Effects of distension on airway inflammation and venular P-selectin expression

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Moldobaeva A, Jenkins J, Wagner E. Effects of distension on airway inflammation and venular P-selectin expression. Am J Physiol Lung Cell Mol Physiol 295: L941–L948, 2008. First published September 19, 2008; doi:10.1152/ajplung.90447.2008.—We previously have shown in mice and rats, enhanced leukocyte recruitment to airway postcapillary venules after excessive distention imposed by the application of positive end-expiratory pressure. Because P-selectin was shown to be essential for this outcome, we sought to establish an in vitro endothelial cell model and determine the mechanisms whereby mechanical distension alters adhesion molecule expression. P-selectin surface expression on mouse jugular vein endothelial cells exposed to cyclic stretch (5 or 20% elongation for 5 min; Flexercell) were compared with static cells. The larger, pathophysiological regimen of cyclic stretch showed a 54% increase in P-selectin expression after stretch compared with static cells. This response was attenuated but confirmed in tracheal venular endothelium (29% increase). We questioned whether these changes were dependent on increases in intracellular Ca2+ through voltage-gated Ca2+ channels. The stretch-induced increase in P-selectin expression was substantially decreased by pretreatment with the T-type Ca2+ channel inhibitor mibefradil (76% inhibition). Furthermore, when the Ca3.1 T-type Ca2+ channel expression was decreased in both endothelial cell subtypes with specific small-interfering RNA, the distension-induced expression of P-selectin decreased to levels less than that observed in static cells. We conclude that P-selectin expression on systemic venular endothelial cells contributes to a proinflammatory phenotype after mechanical stretch and can be selectively modulated by voltage-gated calcium channel inhibition.

positive end-expiratory pressure; vein endothelial cells; tracheal venular endothelial cells

IN BOTH IN VIVO AND IN VITRO SYSTEMS, P-SELECTIN HAS BEEN SHOWN TO BE AN ESSENTIAL ADHESION MOLECULE IN MEDIATING LEUKOCYTE RECRUITMENT DURING INFLAMMATION. ENHANCED P-SELECTIN EXPRESSION SUPPORTS LEUKOCYTE SLOWING AND ROLLING ALONG SYSTEMIC ENDOTHELIAL CELLS. ALTHOUGH P-SELECTIN IS ALSO LOCALIZED IN THE MEMBRANES OF PLATELET α-GRANULES, ITS IMPORTANCE FOR LEUKOCYTE RECRUITMENT STEMs FROM ITS PRIMARY LOCATION IN ENDOTHELIAL CELL WEIBEL-PALADE BODIES. IN ADDITION TO P-SELECTIN, THESE ENDOTHELIAL SECRETORY GRANULES STORE PROTEINS, SUCH AS VON WILLEBRAND FACTOR AND P-SELECTIN BY SEPARATE G PROTEIN-MEDIATED SIGNALING PATHWAYS. INTERESTINGLY, WITHIN 20 MIN, P-SELECTIN CAN BE INTERNALIZED, RECYCLED TO THE GOLGI APPARATUS, AND RETURNED TO WEIBEL-PALADE BODIES (9, 25).

