Platelets Enhance Endothelial Adhesiveness in High Tidal Volume Ventilation

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Although platelets induce lung inflammation, leading to acute lung injury (ALI), the extent of platelet–endothelial cell (EC) interactions remains poorly understood. Here, in a ventilation-stress model of lung inflammation, we show that platelet–EC interactions are important. We obtained freshly isolated lung endothelial cells (FLECs) from isolated, blood-perfused rat lungs exposed to ventilation at low tidal volume (LV) or stress-inducing high tidal volume (HV). Immunofluorescence and immunoprecipitation studies revealed HV-induced increases in cell-surface von Willebrand factor (vWf) expression on FLEC. This increased expression was inhibited by platelet removal from the lung perfusion and by including a P-selectin–blocking antibody in the lung perfusion. The expression was also blocked in lungs from P-selectin knockout (*P sel*^{−/−}) mice perfused with autologous blood, but not with heterologous wild-type blood containing P-selectin–expressing platelets. These findings indicate that in ventilation stress, platelets transfer vWf to the EC surface and that platelet P-selectin plays a critical role in this transfer. Further evidence for such intercellular transfers was the HV-induced FLEC expressions of platelet glycoprotein 1b and of platelet P-selectin. We conclude that in ventilation stress, platelets deposit leukocyte- and platelet-binding proteins on the EC surface, thereby establishing the proinflammatory phenotype of the vascular lining.

Keywords: von Willebrand factor; P-selectin; GP1b; lung; platelets

High tidal volume ventilation (HV) causes lung inflammation (1–3), thereby contributing to morbidity and mortality in acute lung injury (ALI) (4, 5). Since it is increasingly evident that activated platelets determine ALI (6–8), the role of platelets in HV-induced lung inflammation requires further understanding, especially in the context of platelet–endothelial cell (EC) interactions. The EC role is likely to be significant, since inflammatory EC responses, including those relevant to barrier regulation and the expression of proinflammatory receptors, are critical in the development of ALI (4, 5, 9, 10).

Evidence from systemic vessels indicates that primary EC activation increases platelet–EC interactions, leading to proinflammatory conditions on the EC surface (11). Platelets are sentinel cells that roll on EC to monitor vascular viability (12). In systemic blood vessels, oxidized low-density lipoprotein induces tissue factor secretion from EC (13). The resulting coagulation cascade forms fibrin that provides a bed for platelet adhesion and activation (13). Despite this understanding, the relevance of these mechanisms in lung remains unclear. Although alterations in coagulation and fibrinolysis characterize ALI (14), specific platelet-induced mechanisms that induce the proinflammatory

CLINICAL RELEVANCE

We show that high tidal volume causes endothelial transfer of platelet vWf and other adhesive proteins, inducing a proinflammatory vascular lining. In addition, the plasma increases of these proteins may underlie distant organ inflammation.

lung EC phenotype remain undefined, especially in the context of HV.

Platelet–EC interactions involve the proteins von Willebrand factor (vWf), P-selectin, and glycoprotein 1b α (GP1b- α) (11, 15, 16). vWf and P-selectin exocytosis occurs from stores in EC Weibel Palade bodies (WPBs) and platelet α granules (15, 17). Expression of GP1b- α , a member of the membrane protein complex GP1b/V/IX, is exclusively platelet specific (16). GP1b- α ligation of EC vWf causes platelet activation, which induces platelet vWf and P-selectin exocytosis and GP1b- α shedding (18). The extent to which these mechanisms contribute to the development of ALI requires clarification.

In recent reports, we determined the proinflammatory phenotype of freshly recovered lung EC (FLEC) from lungs exposed to HV (19, 20). These studies indicate that despite the lack of overt lung injury, as indicated by the absence of microvascular hyperpermeability, FLEC showed proinflammatory responses, including increased tyrosine phosphorylation, and expression of P-selectin (19, 20). Importantly, these findings indicate that HV-induced EC stretch is itself sufficient to activate an adhesive phenotype on EC. However, the role of other platelet adhesive proteins is not understood. Here, we continued studies in FLEC derived from lungs exposed to moderate HV to determine platelet–EC protein interactions that might play a role in the development of a proinflammatory phenotype on the lung's vascular surface.

