Protective mechanical ventilation does not exacerbate lung function impairment or lung inflammation following influenza A infection

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Submitted 15 April 2009; accepted in final form 4 September 2009

Protective mechanical ventilation does not exacerbate lung function impairment or lung inflammation following influenza A infection. J Appl Physiol 107: 1472–1478, 2009. First published September 10, 2009; doi:10.1152/japplphysiol.00393.2009.—The degree to which mechanical ventilation induces ventilator-associated lung injury is dependent on the initial acute lung injury (ALI). Viral-induced ALI is poorly studied, and this study aimed to determine whether ALI induced by a clinically relevant infection is exacerbated by protective mechanical ventilation. Adult female BALB/c mice were inoculated with 10^4.5 plaque-forming units of influenza A/Mem/1/71 in 50 μl of medium or medium alone. This study used a protective ventilation strategy, whereby mice were anesthetized, tracheostomized, and mechanically ventilated for 2 h. Lung mechanics were measured periodically throughout the ventilation period using a modification of the forced oscillation technique to obtain measures of airway resistance and coefficients of tissue damping and tissue elastance. Thoracic gas volume was measured and used to obtain specific airway resistance, tissue damping, and tissue elastance. At the end of the ventilation period, a bronchoalveolar lavage sample was collected to measure inflammatory cells, macrophage inflammatory protein-2, IL-6, TNF-α, and protein leak. Influenza infection caused significant increases in inflammatory cells, protein leak, and deterioration in lung mechanics that were not exacerbated by mechanical ventilation, in contrast to previous studies using bacterial and mouse-specific viral infection. This study highlighted the importance of type and severity of lung injury in determining outcome following mechanical ventilation.

ventilator-associated lung injury; viral infection; BALB/c

Mechanical ventilation of otherwise healthy lungs may result in lung injury in a process often referred to as ventilator-associated lung injury (VALI) (21, 39). However, in the clinical setting, primary injury resulting in the requirement for mechanical ventilation may make the lung more susceptible to subsequent ventilator-induced inflammatory processes (14, 46, 34). Thus VALI can be considered in terms of a “two-hit” process, whereby lung injury is induced by the initial insult, which may then be exacerbated by the lung trauma associated with mechanical ventilation.

The induction of acute lung injury (ALI), which may then progress to fully developed acute respiratory distress syndrome (ARDS), can result from a number of different insults, such as sepsis (38), acid aspiration (45), chemical exposure (30), and pneumonia (43). Although all these insults in severe cases have the same result, i.e., the need for mechanical ventilation, the state of the lung in terms of inflammation and injury before the commencement of ventilation varies considerably. Understanding how different initial conditions drive the pathogenesis of VALI will aid clinical management and help identify potential therapeutic targets.

One example of a “first hit” that is clinically relevant and may lead to the need for mechanical ventilation is severe viral respiratory infection (5, 23, 28, 37). This has been highlighted by the recent outbreaks of severe ARDS (12) and influenza A/H5N1 (22), where respiratory failure requiring ventilatory support was observed in a number of cases. Similarly, in the pediatric setting, respiratory syncytial virus may result in a requirement for ventilatory support (18, 26). A recent study using a mouse model of pneumovirus (PMV) infection as a model for respiratory syncytial virus demonstrated that mechanical ventilation exacerbates preexisting inflammation as a result of viral infection (6). This observation is consistent with a number of previous studies of bacterially induced ALI, whereby inflammation is exacerbated (2, 29, 41), suggesting that mechanical ventilation can exacerbate ALI induced by bacterial and viral infection. It is unclear, however, whether this observation can be extrapolated to all clinically relevant viral infections or whether the exacerbation of viral ALI is limited to PMV, which is a mouse-specific virus.

The mechanisms by which artificial ventilation can drive lung injury remain unclear, but a number of processes, such as volutrauma (16, 27, 31), atelectrauma (17, 33), biotrauma (15, 42), and epithelial necrosis (11, 21), have been identified. These processes reflect regional overstretch, excessive pressure, and/or release of mediators in response to repetitive mechanical stretch of the lung tissue. To understand the process involved in VALI and to identify the extent of ALI following an insult, it is important to monitor lung mechanics during the ventilation period. We recently developed a technique for measuring lung volume in laboratory rodents that allows us to correlate changes in lung volume with changes in lung mechanics (24). This may aid in understanding the pathogenesis of VALI in the context of preexisting lung injury.

This study aimed to determine whether mechanical ventilation following influenza infection exacerbates preexisting inflammation while lung volume and lung mechanics were monitored during the ventilation period.

