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Increased phospholipase A₂ and lyso-phosphatidylcholine levels are associated with surfactant dysfunction in lung contusion injury in mice

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Abstract

Objective—Surfactant dysfunction is an important pathological disturbance in various forms of acute inflammatory lung injury. Previously we reported the presence of significant alterations in the composition and activity of pulmonary surfactant in blunt trauma-induced bilateral lung contusion (LC) injury in rats. This is extended here to a mouse model of unilateral LC, with a focus on compositional and functional surfactant changes associated with permeability injury and increases in activity of secretory phospholipase A₂.

Results—Surfactant-associated gene expression was not significantly altered in mice with unilateral LC injury based on Affymetrix analysis. LC mice had significant permeability injury with increased albumin and total protein in bronchoalveolar lavage (BAL) at 5, 24, 48 and 72 h post-insult compared to uninjured controls. The percent content of large surfactant aggregates was significantly depleted at all post-injury times, and pulmonary pressure-volume (P-V) mechanics and compliance were abnormal over this period. Surfactant dysfunction was evaluated in mechanistic detail at 24 h, when permeability injury and P-V changes were most prominent. At this time, activity levels of secretory phospholipase A₂ (PLA₂) were increased in BAL, and chromatographic analysis showed that large surfactant aggregates had decreased levels of phosphatidylcholine (PC) and increased levels of lyso-PC. These changes were accompanied by severe detriments in large aggregate surface activity by pulsating bubble surfactometry. Large aggregates from LC mice at 24 h had minimum surface tensions of only 12.6±1.1 mN/m after prolonged bubble pulsation (20 min) compared to 0.7±0.03 mN/m for uninjured controls.

Conclusion—These results document significant detriments in the composition and activity of pulmonary surfactant in LC injury in mice, and suggest that active synthetic phospholipase-resistant exogenous surfactants may have future utility in treating surfactant dysfunction in this clinically-important condition.

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Keywords

lung surfactant; surfactant dysfunction; lung contusion; Phospholipase A₂

INTRODUCTION

Lung contusion (LC) following blunt chest trauma is frequently associated with a need for hospitalization^{1–3}, and is an important and prevalent problem in the care of critically-ill trauma patients. LC is also a prevalent organ injury in blast trauma, which carries a high risk of mortality⁴. LC remains an independent risk factor for the development of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) and as per the ARDS network data, trauma with/without lung contusion is responsible for 7–10% of patients with ARDS^{1–3, 5, 6}. The comprehensive study by Rubenfeld *et al* (2005)⁷ reported mortality rates of 38.5% for ALI and 41% for ARDS in adults with an estimated 74,500 deaths per year and an aggregate 3.6 million hospital days of care in the United States. Conversely, Miller *et al* (2001)³, reported that 25–35% of lung contusion patients deteriorate to ALI/ARDS. In a 2004 study of trauma patients, the incremental hospital cost per patient with ALI or ARDS (\$36,713 or \$59,633, respectively) was much higher than for patients without ALI/ARDS (\$24,715)⁸.

Observational studies by Nakos *et al* (1998)⁹ reported significant alterations of surfactant phospholipid composition in patients with trauma and surgical related-ARDS. This group also found increased levels of secretory Phospholipase A₂ in BAL fluid, which were inversely associated with oxygenation. Their conclusion was that PLA₂ may have a potential role in the induction and exacerbation of ARDS¹⁰.

Active pulmonary surfactant is required to normalize alveolar stability, compliance, and gas exchange^{11, 12}. Because of the essential physiological roles of lung surfactant, its dysfunction can be an important determinant in the severity and progression of ALI/ARDS. In a previous study using a bilateral model of closed-chest LC induced in rats by a falling weight, we documented significant surfactant dysfunction that included decreased large aggregate content, reduced surface activity, and altered aggregate lipid/protein composition¹³. This prior work also showed that surfactant dysfunction in rats correlated with the severity of permeability injury based on protein in Bronchial Alveolar Lavage (BAL) fluid, and with acute inflammation reflected by polymorphonuclear neutrophils (PMNs) in BAL and concentrations of whole lung myeloperoxidase (MPO)¹³. The current study was undertaken to show that significant surfactant dysfunction is also present in mice with unilateral closed-chest LC injury induced by electrical cortical impactor methodology as reported by Hoth *et al* (2007)¹⁴.

