

# High Tidal Volume Ventilation Induces Proinflammatory Signaling in Rat Lung Endothelium

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Alveolar overdistension during mechanical ventilation causes leukocyte sequestration, leading to lung injury. However, underlying endothelial cell (EC) mechanisms are undefined. In a new approach, we exposed isolated blood-perfused rat lungs to high tidal volume ventilation (HV) for 2 h, then obtained fresh lung endothelial cells (FLEC) by immunosorting at 4°C. Immunoblotting experiments indicated that as compared with FLEC derived from lungs ventilated at low volume (LV), HV markedly enhanced tyrosine phosphorylation (TyrP). The tyrosine kinase blocker, genistein, inhibited this response. HV also induced focal adhesion (FA) formation in FLEC, as detected by immunofluorescent aggregates of the  $\alpha_v\beta_3$  integrin that colocalized with aggregations of focal adhesion kinase (FAK). Immunoprecipitation and blotting experiments revealed that HV increased TyrP of the FA protein, paxillin. In addition, HV induced a paxillin-associated P-selectin expression on FLEC that was also inhibited by genistein. However, HV did not increase lung water. These results indicate that in HV, EC signaling *in situ* causes FA formation and induces TyrP-dependent P-selectin expression. These signaling mechanisms may promote leukocyte-mediated responses in HV.

Mechanical ventilation is essential for managing respiratory failure in lung injury. However, the high airway pressures and large tidal volumes, which are often necessary, exacerbate the injury (1–3). Many studies indicate that lungs exposed to mechanical ventilation develop an inflammatory phenotype, characterized by increase of microvascular permeability (3, 4), secretion of cytokines (5, 6), and enhanced leukocyte sequestration (7, 8). These findings provide broad-based evidence that mechanical ventilation causes tissue stress in lungs. However, no studies have addressed the intracellular signaling mechanisms that underlie the inflammatory response to ventilation stress.

Endothelial cells (ECs) lining lung microvessels initiate inflammation by expressing the leukocyte adhesion receptor, P-selectin, which induces leukocyte rolling on the EC

surface (9). In lung endothelium, P-selectin, a constituent of Weibel-Palade bodies (WPB), is probably restricted to extra-alveolar vessels (10–12), although others propose a more extensive expression (13). Nevertheless, a role for the receptor may be indicated in lung pathology, in that P-selectin expression increases in vascular stress (14).

In this article we consider the possibility that high-volume lung ventilation (HV) may induce P-selectin expression in lung EC. Lung expansion during HV stretches the pulmonary vascular bed, and is therefore likely to stretch lung ECs. *In vitro* studies indicate that EC monolayers subjected to stretch enhance protein tyrosine phosphorylation and induce formation of focal adhesion complexes (15, 16). Although the relevance of these EC responses to lung inflammation remains unclear, there are indications from other cell types that tyrosine phosphorylation (TyrP) plays a role in P-selectin responses. Thus, platelet activation is accompanied by P-selectin TyrP (17), and specific tyrosine residues in the cytoplasmic tail of P-selectin determine its sorting to secretory granules in the AtT tumor cell line (18). However, the extent to which TyrP induces EC expression of P-selectin is not known.

A general difficulty is that the understanding of EC signaling mechanisms has been developed through studies *in vitro* that may not apply to mechanisms *in situ*. Here, we used a new approach involving immunomagnetic cell recovery from ventilation-stressed lungs to determine signaling mechanisms in EC *in situ*. Our findings indicate that ventilation stress induces a proinflammatory signaling pathway in lung EC, in which the formation of focal adhesions and the induction of protein TyrP constitute essential components of the regulatory mechanism underlying P-selectin expression.

## Materials and Methods

### Reagents and Antibodies

The following were purchased: trypan blue and phosphate-buffered saline (PBS; GIBCO laboratories, Grand Island, NY); genistein (Sigma, St. Louis, MO); FITC-labeled donkey anti-mouse IgG, TRITC-conjugated goat anti-rabbit IgG, and streptavidin horseradish peroxidase (Jackson ImmunoResearch, Inc., West Grove, PA); affinity-purified polyclonal rabbit anti-phosphotyrosine Ab (ICN Biomedicals, Costa Mesa, CA) and protein A/Protein G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA); anti-factor VIIIIR:Ag/von Willebrand (vWf) factor, anti-CD31 and anti- $\alpha_v\beta_3$  integrin, LM609 monoclonal antibodies (Chemicon, Temecula, CA); anti-FAK (BC3) rabbit polyclonal Ab (Upstate Biotechnology, Lake Placid, NY); Alexa 488-tagged goat anti-mouse IgG and

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**Abbreviations:** endothelial cell, EC; focal adhesions, FA; focal adhesion kinase, FAK; fresh lung endothelial cells, FLEC; high tidal volume ventilation, HV; immunoprecipitation, IP; low tidal volume ventilation, LV; phosphate-buffered saline, PBS; tyrosine phosphorylation, TyrP; Weibel-Palade bodies, WPB.

