High tidal volume ventilation induces NOS2 and impairs cAMP-dependent air space fluid clearance

James A. Frank,1,2 Jean-François Pittet,3,4 Hyon Lee,3 Micaela Godzich,3 and Michael A. Matthay1–3

1Cardiovascular Research Institute and the Departments of 2Medicine, 3Anesthesia, and 4Surgery, University of California, San Francisco, San Francisco, California 94143-0130

Submitted 2 October 2002; accepted in final form 14 January 2003

Frank, James A., Jean-François Pittet, Hyon Lee, Micaela Godzich, and Michael A. Matthay. High tidal volume ventilation induces NOS2 and impairs cAMP-dependent air space fluid clearance. Am J Physiol Lung Cell Mol Physiol 284: L791–L798, 2003. First published January 31, 2003; 10.1152/ajplung.00331.2002.—Tidal volume reduction during mechanical ventilation reduces mortality in patients with acute lung injury and the acute respiratory distress syndrome. To determine the mechanisms underlying the protective effect of low tidal volume ventilation, we studied the time course and reversibility of ventilator-induced changes in permeability and distal air space edema fluid clearance in a rat model of ventilator-induced lung injury. Anesthetized rats were ventilated with a high tidal volume (30 ml/kg) or with a high tidal volume followed by ventilation with a low tidal volume of 6 ml/kg. Endothelial and epithelial protein permeability were significantly increased after high tidal volume ventilation but returned to baseline levels when tidal volume was reduced. The basal distal air space fluid clearance (AFC) rate decreased by 43% ($P < 0.05$) after 1 h of high tidal volume but returned to the prevention rate 2 h after tidal volume was reduced. Not all of the effects of high tidal volume ventilation were reversible. The cAMP-dependent AFC rate after 1 h of 30 ml/kg ventilation was significantly reduced and was not restored when tidal volume was reduced. High tidal volume ventilation also increased lung inducible nitric oxide synthase (NOS2) expression and air space total nitrite at 3 h. Inhibition of NOS2 activity preserved cAMP-dependent AFC. Because air space edema fluid inactivates surfactant and reduces ventilated lung volume, the reduction of cAMP-dependent AFC by reactive nitrogen species may be an important mechanism of clinical ventilator-associated lung injury.

acute respiratory distress syndrome; acute lung injury; alveolar epithelium; ventilator-induced lung injury; pulmonary edema

TIDAL VOLUME REDUCTION during positive pressure mechanical ventilation reduces mortality in patients with acute lung injury and the acute respiratory distress syndrome (ARDS) (2). Although this is the only intervention that has convincingly demonstrated a mortality benefit in this patient population since the original description of the syndrome (37), the mechanism of the protective effect is incompletely understood. Several investigators have reported that ventilation of normal animal lungs with high tidal volume, especially in conjunction with low levels of positive end-expiratory pressure (PEEP), induces interstitial and alveolar edema (6, 34). After experimental acute lung injury, mechanical ventilation with tidal volumes that are safe in the normal lung can worsen the severity of pulmonary edema and alveolar epithelial injury compared with lower tidal volumes (8, 13). One explanation for this finding is the heterogeneity of lung inflation that results from the uneven distribution of alveolar edema. The presence of protein-rich alveolar edema causes the inactivation of surfactant, by both plasma proteins binding to surfactant protein and edema fluid washing away surfactant lipid (39, 40). The loss of surfactant function increases surface tension at the air-liquid interface in alveoli and small airways, resulting in the closing of small airways, alveolar collapse, and a decrease in the effective lung volume. In addition, the presence of edema fluid in the distal airways and alveoli may expose small airway epithelial cells to shear forces as fluid and air move in and out of collapsed and reexpanded airways during ventilation (26).

