
<td>64.7 ± 12.0</td>
<td>3.6 ± 0.7</td>
</tr>
<tr>
<td><strong>Inflated (50 % oxygen)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal (n=7)</td>
<td>109.4 ± 6.4</td>
<td>73.3 ± 2.6</td>
<td>75.4 ± 2.7</td>
<td>97.2 ± 16.7</td>
<td>3.5 ± 1.0</td>
</tr>
<tr>
<td>20h storage (n=7)</td>
<td>93.6 ± 8.31</td>
<td>77.7 ± 3.91</td>
<td>77.0 ± 2.1</td>
<td>72.5 ± 22.41</td>
<td>4.9 ± 1.7</td>
</tr>
<tr>
<td>1h reperfused †</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal (n=8)</td>
<td>n.a.</td>
<td>68.6 ± 5.3</td>
<td>83.3 ± 1.4</td>
<td>57.3 ± 6.5</td>
<td>4.4 ± 1.1</td>
</tr>
<tr>
<td>20h storage (n=5)</td>
<td>n.a.</td>
<td>34.3 ± 5.21</td>
<td>62.5 ± 14.92</td>
<td>24.7 ± 16.21</td>
<td>39.8 ± 11.1</td>
</tr>
</tbody>
</table>

Table values represent mean ± SD
# data from ref 4 (left lung transplants), † data from ref 8 (left lung transplants).
* p < 0.05 versus normal of same experiment (Dunnett’s test)
‡ p < 0.05 versus normal of same experiment (Student’s t-test)
n.a. : not applicable because values are from left lungs

### Results

The composition of surfactant remained remarkably constant during storage of the deflated lungs. The percentage heavy subtype surfactant and the percentage PC relative to total PL did not change (table 1). The SP-A/PL ratio and protein/PL ratio decreased marginally, but these changes are small.
Pulmonary surfactant after cold lung storage for transplantation.

compared to the changes seen in transplanted lungs at 1 hour after reperfusion (published data from a previous study (4) included in table 1). In the lungs that had been stored inflated, the values of surfactant were likewise constant. Here, the decrease of the SP-A/PL reached statistical significance. However, when expressed as a percentage of the normal lungs, the decrease of SP-A/PL was the same as in the experiment where the lungs were stored deflated for 20 hours (74.6 % and 76.7 %, respectively).

The in vitro function of surfactant remained normal; in all groups the minimal surface tension measured in the pulsating bubble surfactometer dropped below 1µN/m.

The amount of SP-A mRNA in the lung tissue did not decrease significantly during lung storage: the amounts of both 1.7 kb and 1.0 kb SP-A mRNA remained within normal ranges in all investigated groups (table 2).

<table>
<thead>
<tr>
<th>SP-A mRNA (% of normal)</th>
<th>1.7 kb</th>
<th>1.0 kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deflated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal (n=4)</td>
<td>100.0 ± 21.7</td>
<td>100.0 ± 27.2</td>
</tr>
<tr>
<td>6h storage (n=4)</td>
<td>93.7 ± 12.9</td>
<td>88.1 ± 13.8</td>
</tr>
<tr>
<td>20h storage (n=3)</td>
<td>78.3 ± 5.5</td>
<td>88.6 ± 4.1</td>
</tr>
<tr>
<td>Inflated (50 % oxygen)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal (n=5)</td>
<td>100.0 ± 13.3</td>
<td>100.0 ± 13.5</td>
</tr>
<tr>
<td>20h storage (n=5)</td>
<td>94.1 ± 19.1</td>
<td>93.9 ± 21.6</td>
</tr>
</tbody>
</table>

values are mean ± SD

Discussion

Our study shows that cold storage of explanted lungs has little impact on pulmonary surfactant. Both composition and function of the surfactant in the explanted lungs were hardly affected, even when storage was extended to 20 hours. When changes were found in the stored lungs, they were negligible compared to changes found in lungs after transplantation (table 1). In contrast, some studies report severe changes of pulmonary surfactant already after the pulmonary flush with preservation solution (0h storage) (3). In our opinion these surfactant changes—in combination with a leak of serum proteins—reflect damage of the lungs caused during the harvesting procedure e.g. by preservation solutions (6).