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acetyl LDL, labeled with the fluorescent probe 1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate (Dil-Ac-LDL) (Molecular Probes, Eugene, OR) and Dynabeads M-450 tosylactivated (Dyna, Oslo, Norway). Mouse anti-rat P-selectin mAb, Rp-2 was a gift of Dr A.C. Issekutz (Department of Pediatrics, Dalhousie University, Halifax, NS, Canada) (14).

### Ventilated Blood-Perfused Rat Lung

The methods for preparing isolated blood-perfused lungs have been described (14). Briefly, we excised lungs from anesthetized, heparinized (1,200 IU heparin/kg) rats (Sprague-Dawley, 600 g), then we pump-perfused them, using autologous blood (37°C, 14 ml/min). We mechanically ventilated the lungs using low (LV) or HV tidal volumes of 6 or 12 ml/kg, respectively. These tidal volumes closely corresponded to those used recently in a clinical trial in which the larger volume was associated with a worse outcome (2). The corresponding inspiratory pressures were 11 and 22 cm H<sub>2</sub>O. Throughout the ventilation period, ventilatory rate and end-expiratory pressure were held constant at 30/min and 5 cm H<sub>2</sub>O, respectively. After 2 h of mechanical ventilation, we chilled the lungs (immersion in PBS, 4°C) to recover fresh lung EC (FLEC) as described below.

### Isolation of Lung Vascular Endothelial Cells

During FLEC recovery, all solutions were maintained at 4°C. The lung vasculature was perfused with 200 ml buffer (PBS, bovine serum albumin 0.1%) to clear blood, then sequentially infused with collagenase (5 ml, 1,000 U/ml, 15 min), trypsin (25 ml, 0.25%, 20 min) and buffer (25 ml, 10 min), and the mixed effluent was collected. To this, we added the lung tissue that was chopped and treated with trypsin (4°C, 30 min) to dislodge remaining EC. The whole sample was filtered (100 µm pore filter; Becton Dickinson Labware, Franklin Lakes, NJ). The filtrate was centrifuged (650 × g, 10 min) and the cell pellet was washed three times by repeated centrifugations in buffer. Cell exposure to collagenase and trypsin was limited to ~ 1.5 h at 4°C.

We next exposed the sample containing a mixture of cells to immune-labeled magnetic beads (19). Briefly, we washed (3×) 5 × 10<sup>6</sup> sheep anti-mouse IgG1 (Fc)-coated, magnetic microbeads (M-450, 4.5 µm; Dynal), exposed them to EC-specific, anti-factor VIIIIR:Ag/vWf (16 µg, overnight, 4°C), and washed again to remove excess antibody. We incubated these beads with lung cells suspended in buffer (150 µl, 1 h, 4°C) and magnetically isolated EC attached to the antibody-labeled beads (magnetic particle concentrator; Dynal). The supernatant contained rejected cells. We determined trypan blue exclusion to indicate cell viability. We determined EC phenotype by immunofluorescence.

### Immunofluorescence

**Labeling for EC phenotype.** To label for EC-specific markers, FLEC were labeled using anti-CD31 (19) or anti-factor VIIIIR:Ag/vWf (20) mAbs (10 µg/ml, 30 min, 4°C), followed by FITC-labeled donkey anti-mouse IgG (15 µg/ml, 30 min, 4°C), then fixed (3.5% paraformaldehyde, 10 min, 4°C). To label the acetylated-low density lipoprotein (Ac-LDL) receptor, FLEC were incubated with 10 µg/ml<sup>-1</sup> Dil-Ac-LDL (21) in PBS (4°C, 3 h), washed with probe-free PBS (10 min), and fixed. Cells were viewed by both brightfield and confocal fluorescence microscopy (LSM 510; Zeiss, Thornwood, NY).

**α<sub>v</sub>β<sub>3</sub> integrin and focal adhesion kinase (FAK) labeling.** FLEC from LV and HV were fixed (3.5% paraformaldehyde, 10 min, 4°C), then immunofluorescently double-labeled using anti-α<sub>v</sub>β<sub>3</sub> mAb, LM609, and rabbit polyclonal anti-FAK antibody BC3 (10

µg/ml of each, 30 min, 37°C), followed by FITC-labeled donkey anti-mouse IgG and TRITC-conjugated goat anti-rabbit IgG (15 µg/ml of each, 30 min, 37°C). Immunofluorescence was detected by confocal fluorescence microscopy (LSM 510; Zeiss).