MATERIALS AND METHODS

Reagents and Antibodies

The following were purchased: PBS (Mediatech Inc, Herndon, VA); sulfo-succinimidobiotin (EZ link sulfo-NHS-biotin) (Pierce Biotechnology, Rockford, IL); anti-mouse IgG-HRP, protein A/Protein G-agarose, mouse monoclonal anti-P-Selectin antibody (CTB201), goat polyclonal anti-vWf antibody (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal IgG₁, rabbit anti-factor VIII:Ag/vWf (Zymed, South San Francisco, CA); mouse anti-human GP1b mAb, CD42a (BD PharMingen, San Diego, CA); Rhodamine 6G, Alexa 488–tagged goat anti-mouse IgG, Alexa 488–tagged donkey anti-goat IgG and Alexa 633–tagged goat anti-rabbit IgG (Invitrogen, Carlsbad, CA); Dynabeads M-270 tosylactivated (Dyna, Oslo, Norway); Strept Avidin HRP conjugate (Jackson ImmunoResearch, Inc., West Grove, PA); anti-rat P-selectin nonblocking and blocking mAbs, RP-2 and RMP-1, respectively, were gifts of Dr. A. C. Issekutz (Department of Pediatrics, Dalhousie University, Halifax, NS, Canada) (21).

(Received in original form September 11, 2007 and in final form April 16, 2008)

This study was supported by National Institutes of Health Grant HL54157 (to S.B.).

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Am J Respir Cell Mol Biol Vol 39, pp 569–575, 2008

Originally Published in Press as DOI: 10.1165/rcmb.2007-0332OC on May 15, 2008

Internet address: www.atsjournals.org

Lung Preparation

Using our reported procedures (19, 20), we isolated and blood-perfused rat (Sprague-Dawley, Taconic Farms, Germantown, NY) and mouse lungs. Mice were wild type (WT, C57BL/6) or genetically deficient in P-selectin (P-selectin knockout [P sel^{-/-}]) (Jackson laboratories, Bar Harbor, ME). Briefly, we pump perfused the lungs with autologous blood at 37°C (hematocrit 10–20%) at 14 and 1 ml/minute, respectively, for rat and mouse at pulmonary artery and left atrial pressures of 12 and 6 cm H₂O. Through a tracheal cannula, we mechanically ventilated the lungs (Harvard Apparatus, Holliston, MA) at low (LV) and high (HV) tidal volumes. The corresponding tidal volumes were 6 and 12 ml/kg for rat, and 6 and 18 ml/kg for mouse. For both species, the corresponding peak inspiratory pressures were 11 and 22 cm H₂O and the mean airway pressures were 6 and 10 cm H₂O. Ventilatory rate and end-expiratory pressure were maintained at 30/minute and 5 cm H₂O, respectively.

Preparation of Blood Perfusates

We gave heparin (200 units/kg) by intracardiac injection to anesthetized animals and withdrew blood 2 minutes later (20 ml for rat, 1 ml for mouse). Unless stated otherwise, we perfused lungs with freshly obtained heparinized blood that was not subjected to separation procedures. To prepare platelet-depleted blood, we centrifuged whole blood (2,000 rpm, 10 min, 4°C) to establish density gradients formed by layers containing red cells, the leukocyte-rich buffy coat, and platelet-rich plasma. We removed the layer of platelet-rich plasma as well as the buffy coat. Next, we resuspended red cells in buffer (4% albumin/PBS, 4°C) and washed them by repeated centrifugation (three times). For control, we performed the centrifugation, but did not remove any layers.

To determine leukocyte and platelet counts, we fluorescently labeled cells (rhodamine 6G, 5 µg/ml, 10 min) in aliquots of blood perfusate obtained at the beginning of the experiment. Leukocytes and platelets were each approximately 90% less in platelet-depleted blood than in whole blood (Table 1).

