Ventilation with Negative Airway Pressure Induces a Cytokine Response in Isolated Mouse Lung

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We tested the hypothesis that, under relatively low tidal volume (V_T) mechanical ventilation, continuing lung de-recruitment induced by negative end-expiratory pressure (NEEP) would increase the lung cytokine response, potentially contributing to lung injury. Mouse lungs were excised and randomly assigned to one of 3 different ventilatory strategies: 1) the zero end-expiratory pressure group served as a control, 2) the NEEP7 group received a NEEP of -7.5 cm H_2O , and 3) the NEEP15 group received a NEEP of -15 cm H_2O . In all 3 groups, a V_T of 7 mL/kg was used. After 2 h of ventilation, lung lavage fluid was collected for measurements of tumor necrosis factor- α , monocyte chemoattractant protein-1, and lactate dehydrogenase. Increases in plateau pressure before and after

mechanical ventilation were significantly greater in the NEEP15 group compared with the zero end-expiratory pressure group or NEEP7 group. Lung compliance was decreased in the NEEP15 compared with the other two groups. Concentrations of tumor necrosis factor- α , monocyte chemoattractant protein-1, and lactate dehydrogenase in lung lavage were larger in the NEEP15 group than in the other groups. Atelectatic lung during repeated collapse and reopening of lung units accentuates the lung cytokine response that may contribute to lung injury even during relatively low V_T mechanical ventilation.

(Anesth Analg 2002;94:1577-82)

Mechanical ventilation has been used as an indispensable life-supportive therapy for respiratory failure (1). However, mechanical ventilation itself can induce a pulmonary inflammatory response and lung damage in the presence or absence of preexisting lung diseases (2). This form of ventilator-induced lung injury (VILI) has been called "biotrauma" (3). Several studies have shown that one of the mechanisms that causes VILI is alveolar overdistension, related to high airway pressure (4-6), or large tidal volumes (V_T) (7-11).

In an isolated rat lung model, Tremblay et al. (2) demonstrated that mechanical ventilation with large V_T and zero positive end-expiratory pressure (PEEP) induced an inflammatory response reflected by the production of cytokines, and lung injury assessed by compliance. Other studies have shown that mechanical ventilation with large V_T results in an increased production of tumor necrosis factor- α (TNF- α) and

interleukin-6 in the systemic circulation (12,13) that may contribute to the development of a systemic inflammatory response syndrome and perhaps distal organ dysfunction (14). We wondered whether lung inflammation could occur when the normal lung is ventilated with normal V_T during repeated collapse and reopening.

Using a mean V_T of 12 mL/kg with a negative end-expiratory pressure (NEEP) of -3 cm H_2O , Taskar et al. (15) examined pulmonary clearance of ^{99m}Tc -diethylenetriamine pentaacetic acid, an index of the integrity of the alveolar-capillary barrier in a rabbit model. These investigators reported an increase in the clearance of ^{99m}Tc -diethylenetriamine pentaacetic acid by the ventilatory strategy associated with a reduced lung volume (15). The same investigators also showed that a V_T of 12 mL/kg and a NEEP of -3 cm H_2O caused leakage of plasma proteins into alveoli when surfactant was depleted, suggesting that surfactant dysfunction makes the lung more vulnerable to repetitive collapse and reopening (16). However, the 12 mL/kg V_T used in those studies (15,16) might not be within a protective zone (i.e., 6-7 mL/kg). Thus, it remains unclear whether there is a correlation between lung derecruitment and injury using a V_T that prevents overdistension. Because VILI is associated with the activation of various inflammatory cells and

Supported in part by Canadian Institutes of Health Research (MOP-44093 to HZ and MA-8558 to AS).

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Accepted for publication February 21, 2002.

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mediators (2), we examined the hypothesis that repetitive collapse and reopening of the lung initiates a cytokine response during normal V_T ventilation. We used an *ex vivo* mouse lung model applying NEEP to test this hypothesis.

Methods

Lung Isolation

The study was approved by the animal use committee of the University of Toronto. Male A/J mice, aged 6 to 8 wk weighing 20 to 30 g were anesthetized with an intraperitoneal injection of ketamine (150 mg/kg) and xylazine (10 mg/kg). A tracheostomy was performed followed by insertion of a 20G angiocatheter (Becton Dickinson, Sandy, UT). The lungs were then excised via a midline sternotomy.

