

Beneficial effects of synthetic KL₄-surfactant in experimental lung transplantation

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This article contains Supplemental Information (SI): SI-Methods, Table S1, Fig. S1, and Fig. S2

ABSTRACT

The aim of this study was to investigate whether intratracheal administration of a new synthetic surfactant that includes the cationic, hydrophobic 21-residue peptide KLLLLKLLLLKLLLLKLLLLK (KL₄), might be effective in reducing ischemia-reperfusion injury after lung transplantation.

Single left lung transplantation was performed in Landrace pigs 22 hours post harvest. KL₄-surfactant at a dose of 25 mg (2.5 ml)/kg total phospholipid was instilled at 37°C to the donor left lung (n=8) prior to explantation. Saline (2.5 ml/kg; 37°C) was instilled into the donor left lung of the untreated group (n=6). Lung function in recipients was measured during 2 hr of reperfusion. Recipient left lung bronchoalveolar lavage (BAL) provided native cytometric, inflammatory marker, and surfactant data.

KL₄-surfactant treatment recovered oxygen levels in the recipient blood (PaO₂/FiO₂ of 424±60 mm Hg vs. 263±101 mm Hg in untreated group; *p*=0.01) and normalized alveolar-arterial oxygen gradient. Surfactant biophysical function was also recovered in KL₄-surfactant-treated lungs. This was associated with decreased C-reactive protein levels in BAL and recovery of surfactant protein A content, normalized protein/phospholipid ratios, and lower levels of both lipid peroxides and protein carbonyls in large surfactant aggregates.

These findings suggest an important protective role for KL₄-surfactant treatment in lung transplantation.

Keywords: ischemia-reperfusion injury, lung surfactant, KL₄-surfactant, lipid peroxidation, protein carbonyls, CRP, inflammation.

INTRODUCTION

Ischemia-reperfusion (I/R) injury associated with lung transplantation clinically manifests with vascular permeability, edema, and hypoxemia [1,2]. Severe I/R injury causes lung damage similar to acute respiratory distress syndrome (ARDS) and represents the prime cause of acute graft dysfunction in the early post-transplant period [1,2]. Inflammatory mediators released by resident donor macrophages and recruitment and activation of circulating recipient neutrophils are believed to play a significant role in the cascade of events leading to lung dysfunction [2]. Alteration of the alveolar surfactant system is another important factor contributing to lung dysfunction after lung transplantation during the early reperfusion period [3-7].

Exogenous surfactant therapy has been investigated as a therapeutic approach to minimize I/R injury, following lung transplantation. In various experimental [8-13] and clinical [14-18] lung transplantation studies, animal-derived surfactants have been administered at different times over the course of the injury: either to the donor (before ischemia) [8,10,12,13,16] or to the recipient (before [9-11,17] or after reperfusion [12-15,18]). Animal-derived surfactants consist of lipid extract preparations obtained from either bovine or porcine sources [19]. Common components in these preparations are phospholipids (PLs), mainly 1,2-dipalmitoylphosphatidylcholine (DPPC), and the hydrophobic surfactant proteins SP-B and SP-C. Currently, efforts are underway to develop synthetic surfactants since surfactants from animal sources raise microbiological, immunological, economic, and purity concerns. New synthetic surfactants consist of combinations of synthetic lipids and either synthetic or recombinant peptides [19]. A synthetic lung surfactant formulation has been developed based upon a cationic and hydrophobic 21-residue lysine (K) and leucine (L) peptide (KL₄) (KLLLLKLLLLKLLLLKLLLLK) [20]. The KL₄-peptide mimics the amino acid sequence of the various peptides taken from SP-B in terms of groups of hydrophobic residues separated by hydrophilic basic residues. The KL₄-peptide, as well as SP-B, appears to function by inducing lateral stability to the surfactant monolayer [21]. The KL₄-surfactant is comprised of DPPC, 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG), palmitic acid (PA), and KL₄. This synthetic surfactant has successfully undergone multicenter clinical trials for the prevention of neonatal respiratory distress syndrome (RDS) [22, 23]. Comparisons between KL₄-surfactant and a non-protein containing synthetic surfactant, colfosceril palmitate (Exosurf[®]),

indicated that the KL₄-surfactant is more effective in preventing the development of RDS, as well as reducing the incidence of bronchopulmonary dysplasia and RDS-related mortality [22, 23].