FLEC Isolation

We isolated FLEC as we previously described (19). Briefly, we terminated the experiment after 2 hours in rat lungs and 1 hour in mouse lungs. We used ice-cold buffer perfusion (0.1% albumin/PBS, 4°C) to clear blood from lungs (200 and 10 ml, respectively, for rat and mouse). Then, with the lung immersed in cold PBS (4°C), we sequentially perfused collagenase, trypsin, and buffer (4°C) to dislodge luminal cells from the lung vasculature. We washed the dislodged cells in buffer (three times), exposed them to magnetic beads (Dynabeads M-270, 2.8 µm; Dynal) labeled with endothelial-specific, rabbit anti anti-vWf antibody (22), and finally recovered them by magnetic separation (magnetic particle concentrator, Dynal). As we reported previously (19), the recovered cells were 97 ± 1% pure for the endothelial phenotype, as confirmed by staining with the endothelial-specific markers, vWf, CD31, and AcLDL. For immunofluorescence studies, since labeling was on endothelium-specific epitopes, P-selectin, and vWf, we used enzymatically dislodged cells without immunomagnetic isolation.

Immunofluorescent Labeling of FLEC

To detect surface epitope expression, we labeled live cells at 4°C to avoid antibody internalization and then fixed the cells. To determine intracellular expression, we fixed (2% paraformaldehyde, room temperature, 10 min) and permeabilized (0.1% triton X, 2 min) cells, then

labeled and fixed them. For immunofluorescent labeling, we used mouse monoclonal Abs against P-selectin and GP1b and goat or rabbit polyclonal anti-human vWf Abs (8 µg/ml, 60 min, 4°C) followed by secondary, fluorescence-tagged IgGs that were respectively, goat anti-mouse, donkey anti-goat, and goat anti-rabbit (10 µg/ml, 60 min, 4°C). For control labeling, we used similar staining procedures, except we used nonimmune IgGs isotype-specific for Abs against P-selectin, vWf, and GP1b. We imaged single cells by confocal microscopy (LSM 510; Zeiss, Thornwood, NY). Fluorescence intensity determinations were obtained separately by two investigators who were blinded to the experimental plan. The correlation coefficient for colocalization was also determined using LSM 5 image examiner software.

Immunoprecipitation

Endothelial P-selectin. We used FLEC from two identically ventilated and perfused rat lungs to ensure adequacy of endothelial protein (150–200 µg) for immunoprecipitation (IP). Thus we used four rats for one paired experiment. We surface labeled FLEC (sulfo-NHS-biotin, 0.5 mg/ml, pH 8.0, 4°C, 30 min), as reported previously (19), lysed cells (4°C, 30 min), and cleared the lysates (13,000 rpm, 15 min). We determined lysate protein concentrations (BCA Protein Assay; Pierce, Rockford, IL) and using monoclonal anti-P-selectin antibody, immunoprecipitated P-selectin from equal quantities of lysate protein (19).

Plasma P-selectin and vWf. We determined protein concentration in plasma from blood perfusates of experimental lungs (centrifugation 13,000 rpm, 10 min). Using monoclonal anti-P-selectin or polyclonal anti-vWf antibodies, we immunoprecipitated P-selectin and vWf from equal quantities of plasma protein (3,000 µg). To confirm antibody specificity, we performed control immunoprecipitations using non-immune IgGs, isotype-specific to the anti-P-selectin and anti-vWf antibodies. The immunoprecipitates were electrophoresed on 8% SDS polyacrylamide gels (SDS-PAGE) under reducing conditions, transferred to nitrocellulose, and blotted with the specified antibodies. We developed blots using enhanced chemiluminescence.

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 more than two groups. Statistical significance was accepted at *P* < 0.05. The blinded data gave a Pearson correlation coefficient of 0.91 (*P* < 0.001) for inter-observer reliability.

