RESEARCH ARTICLE

The proteomic response is linked to regional lung volumes in ventilator-induced lung injury

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INTRODUCTION

Treatment of respiratory failure is complicated by the interaction between preexisting lung injury and mechanical ventilation (MV). As such, an individual patient’s outcome is a combination of the lung injury, resulting from the initial insult and the susceptibility of the lung to mechanical stretch. The implementation of protective ventilation strategies with low tidal volume has led to an improvement in outcomes; however, mortality remains unacceptably high (20, 32). Moreover, lower tidal volume ventilation strategies may heighten the risk of atelectasis resulting in further lung injury (17, 29).

The impact of ventilator-induced lung injury (VILI) is further complicated by the heterogeneous response of the lungs to MV, leading to coexistence of contrasting pathological processes (e.g., overdistension and atelectrauma) in different regions of the same lung (6, 7). An understanding of these heterogeneities will help clarify how mechanical forces during MV amplify lung injury and, thus, inform the design of optimized ventilation strategies to minimize such injury. Recently, in healthy mouse lungs, we showed that the magnitude of regional tidal stretch in response to MV was linked to the regional expression of inflammatory genes (37). In line with this, studies in sheep have shown that regional tidal strain in response to MV alters markers of metabolism (34). Intriguingly, in this sheep model and our mouse model, preexisting systemic inflammation modified the relationship between regional tidal strain/volume and inflammation (34, 36), which highlights the importance of considering both regional variation and the effect of prior lung injury when examining the effect of MV on the lung.

Several molecular and cellular mechanisms have been proposed to explain VILI, including the release of proinflammatory cytokines, production of reactive oxygen species, complement activation, and mechanotransduction (20, 25, 31). However, it remains to be determined how these signaling pathways respond to MV in the presence of prior injury and whether they are regulated in a spatial pattern. Recent studies have applied protein profiling to tissue samples to map regional expression of molecular pathways involved in VILI. Studies in healthy mice (8) have shown altered expression of coagulant pathways, while studies in the preterm lamb lung have revealed spatiotemporal patterns in the proteome (21) in response to MV. To our knowledge, however, no studies have examined the re-
Regional proteomic response in the adult lung to MV in the setting of prior lung injury.

Acid aspiration is a well-known cause of acute respiratory distress syndrome (ARDS), which leads to respiratory failure and a requirement for MV (24). The purpose of this study was to evaluate the effect of MV in the setting of acid-induced lung injury on the lung volume response, using a four-dimensional computed tomography (4DCT) lung imaging technology (37), and to link this with the regional proteomic response. We hypothesized that preexisting lung injury would alter the lung volume response to MV and that this would result in the dysregulation of multiple molecular pathways in a lung region-specific manner.

MATERIALS AND METHODS

Animal preparation and ventilation. All animal experiments were approved by the Monash University and University of Tasmania Animal Ethics Committees and conformed to the guidelines of the National Health and Medical Research Council (Australia). Adult female BALB/c mice were anesthetized, tracheotomized, and mounted upright in a custom-built holder on a rotating stage, as described previously (37). The animals were randomly assigned to four groups ($n = 7$ per group). The first two groups received intratracheal instillation with either 50 $\mu$L of 0.9% saline, or 0.1 M hydrochloric acid with pH 3.0 (Acid), followed by free-breathing for 30 min before euthanasia [Saline/free breathing (FB) and Acid/FB groups]. The second two groups were subjected to 2 h of MV 30 min after intratracheal saline (Saline/MV) or acid (Acid/MV). Ventilated mice were connected via the tracheal cannula to a small animal ventilator (AccuVent 200, Notting Hill Devices, Notting Hill, VIC, Australia) and ventilated at 225 breaths per minute, with a peak inspiratory pressure (PIP) of 12 cmH$_2$O, and a positive end-expiratory pressure (PEEP) of 2 cmH$_2$O. At the end of experiment, mice were euthanized by overdose with pentobarbital sodium (200 mg/kg), and the lung tissue was collected for proteomic analysis (described below).

Lung imaging. As described previously (37), 4DCT images were captured using a liquid-metal-jet X-ray source coupled with a high-speed detector (23). A three-dimensional velocimetry technique (9) was applied to the dynamic images to calculate regional tidal volume ($V_t$), while Hounsfield Units were used to determine the aeration fraction at end expiration to obtain end-expiratory volume (EEV). The whole lung was segmented into 10 regions (four right lobes and six left regions), and the images were processed to calculate the lung volumes in these regions (37). Total and regional $V_t$ and EEV were normalized by the variation in regional lung size at end expiration, calculated as specific $V_t$ ($sV_t$) = regional $V_t$/regional lung volume, specific end-expiratory volume ($s$EEV) = regional EEV/regional lung volume, and strain $= sV_t/s$EEV.