The objective of this study was to investigate whether intratracheal instillation of KL₄-surfactant to the donor left lung prior to explantation: a) recovers arterial oxygenation of recipients; b) improves the composition, alveolar metabolism, and biophysical activity of surfactant isolated from transplanted lungs; and c) reduces the inflammation of the graft.

MATERIALS AND METHODS

For detailed experimental procedures, see the Supplemental Information: *SI-Methods*.

Animals and Experimental Groups. Single left-lung transplantation procedures were performed in weight-matched pairs of large (17 to 23 kg) Landrace x Large White pigs, as described previously [5,7]. A total of 28 pigs was divided randomly into two groups: 1) untreated group (n=12, 6 donors and 6 recipients) without any treatment, and 2) KL₄-surfactant-treated group (n=16, 8 donors and 8 recipients). KL₄-surfactant was provided by Discovery Laboratories, Inc. (Warrington, PA, USA). Animals (donors and recipients) were sedated with ketamine (20 mg/kg body weight (bw)), diazepam (0.1 mg/kg bw), and atropine (0.02 mg/kg bw). Anaesthesia was induced with intravenous propofol (2 mg/kg bw), midazolam (0.6 mg/kg bw), and fentanyl (5 µg/kg bw). After endotracheal intubation, anaesthesia was maintained with a continuous intravenous infusion of propofol (9 mg/kg/h), midazolam (0.6 mg/kg/h), fentanyl (5 µg/kg/h), and pancuronium bromide (0.4 mg/g/h). Volume control ventilation was used with ventilatory setting: oxygen inspired fraction of 1.0, inspiratory/expiratory ratio of 1:2, tidal volume of 10 ml/kg, peak airway pressure of 12-20 cm H₂O, and positive end-expiratory pressure of 5 cm H₂O. The initial respiratory rate was 12 breaths/min, and was adjusted to obtain an end-tidal CO₂ between 30 and 40 mmHg. All animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals [National Academies Press, Washington, DC, 1996].

Transplantation Operation and KL₄-surfactant Treatment. Briefly, sternotomy, thymectomy, and anterior pericardectomy were performed in donors. Before preservation, KL₄-surfactant (or the same volume of saline in the untreated group) was instilled into the left lung at 37 °C at a dose of 25 mg (2.5 ml; 10 mg/ml total phospholipids) KL₄-surfactant/kg bw. Then retrograde flush of cold (4 °C) University of Wisconsin solution (60 ml/kg bw, under a perfusion pressure

not exceeding 30 cm H₂O) was performed. The lungs were inflated to a pressure of 20 cm H₂O, the trachea stapled, and the heart-lung block stored at 4 °C for 22 hours. Recipients underwent a left posterolateral thoracotomy and the left lung was clamped, excised, and lavaged ex situ. Recipient left lung was used as control lung. Subsequently, the donor left lung was implanted. Upon ventilation of the donor lung, reperfusion was started and time was set to zero. Ventilatory and hemodynamic parameters were measured as in [7] in basal situation (presurgery) and after lung transplantation at 0, 1, and 2 h following reperfusion.

Lung Groups and Bronchoalveolar Lavage (BAL) Processing. Individual lung lavages were obtained from the left lung removed from recipients (control group) and donor left lung transplanted into the recipients in KL₄-surfactant-treated (KL4 group) and untreated (UNT group) animals. Cytometric analyses were done in cells obtained from BAL from each lung as detailed in *SI-Methods*. The volume of the remaining cell-free BAL was recorded and used for i) protein quantification by Lowry's method modified by adding sodium dodecyl sulphate (SDS) [5]; ii) protein oxidation, determined on the basis of their protein carbonyl contents by the dinitrophenylhydrazine (DNPH) spectrophotometric assay [24]; iii) tumour necrosis factor- α (TNF- α), and C-reactive protein (CRP) quantification using a pig TNF- α and pig CRP ELISA kits (Pierce Endogen, Rockford, IL, USA and GenWay, Biotech, Inc., San Diego, USA, respectively); and iv) surfactant isolation.

