Mechanical ventilation with moderate tidal volumes synergistically increases lung cytokine response to systemic endotoxin

William A. Altemeier,1 Gustavo Matute-Bello,1,2 Charles W. Frevert,1,2 Yasunobu Kawata,3 Osamu Kajikawa,2 Thomas R. Martin,1,2 and Robb W. Glenny1

1Division of Pulmonary & Critical Care Medicine and 2Department of Medicine, University of Washington, Seattle 98195; and 3Medical Research Service, Veterans Affairs Puget Sound Healthcare System, Seattle, Washington 98108

Submitted 9 January 2004; accepted in final form 8 May 2004

Altemeier, William A., Gustavo Matute-Bello, Charles W. Frevert, Yasunobu Kawata, Osamu Kajikawa, Thomas R. Martin, and Robb W. Glenny. Mechanical ventilation with moderate tidal volumes synergistically increases lung cytokine response to systemic endotoxin. Am J Physiol Lung Cell Mol Physiol 287: L533–L542, 2004. First published May 14, 2004; 10.1152/ajplung.00004.2004.—Previous animal studies have identified a role for activation of innate immunity in the pathogenesis of ventilator-associated lung injury. These studies have used large tidal volume ventilation to study the effect of alveolar overdistension on induction of inflammatory pathways. We hypothesized an alternative mechanism for the pathogenesis of lung injury in which moderate tidal volume ventilation does not independently cause clinical inflammation but rather interacts with innate immune activation by bacterial products, resulting in an enhanced inflammatory response. We measured cytokine expression and lung injury in normal and lipopolysaccharide (LPS)-treated anesthetized rabbits randomized to either spontaneous respiration or mechanical ventilation. Outcome parameters were analyzed by two-way factorial analysis of variance to identify synergism between ventilation and systemic LPS. Mechanical ventilation alone resulted in minimal cytokine expression in the lung but did enhance LPS-induced expression of tumor necrosis factor-α, the CXC chemokines interleukin-8 and growth-related protein-α, and the CC chemokine monocyte chemoattractant protein-1. Increased mRNA expression and activation of the transcription factors nuclear factor-kB and activator protein-1 accompanied the cytokine responses. We conclude that moderate volume ventilation strategies augment the innate immune response to bacterial products in the lung and may play a role in the development of acute lung injury in patients with sepsis.

acute lung injury; innate immunity; transcription factor-1; nuclear factor-kB

SEPSIS IS THE MOST COMMON risk factor for the development of acute lung injury (ALI) (14); however, many patients with sepsis do not develop clinically overt lung injury. Paralleling this, animal models of bacterial sepsis often do not develop lung injury in the absence of either a second local lung insult or overwhelming bacterial load (9, 24). This suggests that the risk of developing ALI during sepsis is increased by the presence of one or more cofactors.

One potential cofactor is mechanical ventilation. Despite the absence of overt lung injury, mechanical ventilation is a common adjunctive therapy in patients with sepsis because of either ventilatory respiratory failure associated with shock or related to antecedent trauma, surgery, or other illness. Mechanical ventilation is increasingly recognized as having the potential to augment preexisting ALI. In addition to the Acute Respiratory Distress Syndrome Network prospective trial, which found improved survival in patients with ALI who were ventilated with low tidal volumes (2), multiple animal studies have demonstrated the potential for mechanical ventilation to worsen lung injury induced by a variety of methods, including intratracheal hydrochloric acid instillation (7, 11), intratracheal lipopolysaccharide (LPS) instillation with or without concurrent saline lavage (10, 26), or intravenous oleic acid (8). However, few studies have looked at the role of mechanical ventilation interaction with an inflammatory stimulus, such as systemic LPS, that does not independently cause lung injury. Systemic injection of LPS mimics the systemic inflammatory response of sepsis, but at low levels, it does not cause overt lung injury as measured by increased protein permeability of the epithelial barrier (6, 33).

Tremblay et al. (35) evaluated lung injury and lung inflammation in ex vivo rat lungs ventilated with different strategies after pretreatment with LPS and found that, except for TNF-α, LPS-induced cytokine expression was only increased with the largest tidal volume (40 ml/kg). Gurkan et al. (11) compared ventilation with tidal volumes of 17 ml/kg vs. 6 ml/kg in mice with or without HCl instillation and found that larger tidal volumes increased IL-6 and TNF-α expression after acid aspiration. However, all animals treated with HCl had evidence of increased lung injury.

