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Interactions of lung stretch, hyperoxia, and MIP-2 production in ventilator-induced lung injury

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Quinn, Deborah A., Ramzi K. Moufarrej, Alexey Volokhov, and Charles A. Hales. Interactions of lung stretch, hyperoxia, and MIP-2 production in ventilator-induced lung injury. *J Appl Physiol* 93: 517–525, 2002. First published April 15, 2002; 10.1152/japplphysiol.00570.2001.—The use of positive pressure mechanical ventilation can cause ventilator-induced lung injury (VILI). We hypothesized that hyperoxia in combination with large tidal volumes (VT) would accentuate noncardiogenic edema and neutrophil infiltration in VILI and be dependent on stretch-induced macrophage inflammatory protein-2 (MIP-2) production. In rats ventilated with VT 20 ml/kg, there was pulmonary edema formation that was significantly increased by hyperoxia. Total lung neutrophil infiltration and MIP-2 in bronchoalveolar lavage (BAL) fluid were significantly elevated, in animals exposed to high VT both on room air (RA) and with hyperoxia. Hyperoxia markedly augmented the migration of neutrophils into the alveoli. Anti-MIP-2 antibody blocked migration of neutrophils into the alveoli in RA by 51% and with hyperoxia by 65%. We concluded that neutrophil migration into the alveoli was dependent on stretch-induced MIP-2 production. Hyperoxia significantly increased edema formation and neutrophil migration into the alveoli with VT 20 ml/kg, although BAL MIP-2 levels were nearly identical to VT 20 ml/kg with RA, suggesting that other mechanisms may be involved in hyperoxia-augmented neutrophil alveolar content in VILI.

neutrophils; cytokines; rat ventilation; macrophage inflammatory protein-2

ACUTE LUNG INJURY OFTEN REQUIRES the use of mechanical ventilation with high levels of oxygen to adequately oxygenate the brain and other vital organs. However, mechanical ventilation (6) and hyperoxia (27) can both damage normal lung tissue. Acute lung injury is an inhomogeneous disease (9, 25). Mechanical ventilation with large tidal volumes (VT) to recruit diseased areas of the lung with low compliance leads to overdistention of normal areas of lung with normal compliance. In severely damaged lungs in which air space is reduced up to 60% (31), the use of even low VT may lead to the overdistention of the remaining normal lung. In a large clinical trial (800 patients) of large-volume ventilation vs. small-volume ventilation in acute respiratory distress syndrome (ARDS), there were 22% fewer deaths in the patients ventilated with smaller VT (1).

In animal models attempting to duplicate the large stretch administered to the normal compliance areas of humans with ARDS, investigators have used mechanical ventilation of the whole lung with large VT in normal animals to produce ventilator-induced lung injury (VILI) (5a, 48). VILI is characterized by pulmonary edema that appears to be caused by increased microvascular permeability (6). Dreyfuss and associates (5a) experimentally separated the effects of pressure used to deliver a tidal breath from the volume of the tidal breath in mechanically ventilated rats. They used abdominal binders to decrease thoracic expansion, thereby decreasing the amount of stretch on the lung but keeping the pressure high. They found that high inspiratory pressure (45 cmH_2O), if not accompanied by stretch, did not invoke edema. Also, if they used negative pressure ventilation with an iron lung to produce large VT (44 ml/kg), edema was still induced (7). This has led to the feeling that lung stretch is the problem and that the injury should be known as volutrauma rather than barotrauma.

VILI has been associated with release of chemoattractant cytokines (13, 44) and subsequent influx of neutrophils. Healthy baby pigs ventilated at peak inspiratory pressures of 40 cmH_2O for over 8 h had alveolar neutrophil infiltration, whereas pigs ventilated at peak inspiratory pressures of less than 18 cmH_2O had no notable histopathological changes (45). VILI has also been shown to cause neutrophil infiltration in rats (15) and sheep (36).

The effects of hyperoxia on the lung have long been recognized (27). Hyperoxia has been shown to cause alveolar hyaline membrane formation, edema, hyperplasia and proliferation of type II alveolar epithelial cells, destruction of type I alveolar epithelial cells, interstitial fibrosis, and pulmonary vascular remodeling (17, 27). With as little as 3 h of hyperoxia, there was increased transcription of tumor necrosis factor-α (TNF-α) mRNA in alveolar macrophages (14) and activation of transcription factors that regulate gene expression (20, 41).

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The development of VILI is a complex issue and involves many factors. Release of chemoattractant cytokines (34), overdistention of normal lung (9), and the presence of prior lung damage (8) have all been found to play a role. Hyperoxia has been shown to cause neutrophil infiltration and pulmonary edema. High levels of oxygen, especially in the first few hours after intubation, are often required to treat patients with ARDS. Little is known about the effect of hyperoxia on VILI. We hypothesized that hyperoxia in combination with large V_T would accentuate noncardiogenic edema and neutrophil infiltration in VILI and be dependent on stretch-induced macrophage inflammatory protein-2 (MIP-2) production, which in rats is the equivalent of IL-8 in humans. To test this hypothesis, we determined the extent of VILI and production of MIP-2, a chemoattractant for neutrophils in rodent lungs, in bronchoalveolar lavage fluid in rats exposed to 2 h of mechanical ventilation with large V_T of 20 ml/kg (V_T 20 ml/kg), with and without hyperoxia. The degree of VILI was determined by measurements of lung water, bronchoalveolar lavage neutrophil counts, and myeloperoxidase (MPO) assay of total lung neutrophil accumulation. The interactions of hyperoxia and large V_T on pulmonary edema formation, MIP-2 production, and neutrophil infiltration were analyzed. The interactions were examined immediately after ventilation and 6 h after ventilation to allow time for production of cytokines. The length of ventilation was limited to 2 h to give a limited, defined, nonlethal stress. To investigate the role of MIP-2 in attracting neutrophils into the lung and air spaces, neutralizing antibody to MIP-2 was used. We found that lung stretch-induced production of MIP-2 played a role in accumulation of neutrophils in the alveoli in V_T 20 ml/kg with or without hyperoxia. However, hyperoxia augmented pulmonary edema formation and neutrophil accumulation in the alveoli beyond that seen with V_T 20 ml/kg in room air (RA), suggesting that chemokines other than MIP-2 may be activated with hyperoxia to attract the greater number of neutrophils.