SP-A was the only surfactant component that seemed to be affected during deflated storage. We believe that maintenance of the amount of SP-A
after lung transplantation is important because SP-A protects the biophysical function of surfactant against inactivation by leaked serum proteins (7). In a study by Veldhuizen and colleagues (1) the alveolar SP-A was found to drop much more than in our study. We wondered whether the difference between the two studies resulted from the fact that we stored the lungs in deflated state, while Veldhuizen and colleagues (1) used inflated storage. Therefore we performed additional experiments with inflated lungs. Again, SP-A dropped by 25%, which now reached statistical significance due to the larger number of animals in the inflated group (table 1). The finding that SP-A decreased to a similar extent after deflated storage and after storage while inflated with 50% oxygen indicates that 50% oxygen does not cause additional harm to SP-A in pulmonary surfactant during storage. In contrast, 50% oxygen inflated storage has been shown to partially preserve SP-A after reperfusion (8) and to be beneficial for the postoperative function of transplanted lungs (8, 9). Inflation of lungs with 100% oxygen for prolonged storage is not necessary and should be avoided, as it cannot be ruled out that it facilitates proteolysis of SP-A, as is seen in ozone exposure (10).

Apparently, surfactant is well preserved during explantation and cold storage of lung transplants, provided that adequate preservation procedures are used. At the end of the storage period in this study, surfactant was unchanged in composition, it was functioning normally, and mRNA of SP-A was available to produce additional SP-A. This indicates that the surfactant system in the lung transplant is able to support a normal function immediately upon transplantation. However, studies on surfactant in lung transplants shortly after reperfusion (indicated in table 1), show that major changes in surfactant develop quickly once the lung is reperfused. In addition, serum proteins leak to the alveolar space after reperfusion, which is most detrimental for the function of alveolar surfactant. As long as we are unable to prevent inactivation of surfactant upon reperfusion, the ischemia-reperfusion injury will occasionally result in primary organ failure of lung transplants. In the treatment of primary organ failure the instillation of large amounts of surfactant into the transplanted lung has been shown to be effective in restoring the quality of the pulmonary surfactant (4, 11).

Acknowledgments
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Pulmonary surfactant after cold lung storage for transplantation.

References


The Function of Surfactant is Impaired during the Reimplantation Response in Rat Lung Transplants.

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Supported by The Netherlands Asthma Foundation, Leusden, The Netherlands.

Abstract

In this study we investigated the surfactant function in rat lung transplants at the peak of the reimplantation response in experimental groups with increasing warm ischemic times of the lung transplant. The left and right lungs in five groups of rats were assessed 24 hours after left lung transplantation: rats receiving transplants with lung graft ischemic times of 60 (n=4), 90 (n=5), and 120 (n=5) minutes, donor rats with 120 minutes lung ischemia (n=5) and normal (nonoperated) rats (n=6). The reimplantation response was assessed by the ventilation score on chest roentgenograms, measurement of the static lung compliance and the (serum) protein concentration in the bronchoalveolar lavage fluid. Surfactant in the bronchoalveolar lavage fluid was assessed by measuring the amount and the composition of surfactant phospholipids and the in vitro surfactant function in a pulsating bubble surfactometer. We found that longer ischemic times caused a more severe reimplantation response in the left lung grafts. Although the ventilation scores were equally low in the 60-, 90-, and 120-minute ischemia groups, the lung compliances decreased and the (serum) protein concentrations increased stepwise in correlation with longer ischemic times. The amount of surfactant phospholipids during the reimplantation response was not changed, but the percentage phosphatidylcholine decreased progressively in parallel with the severity of the reimplantation response. Finally, the in vitro function of surfactant from the lung transplants decreased in parallel with the prolongation of the ischemic time, whereas the function of surfactant from donor lungs with 120 minutes of ischemia and from native right lungs was not changed. We conclude that the surfactant function is impaired during the reimplantation response as a result of a high concentration of inhibiting serum proteins and a low percentage of phosphatidylcholine.

Introduction

Pulmonary surfactant lowers the surface tension at the air-water interface inside the alveolus. This effect is achieved by adsorption of surfactant phospholipids at the air-water interface, a process controlled by surfactant-associated proteins (1, 2). In this way, surfactant prevents collapse of the alveolus at end expiration, prevents formation of alveolar edema and increases the compliance of the lung.

It is now well established that the surfactant function is impaired in the adult respiratory distress syndrome (ARDS) (3, 5). Several factors in clinical
The function of surfactant is impaired during the reimplantation response

ARDS and in experimental ARDS models are responsible for the impairment of the surfactant function. One factor is the inhibition of the surfactant function by serum proteins that leak into the alveoli (6, 7). Other possible factors are an altered composition of surfactant and an altered surfactant metabolism (3, 8, 9).