Ventilatory Setting

The excised lungs were placed in a 37°C humidified plethysmograph and airway pressure was measured (MPX series pressure transducer; Motorola, Phoenix, AZ) and recorded over the ventilation period. Before initiating ventilation, the lungs were inflated to total lung capacity, defined as a transpulmonary pressure of 25 cm H_2O , and sustained for 10 s. After deflation, a pressure-volume (PV) curve was measured with injection of air in 0.05-mL increments until a peak inspiratory pressure (PIP) reached 25 cm H_2O . The lungs were then ventilated with a small-animal, volume-cycled ventilator (Voltek Enterprises Inc., Ontario, Canada) at a V_T of 7 mL/kg and a respiratory rate of 50 breaths per minute for 3 min, before baseline PIP and plateau pressure (Pplat) were measured.

NEEP was established by connecting the expiratory port of the ventilator to a specially constructed chamber. The chamber consisted of a transparent plastic cylinder 60 cm high and 14 cm in diameter, attached in a vertical position to a circular base. The cylinder was filled about half way with water, and a rigid plastic tube of 3-mm inside diameter entered the chamber through a hole in the center of its circular top cover. An aquarium pump, modified to provide a suction port, was connected to another port on the top of the cylinder. When the end of the tube was placed below the water level, the negative pressure caused air from the outside to bubble through the water. The vertical position of the rigid plastic tube was adjustable to provide the required NEEP. The approximately 6 L of air in the cylinder above the water acted as a pressure buffer, so that the negative pressure during expiration remained nearly constant.

Experimental Protocol

After baseline measurements, the lungs were randomized into three ventilated groups using room air: Group 1 served as a control. The lungs were ventilated with a V_T of 7 mL/kg and zero end-expiratory pressure (ZEEP, $n = 10$). Groups 2 and 3 served as test groups ventilated with a V_T of 7 mL/kg and 2 levels of NEEP. The lungs were ventilated either with a NEEP of -7.5 cm H_2O (NEEP7, $n = 10$) in Group 2, or with a NEEP of -15 cm H_2O (NEEP15, $n = 10$) in Group 3.

Upon completion of 2 h of *ex vivo* ventilation, PIP, Plat, and PV curves were determined. The lungs were then lavaged 3 times by using 0.8 mL of cold normal saline. The lavage fluids were pooled and centrifuged. The supernatants were stored at $-70^\circ C$ for measurement of cytokines and lactate dehydrogenase (LDH). The lungs were then filled with 4% formalin for pathologic examination.

Cytokine Assays

Cytokine analysis (TNF- α , monocyte chemoattractant protein [MCP]-1) from the lung lavage fluid was performed in a blinded manner by using commercially available enzyme-linked immunosorbent assay kits (Biosource International Inc., Camarillo, CA).

LDH Assays

Cell injury was assessed by the measurement of LDH release in the lung lavage. A colorimetric assay of the Cytotoxicity Detection Kit (Boehringer Mannheim GmbH, Germany) was used as described previously (17).

Lung Histology

The lungs were fixed by immersion in 10% buffered neutral formalin and were processed using standard histologic techniques. A pathologist read the morphologic slides in a blinded manner.

Data analysis was performed by using two-way analysis of variance followed by the Tukey-Kramer test. Significance was set at $P < 0.05$. Results were reported as mean $\pm$ SEM.

Results

Body Weight

The body weight of the mice was similar in the 3 groups of animals (23.3 ± 1.9 g in the ZEEP group, 23.7 ± 0.7 g in the NEEP7 group, and 24.0 ± 2.2 g in the NEEP15 group, $P =$ not significant [NS]).

