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Lung Metabolic Activation as an Early Biomarker of the Acute Respiratory Distress Syndrome and Local Gene Expression Heterogeneity

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Abstract

Background—The acute respiratory distress syndrome (ARDS) is an inflammatory condition comprising diffuse lung edema and alveolar damage. ARDS frequently results from regional injury mechanisms. However, it is unknown whether detectable inflammation precedes lung edema and opacification, and whether topographically differential gene expression consistent with heterogeneous injury occurs in early ARDS. We aimed to determine the temporal relationship between pulmonary metabolic activation and density in a large animal model of early ARDS, and to assess gene expression in differentially activated regions.

Methods—We produced ARDS in sheep with intravenous LPS (10ng/kg/h) and mechanical ventilation for 20h. Using positron emission tomography, we assessed regional cellular metabolic activation with 2-deoxy-2-[(18)F]fluoro-D-glucose, perfusion and ventilation with ¹³NN-saline, and aeration using transmission scans. Species-specific micro-array technology was used to assess regional gene expression.

Results—Metabolic activation preceded detectable increases in lung density (as required for clinical diagnosis) and correlated with subsequent histological injury, suggesting its predictive value for severity of disease progression. Local time-courses of metabolic activation varied, with highly perfused and less aerated dependent lung regions activated earlier than non-dependent

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regions. These regions of distinct metabolic trajectories demonstrated differential gene expression for known and potential novel candidates for ARDS pathogenesis.

Conclusions—Heterogeneous lung metabolic activation precedes increases in lung density in the development of ARDS due to endotoxemia and mechanical ventilation. Local differential gene expression occurs in these early stages and reveals molecular pathways relevant to ARDS biology and of potential use as treatment targets.

Introduction

The acute respiratory distress syndrome (ARDS) affects approximately 190,600 patients/year in the United States¹, with mortality up to 45%². Despite this major burden, ARDS diagnosis has seen limited progress since its initial description in 1967³, and management is still based on supportive care with no established therapies targeted at the primary disease processes. Accordingly, there is pressing need for methods of early detection and treatment.

The conceptual model for ARDS portrays an acute, diffuse, inflammatory lung injury with increased pulmonary vascular permeability and lung density². Diffuse alveolar damage is the histopathological hallmark, and bilateral opacities on lung imaging, reflective of edema and loss of aeration, are required for clinical diagnosis². In contrast to those concepts of a diffuse process, local pathogenic mechanisms underlie the onset of ARDS particularly in functionally heterogeneous lungs such as those of humans. Mechanical ventilation, frequently used in patients at risk for ARDS, exposes lungs to spatially heterogeneous injury⁴ due to high tidal strain⁵, cyclic airway and alveolar recruitment⁶, stress concentration⁷, gas-liquid interfaces⁸, and overdistension⁹. Lung perfusion is also spatially heterogeneous in ARDS patients¹⁰, potentially influencing local trafficking of inflammatory cells and mediators¹¹. Consequently, localized inflammatory processes should be present and detectable during early ARDS before a diffuse injury pattern is established. However, investigations on the regional basis of molecular and inflammatory processes underlying that early pathogenic period are scant. If inflammation is the fundamental process leading to pulmonary edema, it should precede lung opacities as ARDS develops. Yet, there is no knowledge on the temporal relationship between detectable inflammatory changes and onset of increased lung density, the current radiographic diagnostic marker for ARDS. Better understanding of these key temporal and topographical processes may contribute to advance diagnostic and prognostic biomarkers, and effective therapies¹².

In studies using exaggerated tidal volumes and driving pressures during short periods (1.5-4h) of mechanical ventilation^{13, 14}, we suggested that lung metabolism reflective of inflammation measured with positron emission tomography (PET) using the tracer 2-deoxy-2-[(18F)]fluoro-D-glucose (¹⁸F-FDG) might be a valuable imaging biomarker of early lung injury. Yet, the significance of such biomarker for ventilatory settings and time frames comparable to those present in clinical practice is unknown. In the present study, we used a clinically relevant sheep model submitted to endotoxemia and mechanical ventilation over a long period (20h) using the standard ARDS-Net ventilation protocol¹⁵, to reprise the ‘two-hit’ injury often seen in patients. This large animal model allowed us to recapitulate the lung functional heterogeneity observed in humans, and account for pathophysiological

changes occurring over time frames relevant to human disease¹⁶⁻¹⁸. We combined multi-tracer PET and gene expression techniques to study molecular processes during the early development of ARDS. We hypothesized that lung cellular metabolism and gene expression manifest spatial and temporal heterogeneity in response to distinct regional mechanisms of injury, and precede density changes detectable with conventional radiography. Our aims were to: (a) investigate whether regional changes in pulmonary metabolic activity precede changes in lung density during early ARDS; (b) characterize the temporal trajectories of the components of that metabolic activity contributing to the regional changes, and the related regional lung dysfunction; and (c) determine whether regions with distinct metabolic activity exhibit corresponding differences in underlying gene expression.