IN ADDITION TO BLOODBORNE STIMULATORY AGONISTS KNOWN TO ACTIVATE WEIBEL-PALADE BODIES, ENDOTHELIAL CELLS ARE EXPOSED TO THE PHYSICAL FORCES OF SHEAR STRESS AND PULSATILE STRETCH. WITHIN THE LUNG, MECHANICAL STRETCH DUE TO OVERDISTENTION OF THE LUNG HAS BEEN SHOWN TO TRIGGER AN INFLAMMATORY RESPONSE (6, 29). OUR LABORATORY HAS SHOWN THAT EXCESSIVE DISTENTION OF THE TRACHEAL AIRWAYS RESULTS IN ENHANCED LEUKOCYTE RECRUITMENT TO THE POSTCAPILLARY VENULES (12, 31). IN THESE STUDIES, THE APPLICATION OF POSITIVE END-EXPIRATORY PRESSURE (PEEP) INDUCED A STIMULUS- AND TIME-DEPENDENT INCREASE IN LEUKOCYTE ADHESION TO POSTCAPILLARY VENULES. OTHERS HAVE SHOWN THAT ENDOTHELIAL CELLS EXPOSED TO EXCESSIVE CYCLIC STRAIN SHOW INCREASED LEVELS OF CYTOSOLIC Ca2+ (38). MECHANICAL STRETCHING CAN DIRECTLY ACTIVATE ION CHANNELS WITH Ca2+ ENTERING THE CYTOSOL FROM BOTH INTRACELLULAR AND EXTRACELLULAR POOLS (4, 18, 22, 24). INTERESTINGLY, ZHOU AND COLLEAGUES (40) SHOWED THAT THE T-TYPE Ca2+ CHANNEL PARTICULARLY WAS IMPORTANT IN CONTROLLING WEIBEL-PALADE BODY EXOCYTOSIS. SPECIFICALLY, THEY SHOWED THAT, IN ENDOTHELIAL CELLS EXPOSED TO THROMBIN, THE Ca3.1 T-TYPE Ca2+ Channel Played A CRITICAL ROLE IN CONTROLLING WEIBEL-PALADE BODY EXOCYTOSIS AND CONSEQUENT VON WILLEBRAND FACTOR RELEASE (40). ADDITIONALLY, WEI AND COLLEAGUES (34) FOUND THAT THE LOSS OF SHEAR STRESS DURING ISCHEMIA INDUCED MEMBRANE DEPOLARIZATION THAT LED TO T-TYPE CHANNEL ACTIVATION IN LUNG ENDOTHELIAL CELLS. THESE STUDIES PROVIDE SUGGESTIVE EVIDENCE THAT THE Ca3.1 T-TYPE Ca2+ CHANNEL IS IMPORTANT FOR Ca2+ ENTRY AND MAY BE IMPORTANT FOR SUBSEQUENT P-SELECTIN EXPRESSION. THEREFORE, IN THE PRESENT STUDY, WE HYPOTHEZIZED THAT 1) THE MECHANICAL STRAIN PLACED ON AIRWAY VENULAR ENDOTHELIUM BY OVERDISTENSION RESULTS IN A Ca2+-DEPENDENT INCREASE IN P-SELECTIN EXPRESSION AND ENHANCED AIRWAY INFLAMMATION, AND 2) THE T-TYPE Ca2+ CHANNELS PLAY AN IMPORTANT ROLE IN THE PROCESS. WE APPLIED INTRAVITAL MICROSCOPIC TECHNIQUES FOR IN VIVO DETERMINATION OF THE Ca2+-DEPENDENCE OF THE INFLAMMATORY RESPONSE TO EXCESSIVE DISTENTION AND DEVELOPED AN IN VITRO MODEL TO DETERMINE DISTENSION-INDUCED CHANGES IN P-SELECTIN EXPRESSION ON THE SYSTEMIC VENULAR ENDOTHELIAL CELL SURFACE. OUR RESULTS CONFIRMED THE IMPORTANCE OF T-TYPE Ca2+ CHANNELS FOR DISTENSION-INDUCED INFLAMMATION IN VIVO AND THAT DISTENSION-INDUCED P-SELECTIN EXPRESSION ON MOUSE VENULAR ENDOTHELIAL CELLS INVOLVES Ca2+ ENTRY PROMINENTLY THROUGH THE Ca3.1 T-TYPE Ca2+ CHANNEL.

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METHODS

Mice

Our in vivo protocol was approved by the Johns Hopkins Animal Care and Use Committee. Eight-week-old male C57Bl/6 mice (20–26 g; Charles River, Wilmington, MA) were anesthetized with a ketamine–acepromazine mixture (10:1 at 1.0 µg/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). Mice were used either to study leukocyte recruitment by intravital microscopy or were used for tissue harvest of venular endothelium. A total of 39 mice (27 for in vivo studies + 12 for endothelial cell isolation) were studied.