METHODS

Experimental animals. Sprague-Dawley viral-free rats weighing between 200 and 300 g were obtained from Charles River Laboratories (Wilmington, MA).

Ventilator protocol. The animals were orally intubated with a 2.42-mm OD (1.67 ID) polyethylene catheter under general anesthesia with intraperitoneal ketamine (50 mg/kg) and diazepam (5 mg/kg) while breathing RA. They were then attached to a Harvard small animal ventilator set to deliver either 7 or 20 ml/kg V_T at a rate of 85 breaths/min for 2 h in either RA or 100% oxygen (hyperoxia). The V_T delivered by the ventilator was checked by fluid displacement from an inverted calibration cylinder. Oxygen was fed into the inspiratory port of the ventilator when needed. Spontaneously breathing animals were exposed to hyperoxia in an enclosed chamber. Intubation tubing was increased in length at 14 and 20 ml/kg V_T to provide adequate dead space to maintain P_{CO_2} at 30–40 Torr. Control, nonventilated rats on RA were anesthetized and killed immediately. Control, nonventilated rats with hyperoxia were placed in an oxygen chamber with oxygen passed through at 15 l/min. The chamber was allowed to equilibrate for 30 min before the start of the experimental period. Oxygen levels were measured by mass spectrometry (Perkin-Elmer 1100 medical gas analyzer, Shelton, CT) and were found to be greater than 95%. Rats were either killed immediately after exposures or extubated to RA and killed 6 or 24 h after exposures.

Analysis of lung water. Lungs were removed en bloc, and large airways were removed. Both lungs were weighed and then dried in an oven at 80°C for 48 h. If there were no changes in the dry lung weight at 24 and 48 h, the weight at 48 h was used. Lung wet-to-dry weight ratio was used as an index of pulmonary edema formation.

Lung lavage. A separate group of animals from those used for analysis of lung water was used for lung lavage. After death, the lungs were removed en bloc, and polyethylene tubing was inserted into the left lung and secured. The left lung was lavaged three times with 2 ml of 0.9% NaCl (43). The effluents were pooled and centrifuged at 2,000 rpm for 10 min. Supernatants were frozen at −80°C.

Myeloperoxidase assay. MPO activity in lung parenchyma was used as a marker enzyme for total neutrophil sequestration in the lung (11). The right lower lobe (0.204–0.536 g) was homogenized in 5 ml of phosphate buffer (20 mM, pH 7.4). One milliliter of the homogenate was centrifuged at 10,000 *g* for 10 min at 4°C. The resulting pellet was resuspended in 1 ml of phosphate buffer (50 mM, pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. The suspension was then subjected to three cycles of freezing (on dry ice) and thawing (at room temperature), after which it was sonicated for 40 s and centrifuged again at 10,000 *g* for 5 min at 4°C. The supernatant was assayed for MPO activity by measuring the hydrogen peroxide (H_2O_2)-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB). In its oxidized form, TMB has a blue color, which was measured spectrophotometrically at 650 nm. The reaction mixture for analysis consisted of 25 μl of tissue sample, 25 μl of TMB (final concentration 0.16 mM) dissolved in dimethylsulfoxide, and 200 μl of H_2O_2 (final concentration 0.30 mM) dissolved in phosphate buffer (0.08 M, pH 5.4) minutes before addition to mixture. The reaction mixture was incubated for 3 min at 37°C, and the reaction was stopped by adding 1 ml of sodium acetate (0.2 M, pH 3.0), after which absorbance at 650 nm was measured. The absorbance was reported as units per kilogram of wet lung weight.

Cell counts. Neutrophil counts were used to measure migration of neutrophils into the alveoli, as previously described (2). Total cell counts in lung lavage fluid were performed by using a hemocytometer. To perform cell differentials, cells were fixed on glass slides by use of cytospin and were stained with geimsa.

Measurement of MIP-2. Rat MIP-2 was measured in BAL fluid by using a commercially available immunoassay kit (Biosource International, Camarillo, CA). Each sample was run in duplicate.

Administration of anti-MIP-2 antibody. Anti-rat MIP-2 antibody (Biosource International) or control rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was administered by direct intratracheal instillation, distal to the end of the endotracheal tube, just before the start of ventilation (16, 43, 44, 46).

Study design. Measurement for lung water was made on the following groups of animals, which were killed immediately after exposure: control, nonventilated rats on RA (n = 6); control, nonventilated rats with hyperoxia (n = 5); V_T 7 ml/kg on RA (n = 4); V_T 20 ml/kg on RA (n = 7); V_T 7 ml/kg with hyperoxia (n = 5); and V_T 20 ml/kg with hyperoxia (n =

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7). In two groups, lung water was measured 6 and 24 h after ventilation: V_T 20 ml/kg on RA (6 h, $n = 5$; 24 h, $n = 5$) and V_T 20 ml/g with hyperoxia (6 h, $n = 5$; 24 h, $n = 5$).