Materials and Methods

Experimental Protocol

The experimental approach to imaging and tissue analysis is summarized in figure 1. Animal experiments were approved by the Subcommittee on Research Animal Care at the Massachusetts General Hospital. Six pathogen-free supine sheep (22.8±1.7 kg) were anesthetized, paralyzed, intubated and mechanically ventilated. Anesthesia was maintained with a continuous infusion of ketamine and propofol, and fentanyl boluse (1-2 mcg/kg). Paralysis was established with pancuronium (0.1 mg/kg bolus) at induction and repeated every 90 min (0.02-0.04 mg/kg). For monitoring and collection of blood samples, we percutaneously cannulated a femoral artery and introduced a pulmonary artery catheter via the jugular vein using sterile techniques.

Animals were supine in the PET scanner (Scanditronix PC4096; General Electric, Milwaukee, WI), with the caudal end of the field of view just superior to the dome of the diaphragm. Final positioning was determined after a recruitment maneuver with airway pressure at 40 cmH₂O for 30 seconds to standardize lung history. Sheep were mechanically ventilated for 20h using the ARDSNet lower positive end-expiratory pressure (PEEP) (low stretch) protocol¹⁵. Initial settings were: volume control mode, V_T=6ml/kg, PEEP=5 cmH₂O, inspired oxygen fraction (F_IO₂)=0.3 (adjusted to maintain an arterial O₂ saturation 90% measured by co-oximetry (OSM3, Radiometer, Copenhagen, Denmark)), inspiratory-to-expiratory ratio=1:2, initial respiratory rate (RR)=18 breaths/minute and adjusted to maintain the arterial carbon dioxide partial pressure (PaCO₂) between 32 and 45 mmHg.

Following collection of baseline physiological and imaging data, an infusion of endotoxin (10 ng/kg/min, *Escherichia coli* O55:B5, List Biologic Laboratories Inc, Campbell, CA) was started and continued for the whole experiment. In order to guarantee cardiovascular stability while maintaining continuity of the extrapulmonary inflammatory stimulus, an algorithm to manage hypotension with Ringers Lactate administration and reduction in endotoxin infusion rate was used (fig. S11). No additional recruitment maneuvers were performed. Physiological data were collected at baseline and at 4-hour intervals.

PET Imaging Protocol and Processing

The PET imaging equipment, protocol and processing methods have been previously presented in detail^{14, 19-21}. We collected 15 PET transverse slices of 6.5-mm thickness, which provide 3-dimensional information over a 9.7-cm-long field of view, estimated to encompass ~70% of the total sheep lung volume²¹. Resulting images consisted of an interpolated matrix of 128×128×15 voxels with a size of 2.0×2.0×6.5 mm each. Three different types of PET images were acquired:

- 1) *Transmission scans*: were obtained at baseline (0h), 6h, and 20h using a rotating pin-source of ⁶⁸Ge for 10 min. Transmission scans were used to correct for attenuation in emission scans and to calculate the fraction of gas (F_{GAS}) of regions of interest (ROIs) from regional tissue density (F_{tissue})²² as $F_{\text{GAS}} = 1 - F_{\text{tissue}}$.
- 2) *¹³N-nitrogen emission scans*: performed at baseline and at the end of the 20h mechanical ventilation period for assessment of regional ventilation, perfusion and shunt as previously reported with the ¹³N-nitrogen-saline method^{21, 23}. The imaging protocol started with a tracer-free lung. The ventilator was turned off at the end of inhalation, and the airway pressure equilibrated to a value equal to the mean airway pressure during ventilation. A 30–40 mL bolus of ¹³NN-saline solution was then centrally injected at a rate of 10 mL/s. Simultaneously, collection of a series of consecutive images was started. After an apnea period of 60s, mechanical ventilation was restarted. The total imaging sequence lasted 4 min and consisted of 8 frames of 2.5s and 4 frames of 10s during apnea, and 6 frames of 10s and 4 frames of 30s during the washout phase.
- 3) *¹⁸F-FDG emission scans*: after ¹³NN clearance, ¹⁸F-FDG dissolved in 8 ml saline (~40 MBq at 0 h, 200 MBq at 6 and 20h) was infused at a constant rate through the jugular catheter over 60 s. Starting at the beginning of ¹⁸F-FDG infusion, sequential PET frames (9×10s, 4×15s, 1×30s, 7×60s, 15×120s, 1×300s, 3×600s) were acquired over 75 min. Pulmonary arterial blood was sampled at 5.5, 9.5, 25, 37, and 42.5 min and plasma activity was measured in a well-counter in order to calibrate an image-derived input function for the three-compartment model²⁴. Regional ¹⁸F-FDG kinetics were fitted to a three-compartment model consisting of an intravascular compartment, a tissue compartment representing the concentration of ¹⁸F-FDG available for phosphorylation (i.e., ¹⁸F-FDG that is a substrate for hexokinase), and a metabolized compartment accounting for the concentration of ¹⁸F-FDG that has been phosphorylated by hexokinase^{25, 26}. In this analysis, the ¹⁸F-FDG net uptake rate (K_i), a measure of cellular metabolic activity, is expressed as: $K_i = F_e \cdot k_3$, where k_3 is the rate of ¹⁸F-FDG phosphorylation and F_e is the fractional distribution volume of ¹⁸F-FDG that is in the tissue but not phosphorylated. In addition, kinetic analysis provides constants k_1 (transfer rate from plasma to tissue), k_2 (transfer rate from tissue to plasma), and F_b (the blood volume fraction in the ROI).