Isolation and Biochemical Analysis of Pulmonary Surfactant Fractions. Cell-free BAL was centrifuged at 48,000g for 1h at 4°C to obtain pellets of large surfactant aggregates (LA), which are the active form of surfactant, and supernatant, which contains small surfactant phospholipid vesicles and proteins present in the alveolar fluid (SA). Protein content and protein carbonylation were determined in LA and SA fractions. Surfactant apolipoproteins (SP-A, SP-B, and SP-C) were measured by Western Blot analysis from LA as described [25]. Lipid extracts of LA and SA were obtained by chloroform/methanol extraction [5] and used to quantify total phospholipids in LA and SA by phosphorus analysis [5] and lipid hydroperoxides by the FOX method [26].

Surface Adsorption Assay. The ability of the active fraction of surfactant (LA) to adsorb onto and spread at the air–water interface was tested in a Wilhelmy-like high-sensitive surface microbalance as described [5,7].

Statistical Analysis. All data represent the mean of individual measurements \pm SD and were normally distributed (Shapiro-Wilk test). For statistical analysis, two-tailed unpaired Student's t test was used for comparison of the two groups of recipients (untreated and KL₄-surfactant-

treated) in the analysis of hemodynamic and gasometric parameters. In studies with different lung groups, differences in means between the three lung types (control, UNT, and KL₄) were evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni adjustment; an alpha level of 5% or less ($p \leq 0.05$) was considered significant.

RESULTS

Physiological responses

Gasometric and hemodynamic parameters were measured in recipients from untreated (UNT) and KL₄-surfactant-treated (KL₄) groups before (basal values) and after lung transplantation. The KL₄-surfactant-treated group showed arterial oxygenation (PaO₂) values significantly higher than those of the untreated group after 2 hr of reperfusion (Fig. 1). The preservation of PaO₂ levels with KL₄-surfactant treatment was associated with maintenance of low alveolar-arterial oxygen gradient [D(A-a)O₂], indicating protection of the alveolar wall from injury during reperfusion. On the other hand, we did not find statistical differences when the hemodynamic parameters of untreated and KL₄-surfactant-treated groups were compared (Table S1- supporting information).

Changes in lung surfactant

Figure 2 shows the phospholipid and protein content of LA and SA isolated from control and transplanted lungs in both KL₄-surfactant-treated and untreated groups. LA represent freshly secreted surfactant membranes from type II cells, whereas SA represent metabolic products of LA formed within the airspace together with proteins present in the alveolar fluid. Fig. 2A illustrates that KL₄-surfactant-treated lungs showed a significant increase in phospholipid content in LA, but not in SA, with respect to control and untreated transplanted lungs. This indicates that instilled KL₄-surfactant reached the alveolus and was efficiently incorporated in large surfactant aggregates. The phospholipid SA-to-LA ratio was significantly higher in lavage material from untreated transplanted lungs as compared to KL₄-surfactant-treated and control lungs (Fig. 2B). An uneven distribution of surfactant subtypes is a marker of lung injury and usually occurs by accelerated LA-to-SA conversion in the inflamed lung due to the presence of proteases and/or oxygen radicals [19]. With respect to protein levels in SA and LA fractions, we found that protein levels in SA from transplanted lungs were significantly higher than control lungs, regardless of the group considered (KL₄-surfactant-treated or untreated) (Fig. 2C). Increased levels of proteins recovered in SA indicate leakage of serum proteins into the alveoli. Protein levels in LA of transplanted lungs (either KL₄-surfactant-treated or untreated) slightly increased with respect to control lungs (Fig. 2C) due to co-isolation of some serum proteins with surfactant membranes. Given that serum proteins are potent inhibitors of surfactant biophysical function [19], we used the protein/phospholipid ratio in LA as a marker of surfactant injury. Fig. 2D shows that the protein/phospholipid ratio significantly increased in LA of untreated transplanted lungs compared with control and KL₄-surfactant-treated transplanted lungs.