Lung Compliance (PIP, Pplat, and PV Curve)

The basal PIP and Pplat were identical in the three groups. After 2 h of ventilation, the PIP increased

Figure 1. The differences of plateau pressure (ΔP_{plat}) 2 h before and after mechanical ventilation with a tidal volume of 7 mL/kg in combination with zero end-expiratory pressure (ZEEP), -7.5 cm H₂O of negative end-expiratory pressure (NEEP7), or -15 cm H₂O of negative end-expiratory pressure (NEEP15). * $P < 0.05$ versus ZEEP.

slightly from 25.0 ± 1.0 under baseline conditions to 26.9 ± 0.7 cm H₂O in the ZEEP group ($P = NS$); from 24.3 ± 0.3 in baseline to 31.0 ± 1.0 cm H₂O in the NEEP7 group ($P = NS$); and increased significantly from 25.0 ± 0.2 to 32.0 ± 0.6 cm H₂O in the NEEP15 group ($P < 0.05$).

P_{plat} changed from 16.8 ± 0.5 to 19.1 ± 0.5 cm H₂O in the ZEEP group ($P = NS$), and from 17.7 ± 0.2 to 21.5 ± 0.5 cm H₂O in the NEEP7 group ($P = NS$), and from 17.3 ± 0.4 to 22.3 ± 0.5 cm H₂O in the NEEP15 group ($P < 0.05$).

The changes of P_{plat} (ΔP_{plat}) values before and after the ventilation were larger in the NEEP15 group than in the ZEEP group (Fig. 1).

The PV curves showed a greater downward shift to the right in the NEEP15 group than in the ZEEP group (Fig. 2). The lung volume at PIP 25 cm H₂O was significantly decreased in the NEEP15 group compared with the ZEEP group (Fig. 2). There was no significant shift of PV curves in the NEEP7 group (data not shown).

Cytokines

After 2 h of ventilation, the concentration of TNF- α and MCP-1 in the lung lavage fluid was significantly increased in the NEEP15 group compared with the ZEEP or NEEP7 group (both $P < 0.05$) (Fig. 3).

LDH

The levels of LDH in lung lavage fluids were identical in the ZEEP and the NEEP7 groups but significantly increased in the NEEP15 group ($P < 0.05$) (Fig. 4).

Lung Histology

There was no significant difference in histologic examination in the three groups with respect to epithelial integrity (data not shown).

Discussion

We examined the effect of continuing lung derecruitment using NEEP on lung cytokine responses to mechanical ventilation using a relatively small V_T . The V_T of 7 mL/kg we used has been suggested to be noninjurious by several studies in animals and in humans (2,13,18). We observed a significantly larger ΔP_{plat} , a decreased lung compliance, an increased production of cytokines, and release of LDH in the NEEP15 group, suggesting an inflammatory response and cell injury. Our data thus indicate that the use of NEEP during normal V_T ventilation increases the release of cytokines. This study also suggests that NEEP is a useful model for studying the effect of continuing derecruitment during repetitive collapse and reopening of the lung. Although the *ex vivo* system has several advantages, such as allowing us to study the direct effects on the lung without the potential complications of cytokine influence from the circulation, this model also has limitations. The lack of a circulation with consequent ischemia may have contributed to the injury observed, but all three groups sustained equal periods of ischemia so that this factor was unlikely to be critical in this model.

Taskar et al. (15,16) used a rabbit model and demonstrated that a NEEP as small as -3 cm H₂O decreased lung compliance and oxygenation, and increased lung permeability (15), which was impaired even more if the lungs were depleted of surfactant

Figure 2. Mean values of pressure-volume curves before (upper curves) and 2 h after mechanical ventilation with a tidal volume of 7 mL/kg and zero end-expiratory pressure (ZEEP) or -15 cm H₂O of end-expiratory pressure (NEEP15). The curve showed a greater down-right shift in the NEEP15 group.

Figure 3. Concentrations of tumor necrosis factor (TNF)- α and monocyte chemoattractant protein (MCP)-1 in lung lavage fluids of the 3 groups that were ventilated for 2 h with a tidal volume of 7 mL/kg in combination with zero end-expiratory pressure (ZEEP), -7.5 cm H₂O of negative end-expiratory pressure (NEEP7), or -15 cm H₂O of negative end-expiratory pressure (NEEP15). * $P < 0.05$ versus ZEEP.