Selection of Voxels for Analysis

Lung masks were created for each animal at three time points (baseline, 6h, and 20h) by automatic threshold selection combining aerated regions from transmission scans and perfused regions from ^{13}NN -perfusion scans, and subsequent manual refinement to exclude main bronchi and large pulmonary vessels. Masks were divided along the gravitational axis into three adjacent regions-of-interest (ROIs) of equal height (dependent, middle and non-dependent) to quantify regional F_{GAS} , ^{13}NN -saline and ^{18}F -FDG kinetics.

Transcriptome-wide Gene Expression Analysis

Tissue samples derived from dependent and non-dependent regions consistent with high and low ^{18}F -FDG uptake as derived from the PET images were analyzed using microarray analysis.

Total RNA was extracted using the Qiagen miRNeasy Mini Kit. RNA quality-control assessment was performed using the Agilent 2100 Bioanalyzer. Transcriptome-wide expression was measured with the Agilent Sheep Gene Expression Microarray²⁷ (Agilent Technologies, Santa Clara CA). This array is customized to assay 15,208 sheep transcripts represented in the NCBI RefSeq (Build 16) and UniGene (Release 27) databases.

For qT-PCR validation, total RNA was isolated from frozen lung tissue samples using an RNeasy Mini Kit (Qiagen) according to the manufacturer's standard protocol. During the isolation process, samples were incubated with DNase I (Qiagen) for 15 min at room temperature. The concentration and purity of the isolated RNA was assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific). TaqMan RT-PCR using QuantiTect Virus Kit (Qiagen) was performed in an ABI Prism 7300 Sequence Real-Time PCR System (Applied Biosystems) using universal thermal cycling parameters. Human-specific TaqMan gene expression assays (Applied Biosystems) were used to measure the mRNA levels for PAI-1, CXCL10 and 18S rRNA. Each sample was run in duplicate. Gene expression was analyzed using the comparative CT method with 18S rRNA as the endogenous control.

Histological Examination and Neutrophil Counts

Following euthanasia by exsanguination, the lungs were excised and filled with Trump's fixative to 27 cmH₂O. After fixation, tissue blocks from nondependent (ventral) and dependent (dorsal) regions were sampled and analyzed. Samples were collected based on images with differential FDG activity (low-nondependent, high-dependent).

Light microscopy—Five-micron hematoxylin-eosin sections were examined by two investigators blinded to the lung region using stereological point-counting technique²⁸ at a magnification of 400× across five random, non-coincident microscopic fields²⁹ for alveolar, septal and total lung neutrophils, alveolar edema and hyaline membrane.

Transmission electron microscopy—Three 2×2×2 mm slices cut from non-dependent and dependent regions were used to analyze (JEOL 1010 Transmission Electron Microscope, Tokyo, Japan) the alveolar capillary membrane, neutrophil mitochondria, neutrophil endoplasmic reticulum, and neutrophil lysosomes. Findings were graded with a 5-

point semi-quantitative severity-based scoring system as: 0=normal lung parenchyma, 1=changes in 1-25%, 2=26-50%, 3= 51-75%, and 4=76-100% of examined tissue²⁹.

Statistical Analysis

Data are mean±standard deviation if normally distributed and median and interquartile interval (IQR 25-75%) otherwise. Global cardiopulmonary variables were compared using repeated measures anova with post-hoc Tukey's test for normally distributed data and Kruskal-Wallis test otherwise (SPSS Statistics™ 21.0 for Mac). Imaging measurements were compared with analysis of variance using time, region and timexregion as main factors. Tukey's HSD was used for post-hoc analysis controlling for multiple comparisons (Matlab version R2015b, Mathworks, Natick, MA). All statistical tests were two-tailed, and significance set at $P<0.05$.

Results

Twenty hours of protective mechanical ventilation and endotoxemia produce global cardiopulmonary dysfunction and inflammation consistent with ARDS

Respiratory system compliance worsened over 20h, and oxygenation reached the range of moderate ARDS at 6h (fig. 2, table 1), despite increases in PEEP and $F_{I}O_2$ according to the ARDS-Net protocol. Total fluids administered were 5.0 (4.6-5.4) ml/kg/h. Circulating neutrophils and monocytes decreased by 6h and recovered by 20h (fig. 2, table S1).

Optical microscopy revealed infiltration of alveolar and septal neutrophils, alveolar edema, and hyaline membranes (fig. 2, fig. S1, table S2). Electron microscopy showed mild defects of the alveolar-capillary membrane, increased neutrophil lysosomes, and changes in neutrophil mitochondria and endoplasmic reticulum consistent with neutrophil activation (fig. 2, fig. S2, table S2).