Intravital Microscopy

Intravital microscopy of tracheal vessels was performed using procedures previously described (31). Briefly, after a neck incision, the trachea was exposed and continuously superfused with warmed (37°C), Krebs buffer without/with pharmacological inhibitors. A single postcapillary venule was selected in each mouse just beyond the end of the endotracheal tube and visualized for up to 2 h, and the images were recorded on videotape. Leukocyte trafficking was quantified off-line 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 venular endothelium if it remained stationary for >30 s. Adherent cells were expressed as the number per 200-µm length of vessel. In the experimental groups, a 1-min period of 8 cmH2O of PEEP was applied five times with a 10-min interval of no PEEP between each PEEP application. In previous studies evaluating the effects of changes in end-expiratory pressure and tidal volume on leukocyte recruitment to postcapillary venules in mice and rats, this protocol was shown to elicit significantly increased leukocyte adhesion (12, 31). Measurements were made at baseline (before PEEP) and every 20 min for 40 min after the last application of PEEP, since we previously showed a maximum response (decreased leukocyte velocity and increased adherence) at this time point (31).

Endothelial Cell Culture

Systemic vein endothelial cells. For ease in acquisition of sufficient numbers of a pure population of mouse systemic vein endothelial cells (VEC), we dissected mouse jugular VEC from C57Bl/6 mice (n = 8) and placed them intima-side-down on Matrigel-coated 35-mm tissue culture dishes. After 4–6 days, endothelial cells that had migrated were replated to a T-25 flask coated with gelatin. Cells were cultured in supplemented DMEM (20% FCS, 15 µg/ml endothelial cell growth supplement, 100 µg/ml penicillin/streptomycin, 0.25 µg/ml amphotericin B, and 0.1 mM MEM with nonessential amino acids). An endothelial cell phenotype was confirmed by immunostaining for platelet endothelial cell adhesion molecule, von Willebrand factor, and uptake of Dil-ac-LDL. Only cells with positive staining were used for further experiments. All experiments were carried out using endothelial cells between passages 2–10.

Tracheal venular endothelial cells. Because heterogeneity may exist between venous endothelial cell subtypes, we confirmed key observations made in VEC also in tracheal venular endothelial cells (TvVEC). Tracheal segments devoid of large veins from C57Bl/6 mice (n = 4) were dissected, minced, and digested in 2 ml of collagenase (1 mg/ml; Sigma, St. Louis, MO) at 37°C for 15–20 min with occasional agitation. The cellular digest was filtered through sterile mesh and centrifuged (440 g for 10 min), and the cell pellet was resuspended (2 ml of complete medium) and placed on a 0.2% gelatin-coated 35-mm dish. After 5–7 days, cells positively labeled with fluorescent Dil-ac-LDL (Molecular Probes, Eugene, OR) as an endothelial cell marker and labeled EphB4 (Santa-Cruz, Santa Cruz, CA) as a marker of venous endothelial cells (32) were selected using the FACSARia cell-sorting system (BD Biosciences, San Jose, CA) and replated to a 0.2% gelatin-coated T-25 flask. All experiments were carried out using endothelial cells between passages 2–10.

Cyclic Stretch

VEC and TvVEC were distended with the Flexercell tension Plus System (FX-4000T; Flexcell International, Hillsborough, NC). Two different regimens of cyclic stretch were applied in VEC as follows: 5% elongation was used to represent physiological levels of ventilatory stretch, and 20% elongation was used to represent pathophysiologic overdistention (1, 28), at a frequency of 12 cycles/min. In preliminary studies in VEC evaluating optimal treatment times, we measured P-selectin expression immediately after a 5-, 10-, 20-, and 30-min period of cyclic stretch. Maximum expression was observed after a 5-min exposure to cyclic stretch. Additionally, in preliminary studies evaluating higher frequencies (24 and 60 cycles/min), the integrity of the endothelial cell monolayer was occasionally compromised at the combined higher frequency and 20% elongation. Consequently, the lower frequency (12 cycles/min) was selected.