**P-selectin labeling.** Freshly isolated lung endothelial cells obtained from ventilated lungs (2 h), perfused in the absence or presence of genistein, were immunofluorescently labeled using anti-P-selectin mAb (100 µg/ml), followed by Alexa-tagged goat anti-mouse IgG (2 µg/ml, 30 min, 4°C for each antibody). Cells were then fixed and viewed by confocal fluorescence microscopy (LSM 510; Zeiss). We digitally imaged single cells to quantify fluorescence intensity for labeling of the α<sub>v</sub>β<sub>3</sub> integrin, focal adhesion kinase (FAK), and P-selectin (MCID-M4; Imaging Research, Brock Univ., St. Catharines, ON, Canada).

### Immunoblotting and Immunoprecipitation

FLEC were lysed, as we reported previously for cultured EC (22) (4°C, 30 min) using lysis buffer (150 mmol/liter NaCl, 50 mmol/liter Tris-base, 2 mmol/liter EGTA, 50 mmol/liter NaF, 0.1% sodium dodecyl sulfate, 1% NP-40, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mmol/liter phenylmethylsulfonyl fluoride, and 1 mmol/liter sodium orthovanadate, the phosphatase inhibitor, pH 7.5). After clearing the lysates (14,000 rpm, 15 min), protein concentrations were determined (DC Protein Assay; Bio-Rad, Richmond, CA). Anti-TyrP immunoblotting was performed as described previously (22). Briefly, lysates containing equal amounts of protein were run in duplicate on 10% SDS polyacrylamide gels (SDS-PAGE) under reducing conditions. After transfer to nitrocellulose, phosphotyrosyl-containing proteins were detected using affinity-purified anti-TyrP antibody (previously derivatized with sulfosuccinimidyl labeled biotin), followed by addition of streptavidin-horseradish peroxidase. Duplicate gels were stained with Coomassie Blue. Blots were developed using enhanced chemiluminescence. Immunoprecipitation was performed using equal protein contents in each lane, as described previously (22).

### Statistics

All data are mean ± SE. Differences between groups were tested by the paired *t* test for two groups and by the Newman-Keuls test for > 2 groups. Statistical significance was accepted at *P* < 0.05.

### Results

#### Isolation of Lung Endothelial cells

By our immunomagnetic separation protocol we isolated 1.7 ± 0.4 × 10<sup>6</sup> EC per rat lung. For each lung, we determined purity of EC recovery by exposing an aliquot of cells to the EC-specific markers anti-factor VIIIIR:Ag/vWf, anti-CD31, and fluorescent AcLDL (Figure 1). Fluorescence determinations on 60 cells/lung for each marker indicated positive EC phenotype in 97 ± 1% (*n* = 9 lungs). Further, 93 ± 0.6% of the cells rejected trypan blue (*n* = 4). These findings indicate that EC were recovered with high purity and high viability. In lysates prepared from FLEC, protein content was 40 ± 5 µg/g lung (*n* = 14). Because approximately two times this amount was required for immunoprecipitation studies (22), we combined FLEC lysates from two lungs for a single immunoprecipitation experiment.

#### Protein TyrP

Because *in vitro* studies indicate that mechanical challenge to cells causes the induction of protein TyrP and the forma-