METHODS

Animals. Adult (8-wk-old) female BALB/c mice (Animal Resource Centre, Murdoch, Western Australia) were housed under specific pathogen-free conditions in HEPA-filtered individually ventilated cages (Sealsafe, Tecniplast, Italy). Food and water were provided ad libitum, and the animals were exposed to a 12:12-h light-dark cycle. All experiments were conducted according to National Health and Medical Research Council guidelines for the Care and Use of Animals for Scientific Purposes (2003).
Medical Research Guidelines Council (Australia) with approval from the Institutional Animal Ethics Committee.

**Viral infection.** Mice (*n* = 7) were inoculated intranasally under light methoxyflurane anesthesia with 10^5.5 plaque-forming units of influenza A/Mem/1/71 (H3N1) diluted in 50 μl of virus production serum-free medium (GIBCO, Invitrogen). Control mice (*n* = 6) received 50 μl of virus production serum-free medium only. All mice were studied 4 days after infection, which, using this protocol, we previously determined to be the peak of inflammation and clinical illness (8). Separate groups of mice were also inoculated with influenza (or medium alone) to study the kinetics of cytokine responses (see details of cytokine measurements in *Bronchoalveolar lavage*).

**Ventilation regimen and sampling protocol.** Mice were anesthetized with a 10 μl/g ip injection containing 40 mg/ml ketamine (Troy Laboratories) and 2 mg/ml xylazine (Troy Laboratories). Initially, mice were given two-thirds of the dose to induce a surgical plane of anesthesia. A tracheostomy was performed, and a 10-mm polyethylene cannula (0.86 mm ID, 1.26 mm OD) was inserted and secured with suture. The anesthetized mouse was placed in a custom-made Perspex whole body plethysmograph (~180 ml) and connected to a small animal ventilator (flexiVent, Scireq, Montreal, PQ, Canada) via an external port connected to the tracheal cannula. Mice were ventilated at a frequency of 360 breaths/min with a tidal volume of 10 ml/kg with a positive end-expiratory pressure of 3 cmH2O. Once the animal was connected to the ventilator, the remaining one-third of the anesthetic was given. Top-up anesthetic doses were given periodically (at ~45-min intervals) throughout the ventilation period when required.

For standardization of lung volume history, mice were subjected to two slow inflation-deflation maneuvers up to 20 cmH2O transrespiratory pressure separated by 5 min of regular ventilation at the start of the ventilation protocol. After lung volume history standardization, baseline lung mechanics and lung volumes were measured (see below). Mice were mechanically ventilated for a further 2 h, as described above, with lung mechanics and lung volume measured every 10 min. Periodic recruitment maneuvers were applied throughout the ventilation period in the form of two sighs every 5 min (immediately after and halfway between lung function measurements) at a volume of 20 ml/kg. Body temperature was monitored with a rectal thermocouple and maintained at ~37°C using a heat lamp. At the end of the ventilation period, serum and lavage samples were collected (see below). Separate groups of mice received influenza or medium inoculations, and serum and lavage samples were collected 4 days after infection without ventilation.

**Lung volume.** Thoracic gas volume (TGV) was measured as described previously (24). Briefly, the ventilator was stopped and the trachea was occluded at end-expiratory lung volume. Inspiratory efforts were induced by stimulation of the intercostal muscles via intramuscular electrodes with an electrical stimulator (model S44, Grass) at 20 V with a pulse duration of 1–2 ms. Six inspiratory efforts were induced over a 6-s recording period. TGV was calculated using Boyle’s law and the relationship between box pressure and tracheal pressure after correction for the plethysmograph impedance (24).

**Lung mechanics.** Lung mechanics were measured using a modification of the wave tube forced oscillation technique, as described previously (20). Briefly, during 6-s pauses in ventilation, a small-amplitude pseudorandom oscillatory signal with a frequency range of 4–38 Hz was delivered by a loudspeaker in box setup through the wave tube to the tracheal cannula at end-expiratory lung volume. A four-parameter model with constant-phase tissue impedance was fit to the measured respiratory system impedance (Zrs) spectrum (19):

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Z_{rs} = R_{aw} + j \omega L + (G + j \omega H) / \omega^2
\]

where Raw is Newtonian (airway) resistance, which in the mouse primarily reflects airway resistance due to the low chest wall impedance (40), I is inertia, which is also negligible when the impedance of the tracheal cannula is taken into account (7), G is the coefficient of tissue damping, H is the coefficient

![Fig. 1. Median and interquartile range for baseline airway resistance (Raw; A), tissue damping (G; B), tissue elastance (H; C), and thoracic gas volume (TGV; D) for BALB/c mice 4 days after inoculation with medium or influenza A (Flu).](http://jap.physiology.org/) 

J Appl Physiol • VOL 107 • NOVEMBER 2009 • www.jap.org
of tissue elastance, ω is angular frequency, and α = (2/π) arctan (H/G) represents the interdependence between the dissipative and elastic properties of the lung. Because of the small contribution of the chest wall impedance, G and H are considered the parameters describing the dissipative and elastic properties of the lungs, respectively.

