Impact of Low and High Tidal Volumes on the Rat Alveolar Epithelial Type II Cell Proteome

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Rationale: Mechanical ventilation with high tidal volumes leads to increased permeability, generation of inflammatory mediators, and damage to alveolar epithelial cells (ATII).

Objectives: To identify changes in the ATII proteome after two different ventilation strategies in rats.

Methods: Rats ($n = 6$) were ventilated for 5 hours with high- and low tidal volumes (V_T s) (high V_T : 20 ml/kg; low V_T : 6 ml/kg). Pooled nonventilated rats served as control animals. ATII cells were isolated and lysed, and proteins were tryptically cleaved into peptides. Cellular protein content was evaluated by peptide labeling of the ventilated groups with ^{18}O . Samples were fractionated by cation exchange chromatography and identified using electrospray tandem mass spectrometry. Proteins identified by 15 or more peptides were statistically compared using t tests corrected for the false discovery rate.

Measurements and Main Results: High V_T resulted in a significant increase in airspace neutrophils without an increase in extravascular lung water. Compared with low- V_T samples, high- V_T samples showed a 32% decrease in the inositol 1,4,5-trisphosphate 3 receptor ($p < 0.01$), a 34% decrease in Na^+ , K^+ -ATPase ($p < 0.01$), and a significantly decreased content in ATP synthase chains. Even low- V_T samples displayed significant changes, including a 66% decrease in heat shock protein 90- β ($p < 0.01$) and a 67% increase in mitochondrial pyruvate carboxylase ($p < 0.01$). Significant differences were found in membrane, acute phase, structural, and mitochondrial proteins.

Conclusions: After short-term exposure to high- V_T ventilation, significant reductions in membrane receptors, ion channel proteins, enzymes of the mitochondrial energy system, and structural proteins in ATII cells were present. The data supports the two-hit concept that an unfavorable ventilatory strategy may make the lung more vulnerable to an additional insult.

Keywords: acute lung injury; alveolar epithelium; corticosterone

Acute lung injury is a major cause of morbidity (1) and mortality, and mechanical ventilation is critical to survival of most patients with acute lung injury. However, mechanical ventilation with high tidal volumes (V_T s) is known to have deleterious side effects. The importance of ventilator-induced lung injury caused by mechanical ventilation has been established by several experimental and clinical studies (2, 3). Avoidance of excessive V_T s

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Mechanical ventilation with high tidal volumes leads to increased permeability, generation of inflammatory mediators, and damage to alveolar epithelial cells that may contribute to ventilator-induced lung injury.

What This Study Adds to the Field

Using a proteomic approach, significant reductions of key proteins in alveolar type II cells from rats were identified after short-term positive-pressure ventilation.

during mechanical ventilation reduces the risk of ventilator-induced injury, and application of a lower V_T strategy to ventilate patients with acute respiratory distress syndrome has been shown to lead to a greater than 20% decrease in mortality (4).

Mechanical ventilation with high V_T s leads to increased permeability, generation of inflammatory mediators, and damage to alveolar epithelial cells, all of which may contribute to the development of ventilator-induced lung injury (3, 5–9). The alterations at the cellular level, especially in protein expression, that accompany and may contribute to development of ventilator-induced lung injury are not known, although some recent work has provided new insights into ventilator-induced stress failure at the cellular level (9). There have been no studies to date that have used a proteomic approach to study the qualitative and quantitative effects of mechanical ventilation on protein expression in the alveolar epithelial cells (9, 10). Identification of changes in protein expression induced by mechanical ventilation is important because it may suggest mechanisms of cell injury that have not been previously revealed or explored.

Therefore, to identify and quantify the changes in protein content of alveolar epithelial type II cells induced by mechanical ventilation, we used the proteomic technique, $^{16}\text{O}/^{18}\text{O}$ labeling isotope ratio mass spectrometry (MS), to compare the protein content of alveolar epithelial type II cells from rats subject to mechanical ventilation with the protein content of alveolar cells from spontaneously breathing rats. To further characterize the validity of the animal model, we performed bronchoalveolar lavage (BAL) with cell differential and protein concentration measurements. Moreover, lung water was determined to quantify pulmonary edema, and the levels of the most important endogenous rat steroid, corticosterone, were measured in BAL fluid (BALF) and serum as a marker of stress response and a well-known mediator of type II cell activation (11).

The method of $^{16}\text{O}/^{18}\text{O}$ labeling isotope ratio MS combines enzymatic isotopic labeling with MS and allows simultaneous

(Received in original form May 5, 2006; accepted in final form March 13, 2007)

Supported in part by National Institutes of Health grants RR01614 (A.L.B.), NHLBI HL 74005, and HL 58516 (M.A.M.), and by Deutsche Forschungsgemeinschaft grant HI 810-1 (J.H.).