We hypothesized that mechanical ventilation with moderate tidal volumes can augment the inflammatory response in uninjured lungs to systemic LPS treatment. We measured markers of lung inflammation and injury in 28 rabbits treated with either saline or intravenous LPS at a dose that did not cause lung injury in preliminary experiments. Within each of these groups, animals were further randomized to either mechanical ventilation with tidal volumes of 15 ml/kg or spontaneous respiration.

METHODS

The Animal Care Committee of the University of Washington approved these experiments. Specific pathogen-free New Zealand White rabbits (Western Oregon Rabbit, Philomath, OR) were prospectively randomized to one of four groups: 1) spontaneous ventilation with placebo (0.9% NaCl) infusion (control); 2) mechanical ventilation with placebo infusion (MV); 3) spontaneous ventilation with LPS infusion (LPS); and 4) mechanical ventilation with LPS infusion (MV+LPS).

LPS preparation. LPS derived from Escherichia coli serotype 0111:B4 (Sigma, St. Louis, MO) was suspended by sonication in...
nuclear proteins. In the remaining lung, extravascular water content
maintaining 5% dextrose and 0.05 meq NaHCO₃/ml of
weight-based protocol. The intravenous boluses of 10 ml/kg at times 0,

Table 1. **PCR product length and primer sequences**

<table>
<thead>
<tr>
<th>Gene (Product Length)</th>
<th>Orientation</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (392 bp)</td>
<td>Sense</td>
<td>5′‐CCACGTAATGCAAAACCCGGAAGT‐3′</td>
</tr>
<tr>
<td>IL-8 (892 bp)</td>
<td>Anti-sense</td>
<td>5′‐TCGGGAAAGTCGACAGGCTGAGT‐3′</td>
</tr>
<tr>
<td>MCP-1 (323 bp)</td>
<td>Sense</td>
<td>5′‐GCCGATTGTCCCAGAGTGCTG‐3′</td>
</tr>
<tr>
<td>GAPDH (330 bp)</td>
<td>Anti-sense</td>
<td>5′‐GATGATTTGACCTGAGGGAGGG‐3′</td>
</tr>
</tbody>
</table>

MCP-1, monocyte chemoattractant protein-1.

was determined by the gravimetric method without correction for
hemoglobin concentration.

Cell counts were performed on both BALF and on whole blood
using a hemacytometer. Slides for differential counts were prepared
using a cytocentrifuge (Cytospin; Wescor, Logan, UT) at 750 rpm for
5 min followed by methanol fixation and staining with a modified
Wright stain.

For one animal in each group, the lungs were removed from the
chest at the end of the experiment and fixed with 4% formaldehyde at
25 cmH₂O for 24 h. The lungs were then separated by lobe and
embedded in paraffin. From each lobe, 4-µm sections were made and
stained with hematoxylin and eosin.

**Immunassays for TNF-α, IL-8, growth-related protein-α, mono-
cyte chemoattractant protein-1, IgM, and albumin.** The lung cytokine
response was determined by measuring TNF-α, IL-8, growth-related
protein-α (GRO-α), and monocyte chemoattractant protein-1
(MCP-1) concentrations in BALF using rabbit-specific immunassays
(ELISA) (17, 18). IgM was measured in BALF with a standard
sandwich ELISA using goat anti-rabbit IgM as the capture antibody
(Accurate Chemical and Scientific, Westbury, NY), biotinylated goat
anti-rabbit IgM as the detecting antibody (Accurate), and rabbit IgM
(Chemicon, Temecula, CA) as the standard. BALF albumin
concentration was measured using a sandwich ELISA and a polyclonal
sheep anti-rabbit albumin antibody as both a capture antibody (unconju-
gated) and a detection antibody (horseradish peroxidase-conjugated;
Bethyl Laboratories, Montgomery, TX). Standard curves were pre-
pared using rabbit albumin (Sigma-Aldrich).