Fig. 3 shows that surfactant lipid and protein components were oxidized after lung transplantation as demonstrated by significant increases in lipid peroxides and protein carbonyls in LA of untreated transplanted lungs. Protein and lipid oxidation was also found in SA of these lungs (data not shown). Remarkably, KL₄-surfactant treatment of the donor left lung protected surfactant lipids and associated proteins from oxidative damage (Fig. 3). Fig. 4 shows the content of surfactant apolipoproteins present in LA (SP-A, SP-B, and SP-C) in control and transplanted lungs. While the amount of both SP-B and SP-C in LA did not change after lung transplantation, the content of SP-A decreased by 54±10 % in surfactant from untreated transplanted lungs. KL₄-surfactant treatment of the donor left lung returned the content of SP-A to normal (Fig. 4).

Surfactant function was determined by measuring the ability of LA to adsorb onto and spread at an air-water interface using a Wilhelmy dipping plate attached to an electrobalance suited to monitor changes in surface pressure [19]. The amount of surfactant phospholipids injected into the hypophase was the same for all samples from the control and transplanted groups. Interfacial adsorption is performed through i) the transport of the material injected through the bulk liquid to accumulate at the air/liquid interface and ii) the spread of the material along the surface, producing the surface pressure we measured. The results indicate that the surface adsorption rate of surfactant from untreated transplanted lungs decreased significantly compared with surfactant isolated from control lungs (Fig. 5). KL₄-surfactant treatment resulted in complete normalization of surfactant interfacial adsorption activity.

To investigate the contribution of ischemia to the alterations observed in surfactant composition and function after I/R injury, we performed biophysical and biochemical studies in LA and SA isolated from preserved lungs, i.e., right lung of the donor. We found no differences in phospholipid and protein content in LA and SA, SP-A content, and surfactant surface adsorption between samples isolated from control and preserved lungs (Fig. S1). Furthermore, normal surfactant function and composition were found in the right lung of recipients (native lung) (data not shown), indicating that surfactant alterations are restricted to the transplanted lung as a result of I/R injury.

Other injury variables in BAL

Increased levels of proteins were recovered in cell-free BAL of transplanted lungs regardless of the group (KL₄-surfactant-treated or untreated) as a consequence of the edema (Fig. S2). We determined the concentration of protein carbonyls in cell-free BAL as an indicator of oxidative stress. Fig. 6A shows that protein oxidation greatly increased after

transplantation, and that KL₄-surfactant treatment of the donor left lung protected against oxidative damage.

CRP is one of the most characteristic acute-phase proteins displaying rapid and pronounced increase in BAL in response to inflammation [5,27,28]. Fig. 6B shows that the content of CRP significantly increased in cell-free BAL of untreated transplanted lungs. KL₄-surfactant treatment decreased CRP content in BAL.

TNF- α is an early response cytokine produced primarily by alveolar macrophages, which promotes the cascade of events that lead to pulmonary inflammation [2]. Fig. 6C shows that TNF- α levels greatly increased in transplanted lungs regardless of the group considered (KL₄-surfactant-treated or untreated). Likewise, neutrophil entry was observed in transplanted lungs. KL₄-surfactant treatment did not influence neutrophil influx into the grafts, which is in line with previous studies using animal-derived surfactant [12].

DISCUSSION

Severe ischemia-reperfusion injury leading to primary graft dysfunction occurs in 15-25% of lung transplant recipients, and contributes to significant morbidity and mortality [1]. We found that transplantation and reperfusion of pig lungs that had been stored for 22 hours at 4°C resulted in severe I/R injury characterized by i) significant decrease in PaO₂ and increase in D(A-a)O₂; ii) increase in plasma protein leakage and neutrophil influx into the alveolar space of transplanted lungs; iii) increase in injury markers, such as TNF- α , CRP, lipid peroxidation, and protein carbonyls in alveolar fluid of transplanted lungs; and iv) inactivation of surfactant biophysical activity. Surfactant disturbances largely contributed to impairment of gas exchange under these conditions, since KL₄-surfactant treatment of the donor left lung, prior to explantation, recovered oxygen levels and maintained normal alveolar-arterial oxygen gradients.