Bronchoalveolar lavage. Bronchoalveolar lavage (BAL) samples were collected at the end of the ventilation period by gentle washing with 0.5 ml of pyrogen-free 0.9% saline solution, which was cycled into and out of the lung three times. BAL samples were then centrifuged, and the supernatant was collected and stored for later analysis. The remaining pellet was resuspended in PBS, and a subsample was taken, stained with Trypan blue, and examined under light microscopy in a hemocytometer to obtain a total cell count. A further sample of this resuspended preparation was spun onto slides, exposed to Leishmann’s stain, and examined under a light microscope to obtain a differential cell count. Levels of the cytokines macrophage inflammatory protein-2 (MIP-2), IL-6, and TNF-α were measured in the supernatant using ELISAs according to the manufacturer’s instructions. TNF-α is thought to contribute to pulmonary inflammation in VALI (46) and is one of the primary cytokines involved in the acute response to influenza infection (35); therefore, it is of particular interest in this two-hit model. Similarly, the recruitment of neutrophils to the lung is linked to the pathology associated with VALI (25) and influenza (8), hence, our interest in MIP-2, which is a mouse homolog for the neutrophil-related chemotactic cytokine IL-8. IL-6 is a relatively nonspecific acute inflammatory cytokine that we have used in previous studies of mouse models of VALI as a marker of ALI (10).

Statistical analysis. Changes in lung function over time and between groups were compared using repeated-measures ANOVA. Measures of lung inflammation (cells and cytokines) and baseline lung mechanics were compared between groups using ANOVA or t-tests. Post hoc multiple-group comparisons were done using Holm-Sidak tests. When the assumptions of normality and equal variance were violated, data were transformed using logarithmic or power transformations. Where this was not possible, equivalent nonparametric tests were used. All statistical analyses were conducted in SigmaStat (version 3.5, SPSS, Systat).

RESULTS

Lung mechanics and lung volume. Influenza-infected mice were significantly smaller than control mice [17.8(SD 1.3) and 19.8(SD 1.5), respectively, P = 0.03] 4 days after infection. All mice survived the ventilation period. Influenza infection significantly increased baseline lung mechanics (P < 0.001 for Raw, G, and H) compared with controls (Fig. 1). There was no significant difference between groups in TGV at baseline (P = 0.12; Fig. 1). Given the significant differences in baseline lung function between groups, changes in lung mechanics and lung volume over time were examined in terms of the percent change from baseline (Fig. 2). In the control mice, there was no significant change in Raw after the initial decrease from 10 min of ventilation to the end of the ventilation period (P > 0.05 for all pairwise comparisons). Raw was significantly lower, as a percentage of baseline, in influenza-infected mice than in controls from 20 min of ventilation to the end of the ventilation period (P < 0.001 for all pairwise comparisons; Fig. 2). Although there was a significant increase in G (P < 0.001) and H (P < 0.001) over time, there was no difference between infected mice and controls (P = 0.80 for G and P = 0.36 for H; Fig. 2).

Since the lung mechanics parameters are volume dependent (40), volume-corrected parameters were calculated in the form of specific Raw (sRaw), specific G (sG), and specific H (sH; Fig. 3). sRaw was significantly higher in influenza-infected mice than in controls throughout the ventilation period (P = 0.05), and there was a significant decrease in sRaw in both groups over the course of the ventilation period (P < 0.001). Similarly, sG (P < 0.001) and sH (P = 0.03) were higher in influenza-infected mice than in controls. After a small, but statistically significant, decrease in both groups in the first 40
Influenza A infection caused a significant increase in the total cell count ($P < 0.001$), number of macrophages ($P < 0.001$), and number of neutrophils ($P < 0.001$) in the BAL (Fig. 4). Total cell counts ($P = 0.01$) and the number of macrophages ($P = 0.001$) in the BAL were significantly decreased in ventilated mice compared with nonventilated mice (Fig. 4). In contrast, ventilation did not have an impact on the number of neutrophils in the BAL ($P = 0.92$; Fig. 4).

