Acute lung injury and lung transplantation influence in vitro subtype conversion of pulmonary surfactant

GAYATRI MAITRA, KEVIN INCHLEY, RICHARD J. NOVICK, RUUD A. W. VELDHUIZEZ, JAMES F. LEWIS, AND FRED POSSMAYER

1Medical Research Council Group in Fetal and Neonatal Health and Development, Departments of Biochemistry and Obstetrics and Gynaecology, University of Western Ontario, London N6A 5A5; 2Transplant-Immunology Group, Robarts Research Institute, and Division of Cardiovascular-Thoracic Surgery, London Health Sciences Centre-University Campus, London N6A 5A5; and 3Division of Respiratory Medicine, Department of Physiology, and Lawson Health Research Institute, St. Joseph’s Health Centre, London, Ontario, Canada N6A 4V2

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PULMONARY SURFACTANT is a lipid-protein complex that stabilizes the lung by reducing the surface tension at the alveolar air-liquid interface. Surfactant recovered by bronchoalveolar lavage consists of subfractions differing in morphological appearance and buoyant density (5, 30). The heavy subtype or surfactant large aggregate (LA) fraction, which contains lamellar bodies, tubular myelin, and large vesicles, is rich in the surfactant proteins [surfactant protein A (SP-A), SP-B, and SP-C] and is highly surface active. The light subtype or small aggregate (SA) subfraction, consisting of small vesicles, contains less surfactant protein and has poor surface activity (24, 28, 31).

The metabolic relation between the LA and SA subfractions has been studied in vivo by pulse-chase experiments (1, 17). These studies indicated that the larger, heavier subfractions (LA) are the metabolic precursors of the smaller, lighter surfactant subtypes (SA). This in vivo alveolar metabolism can be mimicked in vitro by the method of surface area cycling, as described by Gross and Narine (6). In this procedure, a tube containing resuspended large surfactant aggregates is rotated end over end, thereby changing the surface area, as occurs during breathing. The LA and SA can then be separated by differential centrifugation. SA formed during this process are morphologically similar to those obtained from lung lavage (6, 10, 29). Furthermore, SA formed via surface area cycling, like SA fractions obtained from lung lavage, do not reduce surface tension to low values.

Gross and Schultz (7) used surface area cycling to demonstrate that several serine protease inhibitors inhibit the conversion of the heavy subtype to SA. Pretreatment of purified surfactant LA with 10 mM diisopropylfluorophosphate (DFP) inhibited its ability to be converted to SA on subsequent cycling (4, 8). Purified surfactant was subsequently found to contain a single DFP-reactive protein of Mr 70–75. This DFP-binding protein, namely convertase, has been proposed by Gross (5) to be responsible for LA-to-SA conversion.
Recent studies (2, 14) have indicated that convertase is a serine-dependent carboxylesterase. Other studies (11, 28) have suggested that the target for convertase appears to be SP-B. However, it appears likely in altered states of lung function, e.g., in lung injury, that other enzymes/proteases besides convertase may also be involved (15, 18, 22).

A previous investigation (23) by our group using a canine single lung transplantation model suggested a potential role for pulmonary surfactant in lung transplant. These studies demonstrated that treatment with bovine lipid extract surfactant (BLES) ameliorated many of the anticipated detrimental effects associated with prolonged ischemia and transplantation. These promising observations led to examination of the possibility that BLES treatment of the donor might protect the lung against mild lung injuries such as those produced by hyperventilation. Maintaining brain-damaged patients on respirators with high oxygen tensions and vigorous mechanical ventilation can compromise the clinical usefulness of their lungs. The model used involved hyperventilation for 8 h, followed by 16 h of ischemia, single left lung transplantation, and estimation of gaseous exchange during 6-h perfusion. The results demonstrated that pretreatment with BLES minimized lung injury during hyperventilation, resulting in a marked increase in oxygenation during reperfusion compared with the absence of donor treatment (19). Similar overall results were obtained in studies where BLES administration was delayed until hour 4 of hyperventilation.