During the reimplantation response, which is a form of acute lung injury after lung transplantation caused by the complex of denervation, disruption of lymphatic vessels, and ischemia-reperfusion, we expect that the surfactant function is impaired in a similar way as in ARDS. The clinical symptoms of the reimplantation response, including the development of protein rich alveolar edema, a decreased lung compliance, and a diminished gas exchange, are similar to those of ARDS (10, 11, 12). Recently Veldhuizen and colleagues (13) showed that the function of surfactant from canine lung transplants was impaired at 6 hours after reperfusion, which is early in the reimplantation response. The reimplantation response has its peak on the first day after lung transplantation in small experimental animals (14), whereas in larger animals and human beings the reimplantation response can remain severe for a period between two days and one week post transplantation (15, 16). The severity of the reimplantation response differs significantly among individuals; in most cases it is mild and transient, but, occasionally, it is severe and can lead to early postoperative death caused by graft failure (17). This disparity indicates that the reimplantation response is affected by variable factors in the transplantation procedure. We previously showed that in rats the reimplantation response was more severe after a longer ischemic time of the lung transplant (14).

The aim of this study was to investigate the surfactant function at the peak of the reimplantation response and, furthermore, to investigate whether the surfactant function was affected more impaired after a longer duration of graft ischemia, corresponding with a more severe reimplantation response. The surfactant function was investigated in surfactant from rats 24 hours after transplantation of the left lung.
Materials and Methods

Experimental Design.
To investigate the function of surfactant during the reimplantation response after lung transplantation, we transplanted left lungs of rats in three groups with increasing warm ischemic times (60, 90 and 120 minutes). After 24 hours, the left lung transplants and the native right lungs were evaluated separately. The effect of ischemia without subsequent reperfusion of the lung was assessed in donor right lungs after 120 minutes of ischemia. For normal control values, we used normal (nonoperated) rats.

One day after transplantation the left lung graft and the native right lung were investigated. Chest roentgenograms were made and the lungs were taken out for pressure-volume (PV) measurements. Each lung was then lavaged separately to obtain bronchoalveolar lavage fluid (BALF). The following were evaluated as parameters of the reimplantation response: the chest roentgenograms were scored, the static lung compliance was calculated and the amount of serum protein in the BALF was determined. The following were evaluated as parameters for the quality of the alveolar surfactant: the amount of surfactant phospholipids and the phospholipid composition in the BALF were determined. The surfactant function was measured as the ability of surfactant to lower the minimum surface tension in a pulsating bubble surfactometer.

Left lung grafts, native right lungs, donor right lungs and normal lungs.
Inbred Lewis rats weighing 200 to 280 gm were used for donors, recipients, and normal control animals. All animals received humane care in compliance with the Dutch regulations and law. Single left lung transplantation was performed according to the method of Prop et al (14, 18). In brief, the left lung graft was taken out of the donor after a pulmonary artery flush with 50 cc cold (4 °C) saline solution at 20 cm water pressure while the lung was ventilated. Thereafter no further cooling was applied and the lung graft was kept at room temperature in a plastic cover to prevent dessication. During implantation the pulmonary artery and vein were first anastomosed and during reperfusion the left main bronchus was sutured.

Three experimental groups were examined with increasing ischemic times of the left lung graft of 60, 90, and 120 minutes (n= 4, 5, and 5, respectively). From these three experimental groups the left lung grafts, the native right lungs, and the donor right lungs after 120 minutes of ischemia (n = 5) were
The function of surfactant is impaired during the reimplantation response assessed separately. For comparison, left and right lungs of normal rats were studied (n = 6).

**Ventilation score on roentgenograms**
Roentgenograms of the chest were made while the rat was anesthetized with an intraperitoneal injection of chloralhydrate. The severity of the reimplantion response was scored with the use of the ventilation score according to Prop et al (14). In brief, this score is based on the radiographic density of the lung and the total lung area and uses a scoring scale ranging from 6 (for normal lungs) to 0 (for opaque lungs).

**PV measurement**
To obtain the lungs for PV measurement the rats were killed and bled from the abdominal aorta. The thorax was then opened, and the pulmonary vasculature was flushed with saline solution at room temperature via the pulmonary artery after the left atrium was opened. The heart and lungs were taken out en bloc, the trachea was cannulated, and its bifurcation was freed of tissue to allow selective clamping of the left and right main bronchi. The donor right lungs of the 120-minute ischemiagroup were treated according to this protocol, the only difference being that the left lung was removed and the remaining left bronchus stump was ligated. To degas the lungs we placed them briefly in a vacuum chamber. The right main bronchus was then clamped, and a pressure-controlled static volume-pressure measurement of the left lung was performed. For this purpose, the cannula in the trachea was connected to a pressure transducer in open connection with a syringe. The pressure was read from an X-Y recorder after calibration with a water manometer. The left lung was then inflated with the syringe in steps of 5 cm water pressure to a maximum of 35 cm water and then deflated with steps of 5 cm water pressure. At every pressure step the inflated volume was read from the syringe after an equilibration time of 50 seconds. After PV measurement of the left lung, the clamp was moved to the left main bronchus for measurement of the right lung in the same way. The PV measurement took place at room temperature in a moist environment to prevent drying of the lung surface. The volume at 35 cm water pressure was considered as Vmax. The static compliance was calculated with the formula: Vmax/ body weight/ 35 cm H₂O (ml . kg body weight⁻¹ . 35 cm H₂O⁻¹ ). The volumes at 5 and 10 cm H₂O at the deflation limb were used to calculate the lung stability indexes according to Clements and collaborators, (19) (\(V_5/V_{max}\))x 100 %, and Gruenwald,(20) (2 V₅ + V₁₀)/2 V_{max}.
**Bronchoalveolar lavage**

