Gene expression profiling of target genes in ventilator-induced lung injury

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Dolinay, Tamás, Naftali Kaminski, Martina Felgendreher, Hong P. Kim, Paul Reynolds, Simon C. Watkins, Dörte Karp, Stefan Uhlig, and Augustine M. K. Choi. Gene expression profiling of target genes in ventilator-induced lung injury. Physiol Genomics 26: 68–75, 2006. First published April 25, 2006; doi:10.1152/physiolgenomics.00110.2005.—In the lungs, high-pressure mechanical ventilation induces an inflammatory response similar to that observed in acute respiratory distress syndrome. To further characterize these responses and to compare them with classical inflammatory pathways, we performed gene expression profiling analysis of 20,000 mouse genes in isolated blood-free (to exclude genes from sequestered leukocytes) perfused mouse lungs exposed to low-pressure ventilation (10 cmH2O), high-pressure ventilation (25 cmH2O, overventilation), and LPS treatment. A large number of inflammatory and apoptotic genes were increased by both overventilation and LPS. However, certain growth factor-related genes, as well as genes related to development, cellular communication, and the cytoskeleton, were only regulated by overventilation. We validated and confirmed increased mRNA expression pattern of five genes (amphiregulin, gravin, Nur77, Cyr61, interleukin-11) by real-time PCR; furthermore, we confirmed increased protein expression of amphiregulin by immunohistochemistry and immunoblotting assays. These genes represent novel candidate genes in ventilator-induced lung injury.

overventilation; acute respiratory distress syndrome; gene expression analysis

Despite intensive investigations, the mortality of acute respiratory distress syndrome (ARDS) remains high (1a). Patients at risk for ARDS as well as ARDS patients themselves (13, 20) are prone to ventilator-induced lung injury (VILI) and benefit from low tidal volume ventilation (1a). Clinical and animal studies suggest that the release of proinflammatory cytokines and other factors during alveolar overdistension contributes to lung injury in VILI (1a, 9, 12, 17). One important insight in this area is that ventilation with high distending pressures (overventilation) may activate, if by different signaling mechanisms, canonical inflammation pathways that also become activated by well-known proinflammatory stimuli such as bacterial endotoxin (31). For instance, both overventilation and LPS activate nuclear factor-κB (NF-κB) (17, 32) and mitogen-activated protein kinase (MAPK) (33), which subsequently activate chemokines, cytokines, and adhesion molecules (32). One important objective is to identify genes that are specifically activated by overventilation but not by endotoxin. Such genes might provide specific targets to reduce the side effects of mechanical ventilation without interfering with the innate immune system. Microarray technology allows us to study gene expression of a significant fraction of the genome and has been used to identify candidate genes critical to a variety of lung diseases including chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, lung cancer, pulmonary hypertension, and lung transplantation (2, 8, 24, 36, 37). However, application of this approach to ventilator-induced gene expression in vivo is complicated by at least two factors: 1) in intact noninjured animals, alveolar overdistension is limited by both the chest wall and adjacent alveoli, and 2) infiltrating inflammatory cells may alter the gene expression profile. These two problems are circumvented in isolated blood-free perfused lungs. Therefore, in the present study, we have used microarray analysis to study the effect of overventilation (OV) on gene expression and compared this gene expression pattern to that induced by LPS (LPS) and the combined effects of OV and LPS (OV/LPS). We identified five new candidate genes in the OV group and hypothesize that the gene products of amphiregulin (Areg), A protein anchor protein 12, gravin (A kinase anchoring protein-12; Akap12), nuclear receptor subfamily 4, group A, member 1 (Nur77), cysteine-rich protein-61 (Cyr61), and interleukin-11 (Ii11) are involved in the pathogenesis of VILI.