With the present model of unilateral LC, we recapitulate many of the important features of surfactant dysfunction that are present in trauma related ARDS. This has the potential to be highly useful in future investigations of molecular mechanisms of pulmonary contusion due to the known genomics and wide availability of molecular reagents for this animal species in a clinically relevant model¹⁵. A major focus of specific experimentation here is not only to investigate the magnitude of surfactant dysfunction, but also to investigate potential mechanisms. In particular, we examine surfactant-related gene expression in mice with LC injury, and whether increased concentrations of secretory phospholipase A₂ (PLA₂) are present in bronchoalveolar lavage (BAL) from injured mice. Increased concentrations of this lytic enzyme can degrade active phospholipids in pulmonary surfactant, and also produce byproduct lyso-phospholipids that are themselves detrimental to surfactant function^{16–18}. We proposed that lung contusion and its subsequent tissue injury will increase lung

permeability injury and secretory PLA2. Both will affect lung surfactant by degradation of surfactant phospholipids and loss of large aggregates. The functional downstream effect is loss of surfactant tension reducing properties that in consequence may affect lung mechanics. This could potential create a pernicious cycle that can amplify and expand the initial tissue damage given by LC (Figure 1)

MATERIALS AND METHODS

Induction of isolated unilateral lung contusion (LC) in mice

All protocols and procedures received approval from the institutional University Committee on the Use and Care of Animals (UCUCA) at the University of Michigan (Ann Arbor, MI). LC was induced by methods similar to those described by Hoth et al (2007)¹⁴. Briefly, Male, C57/BL6 mice (Charles River Laboratories), 26–30g (8–12wk old) were used. General anesthetic using an admixture of ketamine/xylazine at a dose of 100 mg/Kg and 10 mg/Kg respectively was given by intra-peritoneal injection 5–10 minutes prior to contusion. Once depth of anesthesia was confirmed, mice were placed in the left lateral decubitus position. The right chest was struck 1 cm above the costal margin along the posterior axillary line using an electrical cortical contusion impactor with a piston velocity of 5.8m/sec adjusted to a depth of 10 mm. All animals were allowed to recover spontaneously on a warming pad without the use of ventilatory support.

Mice were then studied at different terminal time points (5, 24, 48 and 72 h) after blunt chest trauma and physiological and biochemical variables were compared with uninjured control mice. These reflect the various attributes of the acute inflammatory response following LC and are reviewed in Raghavendran et al (2005)¹⁹. In summary, the onset of inflammation peaks around 24–48 hr and the acute inflammatory response relays into a sub-acute response by 72 hr following lung contusion.

Lung pressure-volume (P-V) mechanics

At a given time point following the injury, mice were exsanguinated by transection of the abdominal inferior vena cava and an 18-gauge metallic cannula was inserted into the trachea through a midline cervical exposure. Animals were then connected to a SCIREQ® Flexivent (Montreal, Quebec, Canada) that allowed simultaneous ventilation and data capture. Immediate closed chest postmortem ventilation was done with the following parameters: tidal volume 10 ml/kg, respiratory rate of 150 breaths per minute, and positive end expiratory pressure 2 cm H₂O. The SCIREQ® Flexivent ventilator introduced force oscillation measurements through a graphite piston system that controlled circuit pressure/volume displacements. Before data capture, animal lung volumes were normalized by a six second inspiratory hold of 0.6 mL at a pressure of 30 cm H₂O. Three types of ventilatory maneuvers were performed with associated parameters analyzed by company-provided software: Total Lung Capacity (TLC), Snapshot (SS), and Pressure-volume ramp volume regulated (PVRV). During TLC, a single breath was performed at a pressure of 30 cm H₂O with a plateau maintained for several seconds to allow mean displaced volume to be determined (Vend-indicative of quasi-static compliance). In Snapshot, a standardized sinusoidal waveform was recreated and resistance (R) of the total respiratory system (airway, parenchyma and chest wall) was calculated. With PVRV, a controlled inflation and deflation were performed to allow P-V curves to be obtained as well as a maximal vital capacity at 30 cm of H₂O (TLC-30). The deflation curve associated with this maneuver also allowed calculation of the hysteresis of the system (measured by Area Under the Curve). Flexivent measurements in animal models of lung injury have been validated and found comparable to results from other mechanical ventilator systems²⁰.