Bronchoalveolar lavage (BAL) was performed after the remnant air was removed in the vacuum chamber. Again, the lungs were assessed separately by clamping the right and left main bronchi sequentially. Each lung was lavaged five times with fresh saline solution (4 °C) via the tracheal cannula.

To standardize the lavage volume, the first lavage was performed with 80% of the normal mean lung volume as measured at 30 cm H₂O in a group of normal rats (n=8). This standard volume per kg rat was 15.8 ml/kg for the left and 27.6 ml/kg for the right lung. The recovered fluid volume of the first lavage was recorded. In the next four lavages, we lavaged with a volume equal to the recovered volume at the first lavage, to prevent rupture of the lung. This process resulted in total BAL volumes of 63.9 ± 9.1 ml/kg from left lungs and 120.4 ± 12.7 ml/kg from the right lungs, with no difference between the groups. The BAL fluid (BALF) was collected on ice and centrifuged at 150 g for 10 minutes to sediment cells and cell debris. Thereafter, samples were taken out of the supernatant (S₁) for protein and phospholipid determination. The two samples and the remainder of S₁ were stored at -20 °C until further determination.

**Assays in BALF**

The protein in S₁ was measured as a parameter of the reimplantation response with a modified Lowry method on a microtiter plate (21). Proteins in the BALF from the left lung grafts and the native right lungs were separated by SDS PAGE gel electrophoresis (Mini-Protean II Ready Gel 4 to 20%; Biorad, Richmond, Calif.) and compared with the protein spectrum of their serum.

The amount of surfactant phospholipids in the BALF was determined in S₁ according to the procedure of Bhawaganani et al (22) after lipid extraction according to Bligh and Dyer (23) and expressed per kilogram of body weight.

All the surfactant phospholipids had to be pelleted to determine the phospholipid composition and the surfactant function. Therefore S₁ was spun overnight at 100,000 g, 4 °C, to obtain a pellet (P₂) of surfactant. This highspeed centrifugation was performed to have both the large aggregates (lamellar bodies and tubular myeline) and the small aggregates (small vesicles) of surfactant in the surfactant pellet. The large aggregates are highly surface active, and the small aggregates are relatively surface inactive (24).

The phospholipid composition was determined in a sample of P₂. After lipid extraction (23) the phospholipids were redissolved in 100 µl chloroform...
The function of surfactant is impaired during the reimplantation response and applied on a silica thin layer plate (HPTLC 60, Merck, Darmstadt, Germany) and run twice in the same direction with the Touchstone E solvent (25). After drying on air the plate was sprayed with rhodamine-G (Sigma Chemical Co, St Louis, Mo.) for the visualization of the phospholipid bands. With this procedure, we identified lyso-phosphatidylcholine, sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylethanolamine + phosphatidylinositol, phosphatidylglycerol and unknown phospholipids. The phospholipid bands were scraped off the plate, and their phospholipid content was determined for calculation of the relative composition of the phospholipids in surfactant. The surfactant function was measured as the ability of the resuspended P2 surfactant to lower the surface tension with the pulsating bubble method according to Enhorning (26) with a Pulsating Bubble Surfactometer® (Electronetics Co, Amersted, New York). For this purpose the pellet (P2) was resuspended and homogenized in a buffer solution (saline solution 0.9 %, HEPES buffer, 3 mmol CaCl₂) at a total phospholipid concentration of 2 mg/ml. The Pulsating Bubble Surfactometer generates a pulsating bubble in the surfactant suspension between a preset minimum and maximum radius. During pulsation it measures the pressure gradient ΔP (in mN.m⁻²) over the air-water interface of the bubble and calculates the surface tension γ in mN.m⁻¹ with the formula of Laplace (γ = ΔP / 2r). When there is a good surfactant function, the surface tension will be the lowest at minimum bubble size. We took the surface tension at minimum bubble size (0.4 mm) after 1 minute of pulsating as measure of the surfactant function in a series of 100 pulsations in 5 minutes at 37°C. The surface tension at minimum bubble size after 5 minutes of pulsating was used to assess a possible delayed adsorption of the surfactant phospholipids to the air-water interface. During this extra four minute period of pulsating the surfactant phospholipids in the monolayer have the possibility to squeeze out eventual inhibiting proteins, which can disturb the adsorption of surfactant phospholipids (27).

