Effects of airway distension on leukocyte recruitment in the mouse tracheal microvasculature

Elizabeth M. Wagner and John Jenkins
Department of Medicine, Johns Hopkins University, Baltimore, Maryland
Submitted 20 September 2006; accepted in final form 2 January 2007

Wagner EM, Jenkins J. Effects of airway distension on leukocyte recruitment in the mouse tracheal microvasculature. J Appl Physiol 102: 1528–1534, 2007. First published January 4, 2007; doi:10.1152/japplphysiol.01054.2006.—We have shown previously that excessive distention of the rat trachea during mechanical ventilation results in enhanced leukocyte recruitment to the airway (Lim LH and Wagner EM. Am J Respir Crit Care Med 168:1068–1074, 2003). The objectives of this study were to develop a mouse model of positive end-expiratory pressure (PEEP)-induced leukocyte recruitment to the airway and begin to pursue molecular mechanisms that may contribute to the in vivo observation of increased leukocyte adhesion after PEEP exposure. We studied C57BL/6 wild-type mice and mice deficient in P-selectin or intercellular adhesion molecule-1 (ICAM-1) exposed to intermittent PEEP (8 cmH2O) applied five times for a 1-min duration, at 10-min intervals. After the imposed ventilatory stress, during normal ventilation (0.2 ml/breath, no PEEP), leukocyte adhesion in tracheal postcapillary venules was determined using intravital microscopy. PEEP induced a time-dependent increase in leukocyte adhesion that was significantly increased between 0 and 60 min (P < 0.01). Furthermore, PEEP-induced leukocyte adhesion at 60 min was ablated in P-selectin- and ICAM-1-deficient mice. These findings demonstrate the essential nature of both P-selectin and ICAM-1 within airway postcapillary venular endothelium for leukocyte recruitment after airway distension.

Inflammation, a predominant feature of several airways diseases, requires an orchestrated series of molecular events whereby inflammatory cells within the airway vasculature roll, adhere, and migrate within the airway wall. Because there exists little specific information regarding this process in airways in vivo, we previously established methods in rats to study leukocyte recruitment to the airways using intravital microscopy (14, 15). With this technique, our laboratory observed that excessive distention of the airway vasculature results in altered leukocyte kinetics (15). After the intermittent application of positive end-expiratory pressure (PEEP), and when overall endothelial shear stress and red blood cell velocity were normal, rolling velocity of neutrophils specifically was shown to decrease, accompanied by increased neutrophil adhesion and subsequent migration from tracheal postcapillary venules. This response was due entirely to changes in airway pressure on the tracheal vasculature because neither other systemic beds nor unexposed upper airways were altered with the application of PEEP (15). Although the precise mechanism of this response is unknown, these results should be viewed in context with the many studies on the effects of mechanical strain on all cells of the lung (17, 21, 24) and with the specific effects of high tidal volume ventilation on leukocyte recruitment within the lung (4, 18). Work by Kuebler and colleagues (12) demonstrated that mechanotransduction with the application of high levels of intravascular pressure in pulmonary capillaries results in an increase in endothelial cell calcium and exocytosis of the adhesion molecule P-selectin (12). Furthermore, increased tidal volume ventilation also leads to calcium-dependent P-selectin expression in freshly isolated lung endothelial cells (26). Our laboratory’s previous work in rats demonstrated that fucoidan, a pan-selectin pharmacological inhibitor, limited leukocyte recruitment to tracheal postcapillary venules after the application of PEEP (15). In vitro application of varying degrees of distention to endothelial monolayers or blood vessel segments provides information on other factors that may contribute to leukocyte recruitment. Intercellular adhesion molecule-1 (ICAM-1), which binds β2-integrins on leukocytes and is involved in firm adhesion of inflammatory cells, is significantly increased in human saphenous vein grafts after a period of pressure distension (3). Thus the goals of the present study were to develop the mouse model of PEEP-induced leukocyte recruitment to the airway and begin to pursue molecular mechanisms that may contribute to the in vivo observation of increased leukocyte adhesion after PEEP exposure. We demonstrate the importance of endothelial adhesion molecules P-selectin and ICAM-1 on proinflammatory responses of tracheal venular endothelium in this mouse model.