Lung aeration is reduced and regional perfusion and ventilation-perfusion ratios are more heterogeneous after 20h of protective mechanical ventilation and endotoxemia

Whole-lung gas fraction was unaffected from baseline (0.64 ± 0.03) to 6h (0.66 ± 0.04), and decreased by 6% from 6 to 20h (0.62 ± 0.05 , $P<0.05$). Regional gas fractions were also unchanged from baseline to 6h (fig. 3A-B). F_{GAS} decreased in dependent regions only from 6 to 20h ($P<0.05$), despite a significant simultaneous increase in PEEP (fig. 3A-B, table 1). Throughout, dependent regions showed lower F_{GAS} (fig. 3B) and higher specific ventilation (=ventilation/volume, fig. 3C-D) than non-dependent regions. Differences in aeration distributions at the voxel level corroborated the regional heterogeneity in lung inflation (fig. S3). These inhomogeneities in local aeration and ventilation imply underlying regional inhomogeneities in the lung mechanical microenvironment.

Regional perfusion was heterogeneously distributed (fig. 3E-F), even after correcting for tissue density (fig. 3G), highest in dependent regions (fig. 3F-G). This heterogeneity increased with time (interaction effect for tissue normalized perfusion: ROI×time, $P=0.017$). Regional differences in perfusion accounted for significant ventilation-perfusion mismatch,

as mean ventilation/perfusion was lowest and shunt highest in dependent regions at baseline and 20h (fig. 3H-J).

Regional and global increases in pulmonary metabolic activity precede rises in lung density and correlate with histological injury

^{18}F -FDG kinetics revealed whole-lung increases in pulmonary metabolic activity (net ^{18}F -FDG uptake, K_i) already at 6h of endotoxemia and mechanical ventilation, suggestive of early neutrophilic activation. This preceded the increase in whole-lung density, the usual radiographic marker of ARDS, observed at 20h (fig. 4A-B). The rise in whole-lung ^{18}F -FDG phosphorylation rate (k_3 , fig. 4C) was a major factor underlying the increased net uptake.

Distinct temporal patterns of lung metabolism were present at the regional level (fig. 5A-B). At baseline, the initially healthy lungs showed homogenous metabolism, with no regional differences in k_3 (fig. 5C, fig. S4) or K_i normalized by tissue density (K_{iT} , fig. S5-S6). From 0-6h, K_i increased substantially throughout the lungs, particularly in dependent regions (fig. 5A-B), preceding any detectable lung density changes (fig. 5A-B, fig. S4). In contrast, during the 6-20h period, non-dependent and middle regions were the primary sites of increased metabolic activity, as evidenced by higher K_i and k_3 (fig. 5B-C). Of note, those early increases in regional k_3 (0-6h) were associated with later increases in lung density (6-20h, fig. 4D).

Examination of K_{iT} indicated that regional tissue density accounted for a minimal portion of the regional temporal changes in ^{18}F -FDG uptake (fig. S5), but significantly influenced the topographical distribution in ^{18}F -FDG uptake (fig. S5 and S6). Thus, by 20h, tissue-specific measures of metabolic activity (i.e., K_{iT} and k_3) were homogeneously increased in all regions relative to baseline. However, dependent, middle and non-dependent regions reached an elevated state of metabolism through distinct temporal trajectories. The association of these metabolic changes with lung aeration indicated differences in the time scale of injury development in regions of low aeration versus regions of high aeration (fig. 6).

Quantification of the determinants of ^{18}F -FDG-uptake ($K_i = \text{Fe} \cdot k_3$) indicated that its rise at 6h and 20h was due to a larger contribution of increased phosphorylation rate (k_3) (fig. 5C) combined with a rise in the ^{18}F -FDG volume of distribution Fe (fig. 5D). Measures of histological injury at 20h (lung injury score, septal congestion, and neutrophil counts) were correlated with k_3 at 6h, suggesting a value for early phosphorylation to signalize ensuing injury (fig. S7).

Gene expression patterns are regionally heterogeneous in early ARDS

We found regional differential expression of 24 distinct annotated genes of the ~15,000 specific RNA sequences represented in the microarrays (dependent *vs.* non-dependent, $P < 0.001$) (fig. 7A-B). Most of these (75%) showed higher expression in dependent regions. Four genes were previously associated with sepsis (CXCL10 in neonatal sepsis³⁰, CHI3L1³¹, complement 3 (C3)³², and ITGAL³³), potentially consistent with higher dependent lung perfusion and endotoxemia in the studied model. In contrast, three genes with higher non-dependent expression have been previously related to experimental VILI (PAI-1³⁴, AREG³⁵, and THBS1³⁶) suggesting regional activation of mechanotransduction

processes. The gene for CXCL10 demonstrated the most significant differential regional expression (6 separate sequences in the used microarray, systematically higher expression in dependent regions, $P<0.001$). The presence of hyperinflation in non-dependent regions (fig. S3) suggests a role for tissue overdilation in eliciting such cellular responses. In fact, expression levels of several VILI-associated genes were correlated with regional hyperinflation (fig. S8). Such findings support the concept of topographically distinct injury mechanisms during early ARDS. Analysis of differentially expressed genes ($P<0.01$, 174 distinct annotated genes) into gene ontology terms resulted in processes related to regulation of leukocyte activation, regulation of cytokine production, regulation of apoptosis, and immune response (DAVID software³⁷, Table S3).