Statistical analysis
All data are given as mean ± standard deviation. For comparison between the ventilation scores a Mann-Whitney U-test for unpaired non-parametric values was performed. For comparison between the other data of the different groups, single-factor analysis of variance was performed. A difference with p < 0.05 was considered to be statistically significant. Left lung grafts were compared with normal left lungs, and the native right lungs and donor right lungs were compared with normal right lungs.

Results
We considered the ventilation score on the chest roentgenogram, the static lung compliance and the serum protein concentration in the BALF as the parameters for the severity of the reimplantation response; we found that

### Table 1 Ventilation score of lungs on chest roentgenograms

<table>
<thead>
<tr>
<th>Lung</th>
<th>Normal</th>
<th>Ischemia time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>60'</td>
</tr>
<tr>
<td>Left (graft)</td>
<td>6.0</td>
<td>3.1 ± 1.1 *</td>
</tr>
<tr>
<td>Right (native)</td>
<td>6.0</td>
<td>4.8 ± 1.0</td>
</tr>
</tbody>
</table>

Values are mean ± SD. * p < 0.001 and # p < 0.05 compared to normal, with no significant difference between 60', 90' and 120' ischemia groups. p-values were calculated with the Mann-Whitney U-test for unpaired, non-parametric values.

Figure 1. Deflation curves of the pressure-volume measurements of left lungs, of normal non-operated rats (n=6) and left lung grafts 24 hours after transplantation with 60', 90' and 120' ischemia (n=4, 5, and 5 respectively). The symbols represent the mean values of each group. The maximum inflated volume at 35 cm H2O differs between the groups, but the shape of the deflation curves is identical. The volumes are presented per kg BW of the rats.
The function of surfactant is impaired during the reimplantation response. The reimplantation response was more severe after a longer ischemic time. The ventilation score of the left lung grafts, independent of the time of ischemia, was significantly lower on day 1 after transplantation than that of normal lungs (Table I). An ischemic time of only 60 minutes resulted in decrease of the ventilation score from 6 to 3.1 ± 1.3, indicating a severe reimplantation response. The ventilation score did not vary according to the 60, 90, and 120 minutes of ischemia of the left lung graft. The native right lungs of all ischemia groups had somewhat lower ventilation scores than normal.

In comparison with normal lungs, the static compliance of the transplanted left lungs was considerably lower (Table II). Furthermore, the lungs with the longest ischemic time of 120 minutes had a significantly lower static compliance than the lungs after 60 and 90 minutes of ischemic time. No differences were observed in the stability indexes which is visualized by the unchanged shape of the deflation limb of the PV curves in all transplantation groups (Figure 1). The native right lungs and the donor right lungs showed no change in compliance (Table II) or in PV deflation limbs as compared to normal right lungs.

Table 2

<table>
<thead>
<tr>
<th>Ischemia time</th>
<th>Normal</th>
<th>60'</th>
<th>90'</th>
<th>120'</th>
</tr>
</thead>
<tbody>
<tr>
<td>left (graft)</td>
<td>0.59 ± 0.03</td>
<td>0.46 ± 0.07*</td>
<td>0.45 ± 0.05*</td>
<td>0.26 ± 0.12* # *</td>
</tr>
<tr>
<td>right (native)</td>
<td>1.04 ± 0.03</td>
<td>0.95 ± 0.05</td>
<td>0.98 ± 0.04</td>
<td>1.00 ± 0.05</td>
</tr>
<tr>
<td>right (donor)</td>
<td>1.04 ± 0.03</td>
<td>—</td>
<td>—</td>
<td>1.01 ± 0.03</td>
</tr>
</tbody>
</table>

Values are mean ± SD. * p< 0.05 all ischemia groups compared to the normal group, # p< 0.05 120' group compared to 60' and 90' groups. p-values were calculated with single factor ANOVA.