METHODS

Mice. Our protocol was approved by the Johns Hopkins Animal Care and Use Committee. Eight-week-old male C57BL/6 mice (Charles River, Wilmington, MA) were anesthetized with a ketamine-acepromazine mixture (10:1 at 1.0 μl/g body wt ip), intubated (18-gauge intracatheter), and ventilated (HSE-HA MiniVent, Harvard Apparatus, Holliston, MA) at 120 breaths/min (0.2 ml/breath). Additional studies were performed on C57BL/6 mice deficient in P-selectin [P-selectin<sup>−/−</sup>] or ICAM-1 [ICAM-1<sup>−/−</sup>]. Both of these strains were obtained from Jackson Laboratories (Bar Harbor, ME). A total of 51 mice (20–26 g) were studied.

Intravital microscopy. For intravital microscopy of tracheal vessels, a midline incision was made in the ventral surface of the neck in mice, and the trachea was exposed. Care was taken not to disrupt any blood vessels. The exposed tracheal surface was continuously superfused with warmed (37°C), Krebs buffer. A single postcapillary venule was selected in each mouse just beyond the end of the endotracheal tube, it was visualized for up to 2 h, and the images

Address for reprint requests and other correspondence: E. M. Wagner, Johns Hopkins Asthma and Allergy Center, Div. of Pulmonary and Critical Care Medicine, 5501 Hopkins Bayview Circle, Baltimore, MD 21224 (e-mail: wagnerem@jhmi.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.
were recorded on videotape. Leukocyte trafficking was quantified offline by measuring 1) leukocyte rolling velocity and 2) the number of adherent cells in a 200-μm length of vessel per time point. Rolling leukocytes were defined as leukocytes that moved at a velocity less than that of erythrocytes in a given vessel. The rolling velocity of 10 leukocytes entering the vessel was determined by measuring the time required for a cell to move 50 μm along the endothelial wall, and the 10 values were averaged for each time point studied. A leukocyte was defined as adherent to the venular endothelium if it remained stationary for >30 s. Adherent cells were expressed as the number per 200 μm length of vessel. Venule diameters were measured with digital calipers using Image Pro software (Media Cybernetics, Silver Spring, MD). In a subset of protocol-matched control mice (n = 3 mice/group), red blood cell velocity was measured using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station TX), and airway pressure (transducer, Argon Medical, Athens, TX) was recorded (PowerLab, ADInstruments, Colorado Springs, CO).

Protocols. In the experimental groups, a 1-min period of 8-cmH2O PEEP was applied two times or five times with a 10-min interval of no PEEP between each PEEP application (Fig. 1). Measurements were made at baseline (before changes in ventilatory pressures) and every 20 min for 1 h after the last application of PEEP. Control mice were ventilated without PEEP for 120 min. In preliminary studies, transient increases in PEEP to 8 cmH2O resulted in peak inspiratory pressures of ~16 cmH2O. To address the effects of increased peak inspiratory pressure in another group of mice, tidal volume was increased to result in an equivalent increase in peak inspiratory pressure as in the PEEP protocol. In this group, tidal volume was increased from 0.2 to 0.7 ml/stroke for 50 min, after which tidal volume was returned to the control level and measurements were made every 20 min for 1 h. Groups of mice studied are summarized in Table 1 and include those with no PEEP exposure (control mice and mice ventilated with increased tidal volume) and mice exposed to 8-cmH2O PEEP [2 times, 5 times, and in P-selectin(-/-) and ICAM-1(-/-) mice].

Statistical analysis. All data are presented as means ± SE. Differences in leukocyte velocity and adhesion were determined using repeated measures, two-way ANOVA with the post hoc Fisher’s test for least significant difference. We compared the change in vessel diameter with no PEEP and 8-cmH2O PEEP with the Student’s t-test for paired comparisons. Single time point comparisons across groups were made using a one-way ANOVA with the post hoc Fisher’s test for least significant difference. A two-tailed P value of 0.05 was accepted as significant.

<table>
<thead>
<tr>
<th>Study groups</th>
<th>No PEEP</th>
<th>8-cmH2O PEEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>8 (×2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 (×5)</td>
</tr>
<tr>
<td>Increased tidal volume</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>P-selectin(-/-)</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>ICAM-1(-/-)</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are no. of mice. PEEP, positive end-expiratory pressure; P-selectin(-/-), P-selectin-deficient mice; ICAM-1(-/-), intracellular adhesion molecule-1-deficient mice.