Materials and Methods

Isolated, perfused, and ventilated mouse lung. We used male BALB/c mice for the experiments (weight 22–30 g, n = 26). The animals were anesthetized with 160 mg/kg pentobarbital sodium (Nembutal; Wirtschaftsgenossenschaft Deutscher Tierarzte, Hanover, Germany). After, a tracheostomy was performed and a cannula was inserted in the trachea. We ventilated the animals through the cannula with 90 breaths/min with a 200-μl tidal volume of room air using a rotary vane compressor pump (VCM; Hugo Sachs Electronic, March-Hugstetten, Germany). Subsequently, a laparotomy was performed and the diaphragm was removed. The mice were heparinized and exsanguinated, and the abdomen was removed. A ligature was put around the pulmonary artery and the aorta. Cannulas were inserted in the left atrium and the pulmonary artery, and the vessels were constantly perfused with RPMI 1640 tissue culture media supplemented with 4% bovine serum albumin (Biochrom, Berlin, Germany). The flow rate of the perfusate was 1 ml/min, generated with a peristaltic pump (Ismatec MS Reglo). Then the thorax was removed,
and a chamber was placed over the animal. Once the chamber lid was closed, we ventilated the mice with negative pressure for 1 h with an end-expiratory pressure of −3 cm H2O and an end-inspiratory pressure (EIP) of −10 cm H2O. Every 5 min, the lungs were recruited automatically with −20 cm H2O (TCM; Hugo Sachs Elektronik) (34). Subsequently, they were randomized in four groups and ventilated for the next 3 h (n = 5–8/group). Groups are as follows: control (C), ventilation with −10 cm H2O EIP; OV, overventilation with −25 cm H2O EIP; LPS, ventilation with −10 cm H2O EIP in the presence of 1 μg/ml Salmonella enterica Serovar abortus equii (Sigma, St. Louis, MO); OVLPS, LPS treatment and OV (14). The Animal Care and Use Committees of the University of Pittsburgh and the Research Center Borstel approved all experimental procedures. At the end of the experiment, the lungs were harvested, snap frozen, and stored at −80°C. Total RNA and protein were extracted from these tissues as described below.

Microarray analysis. Total RNA was extracted from lung tissue with Trizol (Invitrogen, Carlsbad, CA) (27). Labeled complementary RNA was generated and hybridized to CodeLink Uniset I bioarrays as recommended by the manufacturer [General Electric (formerly Amersham Biosciences), Piscataway, NJ] and previously reported by us (24). All arrays were normalized using the CyclicLoess method (35). For analysis, we filtered out genes that did not pass the manufacturer’s recommendation for quality control. Genes were normalized to their controls and log2 base transformed. In a first set of experiments, we used 10K CodeLink bioarrays containing 10,500 mouse genes (n = 3 replicates/group). For a confirmatory analysis, we used 20K CodeLink bioarrays containing 20,000 mouse genes; n = 4 biological replicates of controls, n = 4 biological replicates of LPS group, n = 3 biological replicates of OV group, and n = 5 biological replicates of OVLPS group. After filtering, a total of 9,137 valid genes were obtained for analysis that were present in both sets. The data from the two gene sets could not be directly merged because the baseline values of gene expression can significantly differ in CodeLink single-color oligonucleotide arrays. We normalized both sets to their controls and present the data side by side in a heat map format.

Statistical analysis was performed by the significance analysis of microarrays (SAM, Stanford, CA) program and Scoregene software package (Scoregene Package, available at http://compbio.cs.huji.ac.il/scoregenes/). In SAM, 200 permutations of the data were generated, and significant differences between treatment groups were determined with t-statistics (P < 0.1). The false discovery rate (FDR; P value < 0.1) method was applied to correct for multiple testing (30). Additionally, we used the nonparametric threshold number of misclassification score (TNOM); genes with TNOM = 0 were considered significantly changed (3). We ran the SAM analysis on each data set separately and chose genes that were significantly changed in both data sets. Two thousand eighty-five genes were found. The data discussed in this publication have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO: http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number GSE4215.

Functional annotation analysis. LPS/C, OV/C, and OVLPS/C ratios were created for 9,137 valid genes in both gene sets and OVLPS/C for the second set gene. Genes that passed t-test (P < 0.05) in fold change were selected using the MultipleLabelTest program from the Scoregene package (25). To obtain information about the function distribution of the gene sets, we downloaded the mouse gene ontology (GO) functional annotations from the Source website (http://www.source.stanford.edu). Using Entrez Gene ID (formerly LocusLink ID), we matched cellular functions with genes present in our gene sets. Statistical significance was determined using a hypergeometric model and corrected for multiple testing using FDR methods (24). A 95% confidence interval was set for the FDR correction to correct for multiple testing.