We hypothesized that the previously observed beneficial effects of BLES treatment might extend to surfactant subtype conversion. Therefore, studies were conducted to examine the effects of mild lung injury due to hyperventilation plus transplant and surfactant therapy on surfactant subtype conversion in vitro. Most of the samples of LA used were obtained from the experimental animals referred to above (19, 23). It was observed that LA from BLES-treated animals converted to SA more slowly than LA from nontreated animals but more rapidly than LA from normal lungs. Unexpectedly, calculations based on radioactive dipalmitoylphosphatidylcholine (DPPC) in the administered surfactant suggested that the endogenous component of the LA from BLES-treated dogs converted more rapidly than the exogenous component. These studies indicate surfactant treatment has an effect on surfactant subtype conversion but that administered surfactant does not equilibrate completely with the endogenous pool.

MATERIALS AND METHODS

Experimental model of lung injury and transplant. The canine model used has been previously described (19, 21). A brief overview of the model is provided here. Conditioned dogs were divided into two weight-matched groups: donor and recipient (Fig. 1). The donors were subjected to mild injury by hyperventilation at 45 ml/kg for 8 h (fraction of inspired oxygen = 1, positive end-expiratory pressure = 5 cmH2O, tidal volume = 45 ml/kg, rate = 16 breaths/min). Random donor dogs were treated with 100 mg/kg of BLES (100 mg/kg) either when hyperventilation was initiated (BLES instilled), or after 4 h (BLES delayed instilled). After hyperventilation, the donor lungs were excised and stored for another 16–18 h to induce ischemic injury. The donor right lung was then lavaged with 0.9% saline. The donor left lung was transplanted into the recipient, and the recipient was ventilated at normal (20 ml/kg) tidal volume for another 6 h. The transplanted lung (i.e., the treated, hyperventilated, and stored donor left lung) and the recipient’s native right lung were then lavaged. The unjured left lung, removed from the recipient before transplantation, was also lavaged and served as a control normal lung. LA were prepared from the lavage of each type of lung.

Exogenous surfactant administration. The surfactant preparation used was BLES (BLES Biochemicals, London, Ontario, Canada) supplied at a concentration of 25 mg of phospholipid/ml. Radiolabeled surfactant was prepared as follows: initially, 0.2 ml BLES was extracted into chloroform according to the method of Bligh and Dyer (3). The upper aqueous phase was discarded, and 10 ml of 1,2-dipalmitoyl-L-3-phosphatidyl-[N-methyl-3H] choline (40–85  Ci/mol; American Life Science, Oakville, Ontario, Canada) was added to the lower chloroform phase. The mixture was then dried under a stream of nitrogen. After the addition of 0.5 ml of 0.9% NaCl and a few glass beads to the dried material, the tube was capped and vortexed at maximum speed for 5 min. The resuspended surfactant was then removed and was added to a tube containing the bulk of the BLES. This tube was inverted several times and gently vortexed to obtain uniform mixing of the radioactive material. A treatment dose of 100 mg/kg of 3H-labeled BLES was instilled into the treatment groups at the appropriate times.

Experimental groups. Animals were randomly assigned to one of the following groups: 1) treatment of donors with instilled BLES immediately after the onset of high volume ventilation (instilled group, n = 5); 2) treatment by BLES instillation at hour 4 of ventilation (delayed instilled group, n = 5); and 3) no treatment to donor lungs (nontreated group, n = 6). Gas exchange studies on some of the dogs in the instilled group have been reported previously (19).

Preparation of LA from lung lavage. Lungs were lavaged with 600–1,000 ml of saline three times. Lavage was centrifuged at 150 g for 10 min at 4°C to remove cells and cellular debris. A LA pellet was obtained by centrifugation of the 150-g supernatant at 10,000 g for 10 min at 4°C. The pellet (LA) was resuspended in saline (0.9%) at a concentration of 10–20 mg of phospholipid/ml and frozen in aliquots at −20°C. Aliquots of the supernatant, which contains SA, were also stored at −20°C.

Surface area cycling. LA were thawed and resuspended in conversion buffer (0.15 M NaCl, 10 mM Tris, 1 mM CaCl2, 1 mM MgCl2, and 0.1 mM EDTA; pH 7.4) at a concentration of 0.25 mg of phospholipid/ml. Aliquots (2 ml) were placed in polystyrene tubes (Falcon 2058), capped, and attached to a rotator (Roto-torque rotator). The tubes were cycled at 40 rev/min at 37°C so that the surface area changed from 1.1 to 9.0 cm2 twice each cycle (6, 28). Samples were cycled for 30, 60, 180, and 360 min. Identical noncycled control samples were kept at 37°C for 360 min. Some experiments utilized the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). PMSF was dissolved in ethanol (500 mM) and added to samples at different concentrations ranging from 0.25 to 10 mg/mL before cycling. An equal amount of ethanol was added to samples not receiving PMSF. In all studies except those with PMSF, cycling was conducted within 2 wk after transplanta-
tion. Control studies have demonstrated similar conversion rates after 4–12 wk of storage at −20°C.