P-Selectin Expression

VEC and TvVEC surface expression of P-selectin was measured by flow cytometry using the BD FACSCalibur. Immediately after endothelial cells were exposed to cyclic stretch, they were placed on ice and detached with 2 mM EDTA/PBS and resuspended in PBS with 0.2% BSA (all solutions were chilled). After being washed, cells were incubated with anti-P-selectin-fluorescein isothiocyanate antibody (BD Pharmingen, San Diego, CA), washed, and analyzed. Mouse isotype IgG served as a negative control. Mean channel fluorescence for each experiment was normalized to static control endothelial cells exposed to the same treatment. To confirm our results, using nonfluorescent antibodies in VEC, we also used an enzyme-linked immunosorbent assay (ELISA) as done previously (26). After cyclic stretch, cells were fixed with paraformaldehyde (1%), washed, and incubated with anti-P-selectin antibodies (R&D, Minneapolis, MN). Afterward they were washed and incubated with horseradish peroxidase-conjugated anti-mouse IgG. After being washed, cells were incubated in the dark with H2O2 (0.003%) and 3,3′,5,5′-tetramethylbenzidine (Sigma) as substrate. The reaction was stopped by adding H2SO4 (8N) and read on a plate reader (Bio-Rad, Hercules, CA) at 450 nm.

T-Type Calcium Channel RNA Expression

The relative expression of the three T-type Ca2+ channels Ca3.1, Ca3.2, and Ca3.3 in mouse VEC and TvVEC was determined by RNA isolation and quantitative real-time PCR. Total RNA (2 µg) was extracted from both types of endothelial cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) with standard procedures, as well as brain tissue that served as a positive control (39). The amounts of RNA were determined by measuring the optical density at 260 and 280 nm. The Quantitect Reverse Transcription Kit (Qiagen) was used for the first-strand cDNA synthesis according to the manufacturer’s protocol. The primers used for amplification are listed in Table 1. PCR reactions were performed with QuantiTect SYBR Green PCR Master Mix (Qiagen) and the IQ5 Multicolor real-time PCR Detection System (Bio-Rad Laboratories) using 500 ng of cDNA as the template in each 25-µl reaction mixture. The absolute copy number of mRNA of interest was determined by interpolation of the standard curve with the threshold cycle value of each sample. A melt-curve protocol was performed following the RT-PCR and revealed a single clean melting peak corresponding to a single PCR product for each of the T-type Ca2+ channel transcripts.
peak for all samples tested. Data were normalized to the quantity of β-actin mRNA in individual samples.

Depletion of Endogenous Ca3

Predesigned specific small-interfering RNA (siRNA) for Ca3.1, Ca3.2, and Ca3.3 were purchased from Ambion (Austin, TX) in purified, desalted, and annealed double-strand form. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) siRNA (Ambion) was used as a transfection control treatment. Mouse VEC and TvEC were grown to 70% confluence, and the transfection of siRNA (final concn, 100 nmol/l) was performed using siPORT Amine transfection reagent (Ambion) according to the manufacturer’s protocol. Later (72 h), cells were used for the experiments. The effectiveness of relevant Ca3 depletion was confirmed by Western blot analysis comparing cells transfected with Ca3 siRNA and GAPDH siRNA with untreated VEC and TvEC. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, blocked with 5% BSA in TBS-Tween, and exposed to specific primary antibody (Sigma) and secondary antibody. Immunoblots were compared within the same membrane, and actin served as a loading control.