For the protein concentration in the BALF obtained from left lung grafts after 60 minutes of ischemia was significantly higher values than in the BALF from normal lungs (0.92 ± 0.26 to 0.02 ± 0.02 mg/ml respectively) (Figure 2). A further difference was observed in the left lung grafts after 120 minutes of ischemia when compared with lungs after 60 and

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Figure 2. Protein concentration in BALF of rats that underwent unilateral left lung transplantation after 60', 90', and 120' of ischemia time versus normal (non-operated) animals. Top: left lung grafts, Bottom: native right lungs and donor right lungs. The horizontal lines indicate the mean values of each group. *p < 0.05 versus normal group, #p < 0.05 versus 60' and 90' ischemia group.
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90 minutes of ischemia (1.84 ± 0.17, 0.92 ± 0.25, and 1.12 ± 0.42 mg/ml, respectively). The protein concentration in BALF from the native right lungs in the 60-, 90-, and 120-minute ischemia groups was slightly but significantly higher when compared with the concentration from normal right lungs (0.15 ± 0.08, 0.12 ± 0.05, and 0.29 ± 0.09 to 0.01 ± 0.01 mg/ml, respectively) but it remained much lower than that in the transplanted left lungs (Figure 2). The protein concentration from the nontransplanted 120-minute ischemia donor right lungs was also slightly but significantly higher than that from the normal lungs (0.19 ± 0.13 mg/ml versus 0.01 ± 0.01 mg/ml) (Figure 2).

Figure 3. The protein spectrum of equal amounts of protein on a silver stained 4-20 % SDS gel. In lane 1, serum protein; in lane 2, 3, and 4, lavage protein from 60’, 90’, and 120’ ischemia transplanted left lungs, respectively; in lane 5, lavage protein from a native right lung; and in lane 6, lavage protein from a normal lung. This gel shows that the protein spectrum of left transplanted and native right lungs are similar as the serum protein spectrum.

The spectrum of the proteins from as well the left lung grafts as well as the native right lungs separated with gel electrophoresis was similar to the spectrum of serum proteins (Figure 3).

Parameters for the quality of surfactant as used in this study were the amount of phospholipids in the BALF, the phospholipid composition, and the ability to lower the surface tension. The amount of phospholipids in the
BALF did not differ between the left lung grafts after 60, 90, and 120 minutes ischemia and normal lungs (Figure 4). It is noteworthy that, the amount of phospholipids from all native right lungs after 60, 90, and 120 minutes of ischemia showed significantly higher values compared with normal lungs. The amount of phospholipids from the 120-minute ischemia donor right lungs was normal (Figure 4). The phospholipid composition of the surfactant pellet was clearly changed after lung transplantation. The percentage PC was significantly lower in the left lung grafts after 60 minutes of ischemia than in normal lungs (70.1% ± 4.3% versus 75.5% ± 1.8 %). The percentage PC of the 90 and 120-minute ischemia lungs was even lower ( 56.6 %± 6.8% and 55.3% ± 10.5 % ). In addition to the lower percentage of PC, the percentage of SM was significantly higher for all ischemia groups as compared with normal groups. Furthermore, less consistent changes were found in the other phospholipids; higher percentages of phosphatidylethanolamine + phosphatidylinositol and unknown phospholipids were found in the lungs after 60 minutes of ischemia, and, after 90 minutes all phospholipids apart from PC were significantly higher, whereas after 120 minutes no phospholipids other than PC and SM were abnormal (Figure 5). The surfactant phospholipid composition of the native right lungs and the donor right lungs with 120 minutes of ischemia showed no significant changes (Figure 5).

The minimum surface tension of surfactant recovered from all left lung grafts was significantly higher when compared to the minimum surface tension of surfactant from normal lungs (Figure 6). Within the ischemia groups the minimum surface tension from the lungs after 120 minutes ischemia was significantly higher when compared with 60 minutes of ischemia (37.9 ± 3.7 vs. 28.9 ± 6.8 mN/m) (Figure 6). Surfactant of the 60-, 90-, and 120-minute ischemia native right lungs did not lose the ability to lower the minimum surface tension to normal values and neither did surfactant lavaged from the 120 minutes donor right lungs (Figure 6). The adsorption of surfactant to the air-water interface, assessed by comparing the minimum surface tension after 1- and 5-minute pulsating, was different between the ischemia groups. Surfactant from the normal group reached the minimum surface tension, as expected, within 1 minute (table 3). In the 60 minute ischemia group the minimum surface tension reached normal values between 1 and 5 minutes pulsating whereas the minimum surface tension in the 90- and 120-minute ischemia groups did not decrease in this period.
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Figure 4. Amount of phospholipids in BALF of rats that underwent unilateral left lung transplantation after 60', 90', and 120' of ischemia time versus normal (non-operated) animals. Top: left lung grafts, Bottom: native right lungs and donor right lungs. The horizontal lines indicate the mean values of each group. The larger amount of phospholipids lavaged from the right lung in normal animals can be explained by the larger size of the right lung.