Microarray results for a gene with higher differential expression in non-dependent regions (PAI-1) and another in dependent regions (CXCL10) were validated using the technique of semi-quantitative RT-PCR in samples from three sheep (fig. S9). There was clear agreement in the directions of gene expression ratios with median expression for PAI-1 $\log_2(E_{ND}/E_D)=-2.07$ ($P=0.048$), and for CXCL10 $\log_2(E_{ND}/E_D)=-3.64$ ($P=0.049$). Throughout, use of metabolic imaging to guide tissue sampling improved the sensitivity of the analysis by allowing for paired gene expression comparisons in regions of distinct metabolic temporal trajectories even when inter-animal gene expression variability was large (fig. S10).

Discussion

We demonstrate increased *in vivo* whole-lung and regional metabolic activation within the first 20h of lung injury produced by endotoxemia and mechanical ventilation, which preceded lung density increases routinely utilized for ARDS diagnosis. In lungs of size comparable to those of humans, temporal trajectories of metabolic activation were spatially heterogeneous, and co-registered with topographical heterogeneity in gene expression. Gene expression patterns not only revealed heterogeneous local activation of injury mechanisms previously reported in more homogeneous rodent models, but also identified novel mechanisms and treatment targets potentially relevant to human disease.

Molecular imaging with ^{18}F -FDG-PET quantifies glucose uptake, membrane transport, and metabolism by hexokinase. While increased cellular metabolism (^{18}F -FDG-uptake) has been observed in established ARDS in patients³⁸ and animals³⁹, little is known for conditions mimicking clinical settings in the early stages of lung injury. Our results revealed a marked and heterogeneous increase in lung metabolic activation as early as 6h from the onset of endotoxemia and mechanical ventilation. This contrasted with the lack of changes in global or regional lung aeration by 6h, and a small loss of aeration by 20h. Given that bilateral lung opacities are required for ARDS diagnosis², our results suggest that ^{18}F -FDG kinetics parameters could provide an earlier molecular marker of lung injury than conventional imaging.

The early regional increase in ^{18}F -FDG uptake was due to a rise in both ^{18}F -FDG phosphorylation rate (k_3) and volume of distribution (F_e) in dependent regions. k_3 has been shown to reflect hexokinase activity in tissues⁴⁰, whereas F_e depends on tissue density, neutrophil numbers, and edema²⁶. The contributions of k_3 and F_e to K_i showed substantial

regional variation, with larger k_3 contribution in non-dependent regions, and a balanced k_3 and F_e contribution in dependent regions. The early (6h) reduction in circulating neutrophils that accompanied the increase in ^{18}F -FDG-uptake suggests that those regional metabolic changes reflected increased neutrophil recruitment and activation. These results are consistent with our observed gene ontology, and with findings of regulation of phosphorylation and glucose transport, and neutrophil chemotaxis in a rat model of LPS-induced lung injury³⁴. A smaller metabolic contribution from macrophages, pneumocytes II, and endothelial cells can occur⁴¹. Overall, our findings imply that by the time ARDS is detected with conventional imaging (as required for diagnosis), the underlying inflammatory response may be already fully developed. Therefore, assessment of regional metabolism in the early stages of lung injury with ^{18}F -FDG-PET may be useful to predict disease progression and assess treatment efficacy.

Inflammatory and functional changes from 6-20h differed from those in the first 6h. Indeed, the increase in k_3 in non-dependent regions from 6-20h, indicative of inflammation (fig. 5C), would have been entirely missed if shorter periods had been studied. Accordingly, results from short-term experimental studies, frequently associated with exaggerated non-clinical ventilatory settings^{35, 42}, may be limited in characterizing the development of ARDS over time scales more representative of its clinical progression.

Importantly, structural and functional heterogeneities present in our model, and typical of ARDS patients, raise the possibility that the observed distinct local time courses of metabolism resulted from topographically heterogeneous injury mechanisms. Glycocalyx degradation in regions of increased blood flow and exposure to endotoxin could facilitate early local neutrophil trafficking, surfactant dysfunction, and lung derecruitment⁴³. Such mechanism could compose with cyclic airway and alveolar recruitment⁶, stress concentration⁷, gas-liquid interfaces⁸, high tidal strain⁵, and increased flow of inflammatory cells due to higher dependent lung perfusion in dependent lung regions to produce regional inflammation and injury. The ensuing lung collapse would result in increased regional lung strain in non-dependent lung regions where VILI-related gene expression was observed. Such findings are also consistent with the observed expression of genes associated with sepsis in the dependent regions, with higher perfusion per lung tissue, in this endotoxemia model. Given that most of the FDG-signal represents neutrophilic inflammation^{13,14,41}, we presume that local gene expression in response to injurious events would have occurred at the time the PET signal becomes detectable. Considering our use of a current clinical ventilation protocol, our results suggest that established clinical protective standards for mechanical ventilation¹⁵ do not entirely prevent deterioration of regional lung function and exacerbation of inflammation in the early stages of lung injury.