Tissue extraction and peptide sample preparation. Lung tissue was dissected into 10 regions corresponding to the image segmentation as described above (Supplementary Fig. S1 at https://doi.org/10.6084/m9.figshare.12616757; all supplemental figures may be found at this website). Lung proteins were extracted using T-PER tissue protein extraction reagent (Thermo Scientific, Rockford, IL), and total protein was quantified using a Bradford protein assay kit (Thermo Scientific), according to the manufacturer’s instructions.

Sample volumes corresponding to 50-$\mu$g aliquots were cleaned by precipitation in nine volumes of ethanol. Protein samples were then reduced using 10 mM dithiothreitol overnight at 4°C and alkylated using 50 mM iodoacetamide in the dark for 2 h. Proteins were digested with 2 $\mu$g mass spectrometry-grade trypsin/Lys-C mix (Promega, Madison, WI) on 500 $\mu$g Sera Beads (GE Healthcare, Chicago, IL), as recommended for the SP3 method (15). Peptide samples (1 $\mu$g) were analyzed by data-dependent mass spectrometry using an Ultimate 3000 RSLCnano and Q-Exactive HF (Thermo Scientific), as described in Supplementary Methods S1 (https://doi.org/10.6084/m9.figshare.12616763).

Database searching and criteria for protein identification. Data files were imported into MaxQuant version 1.6.5.0 (https://maxquant.org/) and MS/MS spectra were searched using the Andromeda search engine against the complete Mus musculus UniProt reference proteome. Default settings for protein identification by Orbitrap MS/MS were used, with the match-between-runs function enabled, including a maximum of two missed cleavages, mass error tolerances of 20 ppm, and then 4.5 ppm for initial and main peptide searches, respectively, 0.5 Da tolerance for fragment ions, and carbamidomethyl modification of cysteine and variable methionine oxidation. A false discovery rate (FDR) of 0.01 was used for both peptide-spectrum matches and protein identification. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD013485 (available at http://www.ebi.ac.uk/pride/archive/projects/PXD013485).

Determination of relative protein abundance and statistical analysis. We used MaxLFQ, the MaxQuant algorithm, for peptide intensity determination, and normalization (5), using pairwise comparison of unique and razor peptide intensities and a minimum ratio count of 2. The proteinGroups output files generated by MaxQuant analysis were processed as follows. The normalized label-free quantification (LFQ) intensity values, MS/MS counts and the numbers of razor and unique peptides for each of the identified proteins were imported into Perseus software version 1.5.031 (http://perseus-framework.org/). Protein groups identified either as potential contaminants (prefixed with CON_), identified by modified site only, by reverse database matching, or on the basis of a single matching peptide were removed. LFQ intensity values were then log$_{2}$-transformed and a filter applied to include only proteins detected in a minimum of 70% of the samples. Missing values were replaced with random intensity values for low-abundance proteins based on a normal distribution of protein abundances using default MaxQuant parameters. Mean LFQ values for each treatment group were compared using a two-sided regularized $t$-test with a stabilization ($s_0$) factor of 0.1 (30) and proteins with a permutation-based FDR ($q$ value) $< 5\%$ were considered to be significant.

Bioinformatics analysis. Functional category and Gene Ontology annotation were performed on differentially expressed proteins. The KEGG database was used to identify significantly enriched KEGG pathways. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database of physical and functional interactions was used to analyze the protein-protein interaction of selected proteins. A Benjamini-corrected $P < 0.05$ was used as the cut-off for significantly enriched biological processes.

Data analysis. Differences in regional lung volumes and protein expression between groups and regions were analyzed using two-way ANOVA with main effects, interactions, and repeated measures, as appropriate. Aligned rank transformation was applied for nonparametric data. A Holm-Sidak post hoc test was implemented when ANOVA indicated significance of the main treatment effect or interaction. Total lung volume indices were compared between groups using $t$ tests. SigmaPlot (v. 13, Systat Software, San Jose, CA) was used for statistical analyses. Principal component analysis (PCA) was conducted on proteomic data using SPSS (version 20.0; SPSS, Chicago, IL). Pearson correlation index was calculated to determine the association among different continuous variables. Data are presented as means (SD) or median (range). Differences were considered statistically significant if $P < 0.05$ ($P < 0.1$ for interaction terms).