(16). However, our data showed that a NEEP of -7 cm H₂O did not induce any significant alterations with respect to cytokine responses and lung injury indices. Several factors may explain the apparent discrepancies in these observations. First, Taskar et al. (15,16) used a V_T of 12 mL/kg, which may be relatively injurious compared with the V_T of 7 mL/kg that we used. Second, they compared lung injury indices between NEEP and PEEP (2 cm H₂O) (15,16). The latter may be protective, so that the same injury induced by a NEEP of -3 H₂O might not have been apparent if it had been compared with a ZEEP group as in the present study. Third, they used an *in vivo* model, which may have been associated with hemodynamic compromise during mechanical ventilation that could

have contributed to the lung damage (15,16). We focused on an *ex vivo* lung model to avoid any hemodynamic influences. Finally, there may be species differences that account for these findings.

This is the first report of a significant cytokine response to NEEP ventilation. We chose to measure TNF- α and MCP-1 in the present study because the cytokines are largely involved in lung injury. TNF- α may mediate many of the pathologic changes seen in acute respiratory distress syndrome (ARDS), including initiation of the cytokine cascade and an increase in lung permeability (19). MCP-1, a c-c chemokine family member, can be derived from monocytes and alveolar macrophages. The blockade of MCP-1 attenuates the development of lung injury (20). Because

Figure 4. Values of optical density of lactate dehydrogenase (LDH) in BAL fluids of the 3 groups that were ventilated for 2 h with a tidal volume of 7 mL/kg in combination with zero end-expiratory pressure (ZEEP), -7.5 cm H_2O of end-expiratory pressure (NEEP7), or -15 cm H_2O of end-expiratory pressure (NEEP15). * $P < 0.05$ versus ZEEP.

alveolar macrophages are often involved in lung inflammation, we also measured MCP-1 production. We do not have evidence in this model of which cell type is producing the cytokines, but in a previous study, we found that the epithelial cells serve as a major source of TNF- α and interleukin-6.¹ The cytokine release was not necessarily related to lung structural alterations because no significant lung histologic damage was seen in this model, which may be attributable to the limited study period. However, both lung epithelial cells and inflammatory cells could be the major sources of the increased production of cytokines induced by mechanical stress as previously described in *ex vivo* studies (21,22).

The release of LDH, a product of glucose-pyruvate metabolism reflecting cell injury (23,24), significantly increased only in the NEEP15 group. The sources of LDH could be from lung parenchyma and/or erythrocytes. Because no erythrocytes were found in the bronchoalveolar lavage fluid, we believe that the increased level of LDH was largely produced by lung tissue. Lung histologic examination did not demonstrate significant alterations by NEEP. Because LDH is a stable cytoplasmic enzyme present in all cells, and is rapidly released into the cell culture supernatant upon damage of the plasma membrane, our data suggest that LDH is a sensitive index of NEEP-initiated inflammation and injury at cellular levels during continuing lung decruitment before the development of a significantly altered lung histology. In support of the sensitivity of the LDH measurement with respect to cellular injury, we have previously observed a similar

phenomenon when lung tissue was challenged by lipopolysaccharide (17).

The mechanisms by which NEEP induced lung cytokine response and cellular injury may be attributable to lung instability at end-expiration, resulting in lung injury even under ventilation with low V_T . This suggests that low V_T may be protective only when the alveoli are kept relatively open at end-expiration. This concept is supported by recent studies in which Pelosi et al. (25) and Richard et al. (26) demonstrated that decruitment can occur during low V_T ventilation, and repeated collapse and reopening of alveoli may have a predominant role in causing VILI even with noninjurious ventilation. This may have some relevance to the recently reported ARDSNet trial (18), which found a 22% decrease in mortality in the ARDS patients treated with 6 mL/kg (predicted body weight). A recent study has suggested that one mechanism for decreased injury and mortality may be related to the generation of auto-PEEP in patients who received a high respiratory rate to maintain relatively normal values of $Paco_2$ (27). Thus, it may have been the compensation of low V_T with somewhat increased PEEP that was protective. We do not exclude the possibility that an even lower V_T ventilator setting might otherwise have avoided further VILI.

In conclusion, continuing lung decruitment during repeated collapse and reopening of lung units induced by NEEP accentuates the lung cytokine response and cell injury, even with ventilation using a relative normal value of V_T . This phenomenon may have clinical relevance in atelectasis-prone alveoli during mechanical ventilation in patients.

We thank George A. Volgyesi, P. Eng., for his technical assistance.

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