Using species-specific microarray technology, we detected differential regional expression of genes recently associated with human ARDS⁴⁴, sepsis⁴⁵, VILI³⁵, and LPS-induced lung injury³⁴. The differential expression of PAI-1 and AREG is consistent with activation of coagulation^{46, 47} and growth factor³⁵ pathways during VILI. Gene expression in dependent regions with early increase in FDG uptake pointed to processes associated with inflammation (e.g., CXCL10, C3, CHI3L1), recruitment of inflammatory cells (e.g., ITGAL), and possibly changes to basic cellular machinery that might result in alterations in

metabolism (e.g., FBXO32, LPL, BET1L, Aldh6A1) (all of which potentially reflected by PET). In nondependent later activated regions there was a preponderance of genes related to the matrix/cytoskeleton (e.g., MMP7, TIAM2, THBS), which might reflect distention consistent with colocalized larger gas volumes. Based on previous literature, we speculate that genes related to inflammation and structural components are upregulated^{34, 36, 42}. Instead, those related to the metabolic response may be either upregulated due to endotoxin exposure with higher perfusion in dependent regions³⁴, or downregulated due to stretch in nondependent regions⁴². Because our measurements express the gene expression ratio between the two studied lung regions, we are unable to determine activated vs suppressed genes. Since some identified genes have uncertain function and even those with described function might have additional as yet undetermined roles, the identified genes could serve as targets to be further explored to define gene expression regulation in the early stages of ARDS. Indeed, such identified differentially regulated genes not as yet associated with lung injury may represent novel therapeutic and biomarker targets.

The gene with the most significant differential expression was that of CXCL10, a chemokine which binds to the receptor CXCR3 to stimulate monocytes, natural killer, and T-cell migration, and modulate adhesion molecule expression. Whereas recent studies suggest that CXCL10 directly contributes to neutrophil-mediated pulmonary inflammation in viral and non-viral lung injury^{44, 48} and human mortality following septic shock⁴⁵, this cytokine has not yet been associated with VILI. Our preliminary murine studies (unpublished, R.M. Baron, MD, Department of Medicine - Pulmonary and Critical Care, Brigham and Women's Hospital, Harvard Medical School, Boston, MA; RT-PCR measurements of lung tissue expression of CXCL10 in control and VILI exposed mice) implicate CXCL10 in sterile inflammation resulting purely from mechanical processes, raising the possibility that the regional data in sheep could indicate that those same mechanisms contribute to early injury in dependent lung regions. This is potentially important given the distinct pathways governing mechanotransduction and the few available effective clinical interventions, which include strategies that minimize mechanical forces within the lung. Our results combined with recent findings^{30, 44, 48} suggest that CXCL10 may contribute to the local development of pulmonary neutrophilic inflammation in early ARDS in conditions frequently encountered in intensive care.

We also identified other potentially important molecular pathways, such as the C3 gene. C3 is indispensable in the complement cascade and was recently implicated in rodent VILI, representing a possible treatment target^{49, 50}. Interestingly, our differentially expressed genes overlap minimally with recent findings in exaggerated mechanical ventilation in rats^{46, 51}. This discrepancy may relate to the experimental model, applied mechanical forces (within clinical limits in our study), study duration, exposure to endotoxin, animal species, and tissue sampling locations.

Use of blood or whole-lung lavage samples to detect biomarkers of early ARDS could overlook localized changes in gene expression or metabolism, particularly in view of individual variability in gene expression (fig. S10). Topographic genomic analysis guided by functional and metabolic PET appeared highly effective in identifying genes and pathways with differential regional expression. While CT imaging has been previously used to direct

genomic analysis³⁶, guidance to localized biological processes should ideally be based on molecular imaging techniques sensitive to changes in relevant lung biology. In future studies, such approaches may greatly increase the efficiency and informational yield of gene expression studies on lung injury, potentially establishing a new paradigm for deriving mechanistic information, early biomarkers, and novel targets for drug development. The fact that our imaging methods are immediately applicable to patients^{52, 53} underscores their potential application for individualized patient care.

In conclusion, we show that increases in cellular metabolic activation are topographically and temporally heterogeneous in the early stages of lung injury in sheep with lung sizes and functional heterogeneities comparable to those of humans. The observed metabolic activation preceded increases in lung density, which are routinely used for clinical diagnosis of ARDS, and were accompanied by differential expression of genes and pathways known to be involved in lung injury, as well as potentially novel pathways that could be relevant for further understanding of acute lung injury and use as drug targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Brief Summary Statement

Lung metabolic activation precedes increases in lung density during early lung injury, and relates to the ensuing regional gene expression and histology. This offers a new paradigm for early understanding and detection of clinical ARDS.