Surfactant inhibition or inactivation refers to processes that decrease or abolish normal phospholipid adsorption to form a functional surfactant monolayer at the air-liquid interface film and/or prevent the film from reaching low surface tension upon compression [19]. The significant decrease of surface adsorption rate of surfactant isolated from untreated transplanted lungs can be explained by the following: i)

significant increase in lipid peroxides and protein carbonyls in the active surfactant fraction or LA. Reactive oxygen species released by activated alveolar macrophages and neutrophils may be involved in surfactant oxidation, which results in structural alterations that lead to poor surface activity [29]; ii) high phospholipid SA-to-LA ratio, which suggests accelerated LA-to-SA conversion probably as a consequence of structural alterations in oxidized LA; iii) reduction of SP-A content in LA, which directly affects both surfactant surface adsorption and rate of LA-SA conversion [19]; and iv) significant increase of CRP and other serum proteins in alveolar fluid, which are potent inhibitors of the biophysical activity of surfactant [5, 27, 28]. The reduction of SP-A levels enhances susceptibility of surfactant to be inactivated by CRP, since SP-A binds to CRP and blocks CRP inhibitory effects on surfactant membranes [28]. In sum, we conclude that oxidation of lipids and proteins in surfactant, decreased levels of SP-A, and the presence of CRP and other surfactant protein inhibitors may render the lung susceptible to atelectasis caused by a loss of surfactant function.

Instillation of KL₄-surfactant in donor left lungs prior to explantation significantly improved arterial oxygenation of recipients, likely as a consequence of surfactant protection from oxidative damage and recovery of surfactant composition and biophysical activity. KL₄-surfactant treatment contributed new and fresh tensoactive material that increased the amount of phospholipids in large surfactant aggregates, and normalized the SA/LA phospholipid ratio and the interfacial adsorption rate of surfactant. In addition, KL₄-surfactant treatment prevented production of both lipid peroxides and protein carbonyls in the alveolar compartment, decreased CRP levels, and normalized SP-A. In contrast, KL₄-surfactant treatment did not prevent or reduce plasma protein leakage and neutrophil influx into the alveolar space of the graft, as previously reported in other studies showing potential benefit of exogenous surfactant treatment in I/R injury after lung transplantation [11,12]. Interestingly, these lung injury sequelae, i.e. high alveolar protein concentration and percent of neutrophils in BAL, were absent one week after transplantation in animals treated with surfactant before reperfusion but not observed in untreated rats [9].

The fact that KL₄-surfactant prevented oxidation of lipids and proteins present in the alveolar compartment suggests that KL₄-surfactant might downregulate alveolar cell respiratory burst. This is supported by the fact that neutrophil respiratory burst oxidase

activity is inhibited in vitro by KL₄-surfactant [30], and DPPC, the major lipid component of both KL₄-surfactant and animal-derived surfactants, plays an important role in downregulating monocyte respiratory burst [31]. Oxidative stress and innate immunity have been recently identified as key lung injury pathways that control the severity of acute lung injury [32]. Intratracheal administration of synthetically oxidized surfactant PLs can trigger acute lung injury in vivo, acting through Toll-like receptor 4 [32], and oxidized PL production is a general feature of lethal lung injury in human and other species [32]. These data support the relevance of KL₄-surfactant as a therapy in lung transplantation, given its protective effect from oxidative damage.

The decrease of CRP in BAL of KL₄-surfactant-treated grafts suggests local CRP generation in lung tissue after transplantation, since protein leakage into the alveolar space was not prevented by KL₄-surfactant treatment, and plasma CRP levels were not elevated after reperfusion (data not shown). Thus increased CRP levels in BAL of untreated transplanted lungs might be locally produced by alveolar macrophages [33] and/or epithelial cells [34,35], since production of CRP by these cells is up-regulated by pro-inflammatory stimuli [33-35]. The fact that KL₄-surfactant reduced CRP levels and lipid and protein oxidation in the alveolar compartment suggests that KL₄-surfactant might modulate the activation of alveolar cells and inflammation, as has been previously demonstrated in human airway epithelial cells exposed to hyperoxia [36].