Because both lungs were used for measurement of lung water, separate groups of animals were used for measurement of BAL neutrophils and MIP-2 and lung tissue MPO activity. The left lung was lavaged for neutrophil counts and MIP-2 measurements, and the right lower lobe was used for MPO measurements. BAL neutrophil counts were performed on the following groups of animals killed immediately after exposure: control, nonventilated rats on RA ($n = 8$); control, nonventilated rats with hyperoxia ($n = 5$); V_T 7 ml/kg on RA ($n = 6$); V_T 20 ml/kg on RA ($n = 5$); V_T 7 ml/kg with hyperoxia ($n = 7$); and V_T 20 ml/kg with hyperoxia ($n = 6$). BAL neutrophils were also counted in groups of animals killed 6 h after exposure: control, nonventilated rats with hyperoxia ($n = 9$); V_T 7 ml/kg on RA ($n = 10$); V_T 20 ml/kg on RA ($n = 10$); V_T 7 ml/kg with hyperoxia ($n = 10$); and V_T 20 ml/kg with hyperoxia ($n = 13$). BAL MIP-2 levels were measured on the following groups of animals killed immediately after ventilation: control, nonventilated rats on RA ($n = 7$); control, nonventilated rats with hyperoxia ($n = 6$); V_T 7 ml/kg on RA ($n = 5$); V_T 20 ml/kg on RA ($n = 6$); V_T 7 ml/kg with hyperoxia ($n = 5$); and V_T 20 ml/kg with hyperoxia ($n = 5$). BAL MIP-2 levels were also measured on groups of animals killed 6 h after ventilation: control, nonventilated rats with hyperoxia ($n = 6$); V_T 7 ml/kg on RA ($n = 4$); V_T 20 ml/kg on RA ($n = 7$); V_T 7 ml/kg with hyperoxia ($n = 5$); and V_T 20 ml/kg with hyperoxia ($n = 7$). The total neutrophil influx was measured by MPO assays in animals killed immediately after exposure: control, nonventilated rats on RA ($n = 10$); control, V_T 20 ml/kg on RA ($n = 5$); and V_T 20 ml/kg with hyperoxia ($n = 5$). MPO assay was also performed on groups of animals killed 6 h after exposure: V_T 20 ml/kg on RA ($n = 8$) and V_T 20 ml/kg with hyperoxia ($n = 6$). To examine the effect of intratracheal administration of anti-MIP-2 antibody on BAL neutrophil counts, animals killed 6 h after ventilation were studied: V_T 20 ml/kg on RA with 50 ($n = 2$), 100 ($n = 5$), and 200 μg ($n = 11$) of anti-MIP-2 antibody to serve as a dose response; 200 μg of nonspecific IgG antibody (NSA, $n = 5$) as a control; and the test group with V_T 20 ml/kg with hyperoxia and 200 μg anti-MIP-2 antibody ($n = 8$). MPO assay was performed on V_T 20 ml/kg on RA with 200 μg of anti-MIP-2 antibody ($n = 6$) and V_T 20 ml/kg with hyperoxia and 200 μg of anti-MIP-2 antibody ($n = 7$).

Statistical methods. Analyses were performed using Statview 4.5 (Abacus Concepts, Berkeley, CA; 1998 SAS Institute). The lung wet-to-dry weight ratio, neutrophils in BAL, and MPO activity with and without hyperoxia were compared by ANOVA and then subsequent multiple comparisons by the Scheffé test. Interactions of V_T (7 and 20 ml/kg) and inspired oxygen fraction on lung water, V_T (7 and 20 ml/kg) and inspired oxygen fraction on neutrophil counts in the BAL, and V_T (7 and 20 ml/kg) and time on neutrophil counts in the BAL were compared by use of a two-way ANOVA (8). A significant factor interaction indicates that the two factors act synergistically and not simply additively. Significance was set at $P < 0.05$. All values are expressed as means ± SE.

RESULTS

Lung stretch increased lung water. The wet-to-dry weight ratio for rats ventilated at V_T 7 ml/kg with RA and killed immediately was not different from that for nonventilated rats but was significantly increased with V_T 20 ml/kg ($P < 0.05$, Fig. 1). Hyperoxia had no effect on lung water in control, nonventilated animals but caused a small but significant increase in lung water in animals ventilated at V_T 7 ml/kg ($P < 0.05$, Fig. 1). Ventilation at V_T 20 ml/kg with hyperoxia caused significantly more pulmonary edema than did ventilation for 2 h with V_T 20 ml/kg on RA (Fig. 1). Two-way ANOVA with oxygen and V_T was not significant ($P = 0.7$), indicating that the effect of oxygen was additive, not synergistic. Six hours after coming off the ventilator, lung water had returned toward normal levels, but remained elevated up to 24 h after ventilation (Fig. 1).

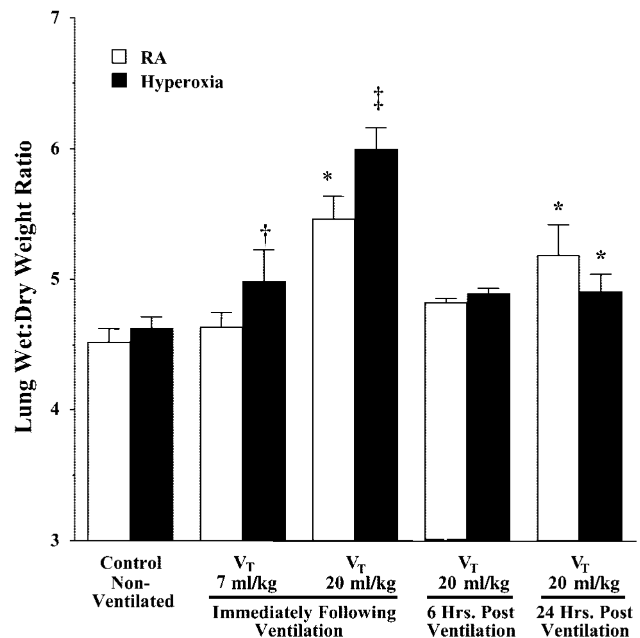

Fig. 1. Lung water measured by lung wet-to-dry weight ratio in control, nonventilated rats; rats ventilated for 2 h at tidal volumes (V_T) 7 or 20 ml/kg for 2 h and killed immediately after exposure; and rats ventilated for 2 h with V_T 20 ml/kg and killed 6 and 24 h after ventilation. Open bars are rats on room air (RA), and solid bars are rats with hyperoxia (means ± SE). *$P < 0.05$ vs. control; †$P < 0.05$ vs. V_T 7 ml/kg RA; ‡$P < 0.05$ vs. all other groups.

Lung stretch-induced MIP-2 production in lung lavage fluid. MIP-2 was not elevated immediately after coming off the ventilator in any group (Fig. 2*A*). MIP-2 was significantly elevated in BAL fluid 6 h after ventilation with V_T 20 ml/kg both in RA and with hyperoxia (Fig. 3*A*). There was no significant difference between groups of rats ventilated at V_T 20 ml/kg with RA or hyperoxia. Thus lung stretch induced production of MIP-2, but the addition of oxygen did not increase MIP-2 production over that seen with 2 h of V_T 20 ml/kg with RA.