Real-time TaqMan PCR and enzyme-linked immunosorbent assay. Quantitative RT-PCR was performed on the first set of samples for five genes with increased expression after OV (n = 3/group). TaqMan PCR was executed as described previously (23). Commercially available Assay-on-Demand primer probe sets (Applied Biosystems, Foster City, CA) were used for Il1β (Mm00434162_m1), Arg1 (Mm00437583_m1), Akap12 (Mm00513511_m1), Nur77 (Mm00439358_m1), and Cyp6l1 (Mm004487498_m1). Gene expression was measured relative to an endogenous reference gene, mouse β-glucuronidase (β-GUS). The results were log2 base transformed, and the arithmetical means of three measurements were compared. Results are presented as means ± SE.

Perfusion buffer was sampled every 30 min, and IL-6 levels were measured for control, LPS, OV, and OVLPS treatments with Quantikine ELISA (R&D Biosystems, Minneapolis, MN) in the second set of samples (n = 3–5/group). Kruskal-Wallis test was performed for both assays for multiple group comparison, and intergroup differences were analyzed with Wilcoxon’s rank sum test with SPSS statistics software (SPSS, Chicago, IL). The significance level was set at P < 0.05.

Tissue immunohistochemical staining. Lung tissues were fixed with 2% paraformaldehyde, merged in 30% sucrose overnight, and snap frozen. Sections of lung were prepared, and immunostaining was performed as described (27). Amphiregulin goat polyclonal IgG primary antibody was used (Santa Cruz Biotechnology, Santa Cruz, CA). A representative picture per group was taken with an Olympus BX51 Fluorescent microscope (Olympus America, Melville, NY). Western blotting. Protein was extracted from whole lung tissue as described previously (23). The total protein concentration was determined with a Coomassie Plus 200 Protein Assay (Pierce, Rockford, IL). Western blot analysis was carried out as previously described for amphiregulin (21). The antibodies were the same as used for immunostaining.

RESULTS

Cytokine release. The isolated perfused and ventilated mouse model permits one to study the effects of high-pressure mechanical ventilation without experiencing significant blood pressure drop or neutrophil sequestration, which may complicate the interpretation of results of in vivo experiments. To illustrate proinflammatory responses in our model, we measured IL-6 levels every 30 min in the perfusate buffer (Fig. 1).

Fig. 1. IL-6 levels in the perfusate buffer. Cytokine levels were measured in 30 min from time point 0 to 240 min. Every time point represents the average measurement ± SE from lungs later used for the second set of microarray experiments. ● Control (C), n = 4; ■ LPS treatment (LPS), n = 5; ▲ overventilation (OV), n = 3; ♦ LPS and OV (OVLPS), n = 5. At 240 min, IL-6 levels were significantly increased in each treatment condition compared with controls: P value < 0.016, LPS vs. C; P < 0.029, OV vs. C; and P < 0.016, OVLPS vs. C.
The IL-6 levels were significantly increased after LPS, OV, and OVLPS treatments compared with controls.

Gene expression profiling. We compared the gene expression patterns of \( /H_1\) 10002, 10 cmH\(_2\)O EIP ventilated animals (controls), LPS-treated animals, and \( /H_1\) 25 cmH\(_2\)O EIP ventilated mice (OV) by microarray analysis. In our first set of microarray experiments, the lungs of the animals in the different treatment groups exhibited distinct gene expression profiles. To confirm these findings, we repeated the microarray experiment on a larger data set and included the cotreatment group (OVLPS). The combined heat map of the two sets of arrays is shown in Fig. 2. Heat map was created using Genomica microarray visualization tool (http://www.genie.rockefeller.edu/genomica). To avoid possible artifacts in expression patterns caused by the use of different batches of bioarrays, we normalized the expression of genes in each set of arrays to its own controls. However, this approach does not permit us to directly merge the two data sets. Our observations are based on identical patterns of gene expression changes in both sets of data. Therefore, when showing expression changes, we mark the data set in which it was detected and we represent data from both data sets to validate our results. Using two different statistical programs, SAM and Scoregene, we identified genes with significantly different expression levels after LPS and/or OV treatment compared with controls. For the complete list of genes, see Supplemental Table E1 (Microsoft Excel 2000 file format; the online version of this article, at the Physiological Genomics web site, contains supplemental data).