The observation that KL₄-surfactant treatment normalized SP-A levels was unexpected since it does not contain SP-A. One possible explanation is that leukocyte proteases are more damaging to oxidized proteins and KL₄-surfactant prevented protein oxidation in LA. Alternatively, this preservative effect of KL₄-surfactant may be attributed to the strong binding of SP-A to KL₄-surfactant membranes [37], which might protect SP-A from degradation.

The therapeutic dose we used for intratracheal administration of KL₄-surfactant (25 mg/kg bw) to donor lungs is approximately 15% of the dose recommended for surfactant instillation in neonatal RDS (175 mg/kg bw) and is much lower than that used for the treatment of patients with ARDS (100-300 mg/kg bw) [19] and that previously used in experimental lung transplantation (50-200 mg/kg bw) [8-13]. We found that a higher dose of KL₄-surfactant (65 mg (2.5 ml)/kg) did not increase the

beneficial effect of this synthetic surfactant (data not shown). It is important to point out that recent studies reporting the beneficial effect of surfactant in clinical lung transplantation were performed with low doses of commercially available animal-derived surfactant (~ 20-45 mg /kg) [14,17,18], and our data indicated that instilled KL₄-surfactant at low doses in the donor left lung was effective in ischemia-reperfusion injury after lung transplantation.

In summary, we conclude that KL₄-surfactant treatment in the donor at low doses protects against oxidative damage, recovers the composition and biophysical activity of surfactant, and causes a parallel improvement of arterial oxygenation, minimizing the damage triggered by ischemia-reperfusion after lung transplantation.

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FIGURE LEGENDS

Figure 1. Arterial oxygen tension (upper panel) and alveolar-arterial oxygen gradient (lower panel) values in untreated (UNT, n=6) and KL₄-surfactant-treated (KL4, n=8) recipients over 2 h of reperfusion. Measurements were made in basal situation (pre-surgery) and after lung transplantation at 0, 1, and 2 hours of reperfusion. N means lung function of the recipient native (N) right lung after left lung explantation. Tx+N means lung function of the transplanted (Tx) and native (N) lungs. Results are presented as the mean \pm SD.

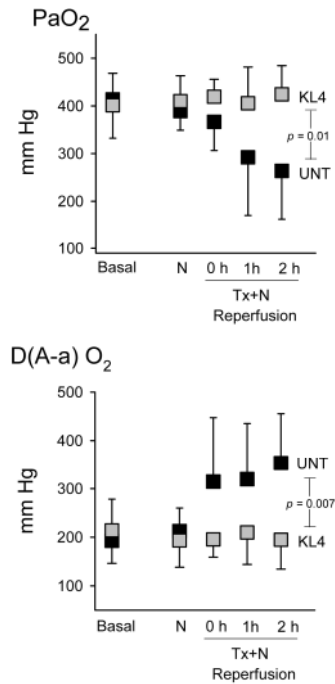


Figure 2. (A) Phospholipid (PL) content in large surfactant aggregates (LA) and small surfactant aggregates (SA) from control (n=10) and transplanted groups, with (KL4, n=8) and without (UNT, n=6) KL₄-surfactant treatment. (B) Average SA-to-LA ratio in transplanted and control lungs. (C) Protein content in LA and SA of control and transplanted lungs. (D) Protein/phospholipid ratio in LA from control and transplanted lungs. Results are presented as the mean + SD. * $p < 0.001$. In C, * $p < 0.001$ UNT and KL4 vs control lungs for SA and LA fractions.