Separation of LA and SA. After cycling, LA and SA were separated by centrifugation at 40,000 g for 20 min at 4°C. Total phospholipid in the LA pellet and in the SA remaining in the supernatant was determined by lipid extraction (3) and phosphorous analysis (25). The treated lungs contained an exogenous surfactant (BLES administered) component and an endogenous surfactant component. SA formation from total LA was based on phosphorous assay. Conversion of the exogenous component was based on radioactive counts. The endogenous component conversion was calculated by subtracting the phosphorous content of the exogenous component from the total phospholipid determined by phosphorous assay. Protein contents were analyzed as described by Lowry et al. (16) with the addition of 0.1% SDS to dissolve lipids.

Data analysis. Results are expressed as means ± SE. Differences in means between conversion of LA from different lung types were tested by ANOVA followed by Bonferroni post hoc analysis. Differences corresponding to \( P < 0.05 \) were considered significant.

RESULTS

The present study investigated the effects of mild acute lung injury induced through hyperventilation, ischemia, and transplantation with the integrity of recovered surfactant LA, as assessed by in vitro surface cycling. Weight-matched dogs were subjected to hyperventilation (45 ml/kg) for 8 h (19). Bronchoalveolar lavage samples obtained after an 8-h hyperventilation period exhibited increased SA-to-LA ratios and increased protein and leukocyte levels indicative of acute lung injury (data not shown). Instillation of BLES before hyperventilation or at hour 4 partially reversed the protein and leukocyte increases and abolished the decreases in gaseous exchange (19). This suggests that BLES treatment can moderate hyperventilation-induced mild lung injury. SA-to-LA ratios in recovered lavage were also reduced, but this effect could result from the presence of BLES, which is primarily in the LA state (19).

Relative physical integrity of the surfactant LA recovered from transplant lungs from BLES instilled and nontreated donors was examined by subjecting LA to in vitro surface cycling and measuring the formation of surfactant SA using phospholipid phosphorous estimation. LA from transplanted lungs (Transplant-LA) converted rapidly during the initial 0.5 h and plateaued between 1 and 3 h (Fig. 2). LA from BLES instilled transplanted lungs also exhibited rapid initial conversion but to a significantly lower extent than Transplant-LA from nontreated animals. Conversion of LA from BLES delayed instilled dogs overlapped with conversion of LA from BLES instilled animals so the
results (BLES treated) were pooled. In all cases, formation of SA was significantly greater than conversion of LA from normal lungs at all time points examined. Furthermore, LA from normal lungs also showed a more gradual increase in SA formation such that conversion did not clearly plateau during the 6-h cycling period.

At least potentially, the slower, decreased formation of SA with LA from BLES-treated lungs could be due to a slower conversion of the treatment surfactant, BLES. Figure 3 compares conversion of BLES itself with the conversion of the endogenous and exogenous surfactant components of LA recovered from BLES-treated lungs (based on [3H]DPPC, see below). Such a dilution effect would require that the treatment BLES converts at a significantly slower rate than the LA obtained from the treated transplanted animals. However, BLES conversion was slightly more rapid than conversion of Transplant-LA from treated lungs at each time point examined (compare transplant treated, Fig. 2, with BLES, Fig. 3). Although these differences were not significant, it appears unlikely that the BLES present in Transplant-LA from treated lungs could account for the observed lower conversion.

Calculations based on the specific activity of [3H]DPPC in BLES allowed comparison of the rates of conversion of the endogenous and exogenous surfactant components of LA recovered from BLES-treated lungs. It should be appreciated that these calculations are based on the premise that conversion of the exogenous surfactant can be represented by transfer of [3H]DPPC from LA to SA. With these transplanted lungs, the recovered LA possessed similar endogenous (51.7 ± 2.1%, n = 10) and exogenous (48.3 ± 2.1%, n = 10) phospholipid pools. Surprisingly, SA formation from endogenous surfactant was greater than SA formation from the exogenous component (Fig. 3). These results suggest that the exogenous instilled BLES did not equilibrate fully with the endogenous surfactant despite being in the lungs for 25–30 h.