RESULTS

Control lung volumes and proteomics at baseline. As expected, the spatial distribution of lung volumes was not uniform in the normal ventilated lung (Saline/MV group) (Fig. 1, A and E;
Supplemental Fig. S2, A and B). Regional sVt was substantially lower in the accessory lobe R4 than other regions, particularly compared with R3 (P = 0.013), L5 (P = 0.009), and L6 (P = 0.013), whereas sEEV did not show notable regional differences. In terms of the baseline proteomic responses, the heat map showed regional heterogeneity in the protein profile (Supplemental Fig. S3A). We used principal component analysis (PCA) to reduce the complexity of the proteomics data and to examine global trends of protein expression in the lung regions (Supplemental Fig. S3B). In the saline group, most of the variance (63.90%) in protein expression was captured by the first three principal components. The PCA analysis suggested that L3, L6, and L1 were significantly different from other regions in relation to proteomic responses. The significant variation in baseline lung function and protein expression provided further rationale for subsequent regional analysis.

Effect of mechanical ventilation and acid on lung volumes. Both acid aspiration (P = 0.012) and 2 h of MV (P = 0.002) caused significant reductions in global sVt (Fig. 2A). However, there was no interaction between acid aspiration and MV on the effect of global sVt (P = 0.84), suggesting that these effects were additive (Fig. 2A).

There was a significant interaction between acid aspiration and MV and their association with global sEEV (P = 0.10). There was no effect of acid on sEEV at the start of MV (P = 0.97) and no effect of MV on sEEV in the saline group (P = 0.92). There was, however, a significant decrease in sEEV in response to MV in the acid aspiration group (P = 0.035). Acid aspiration (P = 0.54) and MV (P = 0.61) had no effect on global strain (data not shown). When assessed regionally, sVt and sEEV in R4 were lower than the other regions (P < 0.001; Supplemental Fig. S2, C and D); however, such variation was independent of acid exposure (P = 0.895).

Identification of differentially expressed proteins. Despite the presence of highly abundant proteins such as albumin and hemoglobin, we achieved a comparable proteome depth (Supplemental Figure S4) to similar studies (27). After data filtering, 1,943 proteins (FDR < 1%) were quantified across all samples relative to the control (Saline/FB) group. On this basis, 46, 73, and 114 proteins were classified as differentially expressed in the Acid/FB, Saline/MV, and Acid/MV groups, respectively (Fig. 3, A–C) based on the q < 0.05 cut-off. Further analysis, after controlling for repeated measurements, identified 27 (upregulation: 20; downregulation: 7) in the Acid/FB group, 62 (upregulation: 53; downregulation: 9) in the Saline/MV group and 89 (upregulation: 64; downregulation: 25) in the Acid/MV group (Supplemental Table S1 at https://doi.org/10.6084/m9.figshare.12616766; all supplemental tables may be found at this site). These protein sets were used for further analysis.
Some of the differentially expressed proteins showed regional heterogeneity in expression level, including five in the Acid/FB group, 43 in Saline/MV, and 61 in Acid/MV with 4 (three upregulated and one downregulated) and eight (all upregulated) proteins showing regional differences that were influenced by treatment (Supplemental Table S2, A–C). Among the differentially expressed proteins, 42 were shared nonredundant proteins in both the Saline/MV and
Acid/MV groups, and 37 were uniquely present in Acid/MV group (Fig. 3D).

Functional characterization of differentially expressed proteins. Functional annotation revealed that 27 of the differentially expressed proteins in the Acid/FB group were involved in transcriptional regulation processes, including methylation, acetylation, and RNA-binding (Supplemental Table S2A). In contrast, the 62 dysregulated proteins in the Saline/MV group were involved in platelet aggregation, hemostasis, inflammation, and fibrinolysis (Supplemental Table S2B). Complement and coagulation cascades (CCC), comprising eight proteins (CfI, Cfd, Fga, Fgb, Fgg, Plg, Serpin1c, and Serpin1d) were the most enriched KEGG pathway (Table 1).