Figure 4. Amount of phospholipids in BALF of rats that underwent unilateral left lung transplantation after 60', 90', and 120' of ischemia time versus normal (non-operated) animals. Top: left lung grafts, Bottom: native right lungs and donor right lungs. The horizontal lines indicate the mean values of each group. The larger amount of phospholipids lavaged from the right lung in normal animals can be explained by the larger size of the right lung.

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Figure 5. Phospholipid composition of surfactant as percentage of total surfactant phospholipids in rats that underwent left lung transplantation after 60', 90', and 120' of ischemia time versus normal (non-operated) animals. Top: left lung grafts, Bottom: right native lungs and right donor lungs. *p < 0.05 versus normal group, #p < 0.05 versus 60’ ischemia group.

Abbreviations: LPC=lyso-phosphatidylcholine, SM = sphingomyeline, PC = phosphatidylcholine, PE+PI = phosphatidyl-ethanolamine + phosphatidylinositol, PG = phosphatidylglycerol and PX = unknown phospholipids
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Figure 6. Minimum surface tension of surfactant measured at minimum bubble size with a pulsating bubble surfactometer from rats that underwent left lung transplantation after 60', 90', and 120' of ischemia time versus normal (non-operated) animals. All samples contained 2 mg/ml phospholipids and the values were taken after 1 minute of pulsating. Top: left lung grafts, Bottom: native right lungs and donor right lungs. The horizontal lines indicate the mean values of each group, "p < 0.05 versus normal group, #p < 0.05 versus 60' ischemia group.
Discussion

We showed that the function of surfactant, measured as the in vitro minimum surface tension, was severely impaired in lung transplants 1 day after transplantation. Simultaneously, these lung transplants showed symptoms of a severe reimplantation response, measured by the low ventilation score, low lung compliance and high serum protein concentration in the BALF all measured as parameters of the reimplantation response. Both the impairment of the surfactant function and the symptoms of the reimplantation response were shown to be more severe in the lung transplant with the longest ischemic time - 120 minutes. We further showed that the surfactant function did not deteriorate during ischemia alone, as the surfactant from ischemic right lungs from donor rats maintained normal function. This finding emphasizes that the function of surfactant is only affected after implantation and subsequent reperfusion of the lung transplant (i.e. during the reimplantation response).

The surfactant in this study was not extracted with organic solvent but was only separated from the cells in the BALF and then pelleted with 100,000 g. Thus it contained both the small inactive and the large active aggregates as well as serum proteins which might explain that the surface tension at minimum bubble size in our normal rats did not reach values below 10 mN/m, as described in other surfactant studies. We believe that the in vitro surfactant function measured in this suspension, of total, not extracted surfactant, is the best representation of the in vivo function of surfactant. This also implicates that the measured impairment of the surfactant function in this study was the overall result of various factors that influence the surfactant function in vivo.

First, the surfactant function might be impaired by the leaked serum proteins. It is well documented that serum proteins inhibit the surfactant function and that this inhibition is concentration dependent (27, 28, 29, 30). In a experimental ARDS model, this inhibition by serum proteins has been shown to be the most important cause of the impaired surfactant function (6). The surfactant phospholipids and the serum proteins in the alveolar lining fluid compete for a place at the air-water interface. When the concentration of serum proteins is not too high, they will be squeezed out of the phospholipid monolayer by the phospholipids; at high concentrations, the serum proteins, especially fibrinogen and globulin, become integrated in the monolayer at the air-water interface (27). The consistent high surface tensions after 1 and 5 minutes of pulsating of the surfactant from the 90- and 120-minute ischemia lungs in our study might indicate that the associated high serum protein
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concentration in these lungs caused such integration within the surfactant monolayer. The decrease of the minimum surface tension between 1 and 5 minutes of pulsating in the 60-minute ischemia group indicates that in that group the serum proteins were disturbing the adsorption of the phospholipids in the monolayer but that they eventually were squeezed out. Our observations support the hypothesis that serum protein inhibition is an important cause of the impairment of the surfactant function in lung grafts.

Another cause for the impaired surfactant function could be the significant changes in the surfactant phospholipid composition, especially the low percentage of PC after transplantation. PC in its dissaturated form (DPPC) is the only phospholipid that enables surfactant to reach a minimum surface tension to near 0 mN/m\(^1\). In one study in patients with ARDS a correlation was found between the percentage of PC and the function of surfactant (3). In this study, impairment of the surfactant function was indicated by a reduction of the hysteresis area measured in surface area-surface tension plots. Furthermore, Klepetko et al (9) found, after lung transplantation in dogs, a decreased oxygenation capacity of the lung graft in association with a decreased DPPC percentage. They suggested that the decrease in DPPC possibly led to an impaired surfactant function. Indeed, in our study, we measured a higher minimum surface tension of the surfactant from the 90- and 120-minute ischemia groups, which had a significantly lower percentage PC as compared with the 60-minute ischemia group. We interpret this observation as an inability of the PC to squeeze out the serum proteins resulting in a high minimum surface tension that could not be lowered by prolonged pulsating. Possibly, the low percentage of PC in surfactant from our lung grafts contributed to the reduced surfactant function.