**BAL cytokines.** Production of protein, IL-6, MIP-2, and TNF-$\alpha$ peaked 2–3 days after influenza infection (Fig. 5). By day 4, when mechanical ventilation was applied at the height of clinical symptoms of disease, unventilated levels of these inflammatory markers had returned to (or were below) starting levels. Protein levels in the BAL were significantly higher 4 days after influenza A infection than at baseline ($P < 0.001$); however, these levels were not altered by ventilation ($P = 0.35$). Neither influenza A infection nor ventilation had an effect on levels of MIP-2 ($P = 0.25$ for infection, $P = 0.64$ for ventilation) or IL-6 ($P = 0.74$ for infection, $P = 0.21$ for ventilation) 4 days after intranasal inoculation. In contrast, in mice infected with influenza A 4 days earlier, TNF-$\alpha$ levels were significantly decreased ($P < 0.001$) in the BAL; again, ventilation had no effect on this cytokine compared with nonventilated mice ($P = 0.80$; Fig. 5).

**DISCUSSION**

Influenza A infection caused physiologically significant deficits in lung function in mice. This deficit in lung function was associated with the infiltration of inflammatory cells into the lung and increased protein leak. In contrast to previous findings using bacterial models (2, 29, 41) or mouse-specific viral infections (6), the inflammation resulting from influenza A infection was not exacerbated by mechanical ventilation. Consistent with this finding, lung function in mechanically ventilated mice following influenza A-induced ALI changed to the same extent as in mechanically ventilated healthy mice.

A number of studies show that mechanical ventilation can exacerbate preexisting lung injury (2, 3, 29). This is perhaps not surprising, given the plethora of data demonstrating that even so-called protective ventilation strategies can cause inflammatory responses in otherwise healthy lungs (9). Recognition of the interaction between preexisting ALI and the effects of mechanical ventilation in its own right led to identification of the two-hit model (44). The two-hit model has been tested in a number of animal studies demonstrating that mechanical ventilation can exacerbate bacterial-induced (2), viral-induced (6), and acid-induced (1) ALI. These results have highlighted the importance of considering the interaction between ALI and VALI in the clinical management of mechanically ventilated patients. In contrast, the present study using a clinically relevant model of viral-induced ALI suggests that the interaction between ALI and VALI may not
be as clear as the consistency of the previous studies has suggested.

It was clear in this study that our model of ALI induced physiologically significant changes in lung mechanics and high levels of cellular infiltration at baseline before mechanical ventilation. Although no direct measures of lung mechanics, other than peak airway pressure, were reported in the only previous study of the association between viral-induced ALI and VALI, there were clear differences in the level of inflammation between our influenza infection model and the inflammation generated by mouse PMV in the previous study (6). Influenza A infection in the present study induced an order-of-magnitude more neutrophils in the BAL and a larger increase in protein leak following viral infection. This suggested that, compared with PMV (6), influenza A infection was a more severe model of viral-induced ALI, which may explain the difference in outcome between the studies. However, there were several subtle, but potentially important, differences between our study and the study of Bem and colleagues (6). These differences primarily relate to the ventilation settings, including our use of a higher positive end-expiratory pressure (3 vs. 2 cmH2O), a higher ventilation rate (360 vs. 150 breaths/min), and a shorter ventilation period (2 vs. 4 h). Consequently, we cannot rule out differences in experimental design as an explanation for the discrepancy in outcomes between the studies. This perhaps highlights one of the difficulties in animal and, to a certain extent, human studies of mechanical ventilation, in that there is no standardization of ventilation settings. Nevertheless, in our hands, relatively mild ventilation settings with regular recruitment maneuvers did not exacerbate influenza-induced inflammation in mice, despite the severity of the initial insult.

As with all complex diseases, the pathways that lead to lung injury in response to mechanical ventilation cannot be completely modeled using a single system. For example, one of the problems associated with tracheal instillation of LPS to induce ALI as a model for bacterial pneumonia is that it induces a large inflammatory response but little alteration of lung architecture. In contrast, models of acid aspiration show considerable structural changes in the lung parenchyma but comparatively low levels of inflammation. It has been suggested that, for effective reflection of the human condition, animal models of ALI/ARDS should demonstrate an increase in epithelial permeability and a neutrophil-dominated inflammatory infiltrate (32). Viral-induced models of ALI, such as models involving direct instillation of live bacteria into the lung (13), appear to generate a neutrophil-dominated inflammatory response and increases epithelial permeability, as indicated by increased protein leak. Consequently, our model of ALI was not only severe, but it also had at least two of the major clinical indicators observed in human patients with ARDS.