On the basis of the larger (second) set of microarrays, LPS treatment upregulated the expression of 396 genes and downregulated 596 genes. OV resulted in the increased and decreased expression of 201 genes and 196 genes, respectively. OVLPS regulated the most genes: 866 genes were up- and 991 were downregulated. Among these, the expression of cytokines, transcription factors, and apoptosis-related genes was increased. LPS and OV reduced the expression of certain well-known protein kinases, cell cycle regulators, transcription factors, and growth factors. Most genes were regulated in the same direction by all three treatments (increased or decreased expression). However, a small group of genes was discordantly regulated by one treatment condition. These genes show a gene expression profile unique to the treatment condition. LPS, OV, and OVLPS regulated discordantly 109, 185, and 224 genes, respectively. The number of genes regulated coordinately and discordantly is shown in Fig. 3. To find genes regulated only by OV in both data sets simultaneously, with no significant change in the LPS treatment group, we used \( t \)-tests (Multiple-LabelTest, Scoregene Package; \( P \) value < 0.05). A total of 27 genes were found. The list of these genes with corresponding fold changes is shown in Supplemental Table E2. Of these genes, the expression of 18 genes was increased and that of 9 genes decreased. Induced were genes of growth factors, intracellular transport, cytokine ligands, transcription factors, cellular matrix proteins, and development. The genes with decreased expression featured developmental and cellular matrix-related genes. Further regulation was observed when LPS was given to overventilated lungs. OVLPS/C fold changes are also shown in Supplemental Table E2. In general, the OVLPS group showed higher expression levels than OV or LPS alone.

![Hierarchical clustering of 9,137 valid genes.](image)

Fig. 2. Hierarchical clustering of 9,137 valid genes. The 4 groups of animals exhibited distinct gene expression patterns. Each narrow column represents 1 animal. Every row represents 1 gene. Orange, genes with increased expression; blue, genes with decreased expression; white, genes without expression change. Column order from left to right: control (C) first set \((n = 3)\), C second set \((n = 4)\), LPS treatment first set \((n = 3)\), LPS second set \((n = 5)\), OV treatment first set \((n = 3)\), OV second set \((n = 3)\), OVLPS second set \((n = 5)\). Visualization of the analysis was done using the Genomica program.

![Genes regulated coordinately and discordantly after LPS and/or OV.](image)

Fig. 3. Genes regulated coordinately and discordantly after LPS and/or OV. LPS, high-pressure mechanical ventilation, and the combination of the 2 treatments differentially regulated genes in the second microarray data set. Intersections of the diagram show the no. of coordinately regulated genes after a combination of treatments. Genes outside of intersection displayed expression changes in the opposite direction, unique to the treatment condition.
These genes were also affected by OVLPS. OV treatment significantly modified the expression of genes involved in growth factor activity. Immune response genes were induced more by LPS than by OV treatment, and LPS primarily regulated more ribosome-related genes. Stress response genes were not affected by OV. The list of cellular functions with the corresponding $P$ values and the number of statistically significant genes per functional annotation found among the 9,137 valid genes is included as Supplemental Table E3.

**Genes with significant changes in expression.** To further access the difference in gene expression between OV and LPS treatments, we decided to validate the RNA expression of five target genes: Areg, Akap12, Cyr61, Nur77, and Il11. These genes had significantly increased expression (MultipleLabelTest, Scoregene Package; $P$ value < 0.05) after only OV. Table 1 shows gene expression fold changes in the LPS and OV treatment groups compared with controls. The expression patterns of both data sets of microarrays agreed well. OVLPS treatment further increased the expression of Areg, Akap12, and Nur77 compared with OV. Cyr61 was not affected by OVLPS, and Il11 induction was similar to that seen with OV.

The increased mRNA expression of these five genes was validated by RT-PCR in the samples from the first set of lungs (Fig. 5). Our findings were further substantiated by immunoblotting for amphiregulin. Amphiregulin protein expression was increased in the OV group compared with the control and LPS treatment groups (Fig. 6). Finally, we used the same antibodies to immunostain the lungs of the three experimental groups (Fig. 7). Lungs subjected to OV showed increased amphiregulin staining compared with controls and LPS-treated lungs with primarily epithelial localization.