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Fig. 1.

Schematic overview of the experimental approach. A two-hit model of the acute respiratory distress syndrome induced by mechanical ventilation and endotoxemia was studied. Positron emission tomography (PET) was used to assess distributions of regional lung aeration (^{68}Ge), ventilation, and perfusion (^{13}NN). ^{18}F -FDG kinetics in co-registered regions were analyzed to estimate parameters describing ^{18}F -FDG transport and metabolism and quantify the in vivo early inflammatory response. Gene expression levels in regions of distinct metabolic activation were measured to determine genes with differential regional expression. Finally, lung function, inflammation (i.e. metabolic activation), and gene expression were compared to determine potential mechanistic relations between these properties.

Fig. 2.

Twenty hours of mechanical ventilation with the ARDS-Net protocol and endotoxin infusion produces deterioration of lung function and gas exchange consistent with ARDS development. Positive end-expiratory pressure (PEEP) levels (A), respiratory system compliance (B), peripheral blood neutrophil counts (C), ratios of arterial O₂ pressure to inspired O₂ fraction (PaO₂/FiO₂) (D), pulmonary vascular resistance (PVR) (E), and blood monocyte counts (F) over 20h of mechanical ventilation and endotoxin infusion (*p<0.05 between time points). Cardiac output decreased at 6h, then recovered in parallel with a significant increase in PVR for similar pulmonary capillary wedge pressures, consistent with a vascular response to endotoxemia. Light microscopy showed hyaline membrane formation (G) as well as alveolar and septal neutrophils (H), consistent with acute inflammation. Under transmission electron microscopy, there was edema of alveolar-capillary membrane (I), and neutrophils showed increased activity and prominence of lysosomes (arrows, J).

Fig. 3.

Spatial heterogeneity in lung function following 20h of mechanical ventilation and endotoxemia in sheep reflects heterogeneous regional dysfunction typical of ARDS patients. Heterogeneous aeration (F_{GAS}) was observed at all time points (A), with lower aeration in dependent (D) regions than middle (M) or non-dependent (ND) regions (B). Distributions of F_{GAS} , and consequently of lung density, were stable over time, with only dependent F_{GAS} decreasing from 6-20h. Specific ventilation (sV) was also heterogeneous (C), with higher values in middle and dependent regions (D). Regional perfusion showed a similar

gravitational gradient to sV (E), which became more pronounced over time (F), even when normalized for tissue (G). At both time points, V/Q ratios were significantly lower in dependent regions (H, I), where the majority of shunt was localized (J). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for regional differences. # $p < 0.05$, ## $p < 0.01$ for differences between time points for the same region.

Fig. 4.

Whole-lung metabolic activation precedes increases in lung density during early experimental ARDS. Lung density, an important diagnostic measure of ARDS, presented a discrete increase from 6 to 20h of endotoxemia and mechanical ventilation (A). In contrast, a notable rise in the whole-lung ^{18}F -FDG net uptake rate (K_i) was already evident at 6h of injury and continued to increase through 20h (B). Increased ^{18}F -FDG phosphorylation rate (k_3) was an important determinant of that rise in K_i (C). Early regional changes in k_3 correlated with subsequent changes in regional lung density (D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 5. Local lung inflammation, assessed from ^{18}F -FDG kinetics parameters, demonstrated distinct temporal trajectories during early experimental ARDS. The intensity and spatial heterogeneity of the ^{18}F -FDG net uptake rate (K_i) increased with time (A). During the first 6h of injury, K_i increased significantly in all lung regions and heterogeneity of K_i became more pronounced than at baseline (A, B). From 6-20h, K_i continued to increase only in non-dependent and middle regions. Quantification of the determinants of ^{18}F -FDG-uptake ($K_i = k_3 \cdot F_e$; k_3 = phosphorylation rate; F_e = ^{18}F -FDG volume of distribution) show the

compound nature of those local metabolic changes. (C) k_3 increased significantly in middle and dependent regions from 0-6h followed by a rise in non-dependent regions from 6 to 20h. These findings indicate an acute early development of inflammation in middle and dependent lung regions, with subsequent activation in non-dependent regions only after 6h. Increases in the distribution volume of ^{18}F -FDG predominated in dependent regions, where they occurred throughout the experiment with larger magnitude (D), associated with lung derecruitment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 6.

Association between early and late change in phosphorylation rate k_3 and regional aeration. During the early phase (0-6h, **A**), regions of low F_{GAS} (averaged over that period) showed larger increases in k_3 relative to baseline values, while during the late phase (6-20h, **B**), regions of higher F_{GAS} showed larger increases in k_3 . This reversal of the dependence of metabolic activity on lung aeration suggests that mechanisms of low-volume injury are associated with the initial inflammatory response followed by mechanisms of high-volume injury. The effect of F_{GAS} during each phase was analyzed using a linear mixed-effects model, including F_{GAS} as a predictor variable and random intercepts for animals. Symbols are: $\square$ = dependent, $\circ$ = middle, and $\triangle$ = non-dependent regions.