Albumin, total protein, and total phospholipid concentrations in cell-free BAL

After P-V measurements, with the metallic cannula still in place, BAL was performed by injecting 2 μ 600 μ l of ice-cold phosphate buffered saline (PBS). Both recovered aliquots were pooled together and centrifuged at $150 \times g$ at 4°C for 10 min to remove cells. Albumin concentrations (μ g/ml) in cell-free BAL were measured by ELISA with a polyclonal rabbit anti-mouse albumin antibody and a HRP-labeled goat anti-rabbit IgG (BD Biosciences Pharmingen, San Diego, CA). Total protein was measured in BAL by the method of Lowry et al.²¹ modified by the addition of 15% SDS to allow accurate quantitation in the presence of lipid. The presence of surfactant and detergent can decrease U-V absorbance basis of the Lowry method underestimating the total amount of protein and their concentrations can appear lower those obtained for albumin, which was quantified by ELISA. Total phospholipid in cell-free BAL was measured by the phosphorus assay of Ames²².

Affymetrix microarray analyses of surfactant protein-associated genes

Microarray analyses examined a subset of genes relating to surfactant proteins (SP) – A, B, C, and D in lung tissue from LC mice at 5, 24, and 72 h post-injury and in uninjured control mice. At the time of sacrifice, the vasculature of the lung tissues were flushed using saline injected through the right ventricle. The RNA extraction from the lung tissue was achieved by using the Qiagen RNeasy mini column. The quality of RNA was then confirmed by spectroscopic analyses. Subsequently the cDNA was then analyzed using Affymetrix® (Santa Clara, CA) GeneChip for mouse, 430, 2.0 in the microarray core facility of the University of Michigan. The expression values for the surfactant protein genes were directly extracted from the CEL files using the robust multi-array average. Confirmatory real-time PCR (polymerase chain reaction) analyses were not performed as the difference in the values of surfactant gene expression were found to be extremely small (Results).

Content and composition of centrifuged large surfactant aggregates

Large surfactant aggregates were sedimented from cell-free BAL by centrifugation at $12,000 \times g$ for 30 min. The content of large aggregates in control mice and LC mice at 5, 24, 48, and 72 h post-contusion was expressed as a percentage of total BAL phospholipid based on the phosphate assay of Ames²². Large aggregates obtained by similar lavage centrifugation conditions have been widely used in lung surfactant research as a reflection of biophysically-active endogenous lung surfactant (Notter RH, 2000 for review)¹¹. The specific large surfactant aggregate fraction studied is closely-analogous to that used in the clinical surfactant Infasurf®, an organic solvent extract of lavaged, centrifuged bovine lung surfactant¹¹. In addition to measuring the content of large surfactant aggregates, thin layer chromatography (TLC) was also used to define aggregate phospholipid class composition including lyso-phosphatidylcholine (lyso-PC) in control mice and LC mice at 24 h. TLC analyses used a solvent system of chloroform: methanol: 2-propanol: triethylamine: water (30:9:25:25:7 by volume)²³.

Pulsating bubble measurements of large aggregate surface activity

The dynamic surface activity of resuspended large surfactant aggregates from uninjured control mice and mice at 24 h post-contusion was assessed during cycling at a physiological rate of 20 cycles/min at $37 \pm 0.5^\circ\text{C}$ on a pulsating bubble surfactometer (General Transco, Largo, FL)²⁴. A small air bubble, communicating with ambient air, was formed in a 40 μ l aliquot of resuspended surfactant aggregates in a plastic sample chamber mounted on the pulsator unit of the surfactometer. The bubble was oscillated between maximum and minimum radii of 0.55 and 0.4 mm (50% area compression for a truncated sphere) while the pressure drop across the air-water interface was measured with a precision pressure transducer. Surface tension at minimum bubble radius (minimum surface tension) was

calculated as a function of time of pulsation from the measured pressure drop at end-compression and the Laplace equation for a spherical interface^{24, 25}. Large surfactant aggregate samples were examined at a uniform phospholipid concentration of 1 mg/ml in 10 mM HEPES, 2 mM CaCl₂, 150 mM NaCl, pH 7.0.

BAL phospholipase A₂ (PLA₂) measurements

Measurements of PLA₂ in cell-free BAL fluid were made at 24 h post-contusion, a time when permeability injury and abnormalities in P-V compliance were prominent. PLA₂ activity in cell-free BAL was measured using the EnzChek® Phospholipase A₂ Assay Kit (Invitrogen, Carlsbad, California). Assays were performed following the manufacturer's protocol in clear-bottom, black 96-well plates (Corning, Corning, NY)²⁶. PLA₂ from honey bee venom supplied with the kit was the positive control. The assay was conducted at 26°C, and fluorescence emission at 515 nm was detected after an incubation step of 10 min by a SpectraMax® M5/M5e Microplate Reader (Molecular Devices, Sunnyvale, CA).