**RNA isolation and RT-PCR.** RNA was extracted from frozen lung
tissue using the RNeasy Midi kit (Qiagen, Valencia, CA) according to
the manufacturer’s instructions and treated with DNase I (Ambion,
Austin, TX) to remove any remaining genomic DNA. Moloney
murine leukemia virus reverse transcriptase (Ambion) was used for
first-strand cDNA synthesis. Taq polymerase in a PCR buffer con-
taining 2.5 mM MgCl₂ (Qiagen) and gene-specific primers (Table 1)
was used for PCR amplification. PCR was carried out for 27 cycles
because preliminary studies showed that this was within the linear
range of amplification for TNF-α, IL-8, and MCP-1 in our samples.
PCR conditions included melting at 94°C for 30 s, primer hybridiza-
tion at 54°C for 30 s, and cDNA elongation at 72°C for 60 s. Negative
control experiments were performed by omitting reverse transcriptase
from RT reactions before PCR.

PCR products were separated by gel electrophoresis and visualized
with SYBR Gold (Molecular Probes, Eugene, OR). Signal intensity
was quantified by densitometry (ImageJ; National Institutes of Health,
Bethesda, MD). Levels of TNF-α, IL-8, and MCP-1 expression were
quantified by GAPDH expression to correct for variability in first-
strand cDNA synthesis.

**Nuclear extract preparation and gel shift assay.** Nuclear protein
elements were prepared by pulverizing frozen tissue submerged in
liquid nitrogen using a mortar and pestle. The tissue powder was
transferred to a Dounce homogenizer and lysed on ice in 4 ml of lysis
buffer (10 mM HEPES, pH 7.9, 10 mM KCl, and 1.5 mM MgCl₂)
sterile, pyrogen-free PBS (Invitrogen, Carlsbad, CA) at a stock
concentration of 5 mg/ml and stored in small aliquots at −20°C. For each
experiment, an aliquot of LPS was thawed and sonicated in an ice
water bath for 10 min. The LPS was then diluted to a final concen-
tration of 2 mg/ml with sterile 0.9% NaCl immediately before use.

**Experimental protocol.** Rabbits were sedated with intramuscular
acepromazine (1 mg/kg) and anesthetized with 4% isoflurane deliv-
ered via facemask. All rabbits were orally intubated with a 3.5-mm
inner-diameter, cuffed endotracheal tube, placed supine, and con-
ected either to a closed ventilation circuit with a bias flow rate of 3
l/min via T-piece or to a volume-controlled mechanical ventilator
(model 665; Harvard Apparatus, Holliston, MA). Mechanically ven-
tlated animals received 15-ml/kg tidal volumes at a rate of 30
breaths/min without positive end-expiratory pressure (PEEP). Anes-
thesia was maintained throughout the experiment for all animals via
inhaled 1.5% isoflurane. Percutaneous 20-gauge angiocatheters were
inserted into a marginal ear vein and an auricular artery for infusions,
blood sampling, and hemodynamic monitoring. Body temperature
was monitored by a rectal thermistor and regulated by a rheostat-
controlled heat lamp. Tidal volume and respiratory rate were moni-
tored continuously in mechanically ventilated rabbits and intermitt-
tently in spontaneously breathing rabbits using a pneumotach (RSS
100; KORR Medical Technologies, Salt Lake City, UT).

Physiological parameters, including blood gas analysis, blood pres-
sure, temperature, tidal volume, respiratory rate, and inspired gas
composition, were recorded hourly. Alveolar-arterial oxygen differen-
tions (A-aDO₂) were calculated using the alveolar gas equation (1).
Carbon dioxide (CO₂) was added to inspired gas as needed to
maintain arterial CO₂ partial pressure (PaCO₂) equivalent to that
observed in spontaneously breathing, LPS-treated rabbits.

All animals received intravenous volume resuscitation via a
weight-based protocol. The intravenous fluid was 0.45% saline con-
taining 5% dextrose and 0.05 meq NaHCO₃/ml of fluid. Fluids were
administered at a constant rate of 10 ml·kg⁻¹·h⁻¹ with additional
boluses of 10 ml/kg at times 0, +4, and +6 h from intubation.

One hour after intubation, a blood sample was taken to measure
total and differential white blood cell (WBC) count and to collect
serum. Two hours after intubation, rabbits were given either 5
µg/kg of LPS or an equivalent volume of 0.9% NaCl intravenously over 30
min. Blood samples for total and differential WBC count and serum
collection were taken 2 h after LPS or placebo treatment (4 h after
intubation) and at the conclusion of the experiment.