Mapping of the 42 overlapping proteins dysregulated in Saline/MV confirmed that these CCC proteins were also present in the double hit model of acid aspiration and MV. Analysis of 37 unique proteins in the Acid/MV group identified an additional six proteins relating to the enriched CCC pathway (C3, C4b, Cfh, Cfb, Kng1, and Serpinf2). Other pathways identified from the set of unique proteins, consisted of seven downregulated proteins that have been linked to Parkinson’s disease, Huntington’s disease, and cardiac muscle contraction. On further examination, these proteins belong to mitochondrial respiratory chain components (MRC), including Ndufa9 (Complex I), Cyc1, Uqcr2c, Uqcr1c (Complex III), Cox5a (Complex IV), and Vdac1 and Vdac2 (mitochondrial porin family). The details for the functional annotation of these overlapping and unique proteins can be found in Supplemental Table S2, C and D.

A regulatory network was established using the differentially expressed proteins (89) in the Acid/MV group (Supplemental Fig. S5). Confirming our pathway analysis above, the network comprised two clear clusters: 1) proteins associating with complement, coagulation, and fibrinolysis as hubs and 2) proteins involving the mitochondrial respiratory chain.

Association between identified protein sets and lung volumes. Because of functional relevance and strong correlation of the proteins belonging to the same pathway (Supplemental Fig. S5; \( P < 0.001 \) for all associations), we employed principal component analysis (PCA) to group these variables. Using this approach, we identified a principal component (PCA 1) for overlapping CCC proteins accounting for 61.07% of the total variance, as well as a PCA 1 for unique CCC proteins (78.60%), and a PCA 1 for unique MRC proteins (80.16%).

PCA 1 for both shared and unique CCC proteins was negatively associated with regional sVt (\( P < 0.05 \)) (Fig. 4A), whereas PCA 1 for the MRC proteins was positively correlated with regional sEEV (\( r = 0.381, P < 0.001 \)) (Fig. 4B). In contrast, regional strain was not associated with the expression of CCC or MRC proteins (\( P > 0.05 \)).

There was a significant interaction between acid aspiration and MV and their association with the PCA 1 score for both overlapping (\( P < 0.001 \)) and unique (\( P < 0.001 \)) CCC proteins (Fig. 4, C and D). For the overlapping CCC proteins, MV increased the PCA 1 score in both the saline (\( P < 0.001 \)) and acid exposed (\( P < 0.001 \)) mice. Acid aspiration had no additional effect on PCA 1 score in the ventilated mice (\( P = 0.07 \)). In contrast, acid aspiration was associated with a decrease in PCA 1 score in the free-breathing mice (\( P < 0.001 \)). For the unique CCC proteins, MV increased the PCA 1 score in both the saline (\( P < 0.001 \)) and acid-exposed (\( P < 0.001 \)) mice. Acid aspiration increased the PCA 1 score in the ventilated mice (\( P = 0.03 \)). In contrast, acid aspiration was associated with a decrease in PCA 1 score in the free-breathing mice (\( P < 0.001 \)). Acid aspiration (\( P < 0.001 \)) and MV (\( P = 0.002 \)) were associated with significant decreases in PCA 1 score for the unique MRC proteins. There was no interaction between acid aspiration and MV and the association with PCA 1 score for the MRC proteins (\( P = 0.86 \)) (Fig. 4E).

Table 1. Pathway analysis of the differentially expressed proteins in different groups