Fig. 7.

Differential regional gene expression between regions of the lung with distinct function and metabolism during early experimental ARDS. Tissue samples from non-dependent (ND) and dependent (D) lung regions were analyzed using species-specific microarrays (A). The heatmap (B) shows relative expression in non-dependent vs. dependent regions (E_{ND}/E_D) on a $\log_2$ scale for genes with differential expression at $p < 0.001$ in six sheep (s1-s6). Positive values (green) indicate higher non-dependent expression and negative (red) values indicate higher dependent expression.

Table 1

Respiratory and cardiovascular variables at baseline and after 6 and 20h of mechanical ventilation and endotoxemia.

	Baseline	6h	20h	P
<i>Respiratory Variables</i>				
F _I O ₂ (%)	40 (40 – 43)	40 (40 – 53)	60 (40 – 80) [¶]	0.04
V _T (mL.kg ⁻¹)	6.5 (6.3 – 7.1)	6.4 (6.2 – 6.9)	6.5 (6.1 – 7.0)	0.70
PEEP (cmH ₂ O)	5 (5 – 8)	7 (5 – 10)	10 (8 – 13) ^{*,¶}	0.01
RR (min ⁻¹)	33 (28 – 36)	35 (33 – 36)	31 (30 – 36)	0.59
P _{Peak} (cmH ₂ O ⁻¹)	17 ± 3	21 ± 4 [*]	28 ± 6 ^{*,¶}	0.01
P _{Plat} (cmH ₂ O ⁻¹)	14 ± 3	18 ± 2 [*]	24 ± 5 ^{*,¶}	0.01
C _{rs} (mL.cmH ₂ O ⁻¹)	19.7 ± 4.6	14.9 ± 2.3 [*]	11.4 ± 3.0 [*]	0.01
pH _a	7.41 ± 0.06	7.32 ± 0.09 [*]	7.28 ± 0.08 [*]	0.01
PaCO ₂ (torr)	46 ± 2	47 ± 13	44 ± 7	0.82
PaO ₂ (torr)	133 ± 45	76 ± 26 [*]	95 ± 29	0.01
PaO ₂ /F _I O ₂	324 ± 122	177 ± 77 [*]	180 ± 100 [*]	0.01
PvO ₂ (torr)	49 (45 – 53)	48 (34 – 67)	48 (46 – 57)	0.96
HCO ₃ ⁻	29 ± 4	24 ± 6	20 ± 4 [*]	0.01
EtCO ₂	43 ± 3	39 ± 5	33 ± 4 ^{*,¶}	0.01
<i>Cardiovascular Variables</i>				
HR (beats.min ⁻¹)	166 ± 35	127 ± 27 [*]	155 ± 22 [¶]	0.02
MAP (mmHg)	96 (89 – 98)	78 (75 – 102)	76 (65 – 89) [*]	0.03
MPAP (mmHg)	17 ± 4	21 ± 4	20 ± 4	0.17
PCWP (mmHg)	4 ± 3	5 ± 2	4 ± 3	0.19
PVR (dynes.sec.cm ⁻⁵)	191 (173 – 281)	400 (374 – 704) [*]	329 (255 – 366)	0.02
CO (L.min ⁻¹)	5.0 ± 1.1	2.8 ± 1.1 [*]	4.3 ± 1.4	0.03
SV	30 (26 – 33)	24 (14 – 28)	25 (21 – 35)	0.15
Lactate (n = 4)	1.8 (0.8 – 3.1)	1.0 (0.8 – 1.9)	1.2 (0.7 – 1.8)	0.63
Temperature (°F)	100.8 ± 0.8	100.3 ± 1.7	98.6 ± 1.6 [*]	0.01

Variables are expressed as mean ± SD for normally distributed variables and median and interquartile range (25 – 75%) otherwise.

F_IO₂ = inspired fraction of oxygen, V_T = tidal volume, PEEP = positive end-expiratory pressure, RR = respiratory rate, P_{Peak} = peak inspiratory pressure, P_{Plat} = plateau airway pressure, C_{rs} = compliance of the respiratory system, PaCO₂ = arterial partial pressure of carbon dioxide,

PaO₂/F_IO₂ = ratio between arterial partial pressure of oxygen and F_IO₂, PvO₂ = mixed venous partial pressure of oxygen, HCO₃⁻ = bicarbonate, EtCO₂ = end-tidal carbon dioxide, HR = heart rate, MAP = mean arterial pressure, MPAP = mean pulmonary arterial pressure, PCWP = pulmonary capillary wedge pressure, PVR = pulmonary vascular resistance, CO = cardiac output, and SV = stroke volume

^{*} p 0.05 (vs. baseline),

$\frac{f}{p} = 0.05$ (vs. 6 h).

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