We recently reported that tidal volume reduction preserves distal air space fluid clearance (AFC) in a rat model of ventilator-associated lung injury (13). In patients with acute lung injury, the presence of preserved air space edema fluid clearance correlates with improved outcomes, including survival (23, 36). Therefore, air space edema formation and impaired edema clearance are partly the result of injurious ventilation and a mechanism by which ventilator-induced lung injury (VILI) may be propagated. In a previous study from our research group, we reported that air space reactive nitrogen species impair cAMP-dependent AFC in a rat model of hemorrhagic shock (3, 4, 27). Reactive nitrogen species form when nitric oxide (NO) combines with oxygen or superoxide (18). In the setting of acute lung injury, air space NO is derived primarily from the inducible form of nitric oxide synthase (NOS2). The effect of high tidal volume ventilation on NOS2 expression and air space reactive nitrogen species has not been previously reported.

Address for reprint requests and other correspondence: J. A. Frank, Cardiovascular Research Inst., Univ. of California, San Francisco, San Francisco, CA 94143-0130 (E-mail: frankja@itsa.ucsf.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
The primary objective of this study was to investigate the mechanisms responsible for the reduction in air space edema fluid clearance due to high tidal volume ventilation in an in vivo rat model of VILI. The second objective was to determine the time course of the changes in fluid transport and permeability associated with high tidal volume ventilation and to determine whether these changes were reversible. The third objective was to determine whether AFC could be restored with β-adrenergic agonists following high tidal volume ventilation in this model. The fourth objective was to determine the role of NOS2 activity in the reduction of air space epithelial fluid transport rates following high tidal volume ventilation.

METHODS

Animal preparation. Experiments were conducted in accordance with the National Institutes of Health guidelines for the use of research animals. Sprague-Dawley rats weighing 300–340 g were anesthetized with an intraperitoneal injection of pentobarbital (75 mg/kg). Rats were placed supine and ventilated through a tracheotomy tube (15-gauge luer stub adapter; Becton Dickinson, Sparks, MD) with a volume-controlled ventilator (model 683; Harvard Apparatus, South Natick, MA) at the following settings: tidal volume 6 ml/kg, PEEP 10 cmH2O, fraction of inspired oxygen 1.0, respiratory rate 55 breaths/min. Respiratory rate was adjusted to maintain arterial pH between 7.35 and 7.45. Airway pressures were monitored continuously (MX860 transducer; Medex, Hilliard, OH), and data were recorded on a computer-based data acquisition system (MP100; BIOPAC Systems, Santa Barbara, CA). A catheter (PE-50 tubing; Becton Dickinson) in the right common carotid artery was used to continuously monitor blood pressure and to obtain arterial blood gases and blood samples. A central venous catheter was placed in the right internal jugular vein for administration of NOS2 inhibitors. Anesthesia was maintained with pentobarbital (500 μg·kg⁻¹·h⁻¹ iv), and muscle relaxation was maintained with pancuronium (2 mg·kg⁻¹·h⁻¹ iv).

Experimental protocol. After a 30-min stable baseline period, rats were ventilated for 1 h (n = 18) or 3 h (n = 18) with a tidal volume of 6 ml/kg and PEEP level of 10 cmH2O; with high tidal volume (10.0 ± 0.1 ml such that the initial peak inspiratory pressure was 30 cmH2O), zero PEEP for 1 h (n = 30); or with the same high tidal volume for 1 h followed by 2 h of ventilation with 6 ml/kg and PEEP 10 cmH2O (n = 27) (Fig. 1). During high tidal volume ventilation, the respiratory rate was reduced to 20 breaths/min, and ~5 ml of dead space was added to the ventilator circuit. Arterial blood gases (model 248; Chiron Diagnostics, Norwood, MA) were obtained every 30 min, and the amount of added ventilator circuit dead space was adjusted to maintain an arterial pH of 7.35–7.45. To determine the effect of NOS2 on the observed changes in AFC, we gave additional groups of rats one of two specific inhibitors of NOS2: L-NAME (1-iminoethyl)lysine (L-NIL, 3 mg·kg⁻¹·h⁻¹ iv) beginning 30 min before the protocol, or N-(3-aminomethyl)benzyl)acetamide (1400W, 5 mg/kg iv) 30 min before the protocol (15, 25). Other rats were given similar volumes of saline. Thirty minutes before the start of the experimental protocol, additional rats were given N°-nitro-L-arginine methyl ester (L-NNAME, 30 mg·kg⁻¹·h⁻¹), a nonspecific NOS inhibitor that inhibits the constitutive and inducible isoforms of NOS.