Statistical analyses

Data are expressed as mean ± S.E.M. unless stated otherwise. One way analysis of variance (ANOVA) with Bonferroni's *post hoc* test was used for all intergroup pairwise comparisons. Significance values were calculated and graphics generated using GraphPad Prism 5.0 (La Jolla, CA). Statistical significance for intergroup comparisons was considered when a *p* value of less than 0.05 was obtained. For direct comparison of surfactant and phospholipid composition at 24 h Student t-test with Welch correction was performed and a *p* value of less than 0.05 was considered significant.

RESULTS

Surfactant protein gene expression by Affymetrix microarray

Genes related to all four surfactant-associated proteins SP-A, SP-B, SP-C, and SP-D were assessed by Affymetrix microarray methods at 5, 24, and 72 h post-contusion relative to uninjured controls (Table 1). Measurements of surfactant gene cDNA indicated no significant changes in expression for any of the genes at these post-LC time points. These findings indicate that the changes in surfactant aggregate content, composition, and activity shown in subsequent sections reflect acquired surfactant dysfunction as a result of LC-induced permeability injury and inflammation.

Permeability injury in mice with unilateral LC

Mice with LC injury had permeability injury with greatly increased amounts of total protein and albumin in cell-free BAL at 5, 24, 48, and 72 h post-contusion (Table 2). The tabulated concentrations of albumin and total protein were measured by different assays (ELISA for albumin and the modified Lowry assay for total protein) so that the quantitative amounts are not directly comparable. However, both measurements showed a similar pattern of significantly increased BAL protein relative to uninjured control mice at all injury times studied. Moreover, the specific presence of substantial BAL albumin concentrations by ELISA directly indicates that permeability injury existed to allow this large plasma protein to be in the alveoli.

Large surfactant aggregate depletion in LC mice

Mice with LC injury had significant depletions in the percent content of large surfactant aggregates in BAL (Figure 2). Large aggregate depletion was most severe early in the course of injury, and improved somewhat at later time points (although aggregates were still reduced compared to control). At 5 and 24 h post-LC, the large aggregate fraction of BAL

made up only $27 \pm 2\%$ and $33 \pm 1\%$ of total BAL phospholipid, respectively, compared to $51 \pm 1\%$ for uninjured controls. Significance levels for aggregate depletion relative to control were $p < 0.05$ at 5 h (due to the small sample size at this time point) and $p < 0.001$ at 24 h. The percentage of large aggregates in BAL at 48 and 72 h post-LC was also significantly reduced compared to uninjured control mice ($38 \pm 1\%$ at 48 h and $42 \pm 2\%$ at 72 h, $p < 0.001$, Figure 2). However, large aggregate percentages at these later times were significantly higher compared to the 24 h injury time point ($p = 0.01$).

Changes in P-V curves and compliance in LC mice

Abnormalities in lung ventilatory mechanics were found throughout the 72 h time course of study following unilateral LC. P-V curves demonstrated a lowered and rightly displaced inflation curve at all injury times compared to uninjured controls (Figure 3). Abnormalities in P-V mechanics were particularly pronounced at 24 h based on the inflation-deflation loops in Figure 2 and on other calculated respiratory volume parameters (Figure 4). At 24 h post-injury, the volume to which the lung was inflated at 30 cm H₂O pressure (TLC-30) was the smallest (0.45 ± 0.02 mL vs. 0.56 ± 0.01 mL in controls; $p < 0.001$) (Figure 4A). The maximal volume to which the lung could be inflated after a total lung capacity perturbation maneuver (Vend) that is indicative of quasi-static compliance was also most diminished at this time (0.63 ± 0.01 mL vs. 0.74 ± 0.01 mL in uninjured controls; $p < 0.01$) (Figure 4B). Snap shot maneuvers assessed with the SCIREQ Flexivent® system also showed increases in the total respiratory resistance (R-SS) that were maximal at 24 h (Figure 4C) in association with diminished Compliance. Although P-V abnormalities were most prominent at 24 h, they were also present at other injury times. AUC (area under the curve) indicative of hysteresis of the respiratory system was diminished at each time point following LC but values achieved statistical significance at 5 and 72 h (Figure 4D). Also, the total dynamic compliance of the respiratory system (C-SS) was significantly decreased at 48 h, and the reciprocal parameter of total system elastance (E-SS) was significantly increased at 48 h (data not shown).