Inhibitors

In vivo, the T-type channel inhibitor mibefradil (10–5 M) and L-type channel inhibitor verapamil (10–5 M) were delivered by superfusion to the exposed trachea for 10 min before and throughout the experimental application of PEEP. These concentrations were selected based on work by Nebe and colleagues (19) and Wu et al. (36). Superfusion with normal Krebs buffer solution was resumed after the last PEEP maneuver. In vitro, before exposure to cyclic stretch, VEC were pretreated with the following inhibitors: the voltage-gated T-type channel inhibitor mibefradil [10 μM, 20 min (36)], the voltage-gated L-type channel inhibitor verapamil [1 μM, 20 min (37)], BAPTA (25 μM, 60 min), and EGTA [1 mM, 60 min in PBS (33)].

Statistics

All data are presented as means ± SE. Changes in cell adhesion were evaluated by one-way ANOVA. Relevant within-group comparisons were made using the Newman-Keuls Multiple-Comparison Test. Because endothelial cell P-selectin expression was normalized to the expression in static cells with the same treatment (% static), a log transform of data points was performed before ANOVA. A P value ≤0.05 was accepted as significant.

RESULTS

Intravital Microscopy

Results of in vivo experiments assessing the importance of calcium to leukocyte recruitment to tracheal postcapillary venules after the application of PEEP are presented in Fig. 1 (control: n = 4, PEEP: n = 8, PEEP + T-type channel inhibitor mibefradil: n = 8, PEEP + L-type channel inhibitor verapamil: n = 7). Confirming our previously published work (31), mice exposed to repetitive stimulation with an elevated level of PEEP (8 cmH2O) demonstrated enhanced leukocyte adhesion relative to control mice undergoing normal ventilation with no PEEP (P < 0.05). Leukocyte velocity in the four groups did not differ at baseline (P = 0.35) and averaged 41 ± 1 μm/s. Only in the PEEP group was velocity significantly decreased compared with all other groups (27 ± 1 μm/s; P < 0.05). When mice were treated locally with a superfusion solution that contained the T-type calcium channel inhibitor mibefradil, there was a significant reduction in the number of firmly adherent leukocytes (P < 0.01 from PEEP). However, treatment with the L-type calcium channel antagonist verapamil did not significantly alter leukocyte adhesion after PEEP (P > 0.05 from PEEP). These results suggest the importance of T-type calcium channels for subsequent leukocyte recruitment after excessive airway distension from the application of PEEP.

Endothelial Cell Responses to Cyclic Elongation

To explore more specifically the effects of distension on venular endothelium, experiments were performed to develop an in vitro model system. Because the first step in leukocyte recruitment requires P-selectin expression and our previous in vivo results after airway distension demonstrated the essential nature of P-selectin (31), experiments were designed to measure P-selectin expression after VEC elongation. Figure 2 shows the effects of 5 and 20% elongation on P-selectin expression in VEC (n = 15; results of all experiments using endothelial cell isolations from 8 mice). The greater level of elongation caused a statistically significant increase in the level of P-selectin surface expression (54% increase; P = 0.01). Endothelial P-selectin surface expression after the application of 5% elongation did not differ statistically from static control cells.

To evaluate the effects of Ca2+ channel inhibition on P-selectin expression after VEC elongation, cells were pretreated

![Fig. 1](https://example.com/f1.png)

Fig. 1. Leukocyte adhesion was significantly attenuated after airway distension with positive end-expiratory pressure (PEEP) when the vasculature was superfused with the T-type calcium channel antagonist mibefradil. However, the L-type channel antagonist verapamil was without effect (n = 4–8 mice/group; *P < 0.05 vs. control, #P = 0.01 vs. PEEP).
with the T-type Ca\(^{2+}\) channel inhibitor mibefradil or the L-type Ca\(^{2+}\) channel inhibitor verapamil before cyclic stretch. The results of these experiments are presented in Fig. 3 (n = 6 experiments/group; P = 0.02). Mibefradil-treated cells showed a substantial, significant decrease in P-selectin expression compared with vehicle-treated cells (P < 0.05). P-selectin expression in verapamil-treated cells showed a smaller decrease in expression (P < 0.05). Cells exposed to inhibitors and 5% cyclic elongation did not differ from static controls (P > 0.05; data not shown).