Endothelial permeability to albumin. At the start of the experimental protocol, 1 μCi of 125I-labeled albumin in 1 ml of saline was infused intravenously (10). After 5 min and then every hour, plasma samples were obtained. Permeability to albumin, expressed as extravascular plasma equivalents (EVPE) in microliters, was calculated using the following equation

\[
\text{EVPE} = \frac{[C_H - (C_{\text{end}} \times Q_0)]/C_{\text{pl}}}{Q_1}
\]

where \(C_H\) represents the 125I counts·min⁻¹·mg⁻¹ in the homogenized left lung, \(C_{\text{end}}\) represents the counts·min⁻¹·mg⁻¹ in plasma at the end of the experiment, and \(C_{\text{pl}}\) represents the average counts·min⁻¹·mg⁻¹ in all of the plasma samples collected during the experiment. \(Q_0\) is the blood volume in the lungs determined by the gravimetric method (10). In some of the 3-h experiments, permeability was measured over the last hour of the protocol (tracer given intravenously 1 h before the end of the experiment). In all of the permeability experiments, the left lung was removed for determination of EVPE, and the right lung was lavaged three times with 2 ml of normal saline. Radioactivity of the air space lavage fluid was used as an index of epithelial permeability to albumin (24).

Measurement of AFC. As in our previous studies (28–30), we determined distal AFC by measuring the increase in protein tracer activity over 30 min following intratracheal instillation of an isoosmolar, 5% albumin solution containing 1 μCi 131I-labeled albumin. The instillate was prepared with bovine albumin (ICN Biomedicals, Aurora, OH). The increase in protein tracer concentration provides a good estimate of the liquid volume removed from the distal air spaces of the lung. AFC was measured at the end of the experiments in the absence of blood flow by a previously described in situ model (13), a method that has been used in many mouse, rat, and human studies (22). Rats were exsanguinated, and then 6 ml/kg of the 5% albumin solution warmed to 37°C were instilled into the trachea. Ventilation was stopped, and an airway pressure of 10 cmH2O was maintained with 100% oxygen. Five minutes after instillation, a baseline sample (0.1 ml) was aspirated from the distal airways via PE-50 tubing (T or time = 0 sample). This allowed for the determination of tracer dilution by edema fluid present in the lungs. Thirty minutes later, another sample was collected from the distal airways. Body temperature was maintained at 37°C...
throughout the 30-min period with an external heating lamp and warming blanket. Core body temperature was monitored with a digital thermometer probe placed in the midoesophagus (Fisher Scientific, Houston, TX). AFC, expressed as the percentage of alveolar fluid volume cleared in 1 h, was determined by the following equation:

$$AFC = \frac{2(1 - (P_t/P_r) \times 100)}{}$$

where $P_t$ is the radioactivity (counts/g) at $T = 0$, and $P_r$ is the activity 30 min later. In some experiments, terbutaline ($10^{-5}$ M), amiloride ($10^{-3}$ M), or dibutylryl cAMP (dcAMP) ($10^{-3}$ M), and aminophylline ($10^{-4}$ M) were added to the instillate.

**Plasma epinephrine levels.** Because endogenous epinephrine can stimulate AFC (12, 20), plasma epinephrine levels were measured in samples obtained during the baseline period and at the end of the experiment by an ELISA (IBL, Hamburg, Germany) as previously described (13).

**Lung lavage nitrite levels.** Lung lavage concentrations of total nitrite and nitrate (NO$_X$) were measured with a colorimetric nitric oxide assay kit (Calbiochem, San Diego, CA). Samples (85-$\mu$l) of bronchoalveolar lavage (BAL) fluid were incubated for 20 min at 25°C with nitrate reductase (0.01 units) and NADH. After the addition of Griess reagent, absorbance was read at 540 nm and compared with a nitrate standard as in our previous experiments (27, 41).