LARGE & SMALL SURFACTANT AGGREGATES

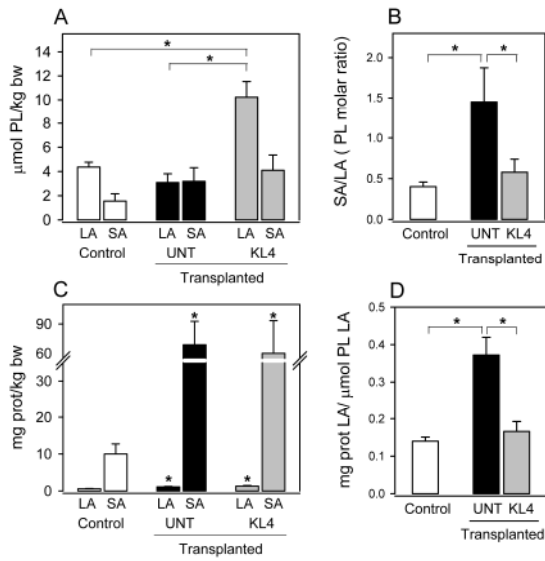


Figure 3. Oxidation of lipids and proteins in LA from control (n=10) and transplanted lungs (KL4, n=8; UNT, n=6). The final concentration of PLs in lipid peroxidation experiments and proteins in carbonylation experiments were similar for all surfactant samples. Results are presented as the mean + SD. * $p < 0.05$.

LARGE AGGREGATES

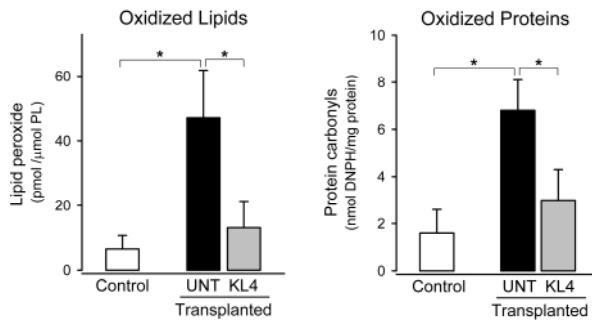


Figure 4. Levels of immunoreactive SP-A, SP-B, and SP-C in large surfactant aggregates isolated from control and transplanted lungs with and without KL₄-surfactant treatment. Two micrograms of proteins from LA of control (n=10) and transplanted lungs (KL4, n=8; UNT, n=6) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blotted with polyclonal antibodies against SP-A, SP-B, or SP-C. Quantitation of surfactant apolipoproteins was achieved by densitometric evaluation of the immunoreactive bands. Results are presented as the mean + SD. * $p < 0.001$.

LARGE AGGREGATES

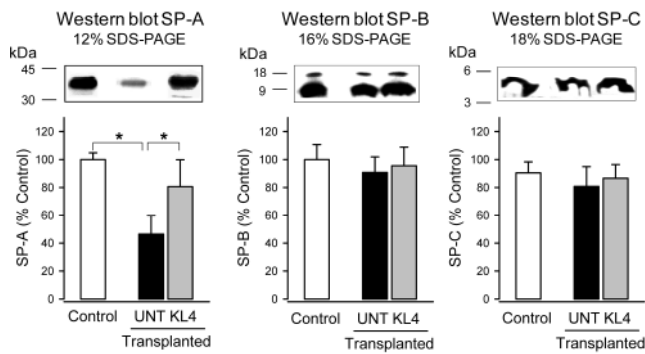


Figure 5. Interfacial adsorption kinetics of large surfactant aggregates from control and transplanted lungs, with and without KL₄-surfactant treatment. The final concentration of phospholipids in the hypophase was 83 nmol/ml for all surfactant preparations obtained from control (n=10) and transplanted lungs (KL₄, n=8; UNT, n=6). Π means surface pressure. Results are presented as the mean \pm SD. Differences among control and KL₄-surfactant-treated transplanted lungs versus untreated transplanted lungs were significant (* $p < 0.001$).

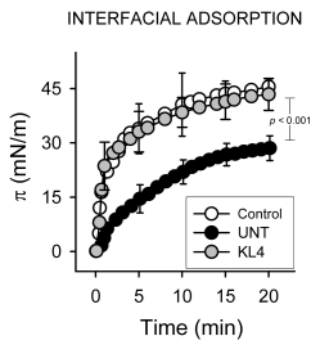


Figure 6. Inflammatory markers in BAL isolated from control (n=10) and transplanted lungs (KL₄, n=8; UNT, n=6). (A) Protein carbonyls/mg proteins, (B) levels of immunoreactive CRP and (C) TNF- α , and (D) percentage of neutrophils. Results are presented as the mean + SD. * $p < 0.05$.