<table>
<thead>
<tr>
<th>KEGG Pathway</th>
<th>Count, %</th>
<th>( P )</th>
<th>Benjamini (( P ))</th>
<th>Proteins Involved</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proteins Identified from Comparison of Saline/MV and Saline/FB</strong></td>
<td></td>
<td></td>
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<tr>
<td>Complement and coagulation cascades</td>
<td>8 (12.9%)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>Cfi, Cfd, Fga, Fgb, Fgg, Plg, Serpin1c, Serpin1d</td>
</tr>
<tr>
<td>Staphylococcus aureus infection</td>
<td>6 (9.7%)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>Cfi, Cfd, Fgg, H2-Ea-ps, Igb2, Plg</td>
</tr>
<tr>
<td>Phagosome</td>
<td>7 (11.3%)</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>Coro1a, H2-Ea-ps, Igb2, Igb3, Mpo, Thbs1, Tubb1</td>
</tr>
<tr>
<td>Platelet activation</td>
<td>6 (9.7%)</td>
<td>&lt;0.001</td>
<td>0.005</td>
<td>Fermt3, Fga, Fgb, Fgg, Iga2b, Igb3</td>
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<tr>
<td><strong>Shared Proteins in Saline/MV and Acid/MV Groups</strong></td>
<td></td>
<td></td>
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<tr>
<td>Complement and coagulation cascades</td>
<td>7 (16.7%)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>Cfi, Fga, Fgb, Fgg, Plg, Serpin1c, Serpin1d</td>
</tr>
<tr>
<td>Staphylococcus aureus infection</td>
<td>4 (9.5%)</td>
<td>&lt;0.001</td>
<td>0.013</td>
<td>Cfi, Fgg, Igb2, Plg</td>
</tr>
<tr>
<td>Platelet activation</td>
<td>5 (11.9%)</td>
<td>0.001</td>
<td>0.012</td>
<td>Fermt3, Fga, Fgb, Fgg, Igb3</td>
</tr>
<tr>
<td><strong>Unique Proteins in Acid/MV Group</strong></td>
<td></td>
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</tr>
<tr>
<td>Complement and coagulation cascades</td>
<td>6 (16.2%)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>C3, C4b, Cfi, Cfb, Kng1, Serpin1f2</td>
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<tr>
<td>Parkinson’s disease, Huntington’s disease</td>
<td>7 (18.9%)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>Ndufa9, Cox5a, Cyc1, Uqcr2, Uqcr1, Vdac1, Vdac2</td>
</tr>
<tr>
<td>Staphylococcus aureus infection</td>
<td>4 (10.8%)</td>
<td>0.001</td>
<td>0.008</td>
<td>C3, C4b, Cfb, Cfi</td>
</tr>
<tr>
<td>Oxidative phosphorylation</td>
<td>5 (13.5%)</td>
<td>0.001</td>
<td>0.011</td>
<td>Ndufa9, Cox5a, Cyc1, Uqcr2, Uqcr1</td>
</tr>
<tr>
<td>Cardiac muscle contraction</td>
<td>4 (10.8%)</td>
<td>0.002</td>
<td>0.015</td>
<td>Cox5a, Cyc1, Uqcr2, Uqcr1</td>
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FB, free-breathing; MV, mechanical ventilation.

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DISCUSSION

The aim of our study was to assess the lung volume response to mechanical ventilation (MV) with and without prior acid-induced lung injury and to link this to the regional proteomic response. We found that combined acid exposure and MV reduced tidal volume (Vt) and end-expiratory volume (EEV), while MV alone (Saline/MV group) had minimal effect on these parameters. Proteomic analysis revealed a number of differentially expressed proteins, some of which were unique to exposure to acid, MV, and the combination of both. Mapping of the overlapping proteins showed significant enrichment of complement and coagulation cascades (CCC), while analysis of the unique proteins in the Acid/MV group identified additional proteins related to CCC and the mitochondrial respiratory chain (MRC). Importantly, regional MRC protein levels were positively correlated with sEEV, while the CCC protein levels were negatively associated with regional sVt.

This study suggests that preexisting acid injury aggravates the deleterious response of the lung to MV by decreasing lung volumes. The regional loss of EEV is linked to downregulation of mitochondrial respiration-associated proteins, while the regional decrease in Vt is linked to activation of complement and coagulation pathways.

Although acid aspiration is a common cause of direct lung injury that can lead to respiratory failure and a requirement for MV, data are scarce on the interactions between acid and MV, particularly the fundamental molecular mechanisms of the potential synergistic effects of these respiratory insults. In analyzing the separate effects of acid and MV, our data showed that acid aspiration caused a decrease in Vt. The deleterious effects of acid aspiration on lung function are well described and include decreasing lung volume (4), which is consistent with decreased lung compliance as a result of pulmonary edema (13). The rapid respiratory response to acid, as indicated...
by the difference in Vt at baseline within 30 min of acid instillation, is also consistent with the previous observations, whereby the greatest impairment in pulmonary gas exchange occurs 20 min after acid aspiration (26). This is probably linked to the rapid development of acid-induced pulmonary edema before subsequent recruitment of inflammatory cells, including neutrophils (14). We found that the low tidal volume ventilation strategy induced minimal changes in lung volume, which is consistent with previous studies (25, 35) and the clinical observation that low tidal volume plus moderate PEEP, minimizes lung injury in the acute respiratory distress syndrome (10). However, when combined with acid-induced lung injury, there was evidence of a pronounced reduction in lung volumes without any change in lung strain after 2 h of MV, suggesting that even so-called “protective” mechanical ventilation has the capacity to exacerbate acid-induced lung injury. It is interesting to note that this is in clear contrast to our previous study (9) using a model of endotoxemia-induced lung injury, in which we observed an increase in sEEV in response to lung injury; likely due to gas trapping at end-expiration. This discrepancy may be related to the difference in the nature of the injury that was induced before MV in the two studies and highlights the importance of understanding the contribution of preexisting injury to the deleterious effects of MV.