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 175, pp 1006–1013, 2007

Originally Published in Press as DOI: 10.1164/rccm.200605-621OC on March 22, 2007
Internet address: www.atsjournals.org

identification and comparative quantification of proteins in samples from two different sources (12–15). This method involves labeling peptides in one of the samples by incorporating two ^{18}O atoms into the carboxyl terminus of all tryptic peptides during proteolytic cleavage, whereas the peptides from the other sample contain ^{16}O atoms. This labeling technique leads to a mass increase of 4 Da in peptides in the ^{18}O -labeled sample, which can then be discriminated by MS from the peptides in the ^{16}O -labeled sample that will not display a mass change. We have previously used an isotope ratio MS approach on liver cells, and found reliable quantitative results (16). We anticipated that this approach could provide new insights into the effects of ventilation on the proteomics of alveolar type II (ATII) cells and suggest mechanisms of cell injury.

METHODS

Supplemental Information

A detailed description of the experimental procedures is provided in PART 1 of the online supplement. PART 2 provides Tables E1–E4, with results of the tests for normal variability and interexperimental variability. PART 3 contains Table E5, with the quantitative information for all proteins.

Ventilation Procedure

Male Sprague-Dawley rats were anesthetized with pentobarbital and mechanically ventilated for 5 hours at 21% $\text{F}_{\text{I}\text{O}_2}$. Six rats were ventilated with a high- V_T strategy (high V_T : 20 ml/kg without positive end-expiratory pressure), a second group of six animals was ventilated with a low- V_T strategy (low V_T : 6 ml/kg with a positive end-expiratory pressure of 4 cm H_2O). The control group consisted of pooled cells from rats that were not mechanically ventilated (i.e., pooled spontaneous ventilation [PSV]; $n = 24$).

Measurement of Extravascular Lung Water

Extravascular lung water was determined using the wet-to-dry ratio of the lungs, as described previously (17, 18). In brief, the left lung was homogenized and the extravascular lung water was determined by measuring the extravascular water-to-dry weight ratio (gram water/gram dry lung).

Cell Isolation

After the experiment, the rats were killed by transection of the abdominal aorta. BAL was performed in all animals with 7 ml of normal saline. ATII cells were isolated as previously described (19).

Sample Preparation

For the ventilation groups, cells from two animals were pooled, resulting in six independent ^{18}O labeling and cation exchange chromatography experiments. To obtain data on the variability without intervention, 40×10^6 ATII cells from two individual, nonventilated Sprague-Dawley rats (INC-1 and INC-2) were individually isolated, lysed, and compared with an equal number of cells from the pooled normal cells (PSV).

After thawing, 80×10^6 cells were used per experiment. ATII cells from all control animals were pooled. A control sample containing an equal number of cells in the post-thawing cell counts was assigned to each sample.

$^{16}\text{O}/^{18}\text{O}$ Labeling

$^{16}\text{O}/^{18}\text{O}$ labeling was performed using H_2^{18}O based on the method described by Stewart (15).

Samples were reduced by the addition of guanidinihydrochloride and Tris(2-carboxyethyl)phosphine hydrochloride at 56°C for 60 minutes, followed by alkylation with iodoacetamide at 37°C for 1 hour. After dilution in double-distilled H_2O and adjustment to pH 7.4, tryptic digestion was initiated at 37°C for 12 hours. After acidification to stop tryptic cleavage, the proteins were cleaned up using C18 SepPack cartridges (Waters, Milford,

MA), and water was extracted from the samples with a speedvac device. The samples from the ventilated groups were resuspended in a buffer containing trypsin 5% wt/wt in H_2^{18}O . The same buffer in H_2^{16}O was used for the nonventilated control animals. After titration to pH 7.4 and incubation for 24 hours at 37°C , the enzymatic reaction was halted by lowering the pH to 3 with 1% formic acid, and the samples were brought to complete dryness.

After resuspension in 1% formic acid, each of the six sample-control pairs was fractionated into 52 subfractions by cation exchange chromatography. For the normal control samples, each sample was fractionated into 26 fractions, and the samples were then brought to complete dryness and resuspended in 1% vol/vol formic acid. Cleanup was performed using a homemade column packed with Jupiter C18 resin (Phenomenex, Sutter Creek, CA).

MS Measurements

A total of 26 of the 52 fractions of each of the six labeled-unlabeled sample-control cation exchange chromatography runs were further separated by reverse phase chromatography. For the INC samples, 13 out of 26 fractions were used. Tryptic peptides were subjected to liquid chromatography-tandem MS analysis on a QSTAR electrospray mass spectrometer (Applied Biosystems, Framingham, MA) that was connected in line with the chromatography unit, as described elsewhere (20). After the initial runs, an exclusion list based on the previously acquired peptides was built, and the sample was run again with exactly the same settings. A total of three runs were performed for low- V_T and high- V_T samples. The exclusion lists were not applied in the INC samples.

The resulting peak lists were searched against the Swissprot rodent protein database using the ProteinProspector 4.11 software package (University of California, San Francisco, CA; <http://prospector.ucsf.edu/>) (21, 22).

Quantification was done by calculation of the ratio of the areas under the peaks between the light and the heavy isotope using the SearchCompare tool from the ProteinProspector suite of programs (University of California, San Francisco, CA).

Statistical Analysis

For each identified protein, the mean and SD of the peak intensity ratios from all peptides assigned to this protein was calculated using Microsoft Excel (Microsoft Corporation, Redmond, WA).