Conversion of LA from the donor’s stored right lung (Donor Right-LA) and from the recipient’s own native right lung (Native Right-LA) were also measured by in vitro cycling (Fig. 4). Conversion of Donor Right-LA and Native Right-LA from nontreated dogs was significantly greater than conversion of LA from corresponding lungs in the BLES-treated group. Conversion of Donor Right-LA and Native Right-LA from the nontreated and treated groups was also greater than that
for LA from the normal lungs. These results suggested that BLES treatment ameliorated, but did not abolish, the effects of acute lung injury and reperfusion.

Figure 5 compares surfactant subtype conversion of exogenous (A and C) and endogenous (B and D) surfactant components from donor right and native right lungs with the conversion of Transplant-LA. Total Transplant-LA (from BLES-treated lungs) converted more rapidly than Donor Right-LA or Native Right-LA, but the differences were not significant (data not shown). Figure 5A compares actual SA formation in micrograms of phospholipid from the exogenous LA component of the three lung surfactants. Not surprisingly, very little absolute conversion was observed with the exogenous component recovered from the recipient’s native lung. In contrast, the endogenous component from Native Right-LA converted to a significantly greater extent than the endogenous component from Transplant-LA or Donor Right-LA (Fig. 5B). The more rapid conversion of endogenous Native Right-LA is likely related to the small proportion of exogenous surfactant in the recipient’s native lung, which accounted for 6.08 ± 1.5% of the total Native Right-LA. However, the more rapid conversion could also be related to the effects of transplantation, because total Native Right-LA converted more rapidly than LA from normal organs (Fig. 4).

Donor Right-LA contained 44.3 ± 2.4% exogenous surfactant, a level slightly lower than the 51.7 ± 2.1% in Transplant-LA. The effects of the different pool sizes were minimized by expressing the relative conversion of each component as a percentage of its amount in the cycled sample (Fig. 5, C and D). Interestingly, when plotted as a percentage of total (Fig. 5C), the exogenous components from all three LA surfactants converted in an identical manner. Comparison of the relative conversion of the endogenous components (Fig. 5D) revealed Transplant-LA converted significantly more rapidly than the endogenous components of Native Right-LA or Donor Right-LA except at the hour 6. The endogenous components from Transplant-LA, Donor Right-LA, and Native Right-LA converted significantly more rapidly than their corresponding exogenous components except for Donor Right-LA and Native Right-LA at 1 and 6 h. This observation suggests that the exogenous components of the transplanted, stored, and recipient’s native lungs did not equilibrate completely with the endogenous surfactant.

In an attempt to clarify the nature of the altered integrity of Transplant-LA from the nontreated group, the effect of PMSF, an active serine-dependent enzyme inhibitor, on in vitro conversion of Transplant-LA and LA from normal lungs was examined (Fig. 6). Low PMSF had little effect on conversion, but ~60% inhibition was observed at high inhibitor levels. A distinct difference in the inhibitory profile was observed with Transplant-LA being significantly more susceptible at lower PMSF concentration.

Subtype conversion can be accelerated by serum proteins. Figure 7 shows LA from experimentally ma-

![Fig. 5. Comparison of the absolute and relative conversion of the exogenous (BLES derived) and endogenous surfactant components from transplanted (n = 10), donor right (n = 10), and native right canine lungs (n = 10). A: SA formation from exogenous components; B: SA formation from endogenous components; C: relative (% of itself) conversion of exogenous components; D: relative conversion of endogenous components. P < 0.05, P < 0.01, and P < 0.001, significantly different from normal lungs; P < 0.05, P < 0.01, and P < 0.001, significantly different from LA from transplanted lungs; *P < 0.05, **P < 0.01, and ***P < 0.001, significantly different from LA from normal lungs.](http://ajplung.physiology.org/).
n manipulated lungs possessed high total protein levels, with no significant difference between groups. Much lower total protein remained associated with LA from control normal lungs.