**NOS2 protein expression.** NOS2 protein expression was measured by Western blot analysis as previously described (27). Briefly, equal amounts of protein from frozen whole-lung homogenates were separated by gel electrophoresis. Separated proteins were transferred to nitrocellulose membranes. The membranes were then immunoblotted with monoclonal anti-mouse NOS2 antibody (1:500 dilution) (Transduction Laboratories, Lexington, KY) overnight at 4°C. The blot was then incubated with labeled secondary antibody (goat anti-mouse Ig, 1:2,000 dilution). Protein bands were quantified by densitometry.

**Statistics.** Comparisons between two groups were made using an unpaired, two-tailed Student's t-test. Comparisons among three or more groups were made using one-way analysis of variance and Tukey's correction for multiple comparisons. $P$ values $>$0.05 were considered significant. Data are expressed as means $\pm$ SE.

**RESULTS**

**High tidal volume ventilation for 1 h reversibly increases lung endothelial and epithelial protein permeability.** Lung endothelial permeability to albumin was increased in rats ventilated with high tidal volume for 1 h (Fig. 2A). When tidal volume was reduced, endothelial permeability to albumin significantly decreased and was comparable to that in rats ventilated with low tidal volume for 3 h ($P > 0.05$). Lung epithelial permeability to albumin similarly increased after 1 h of high tidal volume ventilation but returned to a level comparable with the low tidal volume group by 2 h after tidal volume reduction (Fig. 2B). There was a nonsignificant trend toward a small increase in epithelial protein permeability by 3 h in the rats ventilated with low tidal volume only (Fig. 2B). Pretreatment with L-NAME, L-NIL, or 1400W did not preserve alveolar epithelial or endothelial permeability (data not shown). Ventilation with 6 ml/kg and a PEEP of 10 cmH$_2$O did not induce alveolar edema as measured by the initial dilution of the intratracheal instillate; however, high tidal volume ventilation followed by 2 h of low tidal volume ventilation induced mild air space edema (34.7 $\pm$ 8.1 $\mu$l).

**High tidal volume ventilation for 1 h reversibly decreases basal air space edema fluid clearance.** Ventilation with high tidal volume and no PEEP for 1 h reduced AFC by 43$\%$ ($P < 0.05$ compared with low tidal volume ventilation) (Fig. 3). If the tidal volume was then reduced to 6 ml/kg and a PEEP of 10 cmH$_2$O was added, basal AFC returned to preventilation levels 2 h later (at 3 h) (Figs. 3 and 4). The recovery of basal air space fluid transport correlated with the restoration of lung endothelial and epithelial protein permeability to
near baseline levels (Fig. 2). Amiloride-sensitive AFC after 1 h of high tidal volume ventilation was 7.2 ± 1.2%/h (29% decrease from basal AFC, n = 6). Amiloride-sensitive AFC after 1 h of low tidal volume ventilation was 13.4 ± 1.7%/h (33% decrease from basal AFC, n = 9). Amiloride-sensitive AFC after 1 h of high tidal volume and 2 h of low tidal volume ventilation was 8.5 ± 1.4%/h (55% decrease from basal AFC, n = 5) compared with 12.7 ± 2.2 (31% decrease from basal AFC, n = 3) after 3 h of low tidal volume ventilation. The differences in amiloride-sensitive AFC were not statistically significant at either time point (P > 0.05).

High tidal volume ventilation induces a sustained reduction in cAMP-dependent AFC. In rats that underwent 1 h of high tidal volume ventilation, cAMP-dependent AFC was reduced compared with rats ventilated with low tidal volume (Fig. 4). Terbutaline (10⁻⁵ M)-mediated cAMP-dependent AFC was 19.2 ± 1.4%/h following 1 h of high tidal volume ventilation. In rats ventilated with low tidal volume, cAMP-dependent AFC was 26.0 ± 3.1%/h. However, in rats ventilated with high tidal volume for 1 h followed by low tidal volume ventilation for 2 h, cAMP-dependent AFC was not different from basal AFC rates (17.7 ± 2.3% compared with 19.2 ± 1.4%/h, P = not significant) (Fig. 4). Plasma epinephrine levels were low and did not differ among the groups at any time point (data not shown).