**DISCUSSION**

Cumulating experimental and clinical evidence suggests that VILI is triggered by mechanical overstretch of the alveoli, frequently facilitated by other causes of acute lung injury such as microbial agents (9, 12, 17). However, the extent to which overstretch and microbial agents activate similar or separate intracellular mechanisms is only partly established. We approached this problem by comparing the gene expression profiles of overventilated and LPS-treated mouse lungs. We identified genes whose expression was modified by OV and/or LPS. Many genes, in particular those that code for cytokines, transcription factors, and apoptosis-related proteins, were altered by both OV and LPS, suggesting the presence of common intracellular signaling pathways. However, the expression of some genes including growth factors, metabolizing enzymes, mediators, and cytoskeletal proteins was modified by alveolar

![Functional grouping of genes after lung injury.](image)

**Table 1. Gene expression fold changes of 5 target genes**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Fold Change LPS/C First Set</th>
<th>Fold Change OV/C First Set</th>
<th>Fold Change LPS/C Second Set</th>
<th>Fold Change OV/C Second Set</th>
<th>Fold Change OVLPS/C Second Set</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphiregulin</td>
<td>2.47</td>
<td>9.35</td>
<td>2.11</td>
<td>5.03</td>
<td>6.43</td>
</tr>
<tr>
<td>A protein anchor protein (gravin)-12</td>
<td>3.83</td>
<td>10.07</td>
<td>3.4</td>
<td>2.85</td>
<td>5.35</td>
</tr>
<tr>
<td>Cystein-rich protein-61</td>
<td>1.1</td>
<td>3.18</td>
<td>1.11</td>
<td>3.35</td>
<td>1.93</td>
</tr>
<tr>
<td>Nuclear receptor subfamily-4, A, member 1</td>
<td>0.85</td>
<td>2.7</td>
<td>1.15</td>
<td>4.47</td>
<td>6.51</td>
</tr>
<tr>
<td>Interleukin-11</td>
<td>1.74</td>
<td>4.08</td>
<td>1.22</td>
<td>5.25</td>
<td>3.89</td>
</tr>
</tbody>
</table>

Changes were significant only after overventilation (OV/C) and not LPS (LPS/C) in both data sets. The combined effect of overventilation and LPS (OVLPS/C) is also shown.
distension alone. It is tempting to speculate that these genes activate mechanisms different from the well-known lung inflammatory pathways that have been established for microbial infections.

The analysis for common cellular functions (Fig. 4) provided important insights into the types of genes that are affected by OV and/or LPS. Of particular interest is the fact that many of the genes regulated by OV and/or LPS are involved in the regulation of immune responses, inflammation, stress responses, and cytokine activity. These findings lend further evidence to the biotrauma hypothesis (9). Another group of genes affected by OV was related to apoptosis. Epithelial apoptosis has been observed in both in vitro and in vivo models of VILI (18, 19). The genes identified in this study include genes involved in the FAS ligand and the caspase pathway. Other responsive affected genes are related to the nervous system, pointing to a possible neuroinflammatory axis. For example, expression of the neuropeptide preprotachykinin-1 was related to acute lung injury in a mouse VILI model (5). Furthermore, Hayashi et al. (16) have reported that, in

Fig. 5. mRNA expression by microarray and RT-PCR. A: amphiregulin (Areg). B: A protein anchor protein (Akap12). C: cystein-rich protein-61 (Cyr61). D: nuclear factor subfamily 4, group A, member 1 (Nur77). E: Il-11 expression was significantly increased after OV compared with control. Samples from the first set of microarray are used (n = 3/group). Values represent means ± SE. *Statistical significance, OV vs. C: P < 0.05.
patients with spinal muscular atrophy, mechanical ventilation appears to contribute to neurodegeneration.

Validation of the data and comparison with previous studies. Microarray analysis generates a huge amount of data, and hence validation is an important part of the analysis. In the present study, RT-PCR analysis further substantiated the expression pattern of five genes. The expression of amphiregulin was assessed on the protein level as well. Another form of validation is possible by comparison with previous studies. The isolated perfused mouse lung model is well characterized, and production of IL-6, tumor necrosis factor (TNF), granulocyte monocyte colony-stimulating factor (GM-CSF; unpublished data), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), cytokine-induced neutrophil chemoattractant (KC), and MIP-2α in response to OV has been documented (31). According to the gene array, the induction of these genes by OV in relation to controls was as follows: MCP-1 (Ccl2), 3.15-fold; MIP-1α (Ccl3), 6.74-fold; IL-6, 4.71-fold; GM-CSF (csf2), 2.76-fold; and TNF, 1.18-fold (MIP-2α was not present on the chip). Thus the protein release data are well corroborated by the gene expression data, although, due to the small number, statistical significance was not reached in both data sets. The low expression of TNF-α in the array is explained by the fact that this gene is only transiently expressed after OV and is already on the decline after 180 min.