RESULTS

vWf and P-Selectin Expression

To address EC responses to HV, we determined immunofluorescence of vWf and P-selectin in FLEC by confocal microscopy. At the cell surface, P-selectin and vWf were each weakly expressed in LV (Figures 1A and 1B). However, HV markedly increased cell-surface expression for both proteins (Figures 1A–1C). Control staining with nonimmune IgG resulted in dark images (Figure 1A, *left*), ruling out nonspecific effects. As indicated by fluorescence overlay (Figures 1A and 1B, *bottom rows*), HV also enhanced colocalization of the proteins (Figure 1D). Importantly, these HV-induced effects were blocked by perfusion with reconstituted blood lacking platelets (Figures 1B–1D). Not shown are experiments in which we perfused lungs with platelet-containing reconstituted blood (*n* = 3), since the HV-induced effects were identical to the above effects of whole blood perfusion. In separate experiments, we controlled for leukocyte-depletion effects by adding back leukocytes to the platelet-depleted perfusion. In the presence of leukocytes, inhibition of P-selectin and vWf expressions were 20 ± 5% and 13 ± 1% less, respectively (*P* < 0.05, *n* = 3), indicating that leukocytes enhance platelet-mediated endothelial adhesion molecule expression.

In permeabilized cells, intracellular protein was evident by fluorescence that extended well into the cell interior (Figure 1E). As shown by the single cell (Figure 1E) and group (Figures

TABLE 1. LEUKOCYTE AND PLATELET COUNTS IN PERFUSATE BLOOD

	WB	DB
Leukocytes (× 10 ⁹ /L)	3.5 ± 0.1	0.3 ± 0.01*
Platelets (× 10 ⁹ /L)	1110 ± 146	109 ± 6*

Definition of abbreviations: DB, leukocyte- and platelet-depleted blood perfusate; WB, whole blood perfusate.

Data are shown as mean ± SE.

* *P* < 0.05 compared with WB.

Figure 1. Confocal images show platelets enhance high tidal volume (HV)-induced lung endothelial P-selectin and von Willebrand factor (vWf) expression. LV, low tidal volume ventilation; WB, whole blood; PDB, platelet-depleted blood; perm, permeabilized; mag, magnification. We obtained freshly recovered lung endothelial cells (FLEC) from WB-perfused rat lungs exposed to LV or HV (2 h) or PDB-perfused lungs exposed to HV. We labeled FLEC with anti-P-selectin mAb and rabbit polyclonal anti-vWf antibodies or isotype-specific nonimmune IgGs and Alexa 488–goat anti-mouse and Alexa 633–goat anti-rabbit fluorescent antibodies. (A) Images show IgG (left) or vWf and P-selectin labeling on unfixed cells at low mag (middle and right), (B) single cells at high mag, (C) fluorescence intensity, and (D) co-localization. (E) Images show vWf and P-selectin labeling on fixed, permeabilized cells, (F) fluorescence intensity, and (G) co-localization. For each bar, $n = 100$ cells from three lungs. Mean $\pm$ SE. * $P < 0.05$, compared with bar on left by paired t test. C, D, F, and G: open bars, LV + WB; solid bars, HV + WB; shaded bars, HV + PDB.

1F and 1G) data, the intracellular protein responses were different than the cell surface responses in that HV markedly increased P-selectin fluorescence, but not vWf fluorescence. Platelet-depleted perfusion inhibited P-selectin and vWf expressions. Intracellular colocalization was minimal under all conditions. Adding leukocytes back to the platelet-depleted perfusion did not affect the intracellular expressions. Taken together, these findings indicated that the HV-mediated P-selectin and vWf expressions and co-localization at the cell surface were largely platelet dependent. Moreover, HV increased intracellular P-selectin more than vWf.

To further define P-selectin–vWf interactions in HV, we immunoprecipitated P-selectin from lysates of surface-biotinylated FLEC. Electrophoresis, transfer, and streptavidin blotting revealed bands at 140 and 176 kD (Figure 2A). In platelet-depleted blood perfusion these bands were approximately 50% weaker than in whole blood perfusion (Figure 2B), affirming the fluorescence data that the surface expression of these proteins was platelet dependent.

To determine total cellular content, we reprobed the immunoblot membranes with mAb to P-selectin and polyclonal anti-vWf. Reprobing also indicated that platelet-depleted blood perfusion reduced total P-selectin as well as the vWf that associated with it (Figure 2A, middle and right panels). vWf association with P-selectin was 33% less in platelet-depleted than in whole blood perfusion (Figure 2B). These P-selectin responses indicated that leukocytes and platelets increased both surface and total endothelial P-selectin, as well as the association of vWf with P-selectin.