Lung stretch-induced neutrophil migration into lung lavage fluid. In rats ventilated with RA at V_T 7 or 20 ml/kg, there was no immediate increase in BAL neutrophils compared with control, nonventilated animals (Fig. 2*B*). Six hours later in the rats ventilated at V_T 20 ml/kg, but not at 7 ml/kg, there was a significantly increased BAL content of neutrophils (Fig. 3*B*). Analysis with two-way ANOVA indicated that time plus V_T ($P < 0.05$) acted synergistically. The migration of neu-

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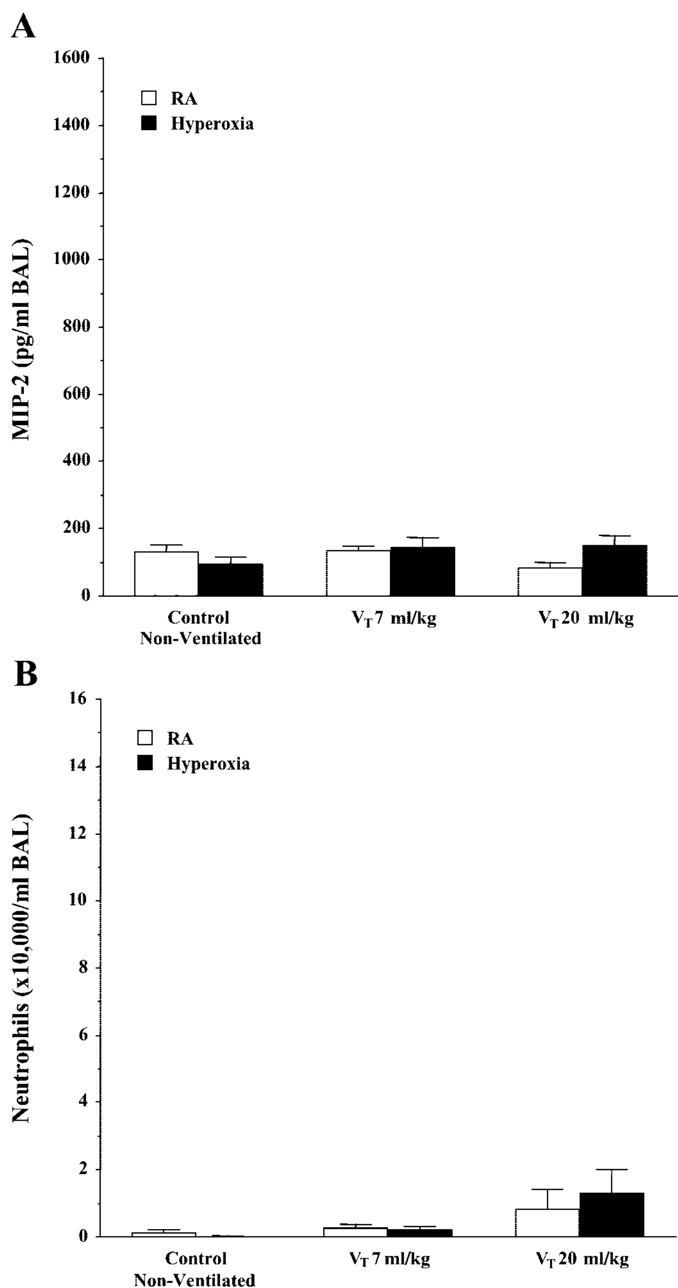

Fig. 2. Measurements of macrophage inflammatory protein-2 (MIP-2; *A*) and neutrophils (*B*) in bronchoalveolar lavage (BAL) fluid in control, nonventilated rats and rats ventilated for 2 h at 7 and 20 ml/kg for 2 h and killed immediately after ventilation. Open bars are rats on RA, and solid bars are rats with hyperoxia (means ± SE). No significant increase occurred in MIP-2 in BAL MIP-2 or neutrophils.

trophils into the alveoli of the RA-ventilated rats corresponded to the rise in MIP-2, suggesting that MIP-2 production over time may be responsible for influx of neutrophils.

Hyperoxia in nonventilated controls and with ventilation at V_T 7 ml/kg did not increase BAL content of neutrophils (Figs. 2*B* and 3*B*). The addition of oxygen to the rats ventilated at V_T 20 ml/kg markedly increased the BAL content of neutrophils 6 h after ventilation ($P < 0.05$, Fig. 3*B*), but not immediately after ventilation (Fig. 2*B*). Analysis with two-way ANOVA indicated that oxygen plus V_T ($P < 0.05$) acted synergistically.

Inhibition of neutrophil migration into the alveoli by anti-MIP-2 antibody. To define the role of MIP-2 production in stretch-induced neutrophil migration into the alveoli, anti-MIP-2 antibody was used. Only animals killed 6 h after ventilation with V_T 20 ml/kg were studied because they had significant increases in BAL MIP-2 and neutrophils. Intratracheal insufflation of anti-MIP-2 antibody produced a dose-dependent decrease in BAL neutrophil counts (Fig. 4). In contrast, in