Statistical tests were only performed if quantitative information for the protein was obtained from 15 peptides or more per comparison. One-sample t tests were used to calculate the p values for the rejection of the null hypothesis that the mean ratio of the peak areas is 1.0 (signifying no difference in the areas under the peptide peaks). Correction for multiple comparisons was done according to the false discovery rate method described by Benjamini and colleagues (23). To determine statistically significant differences between the groups, low- V_T :PSV and high- V_T :PSV ratios were compared with each other using the univariate analysis of variance procedure in SPSS (SPSS, Chicago, IL). The threshold (α) p value calculated according to the false discovery rate method for these comparisons was 0.05. Statistical comparison of the wet-to-dry ratio measurements, neutrophil counts, and protein concentrations were done using the t test for independent samples in SPSS.

RESULTS

Morphology and BAL Changes

Ventilation with either high or low V_T did not result in clearly visible morphologic changes in the lungs. BAL was performed in a standardized way, using 7 ml of phosphate-buffered saline without statistically significant differences in the recovered volume between the groups (data not shown). There were no significant changes in the wet-to-dry ratio or the BAL protein concentration between the three groups (Table 1). The average

TABLE 1. BRONCHOALVEOLAR LAVAGE CELL COUNTS AND EXTRAVASCULAR LUNG WATER IN THE THREE VENTILATION GROUPS

	BAL Cell Counts				BALF Protein (mg/ml)	Lung Water (wet:dry ratio)
	Total Cells ($\times 10^3$)	Macrophages (%)	Neutrophils (%)	Others (%)		
Spontaneous ventilation	526 $\pm$ 168*	98 $\pm$ 3*	5 $\pm$ 3*	2 $\pm$ 1*	11.40 $\pm$ 1.23*	3.56 $\pm$ 0.18†
Low-V _T ventilation	792 $\pm$ 237‡	73 $\pm$ 30‡	27 $\pm$ 30‡	1 $\pm$ 2‡	11.94 $\pm$ 1.79‡	3.72 $\pm$ 0.46†
High-V _T ventilation	819 $\pm$ 592‡	10 $\pm$ 6‡§	88 $\pm$ 6‡§	2 $\pm$ 3‡	13.28 $\pm$ 2.40‡	4.04 $\pm$ 1.00†

Definition of abbreviations: BAL = bronchoalveolar lavage; BALF = BAL fluid.

Data represent mean $\pm$ SD.

* n = 12.

† n = 3.

‡ n = 6.

§ p < 0.01 compared to spontaneous ventilation.

percentage of neutrophils was significantly increased in the high-V_T group compared with spontaneously ventilating animals from the control group (Table 1). There was no significant difference in the percent of neutrophils in the low-V_T group compared with the control animals.

Cellular Protein Content

Lysis of 80×10^6 ATII cells from high-V_T, low-V_T, and PSV groups resulted in a comparable protein content of high V_T (707 ± 124 μ g; p = 0.71 vs. PSV), low V_T (762 ± 100 μ g; p = 0.59 vs. PSV), and PSV (740 ± 62 μ g). The cells from the nonventilated Sprague-Dawley rats (INC) were individually isolated, lysed, and compared with an equal number of cells from the pooled normal cells (PSV). A cellular protein content of 482 (± 35) μ g in INC and 478 (± 14) μ g in PSV was measured.

MS Measurements and Statistical Evaluation

Quantitative information was obtained for a total of 383 proteins. A complete list is provided in the online supplement, PART 3, Table E5. Further analysis, including statistical tests, were performed for the 59 proteins in the low-V_T group and 66 proteins in the high-V_T group that were identified by more than 15 individual peptides. After correction for multiple comparisons, a total of 14 proteins after high V_T (Table 2) and 10 proteins after low V_T (Table 3) were significantly different from the normal PSV control animals.

In the two INC samples, quantitative information was obtained for a total of 62 (INC-1) and 97 (INC-2) proteins. The threshold for quantitative analysis was set to three peptides per protein. These adjustments resulted in quantitative information in the INC groups for 16 (INC-1) and 22 (INC-2) proteins. Quantitative analysis determined a mean protein content ratio of 1.02 (± 0.33 ; INC-1:PSV) and 0.96 (± 0.53 ; INC-2:PSV). Four of the proteins with statistically significant differences after mechanical ventilation were quantified in the INC groups without statistically significant differences. Detailed results on these proteins are included in the online supplement, PART 2, Table E1.

High-V_T Ventilation

High-V_T ventilation induced a significant reduction in the expression of several membrane, mitochondrial, and structural proteins.

Cell Membrane Proteins

There was a 32% reduction in the content of the inositol 1,4,5 triphosphate (IP3) membrane receptor in the high-V_T group

compared with the PSV group (p < 0.01; Table 2, Figure 1). When we compared the ratios for this protein between the high-V_T:PSV and low-V_T:PSV groups, these results was also significant (p < 0.02; Table 4).

The cellular content of the Na⁺, K⁺-ATPase α -1 chain precursor was reduced by 34% in the high-V_T group compared with the PSV group (p < 0.01) (Figure 2 and Table 1). The α -2 and α -3 chains of this enzyme were identified and quantified in both ventilation groups, but statistical comparisons were not done due to the smaller number of peptides (< 15).