So far, only three gene array studies on ventilator-induced gene expression have been published (1, 10, 15). It should be noted that all of these studies have been performed in vivo, where it cannot be excluded that at least some genes that appear to be increased in the lung tissue are in fact only increased because they are present in infiltrating leukocytes. Thus our study is complementary to those previous studies in that it provides a list of genes that are regulated independently of leukocyte infiltration and also independently of the central nervous system. In future work, it will be important to dissect these differences.

Grigoryev et al. (15) analyzed lung tissue from mice ventilated in vivo for 2 h with 15 ml/kg. Of the 69 genes for which they provided detailed data, 62 were also detected by our analysis (15). The correlation for the mouse data between these two studies is highly significant (Rho = 0.57, P = 0.02). Correlation is detailed in Supplemental Fig. E1, and corresponding fold changes are shown in Supplemental Table E4 (Microsoft Excel 2000 file format). Whereas only a few genes reached statistical significance in both studies if analyzed within the context of the array, taken together, these studies provide a robust set of genes induced by OV including amphiregulin, activation transcription factor-3 (ATF3), MCP-1 (Ccl2), tissue factor (F3), growth arrest and DNA damage-inducible 45A (GADD45A), glutamate-cysteine ligase, heat shock protein A8 (HSPA8), IL-6, urinary plasminogen activator receptor, and cyclooxygenase-2. Consistent downregulation

Fig. 6. Amphiregulin protein expressions. Amphiregulin protein expression increased after OV compared with LPS and control treatments.

Fig. 7. Immunohistochemical staining of lung tissue. Lung sections were stained for amphiregulin. Red stain, immunofluorescent-labeled amphiregulin antibody; blue stain, nuclear staining. Scale bar = 50 μm. OV led to increased staining compared with LPS and control treatments.
was observed for the adrenomedullin receptor, transcription factor-21 (TCF21), and recoil factor-2 (TFF2). Further studies on these proteins should provide deeper insight into the mechanisms of VILI.

Recently, Altmeier et al. (1) described the additive effect of LPS and mechanical ventilation with moderate tidal volumes in a mouse model of ventilator-induced lung injury. Four hours of mechanical ventilation altered the expression of 6,136 genes (SAM program, FDR 1%). Similar to their findings, in our data set, most genes were regulated by the combined treatment of LPS and mechanical ventilation. Although we employed higher tidal volumes in our study, we confirmed their basic finding that OV and LPS regulate many genes in an additive manner. We intersected their list of genes with more than threefold average expression changes (135 genes in their online supplement) and found that 59 of those were significantly changed in the OVLPS group in our study. Among them were the genes for Gadd45γ, IL-6, IL-1β, Cxcl2, and Ccl3 that were confirmed by PCR in that in vivo study. One aim of our study was to find genes specifically activated by OV. This may explain why, except for Akap12, our five target genes not appear in their study.

Copeland et al. (10) studied overventilated rats for 30 min in what is now sometimes called a macroarray (590 genes). In accordance with our findings, these authors observed increased expression of B cell translocation gene-2 (Btg2) (upregulated 2.18-fold by OV), epidermal growth factor-1 (Egr1) (6.2-fold), and Nur77 (2.04-fold), adding these genes to the list of candidate genes for ventilator-induced lung injury.

**Differences between OV and LPS.** Thus a number of important target genes were recognized by comparing arrays from different studies. Another approach is the use of statistical methods to identify likely candidate genes within a given study. Herein we have used the combined statistical power of Scoregene and SAM. Applied to our data, 90% of the genes identified by these two procedures were identical, summing up to 2,085 genes regulated by OV and/or LPS. Many genes, in particular those related to inflammation, immune responses, and stress responses, were regulated in an additive manner by concomitant treatment with OV and LPS (e.g., IL-6, GM-CSF, MIF, IL-1β, MCP-1). Of particular interest were genes that were upregulated by OV but not by LPS (Supplemental Table E2). Most of these genes are unknown to the field of acute lung injury, indicating the existence of specific mechanotransduction pathways related to cytokines (IL-11, IL-1 receptor), growth factors, cellular matrix proteins, and developmental genes (Supplemental Table E2).