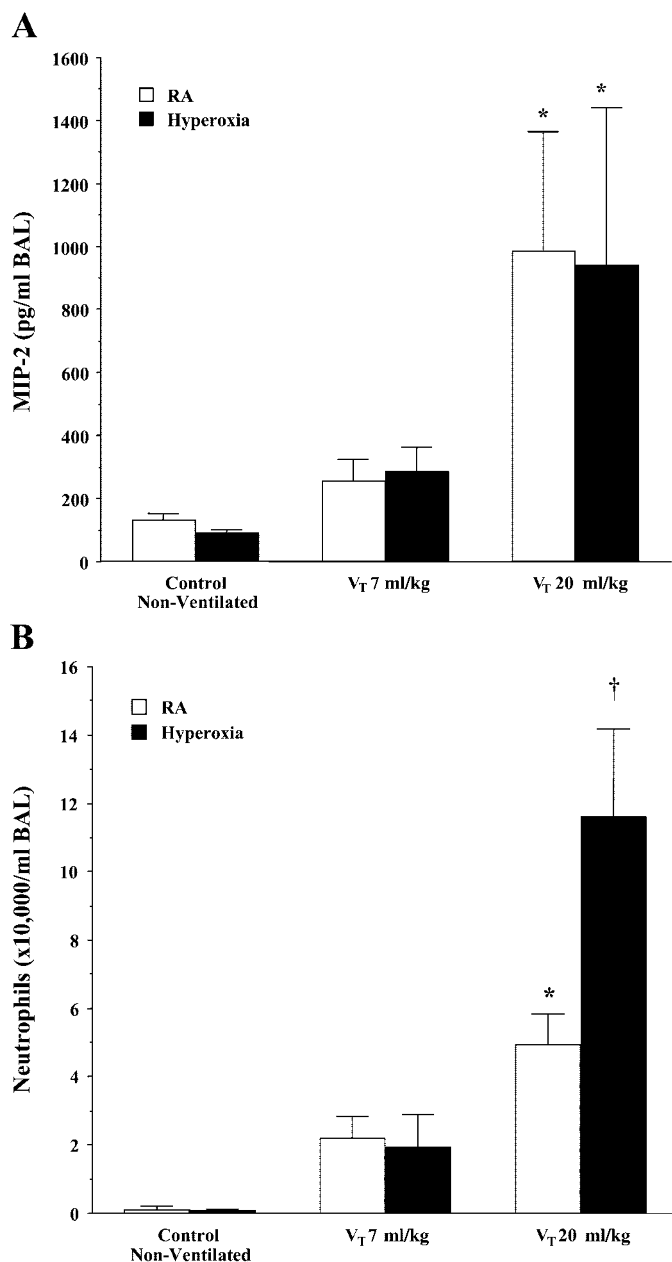

Fig. 3. Measurements of MIP-2 (*A*) and neutrophils (*B*) in BAL in control, nonventilated rats and rats ventilated for 2 h at 7 and 20 ml/kg and killed 6 h after ventilation. Open bars are rats on RA, and solid bars are rats on hyperoxia (means ± SE). *$P < 0.05$ vs. control, nonventilated animals; †$P < 0.05$ vs. all other groups.

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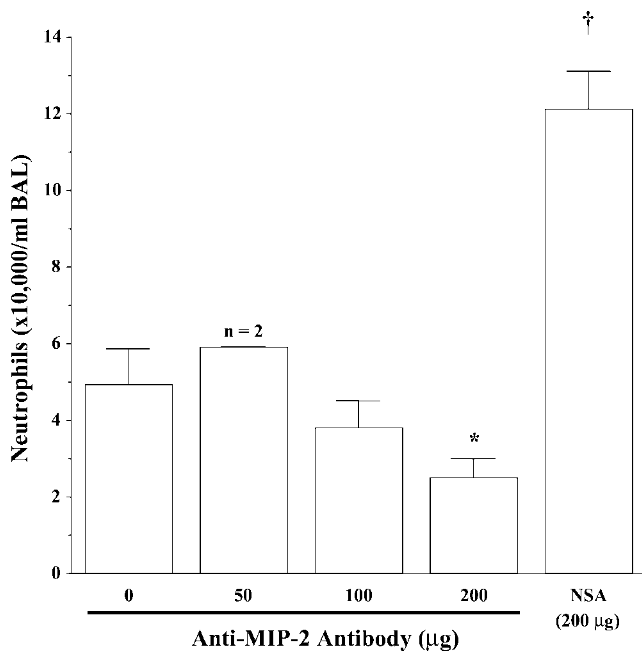

Fig. 4. Neutralization of MIP-2 in BAL by intratracheal administration of anti-MIP-2 antibody. Rats were ventilated with VT 20 ml/kg on RA for 2 h with 50, 100, and 200 μg of anti-MIP-2 antibody or 200 μg of nonspecific antibody (NSA). Animals were killed 6 h after ventilation (means ± SE). **P* < 0.05 vs. 0 μg anti-MIP-2 antibody; †*P* < 0.05 vs. all other groups.

animals treated with control rabbit IgG before ventilation at 20 ml/kg on RA, there was an increase in the number of neutrophils in the BAL compared with animals ventilated without control antibody pretreatment and with anti-MIP-2 antibody ($P < 0.05$). These data show that inhibition of neutrophil migration by anti-MIP-2 antibody was not a nonspecific effect.

The administration of anti-MIP-2 antibody (200 μg) caused a 51% reduction in the rise in BAL neutrophil counts 6 h after ventilation at VT 20 ml/kg on RA (Fig. 5*A*). The neutrophil migration in VT 20 ml/kg on RA with anti-MIP-2 was not significantly different from VT 7 ml/kg on RA ($P = 0.63$) and control, nonventilated animals on RA ($P = 0.20$). The neutrophil migration in VT 20 ml/kg plus hyperoxia with anti-MIP-2 antibody was significantly lower than in the group receiving VT 20 ml/kg plus hyperoxia without anti-MIP-2 antibody ($P < 0.0001$), a 65% reduction (Fig. 5*A*). However, the neutrophil migration was still significantly higher than VT 7 ml/kg plus hyperoxia ($P < 0.05$) and control, nonventilated animals in hyperoxia ($P < 0.02$). Thus stretch-induced neutrophil migration into the alveoli was dependent on MIP-2 in BAL, but the markedly increased neutrophil migration into the alveoli with hyperoxia plus high-volume ventilation may involve another mechanism or an undetected difference in the MIP-2 levels with hyperoxia.

Neutrophil migration into the alveoli vs. total neutrophil infiltration in the lung. To compare migration of neutrophils into the alveoli to total neutrophil infiltration in the lung, MPO assay was used to quantitate total lung neutrophils, i.e., neutrophils marginated in the vasculature, located in the parenchyma and in the alveoli (Fig. 5*B*). MPO was not significantly elevated immediately after mechanical ventilation with VT 20 ml/kg with RA or VT 20 ml/kg with hyperoxia compared with control, nonventilated animals with RA (0.13 ± 0.01 U/kg lung wt, $P = 0.7$). However, by 6 h after mechanical ventilation, MPO activity was elevated