In the next series of experiments, an essential role for extracellular Ca\(^{2+}\) for P-selectin expression after VEC elongation was confirmed (n = 4 experiments/treatment). Elongation (20%) was imposed on VEC pretreated with the membrane-permeable Ca\(^{2+}\) chelator BAPTA, with Ca\(^{2+}\)-free PBS, and with the extracellular Ca\(^{2+}\) chelator EGTA. All of these treatments that limited Ca\(^{2+}\) entry into VEC inhibited P-selectin expression after elongation such that there were no significant differences in P-selectin expression from static cells (BAPTA: 96%, PBS: 100%, EGTA: 94% of static control on average).

Because T-type channel blockade altered leukocyte adhesion in vivo and P-selectin expression in vitro, experiments were performed to determine the relevant channel subtypes present in mouse venous endothelial cells. The relative mRNA expression of the three T-type Ca\(^{2+}\) channels Ca3.1, Ca3.2, and Ca3.3 was measured in VEC and TvEC. Table 2 shows the average copy number of mRNA transcript normalized to the β-actin of each sample for Ca3.1, Ca3.2, and Ca3.3 in VEC and TvEC (n = 5 experiments/cell type). As can be seen, only Ca3.1 is evident in mouse VEC and TvEC and with similar relative abundance. Brain tissue, the positive control for the three channels, demonstrated an ~100-fold greater expression of each of the three Ca\(_v\) channels than the level of Ca3.1 observed in VEC and TvEC.

Gene-silencing techniques were next applied to the Ca3.1 channel to limit Ca\(^{2+}\)-dependent P-selectin expression. Ca3.1 protein expression was first confirmed in VEC and TvEC, as well as the effectiveness of gene silencing. Figure 4A shows representative immunoblots of Ca3.1 protein in control VEC and TvEC and in those treated with the transfection control GAPDH siRNA (n = 4). Ca3.1 protein expression is essentially eliminated after cells had been transfected with Ca3.1 siRNA in both cell types. To determine the importance of this channel for Ca\(^{2+}\)-dependent P-selectin expression in a series of experiments, P-selectin expression of endothelial cells exposed to 20% elongation were compared with those exposed to cyclic stretch and transfected with either GAPDH siRNA (transfection control) or transfected with Ca3.1 siRNA (n = 4 experiments/treatment group) in VEC. Figure 4B shows the results of these experiments (P = 0.02). VEC (20% elongation + medium) showed a 34% increase in P-selectin expression, and control transfected cells (20% + GAPDH) showed a 20% increase in P-selectin expression. P-selectin expression in VEC exposed to 20% elongation after transfection with Ca3.1 siRNA did not increase P-selectin expression and was significantly less than either of the control groups (P < 0.05 vs. medium or GAPDH siRNA transfected). To further confirm stretch-induced P-selectin expression is dependent on Ca3.1, P-selectin surface expression after cyclic stretch was measured by ELISA and compared with the expected thrombin-induced increase in P-selectin expression. Figure 4C shows the results of this confirmatory series of experiments (n = 3 experiments/treatment group). P-selectin expression in untreated cells exposed to 20% elongation was essentially the same as that measured using fluorescent antibodies and FACS analysis (37% increase vs 34% increase; P > 0.05). Furthermore, P-selectin expression after 20% elongation in vascular cells transfected with Ca3.1 siRNA was essentially eliminated (P < 0.001 vs. medium or GAPDH siRNA transfected). Additionally, when static cells were transfected with Ca3.1 siRNA and exposed to thrombin (0.1 U/ml), VEC showed a significant decrease in P-selectin expression (P = 0.001 vs. medium or GAPDH transfected). The thrombin concentration was selected to give an equivalent P-selectin expression as stretched cells. These results confirmed that P-selectin expression induced both by exposure to 20% cell elongation and thrombin treatment is dependent on Ca\(^{2+}\) entry through Ca3.1 T-type channels. As shown in Fig. 4D, 20% elongation of TvEC (n = 4 experiments/treatment group) showed an approx-