P-Selectin–Induced vWf Expression

To determine the extent to which platelet P-selectin determines vWf expression on EC, we added the the P-selectin function-blocking mAb, RMP-1 (21) to the whole blood perfusion. RMP-1 markedly inhibited the HV-induced vWf expression (Figures 3A and 3B). Since this inhibition was similar in extent to that in platelet-depleted perfusion (see Figures 1B and 1C), the finding suggested platelet P-selectin was critical to EC vWf expression. However, a role for endothelial P-selectin was not ruled out.

To better distinguish between the roles of platelet and EC P-selectin, we used the P-sel^{−/−} mouse. During perfusion of lungs with WT blood, vWf expression on FLEC was not different between P-sel^{−/−} and WT lungs (Figure 3C). By contrast, in P-sel^{−/−} lungs perfused with P-sel^{−/−} blood, FLEC vWf expression was approximately 50% of the WT expression. FLEC labeling with nonimmune IgG resulted in dark images (not shown), ruling out nonspecific responses. These findings affirm that HV-induced vWf expression on the EC surface was critically determined by platelet P-selectin.

GP1b

Since platelet activation triggers platelet GP1b- α shedding (18), we determined whether shed GP1b was detectable on the FLEC surface. Under control conditions (non-HV lungs), GP1b and vWf were either nonexistent or weakly expressed on the cell surface (Figures 4A and 4B). However, HV, with whole blood perfusion, markedly increased the expressions as well

A IP: P-selectin

Figure 2. Immunoprecipitation shows HV-mediated endothelial P-selectin expression and vWf co-association are platelet dependent. IP, immunoprecipitation; SA-HRP, streptavidin-horseradish peroxidase; WB, whole blood; PDB, platelet-depleted blood; HV, high tidal volume ventilation. We surface biotinylated (4°C) FLEC obtained from rat lungs exposed to HV (2 h) during WB or PDB perfusion. We immunoprecipitated P-selectin using equal quantities of lysate protein and subjected immunoprecipitates to SDS-PAGE, transfer, and sequential blotting, as indicated. (A) Blots from a single experiment. Molecular weights are indicated on the left. (B) Corresponding densitometric data for four experiments. Mean $\pm$ SE. * P < 0.05, compared with bar on left by paired t test. Solid bars, WB; open bars, PDB.

their co-localization (Figure 4C). EC labeling with isotype-specific, nonimmune IgG resulted in dark images (Figure 4A, left), ruling out nonspecific responses. These findings indicate that in HV, platelets shed GP1b, which bound vWf on the EC surface.

Plasma Immunoprecipitation

Although in LV, immunoblots showed weak plasma expression of P-selectin and vWf, HV markedly enhanced both expressions (Figure 5A, left and right). However, in platelet-depleted blood perfusion, plasma P-selectin increase was blunted and plasma vWf markedly reduced (Figure 5B, left and right), indicating that platelets were responsible for the HV-induced elevations of plasma P-selectin and vWf.

DISCUSSION

To determine platelet-induced proinflammatory mechanisms in ventilation stress, we considered endothelial expressions of two critical markers of platelet-endothelial interactions, vWf and P-selectin (11, 16, 17). Modest HV exposure markedly enhanced expressions of both markers on the endothelial surface. Platelet involvement was indicated in that in the presence of platelet-free blood, endothelial surface expression for each marker decreased substantially. Also, GP1b, which is uniquely expressed on the platelet membrane (16), was evident on EC, further supporting a platelet role. In the P-sel^{-/-} mouse, EC vWf expression was inhibited, indicating that platelet P-selectin determined vWf secretion. Taking these findings together, we conclude that ventilation stress induced a novel proinflammatory sequence of events in which platelets transferred critical adhesion proteins to the endothelial surface.