Eight hours after intubation, animals were deeply anesthetized with
100 mg of tiopental sodium and killed by exsanguination via cardiac
puncture. The right lung of each animal was lavaged with
100 mg of thiopental sodium and killed by exsanguination via cardiac
intubation) and at the conclusion of the experiment.

Eight hours after intubation, animals were deeply anesthetized with
100 mg of tiopental sodium and killed by exsanguination via cardiac
puncture. The right lung of each animal was lavaged with
100 mg of thiopental sodium and killed by exsanguination via cardiac
intubation) and at the conclusion of the experiment.

Eight hours after intubation, animals were deeply anesthetized with
100 mg of tiopental sodium and killed by exsanguination via cardiac
puncture. The right lung of each animal was lavaged with
100 mg of thiopental sodium and killed by exsanguination via cardiac
intubation) and at the conclusion of the experiment.

Eight hours after intubation, animals were deeply anesthetized with
100 mg of tiopental sodium and killed by exsanguination via cardiac
puncture. The right lung of each animal was lavaged with
100 mg of thiopental sodium and killed by exsanguination via cardiac
intubation) and at the conclusion of the experiment.

Eight hours after intubation, animals were deeply anesthetized with
100 mg of tiopental sodium and killed by exsanguination via cardiac
puncture. The right lung of each animal was lavaged with
100 mg of thiopental sodium and killed by exsanguination via cardiac
intubation) and at the conclusion of the experiment.

Eight hours after intubation, animals were deeply anesthetized with
100 mg of tiopental sodium and killed by exsanguination via cardiac
puncture. The right lung of each animal was lavaged with
100 mg of thiopental sodium and killed by exsanguination via cardiac
intubation) and at the conclusion of the experiment.

Eight hours after intubation, animals were deeply anesthetized with
100 mg of tiopental sodium and killed by exsanguination via cardiac
puncture. The right lung of each animal was lavaged with
100 mg of thiopental sodium and killed by exsanguination via cardiac
intubation) and at the conclusion of the experiment.
with 20 up and down strokes of the pestle. This suspension was transferred to a 15-ml tube and spun at 4°C and 800 g for 1 min to pellet the cellular debris. The supernatant was transferred to a new tube and centrifuged at 4,000 g for 15 min to pellet nuclei. The resultant nuclear pellet was resuspended in 50 μl of extraction buffer (20 mM HEPES, pH 7.9, 420 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 25% glycerol). The nuclear suspension was incubated on ice for 45 min with vortex mixing every 5 min. Finally, the nuclear suspension was spun at 4°C and 15,000 g for 15 min. The supernatant was transferred to a new tube and diluted with 25 μl of dilutional buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, and 20% glycerol). Protein concentration was determined by the Bradford assay (Pierce Biotechnology, Rockford, IL), and the remaining nuclear extract was stored at -80°C in small aliquots. Protease inhibitors DTT (50 μM), PMSF (50 μM), and leupeptin hydrochloride (20 μM) and phosphatase inhibitors sodium molybdate (0.1 mM), -glycerol phosphate (10 mM), sodium fluoride (10 mM), sodium orthovanadate (0.1 mM), and -nitro-phenylphosphate (3 mM) were added to the lysis and extraction buffers immediately before use. DTT (50 μM) and PMSF (50 μM) were added to the dilutional buffer immediately before use.

Gel shift assays for nuclear factor (NF)-κB and activator protein-1 (AP-1) were performed using a Promega gel shift assay system (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, oligonucleotides containing the NF-κB consensus binding sequence (sense 5'-AGT TGA GGG GAC TTT CC AGG C-3', anti-sense 5'-G CCT GGG AAA GTC CCC TCA ACT-3') and the AP-1 consensus binding sequence (sense 5'-CGC TGG AAA GTC CCC TCA ACT-3', anti-sense 5'-TTC CGG CTG ACT CAT CAA GCG-3') were end labeled with [γ-32P]ATP. Binding reactions were prepared with 5 μg of nuclear protein and 35 fmol of labeled oligonucleotide in Tris-HCl (pH 7.5) with 4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, and 1 μg poly(dI-dC)-poly(dI-dC) and incubated for 20 min at room temperature. Binding reactions were then separated by nondenaturing 6% polyacrylamide gel electrophoresis. The gel was dried and imaged using a phosphorimager (Packard Bioscience, Meriden, CT). Densitometry was performed using Optiquant software (Packard Bioscience).