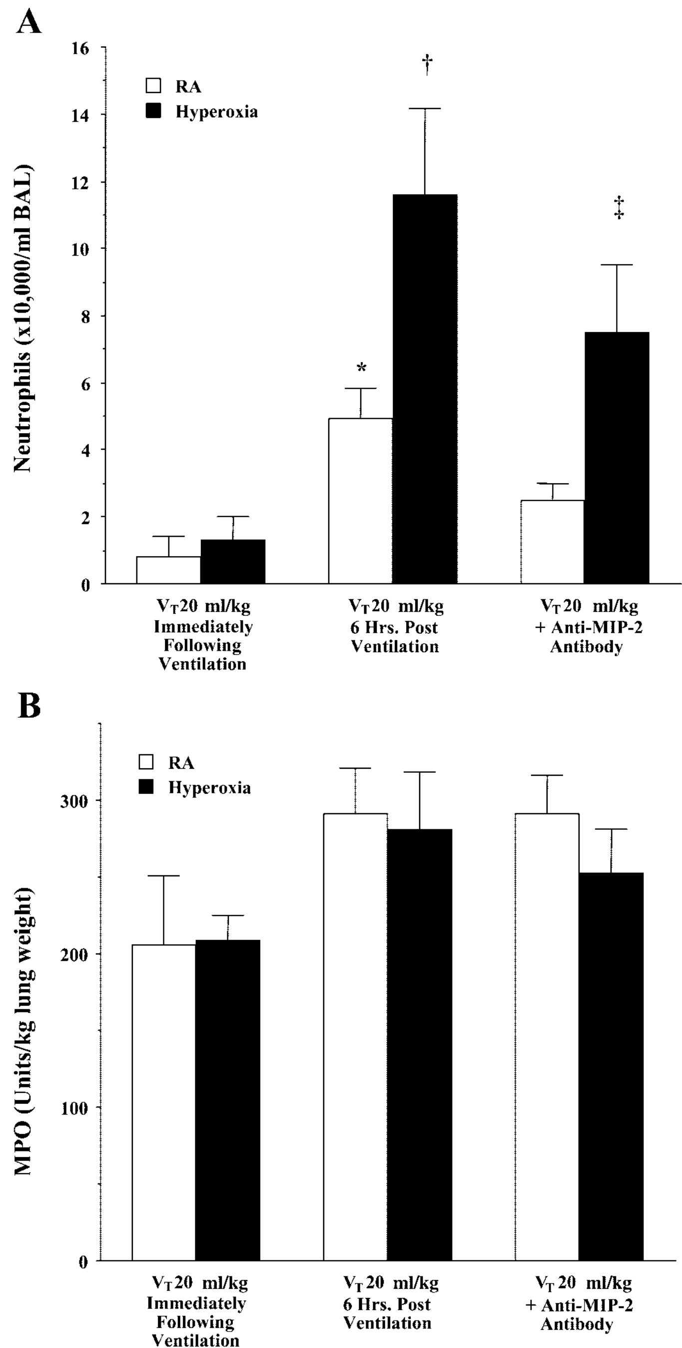

Fig. 5. Effect of pretreatment with intratracheal anti-MIP-2 antibody on neutrophil content (*A*) in BAL and myeloperoxidase (MPO) assay of lung tissue (*B*) in rats ventilated for 2 h at VT 20 ml/kg on RA and with hyperoxia and killed 6 h after exposure (means ± SE). Levels are compared with rats killed immediately after ventilation. **P* < 0.05 vs. VT 20 ml/kg RA, immediately after ventilation; †*P* < 0.05 vs. all other groups; ‡*P* < 0.05 vs. VT 20 ml/kg with hyperoxia.

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both in rats ventilated at V_T 20 ml/kg on RA and with hyperoxia. There was no significant difference between the two groups (Fig. 5*B*). Thus lung stretch increased total lung neutrophil influx during mechanical ventilation, which was not affected by hyperoxia. Lung edema preceded the detectable influx of neutrophils.

Intratracheal administration of anti-MIP-2 antibody (200 μl) did not affect total lung neutrophils in the groups of rats ventilated with V_T 20 ml/kg in RA or with hyperoxia, as measured by MPO assay (Fig. 5*B*). Intravenous administration of 50 μg of anti-MIP-2 antibody before ventilation at V_T 20 ml/kg in RA (n = 3), like intratracheal administration of 200 μg of anti-MIP-2 antibody, had no effect on lung MPO activity (200 μg intratracheal, 291 U/kg lung wet wt vs. 50 μg intravenous, 258 U/kg lung wet wt). Intravenous administration of 100 μg of anti-MIP-2 antibody was lethal in three of four rats. Thus lung stretch led to the accumulation of neutrophils in the lung and stretch-induced MIP-2 production. Inhibiting MIP-2 with an antibody in the airway or via the circulation did not reduce total lung accumulation of neutrophils.

Effect of hypotension on ventilator-induced neutrophil infiltration and migration. Ventilation of rats with large V_T led to hypotension. In our model, there was a period of hypotension for 2 h (mean arterial pressure as low as 60 mmHg) followed by a 6-h period when the arterial pressure was allowed to rise. To rule out any possible effect of ischemia-reperfusion, in a separate group of animals we infused 1 ml of 0.9% NaCl to prevent hypotension. Infusion of saline maintained mean arterial pressure at baseline values for the 2-h period of ventilation. The MPO activity of the right lower lobe and the neutrophil count in the lung lavage fluid were compared in ventilated animals with (n = 6) and without hypotension (n = 5) 6 h after ventilation. There was no difference in the total lung neutrophil migration as measured by MPO activity (in U/kg lung wt, 95 ± 0.20 hypotensive vs. 90 ± 0.16 nonhypotensive, $P = 0.84$) or lung lavage neutrophil counts (in number/ml of BAL × 10,000, 13.1 ± 2.6 hypotensive vs. 10.5 ± 0.8 nonhypotensive, $P = 0.36$) between hypotensive and nonhypotensive animals. Thus the period of hypotension did not contribute to the delayed neutrophil infiltration and migration in the lung.

DISCUSSION

In our rat model of VILI, we found a significant increase in pulmonary edema with high-V_T ventilation (Fig. 1). Hyperoxia further significantly increased pulmonary edema formation (Fig. 1). The effect of V_T and hyperoxia was additive. By 6 h after mechanical ventilation at high-V_T ventilation, lung water had returned toward baseline levels but remained elevated up to 24 h after ventilation (Fig. 1). MIP-2, a chemoattractant for neutrophils in rodent lungs, was not significantly elevated immediately after ventilation with large V_T (Fig. 2*A*) but was significantly elevated in BAL fluid in rats 6 h after ventilation with large V_T (Fig. 3*A*). There was no significant difference between RA and hyperoxia. BAL neutrophils also were not increased immediately after 2 h of mechanical ventilation (Fig. 2*B*). However, by 6 h after mechanical ventilation, there was a significant increase in BAL neutrophils in rats ventilated at V_T 20 ml/kg with RA, but dramatically more so if ventilated with hyperoxia (Fig. 3*B*). There was a synergistic affect between V_T and hyperoxia on neutrophil migration into the alveoli. Neutralization of alveolar MIP-2 by intratracheal insufflation of anti-MIP-2 antibody diminished neutrophil migration into the alveoli during high-V_T ventilation on RA (Figs. 4 and 5A) and with hyperoxia (Fig. 5*A*). MPO assay for total lung neutrophils (alveolar, interstitial, and alveolar) was not increased at the end of the 2-h ventilation period (Fig. 5*B*), even though the rats ventilated at V_T 20 ml/kg had edematous lungs at this time point (Fig. 1). Six hours later, the rats ventilated at 20 ml/kg for 2 h showed a significant and similar rise in MPO, whether ventilated on RA or with hyperoxia (Fig. 5*B*). These data suggest that lung stretch-induced MIP-2 production in the alveoli and airways mediates neutrophil migration into the alveoli in response to large V_T but does not affect total neutrophil influx into the lung with VILI or the augmentation of cell migration by hyperoxia. Total neutrophil sequestration in the lung and hyperoxic augmentation of neutrophil migration from the interstitium into the alveoli may involve other mechanisms besides stretch-induced MIP-2 production in the alveoli and airways.