Mitochondrial Proteins

The high-V_T-treated group showed a 39% decrease of the content in the β -chain of the mitochondrial ATP synthase relative to the low-V_T group (p < 0.0001) (Figure 3). There was also a

TABLE 2. SIGNIFICANT CHANGES AFTER HIGH-V_T VENTILATION COMPARED WITH POOLED SPONTANEOUSLY VENTILATING CONTROL ANIMALS

Protein	n	Mean (SD)	p Value
Membrane proteins			
IP3 receptor type 3 (type 3 IP3 receptor)	22	0.68 (0.50)	0.0069
Na ⁺ , K ⁺ -ATPase α -1 chain precursor	19	0.66 (0.48)	0.0066
Mitochondrial proteins			
ATP synthase β chain, mitochondrial precursor	39	0.61 (0.45)	0.0001
ATP synthase α chain, mitochondrial precursor	34	0.72 (0.45)	0.0011
GDH, mitochondrial precursor	15	0.39 (0.37)	0.0001
MMSDH (acylating), mitochondrial precursor	15	0.57 (0.34)	0.0002
NSL-TP	15	0.64 (0.29)	0.0003
Structural proteins			
Actin, cytoplasmic 1 (β -actin)	35	0.74 (0.51)	0.0045
Annexin A2 (annexin II)	22	0.65 (0.50)	0.0037
DYHC	31	0.57 (0.40)	0.0001
Lamin A	32	0.62 (0.42)	0.0001
Lamin B1	29	0.74 (0.50)	0.0093
Spectrin α -chain (spectrin, nonerythroid α -chain)	73	0.74 (0.54)	0.0001
Other proteins			
Peroxisomal MFE-2	19	0.60 (0.48)	0.0021

Definition of abbreviations: DYHC = dynein heavy chain, cytosolic; GDH = glutamate dehydrogenase; IP3 = inositol 1,4,5-trisphosphate; MFE = multifunctional enzyme; MMSDH = methylmalonate-semialdehyde dehydrogenase; NSL-TP = nonspecific lipid-transfer protein, mitochondrial precursor.

Values indicate the overall number (n) and means (SD) of peptides in all groups combined. The p value refers to the one-sample t test between these numbers and the expected value for the ratio between high-V_T ventilation and pooled spontaneously ventilating control animals (1.00). The threshold (α) p value calculated according to the false discovery rate method for these comparisons was 0.0093. All p values less than or equal to this threshold value were considered statistically significant.

Figure 1. Inositol 1,4,5-trisphosphate (IP3) receptor type 3 content after high-V_T (HTV) and low-V_T (LTV) ventilation. There was a significant difference in the content of the IP3 membrane receptor type 3 between the high-V_T and pooled spontaneously ventilating control (PSV) groups and between low-V_T/PSV and high-V_T/PSV (see Tables 3 and 4). The data are plotted in a binary logarithmic scale to obtain equal distances for corresponding ratios below and above the equal concentration threshold (ratio = 1.0). **p* < 0.01 against normal control animals and a *p* < 0.02 between low-V_T/PSV and high-V_T/PSV. Data are means ($\pm$ SD) of the peptide ratios (data points) in all groups combined.

significant difference between the high-V_T and low-V_T groups (*p* < 0.05; Table 4). The α -chain of the mitochondrial ATP synthase followed the same pattern and was decreased by 28% in the high-V_T group compared with the PSV group (*p* < 0.01). There was also a 61% reduction in the mitochondrial precursor of the glutamate dehydrogenase (*p* < 0.0001) in the high-V_T group compared with the PSV group (Table 2). The same pattern was observed for the mitochondrial precursor of the acylating

TABLE 3. SIGNIFICANT CHANGES AFTER LOW-V_T VENTILATION COMPARED WITH POOLED SPONTANEOUSLY VENTILATING CONTROL ANIMALS

Protein	n	Mean (SD)	p Value
Mitochondrial proteins			
Pyruvate carboxylase, mitochondrial precursor (pyruvic carboxylase)	19	1.67 (0.96)	0.0072
HSPs			
HSP 90- β (HSP 84)	18	0.44 (0.34)	0.0001
Heat shock cognate 71-kD protein	18	0.71 (0.35)	0.0027
Structural proteins			
DYHC	57	0.62 (0.43)	0.0001
Filamin A (α -filamin)	40	0.76 (0.51)	0.0040
Lamin A	23	0.62 (0.51)	0.0017
Lamin B1	30	0.69 (0.50)	0.0020
PLTN	77	0.80 (0.60)	0.0039
Moesin	15	0.64 (0.42)	0.0050
α -Actinin 4 (non-muscle α -actinin 4)	19	0.63 (0.47)	0.0030

Definition of abbreviations: DYHC = dynein heavy chain, cytosolic; HSP = heat shock protein; moesin = membrane-organizing extension spike protein; PLTN = plectin 1.