Furthermore, genes that are of great interest and that were upregulated by OV are amphiregulin (see below), Cyr61, gravin (Akap12), and Nur77 (all of them confirmed by RT-PCR analysis). Cyr61 binds to cell surface integrins and thereby induces intracellular signaling events, some of which relate to cell proliferation and angiogenesis (7). Interestingly, LPS or the combination of LPS and OV did not affect Cyr61. However, ventilation alone significantly increased its expression. The significance of these finding needs to be further investigated. Moreover, we identified several factors pointing to novel intracellular signaling pathways such as Akap12 and Nur77 (Nr4a1) that were activated by OV. AKAPs maintain multivalent signaling complexes by binding additional enzymes, including kinases and phosphatases, to the cytoskeleton and may thus communicate cell distortion inside cells (11). Of note, Akap12 expression was also significantly increased in vivo (1). Nur77, also known as NGFIB or TIS1, represents an orphan nuclear receptor that is involved in cell proliferation and death and may act as an anti-apoptotic factor (26).

**Potential role of amphiregulin.** Stretch can induce growth factors in epithelial cells (29). Tschumperlin et al. (29) demonstrated that epithelial stretch increases epidermal growth factor receptor (EGFR) ligand shedding into the lateral intracellular space, leading to intracellular signaling. The ligands bind to the EGFR that subsequently induces ERK MAPK activation (29). This mechanism could explain why several growth factor-related genes exhibit modified expression during OV.

One of them is amphiregulin. It is a polypeptide growth regulator and part of the epidermal growth factor family. Together with other EGFR ligands, amphiregulin binds to the extracellular domain of EGFR. EGFR binding leads to the activation of ERK and the transcription factor NF-κB. The EGFR is known to induce cell proliferation and the release of IL-8 and/or MIP-2 cytokines. Recently, amphiregulin was shown to have anti-apoptotic activity in acute liver injury (4). The increased expression of the protein was detected after FAS-mediated liver injury. Berasain et al. (4) found that amphiregulin activates ERK1/2, phosphatidylinositol 3-kinase (PI3K), and signal transducer activator of transcription-3 (STAT3) pathways in liver injury, leading to anti-apoptotic effects. Amphiregulin-deficient mice had diminished JNK activation on FAS ligation. Interestingly, all three signaling factors appear to be activated by OV too: OV induces ERK; inhibition of PI3K ablated OV-induced NF-κB activation and gene activation (32); and, in our larger second data set, Erk1 and Stat3 genes were significantly increased with OV.

Amphiregulin is also expressed in lung tissue, and, in human pulmonary epithelial cells, amphiregulin secretion is induced on exposure to tobacco smoke and fine particulate matter (6). Furthermore, increased amphiregulin secretion also contributes to GM-CSF release. In a mouse model of chronic asthma, ovalbumin-challenged animals displayed increased amphiregulin immunostaining of epithelial cells (22). Our immunostaining shows stretch-induced expression of amphiregulin in epithelial cells, which are exactly those cells in which ERK is activated during OV. The possibility that amphiregulin mediates some of the signaling responses during OV merits further investigation.

Finally, we would like to point out that our model is a model of mechanotransduction but not a model of ventilator-induced lung injury, in particular because it is devoid of neutrophils and major histopathological changes (15). In line with this, the ventilation pressure in the OV group of 25 cmH2O corresponds to the plateau pressure in the low tidal volume arm of the ARDSNet study (1a), which is known to cause no major barotrauma. The design of our experiments allows ascribing the changes in gene expression to mechanical responses of the lungs and excludes a contribution of extrapulmonary organs.

Taken together, our findings show that OV and LPS activate a common set of genes, which may represent a sort of unifying stress response. However, OV also activates specific cellular programs that clearly distinguish it from LPS. In the future, it will be important to further characterize these pathways, which
may finally allow specific interference with the side effects of mechanical ventilation.

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REFERENCES

11. Deutscher Forschungsgemeinschaft. Grant Uh-88/4-2, awarded to S. Uhlig. T. Dolinay is the recipient of a European Respiratory Society Fellowship (Nr. STRF2003-012).