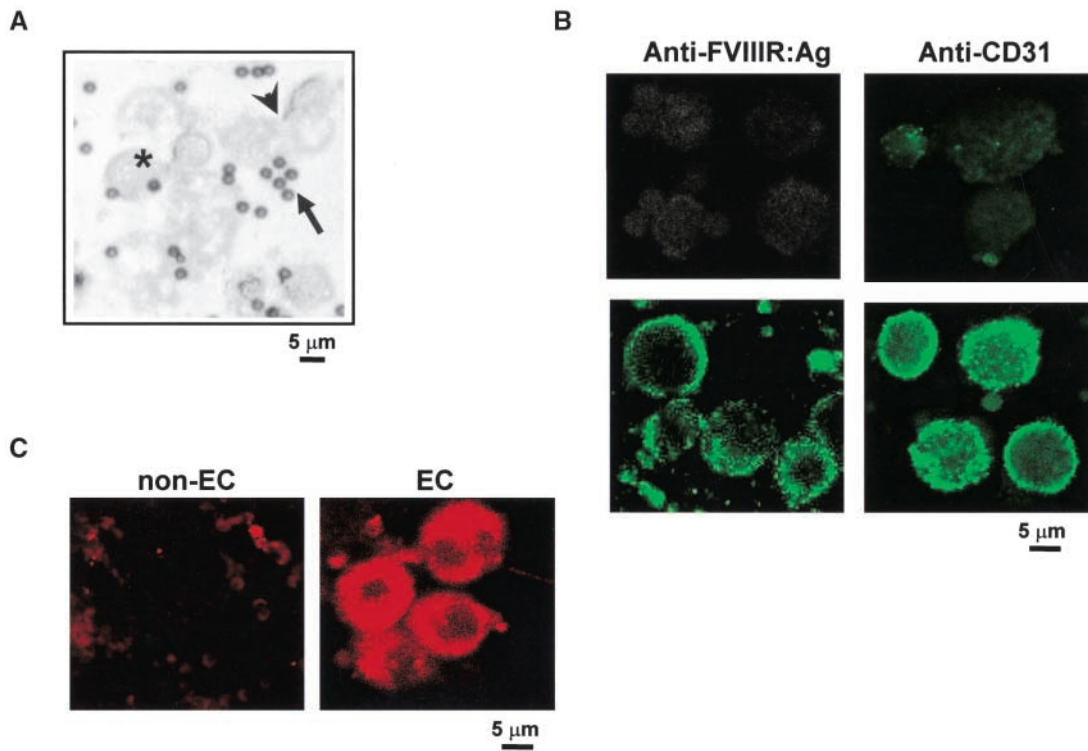


Figure 1. Characterization of FLEC. (A) Brightfield image shows beads (arrow) attached to single (asterisk) and clumped (arrowhead) cells. (B) Cells were first exposed to either IgG (top), or anti-Factor VIII:Ag/vWf (bottom left) and anti-CD31 (bottom right), then fluorescently tagged secondary IgG, fixed. Images show fluorescently labeled cells viewed by confocal microscopy. (C) Cells were exposed to DiI-Acetyl LDL. Images obtained by confocal microscopy show fluorescence in cells retained by immunomagnetic separation (right), but not in cells in the rejected sample (left).

tion of focal adhesions (23, 24), we determined the extent to which these responses occurred in the present experiments. In the LV group, immunoblots of FLEC lysates showed low levels of TyrP for several proteins (Figure 2A). In the HV group, several TyrP bands were markedly enhanced (Figures 2A and 2B). This enhancement was not due to unequal protein loading in gels, because in parallel runs on SDS-PAGE, Coomassie Blue staining was equal for HV and LV (Figure 2A). Because this response suggested that tyrosine kinases were activated, in some experi-

ments we infused the tyrosine kinase inhibitor genistein (25) in the lung's blood flow throughout the experiment. Genistein completely inhibited the enhanced TyrP (Figure 2B). These findings indicated that ventilation stress enhanced protein TyrP in EC.

To assess focal adhesion formation, we determined aggregation of FAK and of the  $\alpha_3\beta_3$  integrin by confocal microscopy. FLEC were fixed and double-labeled with anti-FAK Ab, BC3 and anti- $\alpha_3\beta_3$  mAb, LM609, and then exposed to rhodamine- and FITC-linked secondary Ab, re-

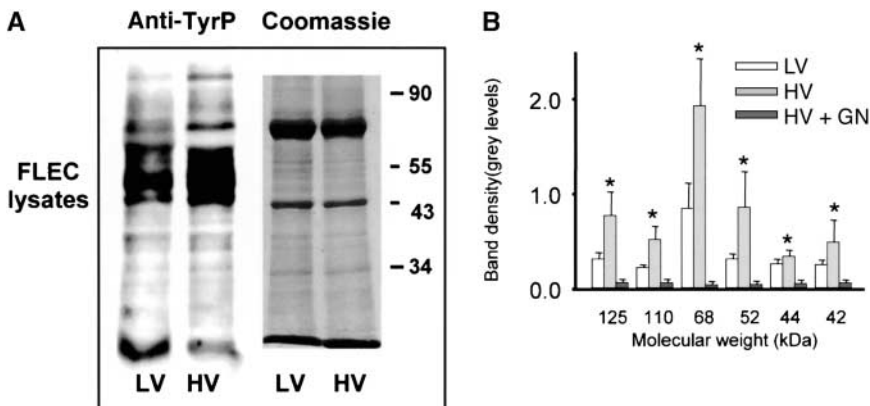
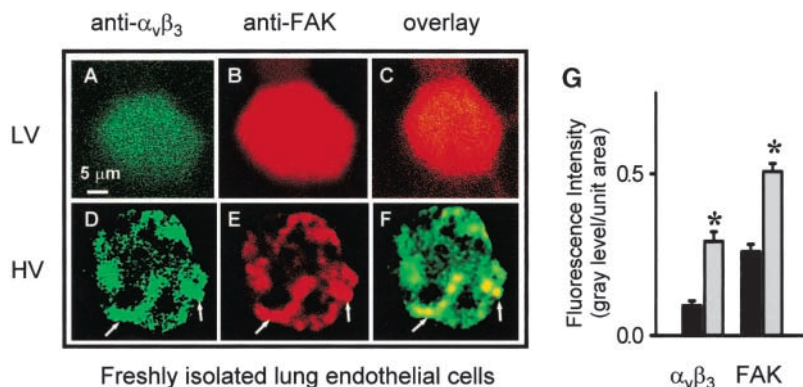