Abnormalities in large aggregate surface activity at 24 h following LC injury

Large aggregates were significantly impaired in surface activity at 24 h post-contusion (Figure 5). Centrifuged large aggregates were resuspended in buffered saline at a uniform phospholipid concentration of 1 mg/mL, and studied for overall dynamic surface tension lowering ability on a pulsating bubble surfactometer at physical conditions relevant for respiration *in vivo* (37°C, 20 cycles/min, 50% area compression). Large aggregates from mice at 24 h post-LC had a greatly increased time course of surface tension lowering at all-time points, and reached minimum surface tensions of only 12.6 ± 1.1 mN/m after prolonged bubble cycling (20 min). In contrast, resuspended large surfactant aggregates from uninjured control mice reached very low minimum surface tensions of 0.7 ± 0.03 mN/m on the pulsating bubble (Figure 5).

Alteration of large surfactant aggregate phospholipid composition at 24 h post-LC

At 24 h, large aggregates had significant abnormalities in phospholipid composition (Table 3, Figure 6). In particular, large aggregates exhibited significantly decreased levels of phosphatidylcholine (PC) and significantly increased levels of lyso-PC compared to control ($p < 0.001$). These changes correlated with an increased level of PLA₂ in BAL, as described below. In addition to altered PC and lyso-PC levels, surfactant aggregates at 24 h also had minor increases in cell-associated phospholipids (sphingomyelin and phosphatidylethanolamine) relative to control (Table 3, Figure 5).

Increased concentration of phospholipase A₂ (PLA₂) in BAL from LC mice at 24 h post-contusion

Because PLA₂ biochemically degrades PC to lyso-PC, we specifically measured levels of this lytic enzyme in BAL from mice at 24 h post-LC (see Methods). Levels of secretory PLA₂ in BAL at this injury time point were significantly elevated compared to uninjured controls (0.60 ± 0.04 Unit/ml vs. 0.413 ± 0.16 Unit/ml in uninjured controls, $p < 0.001$, Figure 6).

DISCUSSION

Results in this manuscript document the presence of substantial surfactant dysfunction in acute unilateral LC injury in mice. No changes in surfactant protein gene expression were found by Affymetrix analysis over 72 h following the initial contusion insult (Table 1). However, significant abnormalities in large surfactant aggregates were present in association with permeability injury and inflammatory PLA₂ activity. The percent content of large surfactant aggregates in BAL was significantly depleted at all post-contusion times studied (5, 24, 48 and 72 h, Figure 2). Concurrently, mice exhibited permeability injury with significantly increased BAL levels of total protein and albumin (Table 2), and pulmonary P-V mechanics were also compromised (Figures 3, 4). Surfactant deficits were examined in further detail at 24 h post-contusion, a time when permeability injury and mechanical abnormalities were most prominent. Activity of secretory PLA₂ was found to be significantly elevated at 24 h post-contusion (Figure 6), and large surfactant aggregates exhibited compositional changes (increased lyso-PC and decreased PC) as a consequence of increased PLA₂ activity. These changes were accompanied by significant decreases in large surfactant aggregate surface activity studied at physiologically-relevant conditions on the pulsating bubble surfactometer (Figure 5). These findings of severe surfactant dysfunction in mice with acute unilateral LC extend prior work by our group showing surfactant abnormalities in rats with bilateral LC¹³. The presence of surfactant dysfunction in unilateral and bilateral closed-chest models of LC involving two animal species and two injury induction methods suggests that it is an important contributor to the pathophysiology of pulmonary contusion. The added demonstration that PLA₂ is increased in LC injury in mice adds further mechanistic understanding.

These impairments in lung surfactant activity and reductions in the content or composition of active large surfactant aggregates, increase activity of PLA₂ and decreased surface tension have been reported in BAL, edema fluid, or tracheal aspirates from patients with ALI/ARDS or other diseases involving acute pulmonary injury^{27–34}. To our knowledge this is the first report of these surfactant derangements for *in-vivo* animal models of acute inflammatory lung injury following LC. Lung surfactant exists in the alveolar hypophase in a size-distributed microstructure of aggregates, the largest of which normally have the greatest surface activity and the highest apoprotein content^{35–37}. Total BAL phospholipid is often unchanged or even increased in amount in animal models of ALI/ARDS, but the percentage of large surfactant aggregates is frequently reduced and the activity of remaining aggregates impaired^{37–40}. Potential mechanistic contributions to large surfactant aggregate changes include biophysical interactions with injury-induced substances in the alveoli, alterations in intra-alveolar aggregate processing, and changes in surfactant reuptake, metabolism or recycling in type II cells^{37–40}. The depletion of large surfactant aggregates found here in mice with unilateral LC (Figure 2), and reported in our prior work in rats with bilateral LC, is likely to be multifactorial and requires further study.