Values indicate the overall number (n) and means (SD) of peptides in all groups combined. The *p* value refers to the one-sample *t* test between these numbers and the expected value for the ratio between low-V_T and pooled spontaneously ventilating control animals (1.00). The threshold (α) *p* value calculated according to the false discovery rate method for these comparisons was 0.0093. All *p* values less than or equal to this threshold value were considered statistically significant.

methylmalonate-semialdehyde and the nonspecific lipid transfer protein (Table 2).

Structural Proteins

The cellular content in β -actin was significantly reduced by 25% in the high-V_T group compared with the PSV group (*p* < 0.01) (Table 2). There was a reduction by 25–40% in the content of annexin A2 (*p* < 0.01) (Table 2). Quantitative data for annexins A1 and A6 indicated a slight reduction without significant differences between high V_T and low V_T and the spontaneously ventilated control animals.

The cellular content in the cytosolic dynein heavy chain was significantly reduced in both the high- and low-V_T groups relative to the untreated control animals (*p* < 0.0001). The same pattern was observed for lamin A and lamin B1 (*p* < 0.01) (Table 2).

Low-V_T Ventilation

There were 10 ATII proteins affected by the low-V_T strategy compared with 14 proteins affected by the high-V_T strategy.

Mitochondrial Proteins

There was a significant, 67% increase in the mitochondrial precursor of the pyruvate carboxylase (*p* < 0.01), which was not present in the high-V_T-treated rats (Table 3).

Heat Shock Proteins

The cellular content in heat shock protein (HSP) 90- β was significantly reduced by 56% in the low-V_T-treated group (*p* < 0.0001) (Table 3). The difference between the two groups was statistically significant (*p* < 0.01) (Table 4). The intracellular content in HSP 90- α followed the same, albeit not statistically significant, pattern. The low-V_T-treated groups showed a 30% decrease in heat shock cognate 71 kD protein (*p* < 0.01) (Table 3), which was not present in the high-V_T-treated group.

Structural Proteins

The cellular content in the cytosolic dynein heavy chain was significantly reduced in both the high- and low-V_T ventilation groups (*p* < 0.0001) (Tables 2 and 3). The same pattern was observed for lamin A and lamin B1 (*p* < 0.01) (Tables 2 and 3). There was 25% less filamin A in the low-V_T group (*p* < 0.01) (Table 3). The cellular content in plectin1 was significantly reduced by an average of 20% in the low-V_T group compared with PSV (*p* < 0.01) (Table 3). The same pattern was found for membrane-organizing extension spike protein (*p* < 0.01) and α -actinin 4 (*p* < 0.01) (Table 3).

ELISA Measurements

Corticosterone as an indicator of stress response was detected in serum and BALF of all samples. The average content of corticosterone in serum was 283 ($\pm$ 106) ng/ml in PSV, 353 ($\pm$ 265) in low V_T, and 528 ($\pm$ 209) in high V_T. Only the concentration difference between PSV and high V_T was statistically significant (*p* < 0.05). In BAL, the average concentration of corticosterone was 4.4 ($\pm$ 2.4) ng/ml in PSV, 7.6 ($\pm$ 4.1) ng/ml in low V_T and 15.9 ($\pm$ 3.7) in high V_T. As in serum, the only concentration difference that was statistically significant was between PSV and high V_T (*p* < 0.01).

Myeloperoxidase and HSP 70 were measured to validate the MS data. The myeloperoxidase ELISA detected myeloperoxidase in 5/6 sample pairs, with an average content of 29 ($\pm$ 9) ng/ml in normal control animals, 19 ($\pm$ 0.7) in low V_T, and 17 ($\pm$ 3) in high V_T. This resulted in average content ratios of 0.71 ($\pm$ 0.03) in low V_T and 0.63 ($\pm$ 0.10) in high V_T. Using MS, myeloperoxidase was quantified in 4/6 sample pairs, resulting in

TABLE 4. SIGNIFICANTLY DIFFERENT PROTEIN RATIOS BETWEEN LOW- AND HIGH-V_T VENTILATION

Protein	Low-V _T :PSV		High-V _T :PSV		p Value
	n	Mean (SD)	n	Mean (SD)	
Membrane proteins					
IP3 receptor type 3	22	1.40 (1.18)	18	0.68 (0.50)	0.0135
Mitochondrial proteins					
ATP synthase β -chain, mitochondrial precursor	39	0.89 (0.73)	55	0.61 (0.45)	0.03608
Structural proteins					
Periplakin	54	1.45 (1.04)	19	0.86 (0.69)	0.00753
HSPs					
HSP 90- β (HSP 84)	27	0.44 (0.34)	18	1.30 (1.25)	0.0064

Definition of abbreviations: HSP = heat shock protein; IP3 = inositol 1,4,5-trisphosphate; PSV = pooled spontaneously ventilating control animals.

Values indicate the overall number (n) and means (SD) of peptides in all groups. The p value refers to the univariate analysis of variance between the low-V_T:PSV and high-V_T:PSV ratios. The threshold (α) p value calculated according to the false discovery rate method for these comparisons was 0.05.

an average content ratio of 0.67 ($\pm$ 0.34) in low V_T and 0.48 ($\pm$ 0.44) in high V_T. There was no significant difference in the paired samples *t* test or the Wilcoxon test between the ELISA and MS sample ratios. The mean bias (difference between the measurements) was 0.11 ($\pm$ 0.29).