RESULTS

Time course of PEEP effects. Average baseline diameter of all the vessels studied was 26.8 ± 0.9 μm. The application of 8-cmH2O PEEP resulted in an average 6.0% increase in venular diameter (P < 0.01). Venular diameters did not differ over the period of observation (P = 0.85). Average leukocyte velocity of all wild-type mice at baseline was 43.1 ± 1.1 μm/s. After the application of PEEP (5 × 8 cmH2O), a significant reduction in leukocyte velocity was observed (Fig. 2A; P < 0.01). The time course of changes in leukocyte velocity (Table 2) and leukocyte adhesion (Fig. 2B) in control mice and those exposed to airway distension by intermittent PEEP is presented. As seen in Fig. 2B, this response was accompanied by an increase in the number of firmly adherent cells throughout the 60-min observation period (P < 0.01). In another group of mice, a lesser stimulus was applied (2 × 8 cmH2O), and leukocyte velocity and adhesion were assessed immediately after the application of PEEP. Although this intervention significantly decreased leukocyte rolling velocity (Table 2), the stimulus was insufficient to result in an increase in the number of firmly adhered leukocytes (Fig. 3). A smaller group of time controls (n = 3 mice) for this series was studied, applying the 2 × 8-cmH2O PEEP protocol and extending the observation time by ~30 min to match the timing of the 5 × 8-cmH2O PEEP protocol. There was no significant change in leukocyte velocity or adhesion over this additional observation time.

Effects of increased inspiratory pressure. In another group of mice, when tidal volume was increased for a sustained time
period, venular diameter increased on average by 3%. Although this change in ventilation resulted in a sustained decrease in leukocyte velocity once normal ventilation was resumed (Table 2), increased leukocyte adhesion was not observed (Fig. 3). Average airway pressures and red blood cell velocity for control, increased PEEP, and increased tidal volume mice are presented in Table 3. No differences in red blood cell velocity were observed among groups, but evident are the expected increases in peak inspiratory pressure that did not differ between tidal volume and PEEP groups. The increase in mean airway pressure [(peak inspiratory pressure / end-expiratory pressure)/2] with the application of PEEP was significantly greater than during the increase in tidal volume (P < 0.01).

Table 2. Leukocyte velocity

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>0 min</th>
<th>20 min</th>
<th>40 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>43.6±2.8</td>
<td>44.8±4.0</td>
<td>42.0±4.1</td>
<td>45.0±4.2</td>
<td>44.4±4.2</td>
</tr>
<tr>
<td>PEEP (5 × 8)</td>
<td>46.1±2.7</td>
<td>33.6±2.5</td>
<td>31.4±0.1</td>
<td>32.2±2.5</td>
<td>42.5±2.6</td>
</tr>
<tr>
<td>PEEP (2 × 8)</td>
<td>39.3±3.4</td>
<td>35.5±2.0</td>
<td>33.4±3.8</td>
<td>31.9±4.2</td>
<td>28.8±6.5</td>
</tr>
<tr>
<td>Tidal volume</td>
<td>40.8±0.8</td>
<td>34.5±2.2</td>
<td>35.2±2.0</td>
<td>35.4±3.2</td>
<td>35.5±3.2</td>
</tr>
<tr>
<td>P-selectin(+/−)</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>ICAM-1(+/−)</td>
<td>46.7±2.0</td>
<td>41.6±2.7</td>
<td>41.5±2.5</td>
<td>41.0±2.2</td>
<td>41.2±1.3</td>
</tr>
</tbody>
</table>

Values are means ± SE. aVelocity was too great to measure. bP < 0.05. cP < 0.01 vs. within group baseline. dP < 0.05. eP < 0.01 vs. control at same time point. fP < 0.05. gP < 0.01 vs. PEEP (5 × 8) at same time point.
**P-selectin**<sup>−/−</sup> and ICAM-1<sup>−/−</sup> mice. In the next series of experiments, the PEEP exposure (5 × 8 cmH₂O) that resulted in a sustained increase in leukocyte adhesion was applied in P-selectin<sup>−/−</sup> mice, the endothelial adhesion molecule essential for leukocyte rolling. Leukocyte velocity in these mice was so rapid that it could not be quantified (Table 2). Furthermore, P-selectin<sup>−/−</sup> mice showed no leukocyte adhesion after the application of PEEP (Fig. 4). In another group of mice, ICAM-1<sup>−/−</sup>, leukocyte velocity was not different from control mice and was not altered by the application of PEEP (5 × 8 cmH₂O; Table 2). Additionally, no adherent leukocytes were observed in this group (Fig. 4; P < 0.01).