In terms of mechanisms contributing to changes in surfactant composition and properties by LC injury, specific interpretations are possible. Chemical degradation of lung surfactant PC by PLA₂ in mice with LC injury not only removes active components of lung surfactant, but

also produces lyso-PC as a byproduct^{34, 41, 42} (Table 3). Lyso-phospholipids, as well as other fluid cell membrane lipids and free fatty acids, are known biophysical inhibitors of surfactant activity. These substances act in part by mixing directly into the lung surfactant film and compromising its ability to reach low surface tension during dynamic compression^{18, 43–46}. In addition, plasma proteins such as albumin present in the alveoli of LC mice as the result of permeability injury (Table 2) are also potent known inhibitors of surfactant activity^{11, 17, 18, 44, 47, 48}. Mechanistically, albumin and other large blood proteins impair surface activity primarily by competitive adsorption that reduces the entry of active surfactant components into the alveolar air-water interface^{45, 47}. Plasma proteins, like lyso-PC, can also directly associate with and incorporate into large surfactant aggregates to impair their intrinsic surface activity^{13, 40}.

Lyso-PC has the potential to play multifactorial roles in the pathogenesis of acute lung injury (Figure 1). In addition to inhibiting surfactant activity as described above, it damages type I cells⁴⁹, exhibits chemotactic activity toward lymphoid and mono-nuclear cells, and induces the expression of vascular adhesion molecules in endothelial cells⁴². A causal relationship between secretory PLA₂ and early surfactant degradation has been demonstrated in LPS-challenged guinea pigs⁴². Although this enzyme was shown to be present in increased concentration levels in LC-injured mice, specific mechanisms for its origin, production and secretion were not studied and remain to be elucidated.

A potential therapeutic significance for the surfactant dysfunction findings here is that they provide a conceptual rationale for exogenous surfactant replacement interventions in LC injury. The presence of phospholipase-induced pathology in mice with LC also has added direct implications for the potential effectiveness such therapy. Native lung surfactant and current clinical exogenous surfactants all contain predominant glycerol-phospholipid constituents that can be degraded by phospholipases. Synthetic exogenous surfactants designed to have molecular structural resistance to phospholipase activity may thus be of particular interest for future therapeutic use in LC. Our prior work has developed several phospholipase-resistant lipids with high surface activity and documented their activity when combined with surfactant peptides in synthetic and semi-synthetic exogenous surfactants (e.g.,^{16, 50–54}). One example of such resistant lipids is DEPN-8, a highly-active C16:0 diether phosphonolipid analog of the major lung surfactant phospholipids dipalmitoyl-PC (DPPC)^{16, 50, 54}. DEPN-8 + 1.5% purified bovine SP-B/C has extremely high surface activity that equals CLSE (the substance of Infasurf®) in the absence of inhibitors, and exceeds its activity in the presence of lyso-PC and phospholipase A₂ (PLA₂)¹⁶. DEPN-8 + 1.5% SP-B/C (DEPN-8/SP) is fully resistant to inhibition by PLA₂, and fully restores excised lung mechanics to normal, whereas CLSE is degraded and loses pulmonary activity when exposed to this enzyme^{16, 50}. DEPN-8/SP also has greater activity in restoring P-V mechanics in surfactant-deficient excised rat lungs in the presence of lyso-PC compared to CLSE^{16, 50}. We are currently exploring the use of DEPN-8 synthetic surfactants in the mouse model of unilateral LC. Alternatively, the use of PLA₂ inhibitors could be explored in our current model. However, there may be potential practical difficulties with such studies. In particular PLA₂ inhibitors have a broad range of lysosomal and other biological effects that are not specific for surfactant metabolism, thus confounding any meaningful surfactant-specific mechanistic conclusion^{16, 51, 55–58}.

Pulmonary contusion remains a clinically prevalent condition, and a common risk factor for the development of ALI/ARDS that carries significant morbidity and mortality. There remains an urgent need to develop improved therapeutic strategies to mitigate LC injury. Based on the evidence presented here, surfactant repletion particularly with phospholipase-resistant artificial surfactant may be a promising alternative to other treatment strategies.