We also assayed platelet-EC protein transfer in terms of the expression of EC GP1b. This protein is uniquely expressed on the platelet membrane and is shed after platelet activation (18). Our finding that GP1b- α was detectable on HV-treated FLEC is evidence that platelets transferred membrane proteins to the EC surface. We suggest that activated platelets come into sufficiently close association with EC as to cause the protein transfer. Since HV induced EC vWf expression, it is possible that platelet activation was initiated by binding interactions

between GP1b on rolling platelets and vWf already present on the EC surface. Such interactions could induce platelet activation, hence further shedding of platelet-derived adhesive proteins on the EC surface.

An important finding was that although the HV-induced increases of surface vWf and P-selectin on FLEC were similar, the intracellular responses were markedly different. Thus in the intracellular compartment, vWf remained unchanged while P-selectin increased. This disparity might be attributable to differences in protein internalization and recycling. Both vWf and P-selectin are internalized by EC, although internalization rates may differ between the proteins in different conditions (23, 24). Since 2 hours of lung endothelial stretch does not increase P-selectin mRNA (25), indicating that P-selectin synthesis is unlikely within this duration, the higher increase of intracellular P-selectin probably reflects its higher cellular uptake.

In HV, platelet depletion markedly blunted endothelial surface expressions of P-selectin and vWf, indicating that platelets were responsible for the bulk of the induced expressions. However, we point out that even under platelet-depleted conditions, these protein expressions were evident to a lesser but to a definite extent. These more modest levels of expression were evidently attributable to endothelial exocytosis that was platelet independent and that resulted directly from endothelial stretch caused by HV. Thus, these endothelial surface expressions were both endothelial and platelet dependent.

Our findings in the P-sel^{-/-} mouse provided insights on the mechanisms underlying vWf deposition on the cell surface. In the knockout mouse, vWf expression was abrogated by approximately 50% during perfusion with P-sel^{-/-} blood. However, perfusing P-sel^{-/-} mouse lungs with WT blood rescued endothelial vWf deposition. This result indicates that endothelial vWf expression is critically determined by platelet P-selectin. This was affirmed by the finding that the P-selectin-blocking mAb, RMP-1, inhibited HV-induced vWf expression. One possibility is that an increase in endothelial surface P-selectin stabilizes platelets (26), permitting the transfer of adhesive proteins between platelets and endothelium. Hence, interactions between platelet P-selectin and endothelial cells was the critical mechanism that led to platelet stabilization and vWf deposition on the endothelial surface.

Figure 3. Platelet P-selectin critically determines surface endothelial vWf expression in HV. anti-P-sel mAb, anti-P-selectin, function blocking mAb, RMP-1 (20 μg/ml); HV, high tidal volume ventilation; WT, wild type, P-sel^{-/-}, P-selectin knockout mouse. (A) In paired rat lungs in HV, the blood perfusate of one also contained anti-P-selectin mAb, RMP-1. FLEC were labeled with goat polyclonal anti-human vWf and Alexa 488–donkey anti-goat antibodies. Images under low (top) and high (bottom) magnification show vWf expression on unfixed FLEC. (B) Fluorescence intensity. For each bar, $n = 150$ cells from four lungs. Mean $\pm$ SE. * $P < 0.05$, compared with bar on left by paired t test. Perfusion: solid bars, blood; open bars, blood + anti-P-sel mAb. (C) In HV, we perfused WT mouse lungs with WT blood and P-sel^{-/-} lungs with WT or P-sel^{-/-} blood (1 h). We obtained FLEC and labeled vWf on unfixed FLEC. Bars show vWf labeling on P-sel^{-/-} FLEC expressed as a percentage of labeling in WT lungs perfused with WT blood (solid bars, WT blood; open bars, P-sel^{-/-} blood). For each bar, $n = 90$ cells from four lungs. Mean $\pm$ SE. * $P < 0.05$, compared with bar on left by paired t test.

These processes might be relevant to the manner in which platelets contribute to leukocyte adhesion on the EC surface. Platelets also transferred GP1b to the EC surface. Since GP1b binds the leukocyte adhesion receptor CD11b (30), it is possible that after platelet–EC transfer, GP1b promotes leukocyte recruitment. In the present study, HV caused co-localizations of P-selectin and GP1b, each with vWf on the EC surface. Such co-localization domains might form adhesive platforms that enable leukocytes and platelets to adhere in close proximity. This sequence of events may contribute to the “secondary capture” that promotes lung endothelial leukocyte adherence in ventilation-induced lung injury and other inflammatory conditions (6). Since platelet adherence to neutrophils causes neutrophil activation (30, 31), the formation of these sites could indicate HV heightens the potential for proinflammatory signaling at the EC surface.