**Tracheal histology.** Examination of specimens of mouse trachea (fixed in 10% formalin, paraffin embedded, and hematoxylin and eosin stained) that had been exposed to PEEP (5 × 8 cmH₂O) demonstrated a preponderance of neutrophils within airway blood vessels (Fig. 5B; ×40 objective, note arrows) compared with a larger tissue area taken from a control mouse (Fig. 5A; ×20 objective).

**DISCUSSION**

The results of the present study corroborate in mice what our laboratory has previously reported to occur in rat airways (15) and provide information concerning the molecular mechanisms of leukocyte recruitment after the imposition of mechanical stress. Excessive airway distension, with repeated intermittent applications of PEEP, resulted in prompt leukocyte recruitment to the airway postcapillary venular endothelium. Furthermore, we showed that endothelial adhesion molecules P-selectin and ICAM-1 are both essential for leukocyte recruitment.

The goals of the present study were to confirm and extend our initial observations in a rat model. Previously, our laboratory showed that the effects of PEEP on increased leukocyte adhesion were dose dependent, not related to any cardiovascular changes that might take place with the application of PEEP, not related to any circulating factors, and that neutrophils were specifically responsive to the stimulus (15). In the present study, we developed an experimental mouse model, which enabled the study of genetically altered mice deficient in specific adhesion molecules. To our knowledge, this is the first application of intravital microscopy to mouse airways and allows the unique opportunity to study airway inflammation in real time. The application of three different ventilatory stresses on leukocyte recruitment confirmed that a threshold level of distension is necessary to activate postcapillary venular endothelium. The level of PEEP selected (8 cmH₂O) was based on our laboratory’s previous study (15). Application of intermittent PEEP five times over the course of 1 h resulted in a decrease in leukocyte velocity and an ever-increasing level of firm adhesion up to 60 min after the stimulus was withdrawn. Because P-selectin is known to be essential for leukocyte rolling in other systemic vascular beds (9, 10) and it rapidly (within 30 min) is shed or internalized back within endothelial cells (7), we questioned whether the 5 × 8-cmH₂O protocol may have obscured an earlier activation and response. Thus, in another group of mice, we applied the 2 × 8-cmH₂O protocol. Although leukocyte velocity decreased significantly with this

---

**Table 3. Airway pressure and red cell velocity**

<table>
<thead>
<tr>
<th></th>
<th>Peak Pressure, cmH₂O</th>
<th>Mean Airway Pressure, cmH₂O</th>
<th>Red Cell Velocity, mm/s</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal ventilation</td>
<td>Distension</td>
<td>Normal ventilation</td>
</tr>
<tr>
<td>Control</td>
<td>8.3±0.4</td>
<td>16.3±0.4*</td>
<td>4.2±0.2</td>
</tr>
<tr>
<td>PEEP</td>
<td>8.7±0.4</td>
<td>17.0±1.2*</td>
<td>4.3±0.2</td>
</tr>
<tr>
<td>Tidal volume</td>
<td>7.0±0.7</td>
<td>17.0±1.2*</td>
<td>3.5±0.4</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.01 from normal ventilation condition. †P < 0.01 from tidal volume distension condition.

---

*Published by the American Physiological Society*
stimulus, it was inadequate to result in a significant increase in the number of firmly adherent cells. However, if the absolute timing of this 2 × 8-cmH2O protocol is evaluated within the time frame of the 5 × 8-cmH2O protocol, leukocyte velocities decreased in a consistent manner in the two protocols. Additional time controls for this 2 × 8-cmH2O series, extending the observation period to match the 5 × 8-cmH2O protocol, did not show any significant differences over the additional 30 min of observation. These results suggest that to achieve firm adhesion, the greater stimulus is required despite the reduction in leukocyte velocity.

To determine whether the level of inspiratory pressure was the relevant stimulus that activated airway postcapillary venular endothelium, we also increased tidal volume to a level that matched peak inspiratory pressure during the application of PEEP (8 cmH2O). Because, in lung parenchyma, high tidal volume ventilation can cause neutrophilic inflammation (4), we expected that a sustained level of increased tidal volume may result in a large increase in firmly adherent cells. However, despite a sustained decrease in leukocyte rolling velocity measured after the return to normal tidal volume ventilation, no significant change in firm adherence was observed. One potential explanation for this observation is that because mean airway pressure was greater in the PEEP group than the tidal volume group (Table 3), a threshold level of mean airway pressure was required to have a significant effect on leukocyte firm adherence and that the relevant stimulus is mean airway pressure.