Hyperoxia alone has been shown to stimulate TNF-α release from alveolar macrophages in 3 h (14) and to increase lung water after 72 h in rats (17). Although the damaging effects of hyperoxia on the lung have been known for many years, the interaction of hyperoxia with mechanical ventilation has received little attention. Gerstmann et al. (10) examined conventional volume ventilation vs. high-frequency oscillation, high-frequency flow interruption combined with oxygen or inspired oxygen concentration as needed to maintain arterial oxygen saturation in premature baboons. They found bronchopulmonary dysplasia only in animals on oxygen. Davis et al. (5) examined the impact of oxygen and V_T of 16–17 ml/kg or of 11 ml/kg for 48 h in neonatal piglets. They found the greatest albumin leak and the most histological evidence of injury in the animals receiving both hyperoxia and a V_T of 16–17 ml/kg. Thus there is some evidence in the developing and newborn lung for an interaction of mechanical ventilation and oxygen. Pulmonary edema has also been observed in adult sheep spontaneously breathing hyperoxia with and without the addition of positive end-expiratory pressure (PEEP), which hyperexpanded the lungs. Sheep breathing hyperoxia with 10 cmH_2O of PEEP died after 54 h of exposure, whereas sheep breathing hyperoxia without PEEP survived for 71 h (21). Our data support these findings and indicate that not only does this interaction persist in the adult, but it is dramatic, occurring with only brief exposure to high-V_T ventilation.

The mechanism of pulmonary edema in VILI has been thought to be hyperexpansion of the lung based

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on the work of Dreyfuss et al. (5a). They used very large VT at 45 cmH_2O and did not measure pulmonary hemodynamics. Thus ischemia-reperfusion could have played a role in this massive VT injury. We have measured hemodynamics in our model and found that mean pulmonary arterial pressure rose from 22 ± 1 to 26 ± 1 mmHg during VT 20 ml/kg (12). Because the airway pressure in our rats ventilated at VT 20 ml/kg was only 25 cmH_2O, it is unlikely that much of the lung was in zone 1, and area in zone 1 was likely to be small and only at peak inspiration. Similarly, there was no difference in the neutrophil infiltration and migration between groups of animals transfused with 0.9% NaCl to prevent hypotension and those animals that were not transfused. Thus ischemia-reperfusion is unlikely to be the basis of injury in the VT 20 ml/kg rats. Parker and associates (29) have suggested, from observations in isolated perfused rat lungs, that endothelial stretch-activated cation channels are the mechanism whereby the leak occurs, because inhibition of these channels with gadolinium prevents the ensuing ventilator-induced edema (30).

The mechanism of inflammation in VILI is not completely understood but appears to involve the production of cytokines. In vitro cell stretch has been shown to induce chemoattractant cytokines including interleukin (IL)-8 (32, 33, 47). Tremblay et al. (44) demonstrated in ex vivo rat lung that positive-pressure ventilation at very large VT of 40 ml/kg produced an outpouring of inflammatory and anti-inflammatory cytokines and chemokines such as MIP-2, TNF-α, IL-1β, IL-6, interferon-γ, and IL-10. These data have recently been questioned by Ricard et al. (35). They found no increase in MIP-2, TNF-α, and IL-1β in their isolated nonperfused lungs. Both studies ventilated for 2 h under the same conditions, suggesting that the isolated nonperfused ventilated lung model may lead to unstable results. MIP-2 production was also found to be increased in overventilated isolated perfused mouse lung. However, the rise in MIP-2 did not start to increase until 2 h and continued to rise at 3 h (13). Like our study on VT 20 ml/kg, the study by Ricard et al. (35) also did not find elevated levels of MIP-2 in intact animals ventilated at VT 40 ml/kg for 2 h immediately after ventilation. They did not measure cytokine levels at later time points. The discrepancy in these findings may be related to the short intervals of follow-up after ventilation. Our data suggest that the inflammatory changes of VILI are delayed events.

We did not find elevated levels of MIP-2 or neutrophil influx immediately after ventilation, although there was significant elevation of lung water at this time point. MIP-2 and neutrophils were present 6 h after ventilation, at a time that lung water had returned to near normal levels. This suggests that the mechanism of noncardiogenic edema formation may be different from the mechanism of cytokine release and inflammatory cell influx and that the neutrophil may not be necessary for initiation of the edema. This does not, however, eliminate the possibility that in situ neutrophils are activated and contribute to pulmonary edema formation in VILI.

Ranieri and associates (34) found that ARDS patients ventilated at VT to maintain normal arterial P_{CO_2} levels (VT 11.1 ± 1.3 ml/kg) had higher levels of TNF-α, IL-1β, and IL-8 in BAL than patients ventilated at VT 7.6 ± 1.1 ml/kg. Previous studies have shown influx of neutrophils (15, 35, 44) and the production of several chemoattractant cytokines. Our study investigated the role of chemoattractant cytokines in the influx of neutrophils and demonstrated the relationship between stretch-induced MIP-2 production and the migration of neutrophils into the alveoli with VILI. We chose to focus on MIP-2, a chemoattractant cytokine for neutrophils. In humans, IL-8, a member of the CXC family of cytokines, is a potent chemotactic factor for recruitment of neutrophils in the human lung (19). Interestingly, no exact homolog of IL-8 has been found in rodents. MIP-2, another member of the CXC family of cytokines, appears to play a related role as a chemoattractant for neutrophils in rodent lungs (38, 39). MIP-2 is produced by alveolar macrophages and binds to the CXCR2 receptor in rodents, which is the homolog of the IL-8 receptor β in humans (3). Stretch-induced production MIP-2 in isolated perfused mouse lungs has been correlated with nuclear factor-κB activation (13).