High tidal volume ventilation induces NOS2 and increases BAL NOₓ. Rats ventilated with high tidal volume for 1 h followed by low tidal volume for 2 h had significantly higher BAL NOₓ levels compared with rats ventilated only with low tidal volume for 3 h (Fig. 5A). Pretreatment with the specific NOS2 inhibitor 1400W prevented the increase in BAL NOₓ (Fig. 5A). There was significantly more NOS2 protein by Western blot analysis in whole lung homogenates from rats ventilated with high tidal volume for 1 h followed by low tidal volume for 2 h, compared with rats ventilated with low tidal volume for 3 h (Fig. 5, B and C). BAL NOₓ levels were nonsignificantly increased after 1 h of high tidal volume ventilation; however, NOS2 protein expression was not increased (not shown).

Inhibition of NOS2 preserved cAMP-dependent AFC following high tidal volume ventilation. Inhibition of NOS2 with 1400W preserved the β-adrenergic agonist-mediated, cAMP-dependent increase in AFC in rats exposed to high tidal volume ventilation for 1 h followed by low tidal volume for 2 h (Fig. 6). Pretreatment with a different selective inhibitor of NOS2, l-NIL, also preserved cAMP-dependent AFC in rats ventilated with high tidal volume for 1 h and low tidal volume for 2 h (25.5 ± 3.6%/h, P < 0.05). Increasing intracellular cAMP directly with dcAMP (10⁻³ M) and aminophylline (10⁻⁴ M) in the intratracheal instillate significantly increased AFC rates in rats ventilated with either high (Fig. 6) or low tidal volumes (not shown). Neither 1400W or l-NIL affected basal or cAMP-dependent AFC in rats ventilated with low tidal volume for 1 or 3 h.

Arterial blood gases, airway pressures, and respiratory mechanics. There were no significant differences in arterial blood pH, PaCO₂, and PaO₂ among the groups at any time point (data not shown). By design, airway pressures were higher and respiratory rate was lower during the high tidal volume ventilation period. The mean peak airway pressure during high tidal volume ventilation was 30.1 ± 0.7 and 19.2 ± 0.3 cmH₂O during low tidal volume ventilation. The PEEP level...
was 0.2 ± 0.1 cmH₂O during high tidal volume ventilation and 10.1 ± 0.1 cmH₂O during low tidal volume ventilation (as measured by the double occlusion technique) (13). Tidal volume was 10.0 ± 0.1 ml during the high tidal volume period and 1.9 ± 0.02 ml during low tidal volume ventilation.

**DISCUSSION**

Although there is now evidence that the use of low tidal volume ventilation for patients with ARDS and acute lung injury significantly reduces mortality (2), the mechanisms of the protective effect are incompletely understood. ARDS is characterized by protein-rich pulmonary edema, hyaline membrane formation, and the infiltration of neutrophils into the air spaces of the lung. Because the presence of edema fluid in the air spaces reduces lung volume by filling alveoli, blocking distal airways, and inactivating surfactant, processes that reduce the rate of edema fluid clearance from the air space contribute to the loss of lung volume. A reduction of lung volume may be an important mechanism for the propagation of lung injury in ventilator-associated lung injury (6). Previously, we found that low tidal volume ventilation preserved air space fluid transport in a rat model of acid-induced acute lung injury (13). In that study, the reduction of tidal volume from 12 to 6 ml/kg at similar levels of PEEP resulted in more preserved AFC. Further reduction in tidal volume to 3 ml/kg resulted in an even higher rate of AFC and a reduction in edema fluid levels of RTI40, a marker of type I cell injury.