In addition to the effects on lung mechanics outlined above, both MV and acid exposure altered the proteome. Exposure to acid alone altered the expression of 27 proteins that are mainly associated with transcriptional regulation processes, consistent with a rapid host response to injury. On the other hand, MV enriched the complement and coagulation cascades (CCC) with additional proteins in this pathway identified in the Acid/MV group. CCC are characteristic of acute lung injury (1, 33) and other inflammatory conditions in the lung including pneumonia, and sepsis (1) and have been shown to be linked to ventilator induced lung injury (VILI) (22, 28), and multiorgan failure (1). In VILI, as a result of tidal stretch, both local and systemic complement systems are activated, resulting in increased vascular permeability and pulmonary edema (18, 22), which can subsequently limit lung expansion, and reduce lung compliance. As such, the CCC proteins are regarded as theoretical therapeutic targets to prevent VILI (22, 28, 33). Consistent with this, we found a negative correlation between the regional expression of CCC and regional Vt after 2 h of ventilation and no association between CCC proteins and regional strain, indicating that the regions with the lowest compliance (i.e., the lowest tidal volume) had the highest expression of CCC.

Interestingly, analysis of 37 differentially expressed proteins in the Acid/MV group led to the identification of seven down-regulated proteins involved in mitochondrial respiratory chain (MRC). Reduction of MRC proteins may decrease ATP generation and energy supply, and lead to excessive reactive oxygen species, which can stimulate pathological processes, including inflammation, cellular damage, mutations, and apoptosis (19). Endothelial mitochondrial depolarization has been proposed as an important mechanism of global loss of endothelial barrier function and pulmonary edema in acid-induced lung injury (14). In addition, evidence linking mitochondrial dysfunction with MV also exists. For example, oxidative mitochondrial DNA (mtDNA) damage occurs during ventilation with high peak inflation pressures (11), and repair of mtDNA damage attenuates oxidative stress and protects against lung injury in moderately severe VILI (12). Recently, Pereira-Fantini et al. (21) reported region-specific mitochondrial alterations in the preterm lamb lung subjected to MV, including an increase in the expression of respiratory chain complex IV (cytochrome-c oxidase) proteins in the nondependent lung and decreased expression of respiratory chain complex III and V proteins in the gravity-dependent lung. In our study, we showed that downregulation of MRC proteins was associated with low sEEV, suggesting that regions exposed to low lung volumes are susceptible to mitochondrial dysfunction. This is consistent with the concept of atelectasis-induced lung injury and the impact of inadequate lung volumes on clinical progression of patients with respiratory failure (17).

One of the limitations of the current study is that we were unable to determine the effect of acid alone at the 2 h time point due to animal welfare concerns. Hence, whether down-regulation of MRC proteins is a result of acid exposure or the interaction between acid exposure and mechanical ventilation is unclear, although the association between MRC expression and lung volumes would suggest the latter. Furthermore, we used acid with a pH of 3.0, which is higher than the pH typically used in these models (16). However, most ICU patients have gastric juice pH in the range of 3.0 to 4.0 (3), and pH 4.0 has been reported to cause lung injury (2), suggesting our model is clinically relevant. It is also important to note that we used pressure-driven MV in our study with no adjustment of tidal volume over the course of ventilation. This differs from the clinical situation which typically involves volume-driven MV, which may have impacted the pathways that were stimulated. Nonetheless, we have shown that low lung volumes (both EEV and Vt) are associated with alterations in protein expression within pathways that are likely to promote lung injury.

In conclusion, our data show that low tidal volume ventilation with moderate PEEP does not exacerbate lung inhomogeneity but predisposes the lung to elevated expression of CCC proteins. The detrimental effects of MV on the lung were magnified by prior exposure to acid, leading to the loss of lung volume. Importantly, we were also able to identify a potential impact of acid exposure, in combination with MV, on mitochondrial function, which was linked to regional end-expiratory volume, suggesting a novel effect of atelectasis in promoting cell injury. Overall, this study provides novel insights into regulatory mechanisms of VILI following acid aspiration and highlights the fact that both tidal stretch and end-expiratory volume may contribute to further lung injury.

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DISCLOSURES

Stephen Dubsky and Andreas Fournas have financial interests in the commercialization of an imaging technology related to this publication. All other authors declare no conflicts of interest, financial or otherwise.
AUTHOR CONTRIBUTIONS

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15. Hughes CS, Murgridge S, Müller T, Sorensen PH, Morin GB, Kri-