The mechanisms responsible for changes in leukocyte velocity and adherence have been extensively studied in other systemic vascular beds (11, 13). Determining the mechanisms of airway inflammation in real time, however, has been difficult to study continuously and without the contributions of the pulmonary circulation. As in most other systemic beds, increased expression of the endothelial adhesion molecule P-selectin is essential to initiate leukocyte rolling along the endothelial surface. P-selectin is known to be stored in Weibel-Palade bodies of endothelial cells. When activated by a number of inflammatory agonists such as histamine, thrombin, and reactive oxygen species, Weibel-Palade bodies translocate, fuse with the endothelial plasma membrane, and release their
contents (16, 23). The results of the present study suggests that another factor, specifically airway distension, either directly or indirectly through the local release of an inflammatory agonist, causes the activation of Weibel-Palade bodies and the exocytosis of P-selectin. That this adhesion molecule is essential for the process of recruitment was demonstrated by the lack of effect of PEEP in P-selectin\(^{-/-}\) mice.

ICAM-1 is the endothelial receptor for the \(\beta_2\)-family of integrins on leukocytes and is known to be involved specifically in firm adhesion and trafficking of neutrophils across the endothelial barrier (2). Normally present in low levels in endothelial cells, ICAM-1 expression is markedly upregulated in response to proinflammatory stimuli such as thrombin (1), TNF-\(\alpha\) (25), and shear stress (22). Cyclic stretching of mesangial cells in vitro demonstrated dose- and time-dependent increases in ICAM-1 mRNA and protein, as well as increased leukocyte adherence (20). Results of the present study demonstrated that ICAM-1 was essential for the PEEP-induced increase in leukocyte adherence (Fig. 3). This result is consistent with that previously shown in a rat model of inflammatory bowel disease (19). Interestingly, however, rolling velocity in the PEEP-exposed ICAM-1\(^{-/-}\) mice was not altered (Table 2). This result suggests an interaction between ICAM-1 and other adhesion molecules that is required for a reduction in rolling velocity that was not present in the ICAM-1\(^{-/-}\) mice and is consistent with those of others that showed no decrement in rolling velocity in ICAM\(^{-/-}\) mice despite exposure to inflammatory stimuli (6, 8).

Another result that suggests the cooperativity of multiple adhesion molecules is the observation that the lesser stimuli, increased tidal volume, and 2 \(\times\) 8-cm\(H_2\)O PEEP resulted in decreased leukocyte velocities yet the stimulus was inadequate for firm adhesion. The linear process of neutrophil rolling, adhesion, and migration may be more aptly described as an integrated response where rolling neutrophils sample endotelial signals and local soluble mediators, and then depending on their level of activation, stop and transmigrate (13). Additional studies with double mutants and/or blocking antibodies are needed to provide further information concerning the relative roles of individual adhesion molecules in the inflammatory response to excessive airway distension.

An acknowledged limitation of the use of intravital microscopy is the inability to differentiate specific leukocyte subpopulations by video imaging. The representative histological section of a mouse trachea after PEEP exposure (Fig. 4) showing numerous neutrophils was anticipated and consistent with the many published studies demonstrating the importance of P-selectin and ICAM-1 for neutrophil recruitment in the systemic vasculature (13), our laboratory’s previously published airway morphometry demonstrating increased neutrophils after PEEP challenge (15), and the present study demonstrating the lack of leukocyte recruitment in P-selectin\(^{-/-}\) and ICAM-1\(^{-/-}\) mice.

Although there is an ever-expanding literature on ventilator-induced lung injury and inflammation, little is known with regard to the airway circulatory response. Our laboratory’s previous observation in the rat was the first to suggest that lung distension caused the activation of airway postcapillary venular endothelium. The present study confirmed these observations and further advanced mechanisms of response. Within the lung parenchyma, both the site and mechanisms responsible for neutrophil recruitment are different from systemic vascular beds (5). Furthermore, the application of PEEP is used as a protective strategy to minimize lung injury (4). The results of the present study suggest that airway inflammation can be a direct cause of excessive endothelial distension and further highlight heterogeneity of lung endothelium.

ACKNOWLEDGMENTS
This work was supported by National Heart, Lung, and Blood Institute Grant HL-10342.

REFERENCES
19. Rijcken E, Kriegstein CF, Antho C, Laukoetter MG, Mennigen R, Spiegel HU, Semninger N, Bennett CF, Schuermann G. ICAM-1 and...