In the current study, we have investigated the mechanisms responsible for the reduction in AFC associated with higher tidal volume ventilation using a rat model.
High tidal volume ventilation reduced basal AFC by 43% in this in vivo model of VILI. We also found that the decrease in basal AFC at the end of 1 h of injurious ventilation was reversible when the tidal volume was reduced and PEEP was added. The reduction in basal AFC at 1 h was largely attributable to the increase in epithelial permeability, since the decrease in AFC correlated with the increase in epithelial permeability. As has been demonstrated in other models (21), increased epithelial permeability results in a reduction in AFC due to the loss of the sodium concentration gradient that drives fluid transport out of the air spaces. Accordingly, the recovery of basal AFC at 3 h when high tidal volume ventilation was followed by 2 h of low tidal volume ventilation correlated with the recovery of epithelial permeability to near baseline levels (Fig. 2B). Although epithelial permeability in the rats ventilated with 1 h of high tidal volume followed by 2 h of low tidal volume was not significantly different from rats ventilated with low tidal volume for 3 h, it is possible that this lack of difference may be the result of a type II error. However, we have previously found that the net rate of AFC may be normal even when epithelial permeability is mildly increased (11, 13, 24). Previous studies of isolated perfused lungs from rats ventilated for 40 min with a tidal volume exceeding total lung capacity (40 ml/kg) reported that active sodium transport was reduced by 50% (19). The effect was due to decreased activity of Na⁺-K⁺-ATPase but not to decreased amounts of the transporter in the membrane. Although we did not examine Na⁺-K⁺-ATPase activity in this study, this may be an additional mechanism by which AFC was decreased at 1 h.

Not all of the effects of high tidal volume ventilation were reversible. Most notable was the absence of cAMP-dependent AFC 2 h after tidal volume reduction. Using isolated perfused lungs from rats exposed to 40 min of high tidal volume ventilation, Saldias and colleagues reported that the administration of β-agonists (33) or dopamine (32) increases AFC to levels comparable with stimulated AFC in nonventilated controls. This effect was prevented by inhibition of cytoskeletal protein polymerization with colchicine, suggesting that the increase in AFC was due in part to increased mobilization of epithelial Na channels (ENaC) or Na⁺-K⁺-ATPase from intracellular pools to the plasma membrane (32). In the present study, we found that β-agonists increased AFC only to basal levels immediately after 1 h of high tidal volume ventilation (Fig. 4). The difference in cAMP-dependent AFC in the current study may be due to differences in the models, including the duration of ventilation and the measurement of AFC in the in situ model instead of isolated perfused lungs. However, we also found that basal AFC spontaneously returned to prevention levels by 2 h if tidal volume was reduced (Fig. 4). Furthermore, there was a complete absence of β-agonist stimulation of AFC 2 h after high tidal volume ventilation (Figs. 4 and 6). This
inhibitory effect on cAMP-dependent AFC was prevented with either 1400W or L-NIL.

To our knowledge, this is the first in vivo rat study to demonstrate that reactive nitrogen species significantly reduce AFC following high tidal volume ventilation. We propose that high tidal volume ventilation reduces AFC by at least two mechanisms. The first is by increasing epithelial permeability and thereby undermining the ion transport gradient across tight epithelia that drives vectorial fluid transport from the apical to the basal surface of the lung epithelium. The second mechanism is inhibition of cAMP-dependent AFC through activation of NOS2 and the formation of reactive nitrogen species. Previous studies from our research group have demonstrated that reactive nitrogen species prevent β-agonist-stimulated AFC following hemorrhagic shock in rats (27). Others have reported a similar decrease in stimulated AFC after prolonged hyperoxia (9). Exhaled NO, derived from NOS2, peaks by 3 h after intratracheal administration of endotoxin (1). This result is consistent with our data on NOS2 expression after high tidal volume ventilation. The current study provides evidence that reactive nitrogen species generated during and following high tidal volume ventilation induce a dissociation between cAMP, because administration of dbcAMP restores AFC (23). Furthermore, nitrite levels are higher in patients with impaired alveolar fluid clearance (35, 41) and impaired AFC correlates with mortality in acute lung injury patients (23, 38). Therefore, the reduction of AFC by reactive nitrogen species may be an important mechanism of clinical ventilator-associated lung injury in patients, potentially contributing to more pulmonary edema and more severe respiratory failure.

This research was supported by National Heart, Lung, and Blood Institute Grants HL-69900 and HL-51854.

REFERENCES