Statistical analysis. For all end points, differences among the four groups were evaluated by a two-way factorial ANOVA that included terms for the main effects of mechanical ventilation and endotoxin.
treatment and a crossed term for the interaction effect between mechanical ventilation and endotoxin (MV/LPS) (31). In this analysis, any interaction between mechanical ventilation and endotoxin treatment beyond that expected by addition of the independent effects (i.e., a synergistic interaction) is identified by a statistically significant crossed effects term (MV/LPS). Because a very strong interaction effect can influence the estimated impact of the main effects acting alone, significant independent effects of mechanical ventilation and endotoxin treatment on outcome endpoints were confirmed by unpaired \( t \)-tests. Comparisons were made between the MV group and the control group and between the LPS group and the control group when a significant interaction was identified by two-way ANOVA.

For temporally repeated data, such as physiological measurements, a repeated-measures ANOVA was used to determine whether or not there were significant differences over time among groups.

For all statistical tests, a \( P \) value \( \leq 0.05 \) was used to determine statistical significance. Results are presented as means \( \pm \) SD. JMP software (SAS, Cary, NC) was used for all statistical analyses.

RESULTS

Thirty rabbits were studied. Results from 24 rabbits (6 per group) are reported. Two rabbits (1 in the LPS group and 1 in the MV/LPS group) died prematurely of cardiovascular collapse and were excluded from the analyses. The remaining four animals were used for histological comparison. There were no significant differences between groups with respect to weight (3.29 \( \pm \) 0.03 kg) or initial peripheral blood WBC count. Serum TNF-\( \alpha \) was not detectable in any of the animals at base.

Physiological response. Mean arterial pressures differed over time among the four groups, with the lowest pressures occurring in the MV/LPS group (Fig. 1A). Over time, blood pressure fell in all animals treated with LPS and rose slightly in animals treated with placebo, regardless of ventilatory strategy.

Initially, nonventilated animals had greater A-aDO\(_2\) compared with mechanically ventilated animals (Fig. 1B). Over time, A-aDO\(_2\) trended higher for animals treated with endotoxin independent of ventilation status. The greatest increases in A-aDO\(_2\) over the course of the experiments were in the MV/LPS group (17.6 \( \pm \) 4.0 mmHg). \( P_{\text{aCO}_2} \) decreased over time in the LPS group (Fig. 1C). \( P_{\text{aCO}_2} \) in the MV and MV/LPS groups were well matched with the \( P_{\text{aCO}_2} \) in the LPS group. The control group maintained normal alveolar ventilation throughout the experiment. There was a trend toward a

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Control</th>
<th>MV</th>
<th>LPS</th>
<th>MV+LPS</th>
<th>MV Main Effect</th>
<th>LPS Main Effect</th>
<th>MV-LPS Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-( \alpha ), ng/ml</td>
<td>0 ( \pm ) 0</td>
<td>0 ( \pm ) 0</td>
<td>86 ( \pm ) 42</td>
<td>71 ( \pm ) 36</td>
<td>n.s.</td>
<td>( P &lt; 0.0001 )</td>
<td>n.s.</td>
</tr>
<tr>
<td>IL-8, ng/ml</td>
<td>1.7 ( \pm ) 0.7</td>
<td>2.9 ( \pm ) 1.8</td>
<td>211 ( \pm ) 104</td>
<td>185 ( \pm ) 90</td>
<td>n.s.</td>
<td>( P &lt; 0.0001 )</td>
<td>n.s.</td>
</tr>
<tr>
<td>GRO-( \alpha ), ng/ml</td>
<td>1.8 ( \pm ) 0.3</td>
<td>1.5 ( \pm ) 0.6</td>
<td>2.0 ( \pm ) 0.2</td>
<td>1.8 ( \pm ) 0.2</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>MCP-1, ng/ml</td>
<td>1.2 ( \pm ) 0.5</td>
<td>1.4 ( \pm ) 1.3</td>
<td>68 ( \pm ) 47</td>
<td>112 ( \pm ) 108</td>
<td>n.s.</td>
<td>( P = 0.001 )</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

MV, mechanical ventilation with placebo; GRO-\( \alpha \), growth-related protein-\( \alpha \); n.s., not significant.
higher pH in the MV group secondary to respiratory alkalosis that was not offset by a metabolic acidosis (Fig. 1D). The pH for the LPS group and for the MV+LPS group was matched with the control group over time.