For HSP 70, the ELISA detected the protein in 5/6 sample pairs with an average content of 17 ($\pm$ 3) ng/ml in normal control animals, 15 ($\pm$ 0.2) ng/ml in low V_T and 17 ($\pm$ 2) ng/ml in high V_T. This resulted in average content ratios of 0.9 ($\pm$ 0.01) in low V_T and 1.02 ($\pm$ 0.1) in high V_T. Using MS and averaging all HSP 70 proteins as one protein, HSP 70 was quantified in 5/6 sample pairs, resulting in an average content ratio of 0.76 ($\pm$ 0.42) in low V_T and 0.81 ($\pm$ 0.14) in high V_T. There was no significant difference in the paired samples *t* test or the Wilcoxon test between the ELISA and MS sample pair ratios. The mean bias was -0.19 ($\pm$ 0.29).

DISCUSSION

Using the proteomic technique of ¹⁸O labeling, chromatographic fractionation and purification, and electrospray ionization tandem MS, alterations in the protein content of rat alveolar cells were identified and quantified in rats after positive-pressure mechanical ventilation over 5 hours.

In summary, ATII cells were isolated from rats ventilated with either high or low V_Ts that were compared with control cells from spontaneously breathing rats. The ventilation model was characterized by significant increases in the percentage of neutrophils in BALF after high-V_T ventilation. Only high-V_T ventilation led to a statistically significant increase in endogenous steroid levels in serum and BAL, a marker of stress response and potential mediator of type II cell activation and pulmonary edema clearance. Conversely, there were no statistically significant alterations in BAL protein concentration and total lung

Figure 2. Na⁺, K⁺-ATPase α -1 chain and dynein decreased with high V_T (HTV). There was a significant parallel decrease in the cellular content of both proteins in the high-V_T group. Dynein is an essential component of microtubules that facilitate the intracellular movement of Na⁺, K⁺-ATPase and incorporation into the plasma membrane. The data are plotted in a binary logarithmic scale to obtain equal distances for corresponding ratios below and above the equal concentration threshold (ratio = 1.0). **p* < 0.01 against normal control animals (PSV). Data are means ($\pm$ SD) of the peptide ratios (data points) in all groups combined.

Figure 3. ATP synthase α - and β -chain. In the high-V_T (HTV) group, there was a statistically significant reduction in the content of the α - and β -chains of the mitochondrial ATP synthase. The data are plotted in a binary logarithmic scale to obtain equal distances for corresponding ratios below and above the equal concentration threshold (ratio = 1.0). **p* < 0.01 against normal control animals (PSV). Data are means ($\pm$ SD) of the peptide ratios (data points) in all groups combined.

water after any type of mechanical ventilation. Using quantitative proteomics, statistically significant alterations in the AII cells from rats after high V_T were detected and quantified. Changes in content were found in proteins involved in cell signaling, alveolar edema fluid clearance, mitochondrial energy metabolism pathways, and cell structure.

Despite these significant changes in crucial proteins involved in cell homeostasis and cell survival, the changes in the overall average protein ratios suggest relatively small changes in the content of the vast majority of the intracellular proteins after 5 hours of positive-pressure ventilation. This result is confirmed by less pronounced differences in these proteins and the smaller SD of the overall protein ratio upon comparison of the INC rats and the PSV. Such a result is in agreement with those of previous studies (24), and indicates that the majority of the intracellular proteins, especially the highly abundant housekeeping proteins that are most likely to be identified, undergo only minimal changes with short-term positive-pressure ventilation.

For the rat proteins that were detected by MS, there is only a limited selection of well validated antibody pairs or ELISA kits available. The results of the comparison between ELISA and MS measurements indicate that these results cannot be used interchangeably. However, antibody detection and MS are different methodologies with a different bias. For example, ELISA might lead to the detection of protein fragments that contain only a part or none of the peptides that are detected by MS. Another potential source of disagreement between the measurements is protein definition: whereas MS detected three different isoforms of HSP 70, there are no discriminatory data available for the ELISA that we used. Given these limitations, the agreement between the two methods of measurement is, in our view, sufficient to validate the MS results.

We found significant variation in the peptide ratios for the individual proteins. Potential causes for this phenomenon includes different effects of mechanical ventilation to different areas of the lung. This could result in differences in cell stimulation and protease release, causing partial proteolytic cleavage and post-translational modifications of proteins and peptides with potential changes in detection and assignment. Therefore, some peptides could potentially be cleavage products of several different proteins. We tried to minimize such bias by using one peptide only for the identification of the highest scoring protein and ignoring less significant matches, and by statistically comparing only quantitative results obtained from at least 15 independent peptides. Moreover, interexperimental and interindividual variations clearly contribute to the variation in peptide ratios, as can be seen from the means and SDs of the comparisons of individual normal rats to the pooled control animals.