Interestingly, the time point we chose (4 days after infection, which corresponds to the peak of neutrophil recruitment and signs of clinical illness in this model (8)) corresponded to the time when all the cytokines that we measured were past their peak in response to influenza infection. Thus there was scope for the cytokine system to respond, and yet there was no increase in these cytokines in response to mechanical ventilation. The apparent lack of generation of additional cytokines suggests that the timing of application of mechanical ventilation following lung injury may vastly alter the outcome. Interestingly, the aforementioned study by Bem and colleagues (6) also demonstrated no increase in TNF-α, IL-6, or MIP-2 production in unventilated control mice infected with PMV. These cytokines were also not altered by mechanical ventilation, and it was not until infection and ventilation were combined that they were significantly increased (6). The lack of effect of PMV on cytokine production suggests that the cytokine response was in a recovery stage similar to that observed

Fig. 4. Median and interquartile range for total cell count (TCC; A), number of macrophages (Mac; B), and number of neutrophils (C) in bronchoalveolar lavage of unventilated and ventilated BALB/c mice 4 days after inoculation with medium or influenza A.
in the present study. The fact that we did not observe an increase in these cytokines in response to mechanical ventilation in the presence of influenza infection, notwithstanding the differences in ventilation strategies already discussed, implies that the nature of the initial insult may affect the immune response to mechanical ventilation.

At the peak of clinical signs of illness in our model of influenza infection, the acute phase of the immune response and neutrophil recruitment by chemotactic activity was over, as demonstrated by the kinetics of MIP-2 production. Recruitment of neutrophils, their products, and the associated cytokines has been implicated in the progression of VALI in a number of studies, as demonstrated by increases in these inflammatory markers following mechanical ventilation (4) and ALI (36). Studies using neutrophil elastase knockout mice have also shown that this neutrophil product is critical in the recruitment of neutrophils during VALI (25). Given the importance of neutrophils and their products in these models, we would predict that a model of ALI, such as the model in the present study, which induced the recruitment of high numbers of neutrophils to the lungs, would show considerable exacerbation of ALI following mechanical ventilation, and this was not the case. Similarly, although influenza infection caused an increase in Raw, G, and H, mechanical ventilation did not appear to exacerbate this initial deficit in lung function, as indicated by the similar percent increase in G and H and stability of specific lung mechanics throughout the ventilation period. The consistency of specific measures of lung mechanics over time suggested that most of the change in the influenza-infected mice and the controls was due to the decrease in TGV, which presumably reflected the progressive loss of lung volume due to atelectasis. The fact that there was no difference in starting lung volume between the groups suggested that influenza A infection by itself did not cause significant atelectasis. Therefore, the protective ventilation strategy used in this study may have been sufficient to maintain lung volume during the ventilation period, despite the preexisting deficit in lung function.

As with all animal models of disease, there are limitations in how they reflect the human condition. For example, as with most animal models of VALI, we used a relatively short ventilation protocol. The short ventilation protocol was chosen, because we previously showed that even short durations of mechanical ventilation can cause significant changes in lung mechanics and inflammatory markers (10), and pilot studies (data not shown) suggested that influenza-infected mice would not have survived a longer ventilation protocol because of the high level of baseline lung injury. One of the other problems with these two-hit animal models is that they often do not result in an injury that requires ventilatory support of the animal. That is, the insult is not severe enough to cause respiratory distress. In our model, on the basis of previous data, all the mice would have recovered within days of the peak of infection, with no lasting changes in lung function (8). Similarly, most other published models of ALI, as mentioned above, produce milder disease than the present study, which would otherwise not require ventilatory support of the animal. Therefore, the inflammatory and immune events, particularly those associated with ARDS, are unlikely to be modeled effectively by any of these insults, and care should be taken when results of such studies are interpreted.

In summary, this study is only the second to examine the role of viral-induced ALI on the outcomes of mechanical ventilation. Using a severe, clinically relevant model of ALI with hallmarks of the human disease, we have demonstrated that the influx of inflammatory cells, production of inflammatory cytokines, epithelial permeability, and initial deficit in lung function were not exacerbated by mechanical ventilation. Future studies in this field should give careful consideration to the model of ALI to ensure that the severity of disease reflects human ARDS, such that the true interaction between ALI and VALI can be identified.
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