Airway pressures were only measured in mechanically ventilated animals. Over time, there was a trend toward increasing peak airway pressures in the MV+LPS group compared with the MV group; however, this difference was not statistically significant (Fig. 2).

Systemic inflammatory response. No baseline differences in peripheral WBC count or serum concentrations of either TNF-α or GRO-α were observed among the four groups. Minor but statistically significant baseline differences in serum concentrations of IL-8 and MCP-1 were present; however, concentrations for both chemokines were greatest in the control group and least in the MV+LPS group.

Two hours after endotoxin or placebo treatment, serum concentrations of TNF-α, IL-8, and MCP-1 were greater in endotoxin-treated animals (Table 2). There was no main effect of mechanical ventilation alone and no synergism between mechanical ventilation and endotoxin treatment identified for serum concentration of any cytokines. Serum cytokine expression at the conclusion of the experiment mirrored these patterns with the exception of TNF-α, which was not detectable in any animals (data not shown).

Lung inflammatory response. Cytokine protein expression in BALF differed significantly among the four groups (Fig. 3). TNF-α was only detectable in BALF from animals in the MV+LPS group (2.2 ± 2.0 ng/ml). Compared with the control group, both IL-8 and MCP-1 expression were modestly increased in the MV-only group (1.6 ± 0.7 vs. 0.3 ± 0.1 ng/ml and 1.6 ± 1.3 vs. 0 ng/ml, respectively). GRO-α concentration did not differ significantly between the control group and the MV-only group. The LPS-only group had greater expression of IL-8 (6.2 ± 3.9 ng/ml) and MCP-1 (10.5 ± 5.8 ng/ml) and of GRO-α (0.5 ± 0.4 vs. 0 ng/ml) compared with the control group. However, when treatment with both mechanical ventilation and endotoxin were combined (MV+LPS group), there was a synergistic augmentation of expression of IL-8 (26.5 ± 12.8 ng/ml), MCP-1 (24.9 ± 8.0 ng/ml), and GRO-α (1.0 ± 0.3 ng/ml) in the BALF.

Total pulmonary RNA was extracted and analyzed from three animals in each group. Relative to the control group, mechanical ventilation alone (MV group) did not alter expression of TNF-α mRNA (Fig. 4A); however, it did increase mRNA expression of IL-8 (5.9 ± 2.0-fold increase, \( P = 0.05 \), Fig. 4B). Additionally, there was a strong trend toward increased expression of MCP-1 (6.1 ± 3.3-fold increase, \( P = 0.06 \), Fig. 4C) in the MV-only group. Relative to the control group, the LPS-only group had increased mRNA expression of...
TNF-α (8.6 ± 4.1-fold, P = 0.03), IL-8 (9.0 ± 1.9-fold, P = 0.002), and MCP-1 (9.7 ± 0.4-fold, P < 0.0001; Fig. 4). The combination of both mechanical ventilation and endotoxin treatment (MV + LPS group) caused the greatest fold increases in mRNA expression relative to the control group for all three cytokines (TNF-α: 22.2 ± 6.5-fold, IL-8: 19.4 ± 2.7-fold, and MCP-1: 16.0 ± 1.9-fold); however, a synergistic interaction between mechanical ventilation and endotoxin treatment was only present for TNF-α and IL-8 expression (Fig. 4).

Nuclear protein extracts were prepared from three animals in each group, except for the MV group in which extracts from two animals were prepared. There was a consistent increase in nuclear NF-κB in the MV, LPS, and MV + LPS groups (Fig. 5A and B). Within these three groups, NF-κB binding was consistently greater in animals treated with LPS. AP-1 binding was also increased in the MV, LPS, and MV + LPS groups above that in the control group (Fig. 5, C and D). In contrast to NF-κB, AP-1 binding was consistently greater in the MV and the MV + LPS groups compared with the LPS group.