Figure 2. Protein tyrosine phosphorylation in FLEC. LV, low tidal volume ventilation; HV, high tidal volume ventilation; anti-TyrP, anti-phosphotyrosine; GN, genistein (100 μmol/liter). (A) FLEC lysates from HV and LV were each subjected to SDS-PAGE in duplicate. Lanes show immunoblots with anti-phosphotyrosine (left) and Coomassie Blue staining (right). Molecular weight markers are shown on the right. (B) Quantification of band densities at the indicated molecular weights from anti-TyrP blots from five experiments. Open bars, LV; shaded bars, HV; filled bars, HV+GN. \*P < 0.05, compared with bars to the left and right. Values are mean ± SE.



**Figure 3.** Confocal microscopy of immunofluorescently labeled  $\alpha_v\beta_3$  integrin and FAK in FLEC. LV, low tidal volume ventilation; HV, high tidal volume ventilation. Images show single, freshly isolated cells from LV (upper panels) and HV (lower panels) labeled for the  $\alpha_v\beta_3$  integrin (A, D) and FAK (B, E) and corresponding overlay images (C, F). (G) Figure shows fluorescence intensity as quantified by digital imaging of 50 cells/lung from three paired LV (solid bars) and HV (shaded bars) experiments. \* $P < 0.01$  compared with bar on left. Values are mean  $\pm$  SE.

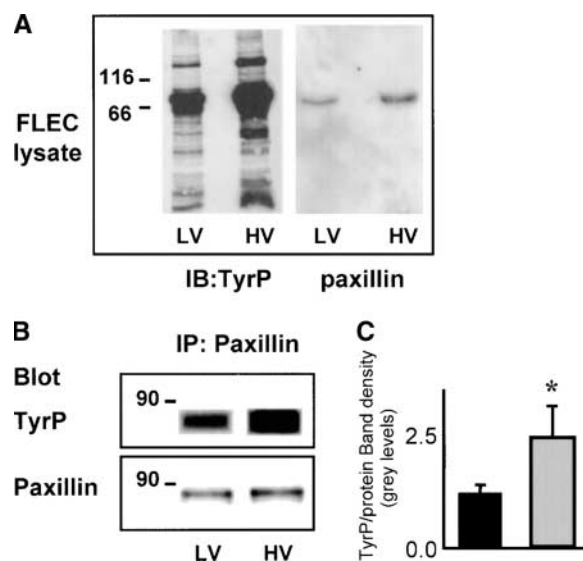
spectively. In LV, cell surface fluorescence was weak and diffuse (Figures 3A–3C). By contrast, in HV, fluorescent aggregates of 2–5  $\mu\text{m}$  diameter were evident over > 50% of the cell surface (Figures 3D–3F). The merged pseudocolors in image overlays indicated that the proteins had colocalized in the presence of ventilation stress. Fluorescence intensity per unit area for both proteins was greater in HV than LV (Figure 3G). Because initial phosphotyrosine blots of FLEC lysates revealed a prominent band at  $\sim 68$  kD that potentially indicated paxillin (Figure 2A), in a separate group we sequentially blotted for phosphotyrosine and paxillin (Figure 4A). Further, we immunoprecipitated paxillin from FLEC lysates (Figure 4B). These experiments revealed that tyrosine phosphorylation on paxillin was two times greater in HV than in LV (Figure 4C).

#### P-Selectin Expression

To detect cell surface expression of P-selectin, we surface-biotinylated FLEC at 4°C, then immunoprecipitated P-selectin from FLEC lysates. Representative findings from sequential immunoblotting of the immunoprecipitates are shown in Figure 5. Immunoblotting with streptavidin revealed a band at 140 kD that was more prominent in HV than in LV. Reprobing the gel with anti-P-selectin mAb confirmed that the 140-kD band was P-selectin, indicating that surface expression of P-selectin was enhanced in HV. This blot, which also provided an assessment of the total amount of P-selectin protein in FLEC, namely that comprising the intracellular as well as the surface-expressed contents, revealed bands that were consistently more pronounced in HV. This finding indicated that HV increased the overall amounts of P-selectin protein in FLEC. We reprobbed the blot of immunoprecipitated P-selectin with anti-paxillin mAb. This blot also revealed a more prominent band in HV than in LV, indicating that ventilation stress increased the association of paxillin with P-selectin. However, the amount of co-associated paxillin differed between experiments.