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Abbreviations

LC	Lung Contusion
BAL	Bronchial Alveolar Lavage
PV	Pressure-Volume curves
PLA₂	Phospholipase A ₂
PC	Phosphatidylcholine
ALI	Acute Lung Injury
ARDS	Acute Respiratory Distress Syndrome
PMN	Polymorphonuclear Cells
SP	Surfactant Protein
PCR	polymerase chain reaction
ANOVA	Analysis of Variance

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FIGURE 1. Potential mechanism of Lung Contusion (LC) induced alteration on surfactant composition and activity

After lung contusion, increased activation of Phospholipase A₂ (PLA₂) will degrade surfactant phospholipids such as Phosphatidylcholine into Lyso-phosphatidylcholine, modifying the qualities of surfactant and directly increasing inflammation. Additionally the loss of large aggregates due to interactions with plasma proteins coming from permeability injury and due to increased activity of PLA₂ will diminish surfactant tension-reducing properties having downstream effects in lung mechanics (i.e reducing compliance).

FIGURE 2. Large surfactant aggregate content in bronchoalveolar lavage (BAL) during evolving lung contusion

Large aggregates were centrifuged at $12,000 \times g$ from cell-free BAL from uninjured control mice and LC mice at 5, 24, 48, and 72 h post-contusion and subsequently quantitated by phosphate assay as a percentage of total lavage phospholipid. Surfactant aggregates were found to be depleted most prominently early in LC injury (5 and 24 h), but remained depleted below control levels throughout the 72 h time period studied. + $p < 0.05$ compared to uninjured control; * $p < 0.001$ or less compared to uninjured control; # $p < 0.01$ or less compared to 24 h injury. Data are Mean $\pm$ S.E.M. with N= 5–9 except for injured mice at 5 h (N=2).

FIGURE 3. Pressure-volume (P-V) curves in evolving lung contusion injury in mice

Pressure-volume curves were generated with the PVrV force oscillation maneuver using a SCIREQ Flexivent® mice ventilator. Compliance was diminished in all post-injury time points compared to uninjured controls, with the greatest decrease in P-V compliance seen at 24 h post-injury. Curves were generated with over 4000 data points and provide a visual for the overall observation of these curves, similar to observations in the currently used mechanical ventilators. For detailed statistical analysis and interpretations from the PV curves, please refer to Figure 3. Tick marks represent major integral of pressures during inflation and deflation.

FIGURE 4. Maximal lung volumes, hysteresis and respiratory mechanics in lung contusion
A. Volume obtained using a standard inspiratory pressure of 30 cm H₂O (TLC-30). TLC-30 was significantly diminished at 24, 48 and 72 h post-injury compared to uninjured control;
B. Maximal volume for a single ramped breath (V_{end}) indicative of Quasi-static compliance, showing significantly diminished end-volume at 30 cm H₂O at post-injury times of 24, 48, and 72 h; **C.** Total resistance of the respiratory system, with resistance increased most prominently at 24 h post-injury compared to control or 48 and 72 h of injury.
D. Area under the Curve (AUC, the area enclosed by the inspiratory and expiratory loops of curves in Figure 3), showing decreased hysteresis post-contusion in consonance with

decreased compliance seen with other maneuvers. One way ANOVA vs. control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; One way ANOVA vs. 24 h injured animals # $p < 0.05$.

Figure 5. Dynamic surface tension lowering of resuspended large surfactant aggregates from mice with LC injury at 24 h post-injury

Centrifuged large surfactant aggregates were resuspended in 0.15 M NaCl + 2 mM CaCl₂ at a uniform phospholipid concentration of 2.5 mg/ml and examined for surface activity on a pulsating bubble surfactometer (20 cycles/min, 37°C, 50% area compression). Surface tension at minimum bubble radius (minimum surface tension) is shown as a function of time of bubble pulsation. Surface activity is reduced at all times of pulsation for mice at 24 h post-injury compared to uninjured controls. Data are Mean $\pm$ S.E.M. for n = 4.

Figure 6. Large surfactant aggregate (LG) content, phospholipase A₂ (PLA₂) concentration in cell-free BAL, and lyso-PC (LPC) content in large surfactant aggregates from uninjured and LC-injured mice

Large surfactant aggregates were obtained by centrifugation of cell-free BAL at $12,000 \times g$ for 30 min, and the aggregate content was determined as a percentage of total BAL phospholipid content based on phosphate assay. Phospholipase A₂ concentration in cell-free BAL was measured with the PLAThe EnzChek® Phospholipase A₂ Assay Kit (Methods). Lyso-PC content in large surfactant aggregates was determined by thin layer chromatography. Data are Mean $\pm$ S.E.M. with $n = 4$ for LG and LPC, and $n = 8$ for PLA₂.