There was no difference among the groups for total WBC count isolated from BALF (Fig. 6A). Differential analysis showed increased polymorphonuclear leukocytes in BALF from mechanically ventilated animals (Fig. 6B) whether or not LPS was present. There was no interaction effect between mechanical ventilation and LPS treatment on either BALF cell count or differential.

**Lung injury.** Compared with the control group, only the combination of both endotoxin treatment and mechanical ventilation increased the wet/dry ratio (5.62 ± 0.21 vs. 5.19 ± 0.07, Fig. 7). There was a significant interaction effect (P = 0.02) between mechanical ventilation and LPS treatment, indicating synergism for extravascular lung water accumulation.
Permeability to albumin was increased in mechanically ventilated animals compared with spontaneously breathing animals regardless of LPS treatment (Fig. 8A). There was no synergism between mechanical ventilation and LPS on albumin permeability. Permeability to very large molecules such as IgM was not different among the four groups (Fig. 8B). Histological comparison did not reveal gross differences in alveolar structure, cellular infiltration, or edema among the four groups (Fig. 9).

DISCUSSION

The goal of this study was to test the hypothesis that mechanical ventilation at moderate tidal volumes modulates the innate immune response to LPS, resulting in augmented pulmonary inflammation. The major finding of this study was that mechanical ventilation at a tidal volume of 15 ml/kg magnified the lung cytokine responses following systemic LPS treatment. The study design enabled us to identify interactions between mechanical ventilation and LPS treatment that were synergistic, rather than additive, in magnitude through the use of a two-way ANOVA model. Additionally, the experimental design reduced potential confounding effects due to differences of posture, intravenous volume resuscitation, anesthesia, or endotracheal intubation.

Previous studies have focused on the effect of ventilation strategy on preexisting lung injury induced by a variety of different insults. Additionally, previous studies frequently used hyperoxia, which can independently potentiate lung injury during mechanical ventilation (29, 32). The current study builds on prior studies by demonstrating that moderate tidal volume ventilation augmented lung inflammatory responses to systemic endotoxin in the absence of preexisting lung injury. Animals treated with only endotoxin demonstrated a modest molecular inflammatory response in the lung but did not develop lung injury as measured by permeability to albumin or IgM, extravascular water accumulation, or histology. The mechanical ventilation protocol used did independently result in modest alveolar neutrophil accumulation and epithelial barrier dysfunction to medium size proteins such as albumin; however, epithelial disruption was not present as demonstrated by both preservation of barrier function to large proteins (Fig. 8B) (13) and by the absence of histological changes. Additionally, clinically significant lung injury as measured by changes in airway pressures or gas exchange was absent.

Despite the significant augmentation in lung cytokine response with the combination of mechanical ventilation and endotoxin treatment, there was minimal evidence for lung injury in any of the four groups. Greater extravascular water accumulation and a trend toward greater impairment in gas exchange during the study period suggests that early lung injury was developing in the MV+LPS group. We speculate that lung injury would continue to worsen in this group with increasing experimental time secondary to the augmented cytokine response. Several studies have found that blocking either CXC chemokines (3, 39) or early response cytokines such as TNF-α or IL-1 (15, 27) can attenuate lung injury, supporting the hypothesis that lung inflammatory responses can directly contribute to lung injury. Because lung injury develops 24 or more hours after intensive care unit admission in ~50% of patients with ALI (14), the experimental model used in this study may provide insight into early changes occurring in those patients.

Understanding the mechanism by which mechanical ventilation augmented cytokine production is an important first step toward developing potential protective interventions. One possible mechanism is that mechanical ventilation increased translocation of endotoxin or systemically derived early response cytokines to the alveolar compartment. Facilitation of endotoxin translocation from the alveolar compartment to the systemic circulation by mechanical ventilation with similar tidal volumes has been reported (26). However, in contrast to the current study, severe lung injury and, presumably, disruption...
of the epithelial barrier, was initially induced by a combination of saline lavage and preventilation with an FI O2 of 1.0. Endotoxin translocation from the systemic to the alveolar compartment is also less likely because of rapid clearance from the liver, estimated at ~90% within 2 min (37).