To determine cell surface expression of P-selectin by immunofluorescence, we sequentially exposed FLEC to anti-P-selectin mAb and fluorescence-tagged anti-mouse IgG. We maintained cells at 4°C to prevent cellular uptake of the antibodies and, thereby, to label surface proteins selectively. In fields of cells viewed at low power by confocal microscopy, immunofluorescence of P-selectin was weak

and diffuse in LV, but extensive and present on > 97% of cells in HV (Figure 6A, panels a and b). Viewed at high magnification, most cells for HV revealed a clumped fluorescence at the cell periphery (Figure 6A, panel d). By contrast, fluorescence was diffuse and weak for LV (Figure 6A, panel c). Quantification by digital image analysis indicated a 2-fold increase of fluorescence in HV (Figure 6B), reaffirming that HV increased cell-surface P-selectin expression. Inclusion of genistein in the perfusate diminished immunofluorescence of P-selectin (Figure 6B) and de-



**Figure 4.** Paxillin tyrosine phosphorylation in lung endothelium. LV, low tidal volume ventilation; HV, high tidal volume ventilation; IP, immunoprecipitation; IB, immunoblotting; TyrP, tyrosine phosphorylation. (A) Lysates of FLEC were subjected to SDS-PAGE, then immunoblotted first with anti-phosphotyrosine (left panel), then reprobbed for paxillin (right panel). Data are representative of three paired LV and HV experiments. Molecular weight markers are shown on the left. (B) Top and bottom panels are anti-TyrP and anti-paxillin blots of immunoprecipitated paxillin. (C) Optical densities from immunoprecipitation data from three paired experiments are expressed as tyrosine phosphorylation/protein ratios. Solid bars, LV; shaded bars, HV. \* $P < 0.05$ , compared with bar on left. Values are mean  $\pm$  SE.

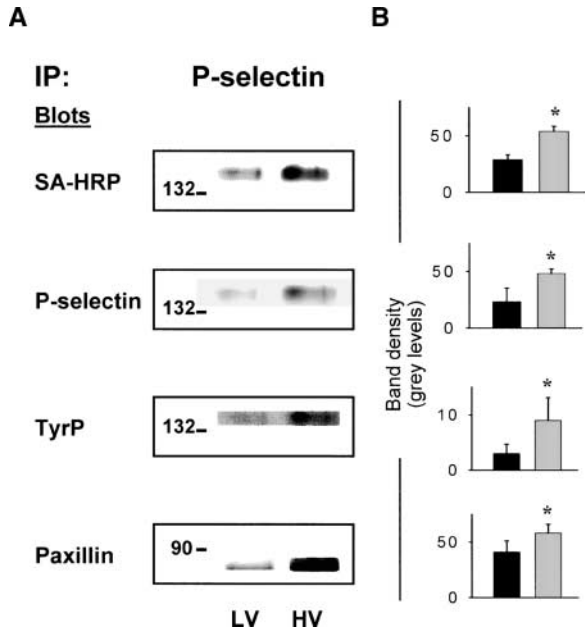


Figure 5. Lung endothelial P-selectin expression and co-association of paxillin. LV, low tidal volume ventilation; HV, high tidal volume ventilation; IP, immunoprecipitation; TyrP, tyrosine phosphorylation; SA-HRP, streptavidin horseradish-peroxidase. P-selectin was immunoprecipitated from lysates of surface biotinylated (4°C) FLEC, then subjected to SDS-PAGE, transfer, and sequential blotting as indicated. Blots from single experiments (A) and corresponding densitometric data for three experiments each (B) are shown. Molecular weight markers are indicated on left. Solid bars, LV; shaded bars, HV. \*P < 0.05, compared with bar on left. Values are mean ± SE.

creased the recovery of cell-surface P-selectin by immunoprecipitation (Figure 6C). These findings indicated that the HV-induced P-selectin expression was blocked by blockade of TyrP.

To assess the extent to which pulmonary edema associated with these responses, we determined extravascular lung water by our standard methods (26). In paired LV and HV experiments, lung water averaged 4 ± 0.1 and 4.1 ± 0.1 g/g dry (mean ± SE; n = 4), respectively. These values were within our established limits of normal (27).

**Discussion**

From these studies in FLEC, we report the novel finding that ventilation stress enhanced protein TyrP, leading to P-selectin expression. Successful application of our newly developed immunochemical and immunofluorescence assays in FLEC revealed that the effect of ventilation stress was to activate several focal adhesion proteins. Thus, the integrin α<sub>v</sub>β<sub>3</sub> and FAK underwent aggregation and co-localization, whereas tyrosine phosphorylation increased on paxillin. These events were associated with increased P-selectin expression and co-localization of paxillin with P-selectin. We ruled out platelets as a source of the present P-selectin expression, because lungs were cleared of blood components with extensive buffer perfusion before cell isolation. Moreover, the high purity of the cell isolate and the phenotypic appearance of P-selectin expressing cells precluded the presence of platelets. The increased P-selectin expression was blocked by inhibition of EC tyrosine phosphorylation. We conclude that in ventilation stress, protein tyrosine phosphorylation constitutes a major pathway for pro-inflammatory signaling in lung EC.