The change in the phospholipid composition after transplantation can be explained by an increase of membrane phospholipids as a result of cell death or an altered phospholipid metabolism. Cell death with subsequent membrane damage as a cause for an altered phospholipid composition of surfactant was shown in a post-mortem study in rats (31). The first change that occurred after death was a decrease in the PC / SM ratio caused by an increase of the SM. Also in our lung transplants the SM percentage was consistently higher than in normal lungs. Because the surfactant composition from donor lungs after 120 minutes of ischemia did not show such changes, membrane damage must only occur after subsequent reperfusion of the graft. A possible changed DPPC metabolism after lung transplantation was found by Klepetko et al.(9). In dogs, the DPPC levels decreased during preservation and reperfusion of lung transplants, which could be prevented by stimulation of the DPPC metabolism with L-carnitine. Their results suggest that the metabolism of
DPPC is down regulated by lung transplantation. In our study we did not investigate the surfactant metabolism systematically, but we found a considerably higher amount of surfactant phospholipids in the native right lungs during the reimplantation response. This finding might indicate that the surfactant metabolism during the reimplantation response is altered indeed. The higher phospholipid content in the native right lungs maybe attributed to hyperventilation of these lungs, as compensation for the less functional transplanted lungs. The relation of hyperventilation and increased surfactant metabolism was shown before by Oyarzun and Clements (32). Studies that investigate the specific surfactant metabolism in the alveolar type II cells during preservation and reperfusion of the lung are necessary to elucidate this unknown area.

A third factor that could be responsible for the impaired surfactant function but that was not analyzed in this study, is a possible shift from the large surfactant aggregates to the small surfactant aggregates in the transplanted lungs. The large aggregates of surfactant, including the multi lamellar bodies and tubular myeline, are highly surface active, whereas the small aggregates, consisting of small vesicles, show only low surface activity. Recently Veldhuizen et al. (13) showed a significant shift toward the small surfactant aggregates after lung transplantation in dogs. Furthermore, they confirmed that suspensions of phospholipids extracted from small aggregates failed to lower the minimum surface tension to a low level that was found for suspensions of the large aggregates. The shift toward small aggregates is possibly caused by a lack of functional surfactant protein-A. (8) This decrease in functional surfactant protein-A might be caused by a reduced production of surfactant protein-A in the type II pneumocytes and Clara cells or by surfactant protein-A inactivation by elastase released from neutrophils (33).

As is shown in this study, a prominent factor impairing the surfactant function appears to be the leaked serum proteins, we consider that prevention of leakage of these serum proteins by better preservation is essential in lung transplantation. Also others have shown that the amount of alveolar protein is increased after lung transplantation and that the amount of protein leakage differs between different preservation techniques (13). An increased permeability for serum proteins is caused by a loss of the integrity of the alveo-capillary barrier. (34, 35) Therefore, strategies that improve the preservation of the alveo-capillary barrier and hence prevent leakage of serum proteins will also improve surfactant function after lung transplantation.

Another approach of improving the surfactant function is supplementation of the alveolar surfactant pool. Surfactant therapy, by intra-tracheal installation or nebulization, is reported to be beneficial in patients with ARDS.
The function of surfactant is impaired during the reimplantation response and also in experimental ARDS models (5, 36, 37). We expect that surfactant therapy may also prevent early lung dysfunction after lung transplantation. Primarily, the supplementation of surfactant may overcome the inhibitory effect of serum proteins on the surfactant function by increasing the phospholipid to protein ratio. In addition surfactant treatment will increase the percentage of PC in the alveolar phospholipid pool, improving the composition and consequently the function of alveolar surfactant in the transplanted lung.

In conclusion this study shows that the surfactant function is impaired during the reimplantation response. Furthermore, the surfactant function is more impaired when the reimplantation response is more severe. This impairment of the surfactant function is caused by serum proteins leaking into the alveolar space and by a low percentage of PC. We expect that improvement of lung preservation methods will prevent the deterioration of the surfactant function during the reimplantation response. The recently achieved beneficial effects of intratracheal surfactant therapy in ARDS lungs are promising for the treatment of surfactant dysfunction in lung transplants.

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References
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