DISCUSSION

In vitro surface area cycling was used to examine the properties of surfactants recovered from BLES-treated and nontreated hyperventilated, stored, and transplanted reperfused lungs compared with surfactant from the recipients’ normal left lungs (Fig. 1). Transplant-LA from nontreated animals converted rapidly and generated a high level of SA. Transplant-LA from BLES-treated lavage converted more slowly than Transplant-LA from nontreated animals but significantly faster than LA from normal lungs. Thus BLES instillation reversed part, but not all, of the effect of hyperventilation, ischemia, and reperfusion on LA integrity during cycling. These observations are consistent with improved lung function due to BLES treatment under these conditions (19).

Theoretically, the increased stability of LA from BLES-treated compared with untreated dogs could be due to the physical properties of instilled BLES. However, BLES itself converted rapidly (Fig. 3) with a rate slightly, although not significantly, higher than Transplant-LA from treated lungs. Donor Right-LA and Native Right-LA converted at a slightly lower (nonsignificant) rate than Transplant-LA. Consequently, it appears unlikely that the properties of BLES alone could explain the lower conversion observed with LA recovered after BLES treatment.

Surprisingly, calculations based on \(^{3}H\)DPPC indicated that conversion of the endogenous component of Transplant-LA from treated lungs was greater than that of the exogenous component. This indicated that exogenous BLES did not equilibrate with the endogenous pool despite having been in the lungs during 8 or 4 h of hyperventilation, 17 h of cold storage, and 6 h of reperfusion. To our knowledge, a difference in the physical or metabolic processing of endogenous and exogenous surfactants has not been previously observed. Other investigators (12, 13) have concluded or assumed that once in the lung for a short period, DPPC and other surfactant components behave similarly to endogenous components. It should be noted that most of the previous work on subtype conversion has been conducted with neonatal or normal animals, not with acute lung injury or transplantation. For a number of reasons, mixing of exogenous surfactant with endogenous alveolar and tissue pools cannot occur instantaneously (12). However, the surfactant turnover times of 8–13 h estimated for normal rabbits and sheep are in the same range as the total nonischemic incubation periods employed in the present experiments. Hence, equilibration within the alveolar environment would be expected. Furthermore, a considerable amount of the administered \(^{3}H\)DPPC should have been taken up and either metabolized or resecreted by type II cells.

The precise nature of the apparent derangement in surfactant processing is not known, but these observations imply the presence of a nonintegrated pool of exogenous surfactant within the lung during a considerable time frame. In addition, the slower in vitro conversion of \(^{3}H\)DPPC in recovered LA compared with conversion of LA from corresponding normal lungs; \(+ P < 0.05\), significantly different compared with the corresponding normal lung sample.

Fig. 6. Effect of phenylmethylsulfonyl fluoride (PMSF) on conversion of surfactant LA from transplanted and normal lungs. The degree of inhibition is plotted. Results are expressed as means ± SE for 3 independent observations. \(\ast P < 0.05\), significantly different compared with conversion of LA from corresponding normal lungs; \(+ P < 0.06\), significantly different compared with the corresponding normal lung sample.

Fig. 7. Proteins associated with LA recovered from surfactant from transplanted, donor right, native right, and normal lungs. Protein levels (in \(\mu g\) protein/mg phospholipid) in LA isolated from experimental lungs were determined by the assay of Lowry et al (16). Total protein in LA from normal lungs was included for comparison [LA from transplanted lungs: nontreated \((n = 5)\), treated \((n = 5)\); LA from donor right lungs: nontreated \((n = 6)\), treated \((n = 5)\); LA from native right lungs: nontreated \((n = 6)\), treated \((n = 5)\); and LA from normal lungs \((n = 18)\).
ministered Survanta. These authors concluded that incorporation of endogenous surfactant apoproteins, particularly SP-A, was responsible for the improved activity (for a review, see Ref. 12). SP-A incorporation could explain the slower conversion of [3H]DPPC observed here. Whether components of exogenous surfactant other than DPPC also exhibit different conversion rates than their endogenous counterparts must still be investigated.