Another potential mechanism for interaction between LPS treatment and mechanical ventilation was activation of complementary proinflammatory intracellular signaling pathways. Mechanical ventilation increased expression of TNF-α, IL-8, and MCP-1 mRNA in both placebo- and LPS-treated animals, suggesting that mechanical ventilation affects transcriptional regulation of these cytokines. In vitro, cyclical stretching of bronchial epithelial cells induces expression of IL-8 mRNA that is blocked by pretreatment with actinomycin D (28). Although we found that mechanical ventilation and LPS each activated the transcriptional factors NF-κB and AP-1, AP-1 activation was greater in the animals treated with mechanical ventilation. These in vivo findings again are supported by the in vitro finding that AP-1 is activated in bronchial epithelial cells undergoing cyclical stretch (28). In contrast, NF-κB activation was the major transcription factor activated in the lung following LPS exposure, consistent with prior reports (4, 5). Concurrent AP-1 and NF-κB activation cooperatively enhances transcription of acute inflammatory cytokines (23, 30, 34). We speculate that the mechanism by which mechanical ventilation and systemic LPS interact, resulting in augmented inflammatory cytokine expression, is through differential activation of the transcription factors NF-κB and AP-1. Other studies support activation of AP-1 by mechanical ventilation. AP-1, a dimeric molecule of basic region leucine zipper proteins, including c-fos and c-jun, is regulated at both transcriptional and posttranslational levels (19). Mechanical ventilation both increases c-fos mRNA (35) and activates MAPKs involved in phosphorylation of c-jun (36). Pretreatment with a specific inhibitor of JNK blocks stretch-induced expression of IL-8 mRNA by A549 cells (22).

Because measurements of transcription factor binding were made at only one time in the current study, an alternative explanation for the findings is that mechanical ventilation resulted in a more prolonged activation of AP-1 and that LPS caused more prolonged activation of NF-κB as opposed to differences in the absolute magnitude of binding activity. This would not change the basic hypothesis that differences in AP-1 activation and NF-κB activation by mechanical ventilation and LPS resulted in an elevated cytokine transcriptional response.

The current study was not designed to identify the specific mechanical stress responsible for the effects of mechanical ventilation. Both alveolar overdistension and cyclical collapse and reexpansion of alveoli have been postulated as mechanisms of ALI. Animals in this study were ventilated without PEEP and with tidal volumes ~2.5 times the reported spontaneous tidal volume of rabbits (38), suggesting that both mechanical stresses may have been present in this model. Other potential confounding factors include differences in systemic blood pressure and arterial pH among the four groups. Hemorrhagic shock followed by volume resuscitation causes lung injury in animal models (12, 16, 25); however, to the best of our knowledge, the role of nonhemorrhagic shock in lung injury has not been studied. Similarly, higher pH in the MV-only group secondary to an uncompensated respiratory alkalosis may have altered the response in this group. We chose to match arterial PCO2 among intervention groups as opposed to pH because of the known effect of hypocapnia on ventilator-associated lung injury (20, 21).

Fig. 9. Representative hematoxylin and eosin-stained 5-μm lung sections at ×400 magnification from 1 animal in each group. Control (A), MV only (B), LPS only (C), and MV+LPS (D) are shown.
In summary, moderate tidal volume mechanical ventilation over 8 h caused minimal cytokine expression in BALF. However, the combination of mechanical ventilation and systemic treatment with LPS resulted in enhanced lung cytokine response and early lung injury in rabbits. Mechanical ventilation synergizes with systemic endotoxin in the development of lung inflammation by a mechanism involving differential activation of the transcription factors AP-1 and NF-κB. Therapeutic strategies aimed at preventing AP-1 activation may be protective in mechanically ventilated patients.

ACKNOWLEDGMENTS
The authors gratefully acknowledge the assistance of Dowon An and Sheng-Sheng Wang in performing the animal studies, Dave Frazer in performing densitometry, and Steve Mongovin in performing immunoassays. The authors thank Dr. Karol Bomzoky for advice and for use of his laboratories and Dr. Nayak Polissar of Mountain-Whisper-Light Statistical Consulting for advice on the statistical methods.

GRANTS
This work was supported by a Parker B. Francis Fellowship from the Francis Family Foundation and National Heart, Lung, and Blood Institute Grants HL-71020, HL-24163, and HL-30542.

REFERENCES