**EC Isolation**

For the first time, these methods allow detection of signaling mechanisms in EC that are freshly isolated from experimen-

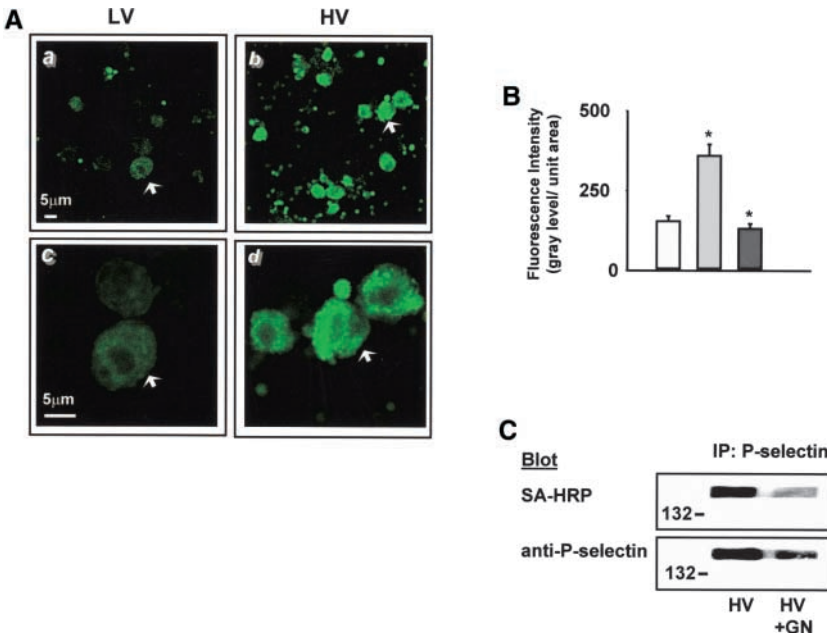


Figure 6. P-selectin expression in FLEC. LV, low tidal volume ventilation; HV, high tidal volume ventilation; IP, immunoprecipitation; SA-HRP, streptavidin horseradish-peroxidase; GN, genistein (100 µmol/liter). (A) Images of FLEC obtained by confocal microscopy show immunofluorescence of P-selectin for LV and HV as indicated. Arrowheads mark cells that were viewed at low (top) and high (bottom) magnifications in each group. (B) Quantification of fluorescence intensity by digital imaging of single cells. Data are mean ± SE for 30 cells viewed per lung in each of three paired experiments. Open bars, LV; shaded bars, HV; solid bars, HV+GN. \*P < 0.05, compared with bar on left. (C) SA-HRP (top) and anti-P-selectin (bottom) blots of immunoprecipitates of P-selectin from lysates of surface biotinylated (4°C) FLEC. Molecular weight markers are indicated on left. Data are representative of three paired LV and HV experiments.

tally treated lungs. Cell recovery at 4°C inactivated enzymatic processes, and thereby preserved phosphorylation responses developed *in situ*. Immunomagnetic protocols for EC recovery have targeted CD31 or bound lectins on the cell surface (28). We avoided these targets because CD31 is nonspecific for EC and is also expressed on leukocytes (29), whereas lectins may cause cell lysis. In EC, vWf is contained in WPB (30) and is secreted by WPB exocytosis (31). Under nonstimulated conditions, vWf is expressed on the surface of nonpermeabilized cells (30) as well as that of unfixed perialveolar capillary endothelium (32). Our finding that vWf is expressed on the surface of FLEC corroborates these reports. Because EC recovery was independent of the numbers of beads attached to them, the extent of vWf expression was not a major determinant of EC isolation. Evidently, to a small extent vWf is constitutively expressed on the cell surface, and this amount is sufficient for targeted cell recovery.

Microscopy of FLEC indicated that our recovery procedure yielded single as well as clumped cells. Because of clumping, these isolated cells were amenable to immunofluorescence and confocal microscopy, but not to fluorescence-activated cell sorting. Using immunofluorescent labeling for three EC-specific markers, we determined that the cells were recovered with high purity and viability. Presently, the vascular sites of origin for these cells remain undetermined. In the pulmonary circulation, extra-alveolar vessels are preferred sites of expression for both vWf (32) and P-selectin (11, 12). Because our recovered EC expressed both proteins, they may be of extra-alveolar origin. This possibility is also supported by the relatively small cell recovery by our isolation methods. Thus, in both LV and HV each lung yielded 1–2 million FLEC, which constitutes a small fraction of the estimated  $410 \times 10^6$  EC in rat lung (33). Because the bulk of lung EC are contained in alveolar capillaries, we attribute the small FLEC yield to the possibility that capillary EC were not accessible to our isolation methods.