The statistically significant reductions in the content of cell membrane-associated proteins, mitochondrial proteins, and cell structure proteins identified in this study may indicate important functional consequences for AII cells in high- V_T ventilation. These results could suggest positive pressure-induced disruptions of mechanisms involved in edema fluid clearance, cell signaling pathways, and energy metabolism, all of which contribute potentially to the cellular injury induced by mechanical ventilation. Moreover, these changes may predispose the lung to further injury by other pathologic stimuli, such as infections, blood transfusions, or aspiration. The potential implications of these results are discussed in more detail the following discussion.

Cell Membrane Proteins

IP3 is a second messenger produced in response to the stimulation of G-protein-coupled receptors or receptor tyrosine kinases. IP3 rapidly releases Ca^{2+} from intracellular Ca^{2+} pools within the endoplasmic reticulum and other cellular membranes by

binding to the IP3 receptor (IP3R), which amplifies and transduces cellular signals, many of which are generated at the plasma membrane. A large number of proteins interact directly with IP3R, which indicates that IP3R plays a role in many other signaling pathways (25). Specifically, the IP3R type 3 identified in this study has been shown to mediate capacitative Ca^{2+} flux across the plasma membrane (26) and nuclear membranes, and to mediate transforming growth factor- β -mediated Ca^{2+} influx (27). A decrease of IP3R in the alveolar epithelial cells damaged by high V_T may, therefore, have important functional implications for cell signaling and transduction at multiple levels.

The principal role of Na^+ , K^+ -ATPase is to maintain the ion gradient between the intracellular and extracellular compartments. The expression and post-translational modification especially of the α -1 chain of this enzyme is known to play an important role in the clearance of pulmonary edema fluid (28, 29). A significant reduction of the cellular content of the α -1 chain precursor after high V_T may potentially contribute to a reduced capacity of the AII cell to remove alveolar edema. This observation is in agreement with previous results from Dr. Snazjder's group, demonstrating the induction of pulmonary edema and diffuse alveolar damage in rats after high- V_T ventilation (30). Because our results do not show overt pulmonary edema, the significant reduction in Na^+ , K^+ -ATPase content after high V_T could be interpreted as an early indication of a defect in alveolar ion and fluid transport capacity. Such an impediment could lead to an impaired alveolar fluid clearance and frank alveolar edema if additional injurious stimuli, such as infection or increased hydrostatic vascular pressure, are present.

The finding of a reduced cytosolic dynein concentration, especially after high V_T , confirms results from a previous study that demonstrated the presence of cytosolic dynein in AII cells (31). It is noteworthy that dynein kinetics after low V_T and high V_T are similar to the kinetics of Na^+ , K^+ -ATPase. Cytosolic dynein is an essential component of microtubules, and disruption of the microtubule network has recently been demonstrated to inhibit vesicle motion toward the plasma membrane and prevent a dopamine-induced increase in the Na^+ , K^+ -ATPase activity in alveolar epithelial cells (32). This decrease in cytoplasmic dynein could contribute to an impaired ability of the injured AII cells to augment Na^+ , K^+ -ATPase activity and the associated fluid clearance in high- V_T ventilation.

Mitochondrial Proteins

Quantitative information for a large number of mitochondrial proteins was obtained. The 28 and 39% decrease in the cellular content of the α - and β -chains of the enzyme mitochondrial ATP synthase in the high- V_T samples may indicate a disruption of energy metabolism in the AII cells by a brief period of high- V_T ventilation.

An increase in the mitochondrial precursor of pyruvate carboxylase was found in the low- V_T (but not the high- V_T) samples. This enzyme catalyzes the ATP-dependent carboxylation of pyruvate to form oxalacetate, which may be used in the synthesis of glucose, fat, the amino acids, aspartate, glutamate, and glutamine, and neurotransmitters, GABA and acetylcholine. The lack of an increased content of pyruvate carboxylase after high V_T could indicate an inability of AII cells after high V_T to replenish the oxalacetate used directly or indirectly via the tricarboxylic acid cycle for biosynthetic purposes (33).

Another important mitochondrial enzyme, glutamate dehydrogenase, was decreased by 61% in the high- V_T group. Glutamate dehydrogenase catalyzes the conversion of L-glutamate and water to 2-oxoglutarate and NH_3 in the presence of NAD^+ . The 60% reduction in the cellular content in glutamate dehydrogenase in the high- V_T group may point toward a reduced glutamate

metabolism after high V_T . In agreement with this finding, the results of a previous study indicate that sepsis induces significant decreases in the lung concentrations of glutamine (37%), glutamate (21%), 2-oxoglutarate (65%), and AMP (18%) in rats (34). Disruptions of glutamate metabolism have been implicated in several clinical disorders in brain, liver, and pancreas (35).

Structural Proteins

Many structural proteins have important roles in cell signaling and microvesicle formation in addition to maintaining the structural integrity of the cells. There were statistically significant reductions in the cellular content of cytoplasmic dyenin, lamin A and B1, annexin A2, actin, and spectrin. These changes were more pronounced in the high- V_T samples, but several proteins were altered in animals after both ventilation modes.

Annexins are a family of calcium- and phospholipid-binding proteins, which are structurally related and appear to be involved in membrane fusion and signal transduction (36). The 35% decrease in the concentration of annexin A2 in the high- V_T samples may therefore have functional consequences for the A2 cell. Recent studies suggest that annexin II is involved in the cytoskeleton reorganization in stimulated type II cells, therefore allowing surfactant-containing lamellar bodies access to the plasma membrane (37).