The rise in alveolar MIP-2, in our study, was similar in rats ventilated with VT 20 ml/kg on RA and with hyperoxia. There was no greater increase in MIP-2 in the presence of oxygen plus high VT, although there was a larger increase in neutrophil migration into the alveoli with high VT with hyperoxia. Anti-MIP-2 antibody blunted the influx of neutrophils in animals exposed to high VT in RA as well as in hyperoxia and did not have a greater effect in hyperoxic rats. Thus MIP-2 has a role in the VT 20 ml/kg accumulation of alveolar neutrophils with or without hyperoxia, but the synergistic effect of hyperoxia and VT in attracting alveolar neutrophils may be mediated by other mechanisms as well. Multiple other possible chemoattractants have been identified. In rabbits with lung saline lavage, high-volume ventilation with hyperoxia also resulted in neutrophil recruitment in the lung. This neutrophil recruitment was blunted by IL-1 blockade (26). Hyperoxia has also been shown to increase production of several cytokines, including TNF-α, IL-6, IL-3, and IL-1β (4), and to increase expression of intracellular adhesion molecule-1 expression (41).

Our study is limited in that we only measured MIP-2 levels in the BAL. There may have been differences in the whole lung MIP-2 between RA and hyperoxia-ventilated animals. Chemokines are basic proteins and bind to negatively charged heparin and heparin sulfate in the interstitium and to human Duffy antigen receptor for chemokines on erythrocytes and endothelial cells (23). These mechanisms are felt to create local concentration gradients and cause migration of neutrophils. Because intratracheal administration of anti-MIP-2 antibody inhibited neutrophil migration into the alveoli in our rats, it appears that the concentration of

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MIP-2 was higher in the alveoli than in the lung interstitium and vessels. Higher concentrations in the lung interstitium than in the circulation may lead to attraction of neutrophils from the blood vessels, and chemokine release in the alveoli may lead to further neutrophil migration into the alveoli. If V_T 20 ml/kg plus hyperoxia caused more tissue matrix binding than V_T 20 ml/kg with RA, then we may be underestimating total MIP-2 based on analysis of BAL MIP-2. We may thus have used insufficient intratracheal antibody to MIP-2 to fully neutralize all the MIP-2 in the groups with V_T 20 ml/kg plus hyperoxia. However, it seems unlikely that the large anti-MIP-2 antibody neutralized MIP-2 in the interstitium unless, because of stretch-induced lung injury, it was able to penetrate the alveolar epithelial lining. Nevertheless we cannot fully exclude the possibility that MIP-2 plays a larger role in regulating lung neutrophil infiltration in synergy with hyperoxia and V_T 20 ml/kg than we have shown. On the other hand, it is also likely that other mechanisms may attract neutrophils into the alveoli with hyperoxia and large V_T.

Neutrophils have been implicated in VILI in animal models of ARDS. In a surfactant-depleted rabbit model of ARDS ventilated with a conventional volume ventilator at 12 ml/kg for 4 h, there was more protein leak, more hyaline membrane formation, worse gas exchange, and much more extensive granulocyte infiltration than in rabbits ventilated with lower V_T and lower mean airway pressure by a high-frequency ventilator. Depletion of granulocytes with nitrogen mustard significantly reduced protein leak and hyaline membrane formation and improved gas exchange in the conventional ventilator animals (18). Neutrophil infiltration has also been found to be increased in a lavaged rabbit model of ARDS ventilated with a conventional ventilator as opposed to a high-frequency ventilator with lower V_T and lower mean airway pressure (40). We found that there was an increase in neutrophil influx with moderately high-V_T ventilation (V_T 20 ml/kg) in normal animals. This occurred without the necessity of adding other forms of lung injury such as surfactant depletion, which previous studies have used.

Lung stretch, with or without hyperoxia, increased lung MPO. Goldblum and associates (11) have shown that lung MPO assesses total lung content of neutrophils, including those marginated in the microcirculation, contained in the lung parenchyma and present in the alveoli. Hyperoxia plus lung stretch did not cause more neutrophils to enter the whole lung than did stretch alone, as measured by MPO activity. This was also found by Blackwell and associates (2). They set up different chemotactic gradients of MIP-2 from the alveoli to the blood by intratracheal injection of endotoxin with and without intraperitoneal installation of endotoxin in rats. They found differences in the migration of neutrophils into the alveoli without changes in total lung MPO activity. Increased migration of neutrophils into the alveoli occurred when a gradient for MIP-2 was present between the alveoli and the serum. Our data are consistent with stretch-induced MIP-2 production in the alveoli serving as a chemoattractant to draw neutrophils into the alveoli.

Because total neutrophil infiltration was not affected, other factors may have been responsible for the sticking of neutrophils in the lung during ventilation. Mechanical ventilation has been shown to increase the transit time of neutrophils in the lungs of rabbits (24) and humans (22). Cyclic stretch has been shown to upregulate intracellular adhesion molecule-1, neutrophil adhesion (37), and production of IL-8 (32, 33, 47). A combination of an increased transit time and increased neutrophil adhesion could lead to increased neutrophil margination in the lung, independent of chemokine production.

We conclude that large-V_T ventilation, for even a short time, caused pulmonary edema and a delayed influx of neutrophils into the lung. Edema formation occurred before significant neutrophil infiltration in VILI. The migration of neutrophils into the alveolar space in VILI was dependent on stretch-induced production of MIP-2, whereas total neutrophil influx into the lung, including vascular margination and interstitial neutrophils in VILI was not solely dependent on stretch-induced production of MIP-2. Hyperoxia augmented ventilator-induced pulmonary edema and migration of neutrophils from the parenchyma into the alveoli but did not increase MIP-2 production beyond that seen with large tidal ventilation alone. Thus factors other than MIP-2 may be responsible for the synergistic effect of hyperoxia and large V_T in attracting neutrophils into the alveoli. In patients with ARDS, even 2 h of positive-pressure ventilation at high V_T and high oxygen levels, as often occurs when a patient is initially intubated, may cause an increase in neutrophil migration into the alveoli.

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