Donor Right-LA and Native Right-LA from non-treated animals converted at a slower rate than Transplant-LA but more rapidly and to a significantly greater extent than LA from normal lungs. Somewhat slower conversion was observed with Donor Right-LA and Native Right-LA from BLES instilled animals, but conversion remained significantly greater than that of LA from normal lungs. Transplant-LA from treated lungs converted more rapidly than Donor Right-LA or Native Right-LA and significantly faster than LA from normal lungs at all time points. Although the differences were not significant, these results are consistent with a gradation in injury between transplanted and donor right or native right lungs. This suggestion was reinforced by the observation that the endogenous component of Transplant-LA converted more rapidly than the endogenous components of Donor Right-LA or Native Right-LA (Fig. 5D). These results indicate that whereas hyperventilation, storage, and ischemia produce acute lung injury, further injury arises during the 6-h reperfusion period. Previous studies (19–21, 29) have also indicated that reperfusion augments the injury arising through ischemia.

Conversion of LA recovered from the recipient’s right lung was similar to the corresponding donor right lungs with both treatment and nontreatment groups. These results are consistent with previous observations showing increased permeability and depressed function of native right lungs after reperfusion (19, 23, 29). The basis of the detrimental effects on the recipient’s native lung are not understood, but we (23) have previously suggested that these could result from cytokines released by the reperfused transplanted lung and/or from volutrauma arising from reduced compliance of the transplanted lung. BLES treatment also led to significant improvement in lung function and subtype conversion with the native right lungs.

The mechanisms by which hyperventilation, ischemia, and transplant affect LA integrity must still be addressed. Studies (5, 27, 28, 30) with isolated LA and reconstitution studies have shown that the conversion of LA to SA requires rapid adsorption to the air-water interface (e.g., surfactant lipids that do not adsorb rapidly do not convert) and repeated alterations in surface area (e.g., LA in tubes positioned on the rotator so that they roll rather than tumble end over end do not convert). Studies by Gross and associates (4, 5, 14) have shown that inhibitors of serine-active enzymes such as DFP, α1-antitrypsin, and dihydrostatin markedly reduce in vitro conversion. Loss of SP-B from canine surfactant during in vitro conversion was originally attributed to protease activity but appears to result from dissociation of SP-B from surfactant lipids (11, 28). Convertase has recently been identified as a 70-kDa carboxyesterase (2, 14), but the manner by which the serine-dependent enzyme influences conversion is not known. SP-A contributes to LA integrity and protease activity on this collectin, e.g., by neutrophil elastase, could contribute to the observed effects of lung injury (5, 22, 30). Studies conducted using LA from the present series of animals revealed in vitro conversion of LA from transplanted or normal lungs is markedly reduced by the active serine-dependent enzyme inhibitor PMSF. Interestingly, conversion of Transplant-LA was more susceptible to inhibition by PMSF than conversion of normal LA. This is consistent with the possibility that the enhanced conversion of Transplant-LA is due to differences in the nature of the serine-dependent enzyme convertase, but other PMSF inhibitable proteases could be involved. Serum proteins have also been shown to affect in vitro conversion, although the mechanism is not clear (9, 26). Protein levels were elevated to about the same extent in LA isolated from all experimental lungs. Thus altered serum levels could account, at least in part, for the more rapid conversion relative to LA from normal lungs but could not explain differences in conversion observed between Transplant-LA and LA from the other experimental injured lungs.

In conclusion, it is evident from these and previous studies that BLES treatment greatly diminishes the deleterious effects of hyperventilation, ischemia, and reperfusion. BLES treatment resulted in lower SA-to-LA ratios in vivo and conversion rates in vitro. The reduced in vitro conversion rate could not be attributed to the physical properties of BLES, which, by itself, converts rapidly in vitro. Interestingly, [3H]DPPC from administrated BLES converted more slowly than the endogenous component in Transplant-LA. This finding indicates that the exogenous surfactant did not equilibrate with endogenous surfactant within the lung. The slow conversion rate further demonstrates that the exogenous surfactant was modified within the lung. The basis for the differences in in vitro processing of endogenous and exogenous surfactants is not known. The exogenous component from Transplant-LA converted more rapidly than those from Donor Right-LA and Native Right-LA. However, the exogenous component of LA recovered from all experimental lungs converted with identical kinetics. This would suggest that exogenous surfactant is not as susceptible to the degree of injury as the endogenous components. The similar relative conversions observed with Donor Right-LA and Native Right-LA from BLES-treated groups were lower than with LA from nontreated groups but higher than LA from normal lungs. Taken together, these studies suggest BLES modulates but does not abolish lung injury under these circumstances.

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