Our results indicate a 26% reduced concentration of the spectrin α -chain in the high- V_T group. Spectrin is known to be involved in secretion, interacts with calmodulin in a calcium-dependent manner, and is thus a candidate for the calcium-dependent movement of the cytoskeleton at the membrane. It contributes as well to clustering and regulation of proteins associated with the plasma membrane, control of morphogenesis and cell proliferation by organization of cell-cell contact regions, protein sorting and trafficking in Golgi apparatus and in intracellular vesicles, architecture of the nucleoplasm, and regulation of transcription factors in the nucleus (38). Also, interestingly, the putative pore-forming subunit of the rat epithelial (amiloride-sensitive) α epithelial Na^+ channel binds to α -spectrin *in vivo* (39).

Periplakin is a component of desmosomes and may serve as a link between desmosomes and intermediate filaments. Protein kinase B, a protein kinase mediating a variety of cell growth and survival signaling processes, is reported to interact with periplakin, suggesting a possible role for this protein as a localization signal in Akt1-mediated signaling (40, 41). A change in the intracellular content of periplakin could be one regulating factor in these signaling events. The increase in concentration we observed in the low- V_T samples could be a consequence of increased cell stimulation. Consequently the lack of such an increase in the high- V_T samples could indicate an inability of the A2 cell to compensate for the higher amount of stress in this condition.

Interestingly, the concentrations of lamins A and B were significantly reduced in both the low- and high- V_T samples. Both of the two main types of lamins that can be discerned (A and B) are intermediate filament proteins. Whereas B-type lamins are ubiquitously expressed in all animal cells, the expression of A-type lamins is low or absent in cells with a low degree of differentiation and/or in highly proliferating cells (42, 43). A-type lamins have been associated with several genetic disorders. Two hypotheses regarding the involvement of lamins have been considered: the structural hypothesis suggests that mutations giving rise to weakened association of lamins with the lamina lead to fragility of the nuclear envelope and its breakage, whereas the gene expression hypothesis proposes that some mutations give rise to altered associations of A-type lamins with transcription factors

(42). Thus, any positive pressure, whatever the magnitude, may have the capacity to affect lamins.

Peroxisomal Proteins

The cellular content in multifunctional enzyme (MFE) type 2 was significantly reduced in the high- V_T group. β -Oxidation of acyl-CoA in mammalian peroxisomes can occur via either MFE-1 or -2, both of which catalyze the hydration of trans-2-enoyl-CoA and the dehydrogenation of 3-hydroxyacyl-CoA. MFE-2 has recently attracted interest because of its role in lipid metabolism (44). Decrease of this enzyme after ventilation with high V_T s may be another indicator for defects in energy metabolism of the A2 cell. To our knowledge, there is no previous data on such a role in lung injury. The significance of these changes is unclear, but illustrates how a proteomics approach may identify proteomic changes that may be relevant in the pathogenesis of a disorder.

Limitations and Outlook

Despite these results, several limitations to this study must be acknowledged. The most important one is the relatively small number of experiments ($n = 3$) and animals ($n = 6$) per group. The experimental design employed was chosen due to the resources that are currently needed for this type of proteomic analysis. This study design, however, provides only limited information on the effects of interindividual and interexperimental variability on the results. Supplement PART 2 provides additional data on interexperimental variation (online supplement PART 2, Tables E2–E4). Of course, this technique provides information on only one cell type in the lung. Moreover, a large number of the cellular responses to stimuli are post-translational modifications of existing proteins that result in little or no changes in cellular protein content. A different sample preparation approach is required to target these, as the approach that we used is not sensitive to these changes (45). However, the results with a chromatography-based MS approach demonstrate that the application of quantitative proteomics to specific isolated cells is a valuable discovery tool for the elucidation of cellular reactions to stimuli. Because of its ability to evaluate changes in the content of a larger number of proteins in a single experiment, this methodology can provide new insights into the interplay of protein cascades. In future studies, protein quantification by stable isotopes could be complemented by a further fractionation of subcellular compartments and immunoprecipitation procedures for the specific comparison of post-translational modified proteins (46).

Conclusions

This study was designed to discover previously unknown alterations following the intracellular biochemical consequences of lung injury induced as a result of mechanical ventilation. After short-term positive-pressure ventilation, significant reductions of key proteins in A2 cells were identified and quantified. The identified proteins are known to be involved in cascades that have important functional consequences regulating intracellular signal transduction, alveolar edema fluid clearance, intracellular energy metabolism, and cell structure. Alterations in the cellular content of these proteins may have an important role in the development of ventilator-induced or -associated lung injury.

Conflict of Interest Statement: J.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.C.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.A.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.J.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. X.F. does not have a financial relationship with a commercial entity that has an interest

in the subject of this manuscript. J.C.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.B. is a director of Searchmaster Ltd. (UK), which has a contract with the University of California, San Francisco to develop ProteinProspector, the software used in this study. A.L.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.A.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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