A potential concern regarding HV-mediated expression of adhesion proteins on the endothelial cell surface relates to the procedural activation of leukocytes and platelets. However, such activation is unlikely, since blood isolation procedures were identical for the HV and LV groups, yet the HV markedly increased endothelial P-selectin and vWf. Hence these effects were not due to nonspecific procedural effects. Further in HV, unprocessed and reconstituted whole blood induced equivalent P-selectin expressions on FLEC, thereby further ruling out procedural activation as a factor.

Figure 4. HV induces surface endothelial GP1b expression and vWf colocalization. HV, high tidal volume ventilation; CTL, control (freshly excised lung). We obtained FLEC from CTL nonperfused or HV-exposed (2 h) rat lungs perfused with whole blood. We labeled FLEC with mouse monoclonal anti-GP1b and rabbit polyclonal anti-human vWf primary antibodies or nonimmune isotype-specific IgGs and Alexa 488–goat anti-mouse and Alexa 633–goat anti-rabbit fluorescent secondary antibodies, respectively. (A) Images show IgG (left) or GP1b and vWf labeling on single unfixed cells (middle and right), (B) fluorescence intensity, and (C) co-localization. For each bar, $n = 100$ cells from three lungs. Mean $\pm$ SE. * $P < 0.05$, compared with bar on left by paired t test. B and C: open bars, CTL; solid bars, HV.

Our findings on the plasma content of these proteins in plasma are potentially relevant to mechanisms underlying systemic organ involvement. It is understood that lung inflammation is capable of generating organ-wide systemic responses that might lead to multi-organ failure (27,28). In HV-treated lungs, plasma levels of both vWf and P-selectin increased in a platelet-dependent manner, implicating platelet activation in HV. Since plasma vWf deposits on surfaces *in vitro* (29), increases in plasma P-selectin and vWf may lead to the deposition of these proteins in other organs, causing platelet-dependent inflammatory signaling. Thus, our findings indicate that modest increase in tidal volume releases proinflammatory factors in plasma that might augment morbidity and mortality in ALI.

In conclusion, our findings indicate that a modest HV-mediated increase in tidal volume activates platelets and lung EC, while inducing proinflammatory conditions in plasma. In lung EC, endothelial activation promotes an adhesive endothelial phenotype in which EC express P-selectin, vWf and GP1b. Platelets contribute to the phenotype, thus indicating they amplify EC adhesiveness. Our results in P-selectin knockout mice indicate the critical role of P-selectin in the transfer of platelet vWf to EC. The HV-induced increases in plasma vWf and P-selectin suggest the induction of mechanisms by which soluble factors released in plasma potentially enhance platelet, leukocyte, and endothelial interactions in distant organs. These mechanisms could account for multi-organ failure in ALI.

Figure 5. In HV, platelets increase plasma P-selectin and vWf. IP, immunoprecipitation; LV, low tidal volume ventilation; HV, high tidal volume ventilation; WB, whole blood; PDB, platelet-depleted blood. We obtained plasma from the WB-perfusates of rat lungs exposed to LV or HV (2 h) and the PDB perfusates of rat lungs exposed to HV (2 h). Using perfusate plasma, we immunoprecipitated P-selectin or vWf with anti-P-selectin or anti-vWf antibodies or isotype-specific, non-immune IgGs. We elec-

trophoresed, transferred, and immunoblotted the immunoprecipitates as indicated. (A) Anti-P-selectin and anti-vWf blots. Molecular weights are indicated on the left. Arrows indicate immunoblotted proteins. (B) Densitometry for the indicated proteins. Each bar represents perfusates from three lungs (open bars, LV + WB; solid bars, HV + WB; shaded bars, HV + PDB). Mean $\pm$ SE. * P < 0.05, compared with bar on left by paired t test (n = 3 paired experiments).

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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