### P-Selectin

A novel finding revealed in immunoprecipitates of surface-biotinylated FLEC from the ventilation-stressed group was the presence of TyrP on P-selectin that was expressed on the cell surface. It is proposed that TyrP of P-selectin regulates its incorporation in secretory granules (18). Consistent with this notion, in our experiments genistein inhibited surface expression of P-selectin in FLEC. Although the specific targets of TyrP remain unclear, taken together with the reported data our findings indicate that protein TyrP is critical in the regulation of P-selectin expression in EC.

P-selectin is stored in WPB adjacent to the EC luminal membrane (34). Activation of WPB exocytosis causes P-selectin expression and is generally attributed to increases of cytosolic  $Ca^{2+}$  (31). In previous studies (35, 36), we showed that exposing EC to the  $\alpha_v\beta_3$  ligand vitronectin causes aggregation of the  $\alpha_v\beta_3$  integrin. Genistein blocks the associated induction of TyrP that leads to ER release of  $Ca^{2+}$ , but not the integrin aggregation itself. These findings suggest that  $\alpha_v\beta_3$  aggregation is not determined by TyrP, although the aggregation event activates tyrosine kinases. To the extent

that these mechanisms apply to the present experiments, we speculate that the present  $\alpha_v\beta_3$  aggregation activated TyrP-dependent pathways to induce the  $Ca^{2+}$  increase required for WPB exocytosis.

The localization of P-selectin with paxillin points to a new mechanism underlying P-selectin expression. Paxillin, which is recruited to focal adhesions following aggregation and autophosphorylation of FAK, is increasingly regarded as an adaptor protein that forms a docking platform for signaling proteins (37). Although such mechanisms remain inadequately understood in the context of inflammation, the enhanced association between paxillin and P-selectin in ventilation stress suggests that paxillin may provide a docking function for P-selectin exocytosis. Although this issue requires further investigation, the tyrosine residue on P-selectin has been previously implicated in the sorting of P-selectin to secretory vesicles (18, 38). Tyrosine kinases are implicated in some forms of exocytosis (38, 39); tyrosine phosphorylated adaptor proteins may act as P-selectin chaperones during WPB exocytosis.

We point out that despite the enhanced P-selectin expression on FLEC, HV for 2 h did not increase lung water. To this extent, our findings agree with those of Matthay and coworkers (1), who showed that mechanical ventilation at tidal volume of 12 ml/kg does not cause pulmonary edema. However, the fact that proinflammatory signaling was induced in the present experiments is an indication that even in the absence of pulmonary edema, these modest levels of ventilation stress may be sufficient to initiate endothelial signaling that primes the microvascular bed for injury. Thus, Matthay and coworkers have shown that mechanical ventilation at 12 ml/kg exacerbates pre-existing lung injury induced by acid instillation.

### Focal Adhesions

Integrins constitute the link between subcellular matrix and cytoskeleton at focal adhesions (37, 40). Distortions attributable to shear and stretch at the cell–matrix interface result in integrin aggregation that, in turn, induces focal adhesions (23, 24, 41). Focal adhesions function as “signaling centers” that impact cytosolic, cytoskeletal, and nuclear responses (37, 40). The extent to which these events may have occurred in the present study is indicated in the immunofluorescence responses of the integrin  $\alpha_v\beta_3$  and the focal adhesion protein FAK. In HV, both proteins co-aggregated. Moreover, TyrP increased on paxillin. These responses are consistent with increased focal adhesion formation. We speculate that to promote cell anchorage, EC *in situ* developed focal adhesions to withstand mechanical stresses induced by HV. The extent to which these responses occur in other forms of lung stresses requires further study.

Two general conclusions may be drawn from these studies. First, it is possible to determine signaling pathways in EC *in situ*, thereby avoiding *in vitro* data that may not reflect conditions existing in the intact organ. Second, the formation of EC focal adhesions and the signaling through TyrP constitutes an important mechanism in the lung’s response to stressful mechanical ventilation. This signaling is widely considered to be important for gene transcription, because signaling events resulting from TyrP induce activa-

tion of nuclear factors (42, 43). We show here a link between TyrP and P-selectin expression. This indicates that activation of tyrosine kinases requires further consideration as a basic mechanism underlying lung inflammatory pathology.

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