*: $p < 0.0001$ compared with uninjured mice; #: $p < 0.001$ compared with uninjured mice; +: $p < 0.01$ compared with uninjured mice.

Table 1
Whole lung genomic analyses performed on LC and uninjured control mice using Affymetrix microarray

Affymetrix cDNA data are shown for all probes related to the four surfactant proteins (SP) – A, B, C, and D contained in the genechip. Tabulated values are individual UniGene readings of different probe sets for 3 control mice and for 3 mice at each of three injury time points (5, 24, 72 hr post-contusion). No significant differences in cDNA were found between data sets for uninjured control mice and LC-injured mice at any of the injury time points studied. See text for details.

Surf-Associated Protein	Entrez Gene	UniGene	uninjured control	uninjured control	uninjured control	LC 5h	LC 5h	LC 24h	LC 24h	LC 24h	LC 72h	LC 72h
A1-probe set #1	20387	Mm.46062	13.29	13.19	13.27	13.15	13.16	13.06	13.26	13.21	13.24	13.45
A1-probe set #2	20387	Mm.46062	12.00	11.99	11.98	12.02	11.82	11.85	11.97	11.87	12.02	12.50
B	20388	Mm.46033	12.66	12.52	12.70	12.62	12.67	12.38	12.61	12.83	12.66	13.03
C-probe set #1	20389	Mm.24040	5.11	4.97	5.56	6.78	4.26	4.88	4.60	4.48	5.81	4.70
C-probe set #2	20389	Mm.24040	13.92	14.02	14.07	14.12	13.85	14.04	13.97	13.88	14.17	13.89
D	20390	Mm.1321	13.13	13.07	13.08	13.08	13.17	13.04	13.10	13.37	13.23	13.67
												13.29
												13.45

Table 2

Increased protein content of whole cell-free BAL from mice at various times after LC injury.

<i>Group</i>	Phospholipid (mg/ml)	Protein (µg/ml)	Protein/PL %	Albumin (ELISA) (µg/ml)
Uninjured control	0.28±0.03	179 ± 9	64 ± 2	98 ± 5
LC (5h)	0.23± 0.01	791 ± 58 *	345 ± 16 *	1174 ± 206 *
LC (24h)	0.26± 0.02	956 ± 80 *	411 ± 49 *	1047 ± 204 *
LC (48 h)	0.24± 0.01	870 ± 213 *	353 ± 81 *	731 ± 163 *
LC (72 h)	0.25±0.01	818 ± 152 *	333 ± 66 *	699 ± 138 *

Data are Mean ± S.E.M with N= 12 for uninjured mice and N=4–18 for LC mice at 5, 24, 48 and 72 h post-insult. See Methods for details on total protein and albumin quantification.

* *p* value <0.001 compared to uninjured controls.

Table 3

Phospholipid class compositions in large surfactant aggregates centrifuged from cell-free BAL from mice at various times after LC injury.

Phospholipid Class	Uninjured Control	Injured (24-h)
Lyso-PC	1.45 ± 0.30	5.52 ± 1.19 [#]
Sphingomyelin (SPH)	2.35 ± 0.16	3.45 ± 0.23
Phosphatidylcholine (PC)	84.99 ± 0.82	78.85 ± 0.76 [#]
Phosphatidylinositol (PI)	4.04 ± 0.57	3.92 ± 0.36
Phosphatidylethanolamine (PE)	1.92 ± 0.25	2.63 ± 0.24
Phosphatidylglycerol (PG)	4.65 ± 0.68	4.45 ± 0.61
Residual	0.60 ± 0.22	1.19 ± 0.39

Large surfactant aggregates were pelleted from cell-free BAL by centrifugation at 12,000 × g for 30 min, and phospholipid class compositions were defined by thin layer chromatography with a solvent system of chloroform:methanol:2-propanol:triethylamine:water (30:9:25:25:7 by volume) (Methods). Phospholipid classes are expressed as weight percent relative to large aggregate total phospholipid content. Data are Mean ± SEM with n = 4 in each group.

[#] $p < 0.001$ or less compared to uninjured control evaluated by Student's t-test with Welch's correction.