### Table 2. T-type channel expression; average copy number of mRNA transcript normalized to the β-actin of each sample for Ca3.1, Ca3.2, and Ca3.3 in VEC and TvEC

<table>
<thead>
<tr>
<th>Channel</th>
<th>VEC</th>
<th>TvEC</th>
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<tbody>
<tr>
<td>Ca3.1</td>
<td>0.32±0.28</td>
<td>0.00</td>
</tr>
<tr>
<td>Ca3.2</td>
<td>0.17±0.09</td>
<td>0.01</td>
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<tr>
<td>Ca3.3</td>
<td>0.00</td>
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Values are means ± SE; n = 5 experiments/cell type. VEC, vein endothelial cells; TvEC, tracheal venular endothelial cells.
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ENDOTHELIAL STRETCH ACTIVATES P-SELECTIN THROUGH T-TYPE CALCIUM CHANNELS

Fig. 4. A: effectiveness of relevant Ca₃.3 depletion was confirmed by Western blot analysis. Cells transfected with Ca₃.3 siRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) siRNA were compared with untreated jugular vein endothelial cells (VEC) and tracheal venular endothelial cells (TvEC). Immunoblots were compared within the same membrane, and actin served as a loading control. B: P-selectin expression after 20% elongation and after transfection with Ca₃.3 siRNA. VEC transfected with Ca₃.3 siRNA did not increase P-selectin expression, yet control transfected cells (20% + GAPDH) showed the expected increase in P-selectin expression after cyclic stretch (mean ± SE, n = 4 experiments; *P < 0.05 vs. medium and GAPDH). C: P-selectin expression was confirmed by enzyme-linked immunosorbent assay (ELISA) after 20% elongation or thrombin and after transfection with Ca₃.3 siRNA. VEC transfected with Ca₃.3 siRNA did not increase P-selectin expression, yet control transfected cells (20% + GAPDH) and medium controls showed the expected increase in P-selectin expression after cyclic stretch. Likewise, thrombin-induced P-selectin expression was similar to control cells transfected with GAPDH siRNA. However, cells transfected with Ca₃.3 siRNA showed a significant reduction in P-selectin expression after thrombin. Both groups treated with Ca₃.3 siRNA were statistically different from medium and GAPDH groups (mean ± SE, n = 3 experiments/group; ***P = 0.001 vs. medium and GAPDH). D: P-selectin expression after 20% elongation and after transfection with Ca₃.1 siRNA. TvEC transfected with Ca₃.1 siRNA did not increase P-selectin expression, yet control transfected cells (20% + GAPDH) showed the expected increase in P-selectin expression after cyclic stretch (mean ± SE, n = 4 experiments; **P = 0.01 vs. medium and GAPDH).

The present study was undertaken to confirm increased P-selectin expression and leukocyte recruitment after airway venular distension and to determine whether this response is Ca²⁺ dependent. Although our results showed that Ca²⁺ T-type channel inhibition led to a decreased response in vivo, it was necessary to link the response specifically to the venous endothelium. Therefore, an in vitro model of endothelial cell distension was established. Although not a direct parallel with our in vivo model, in vitro results confirmed that distension-induced P-selectin expression on mouse venous endothelial cells involves Ca²⁺ entry through the Ca₃.1 T-type Ca²⁺ channel.

The effects of excessive ventilatory strain on the pulmonary endothelium have received considerable attention over the past several years (23, 27, 29). Increased numbers of inflammatory cells are recruited to the lung after a 1-h exposure to high tidal volume ventilation (2). This response has been shown to be both microvessel and leukocyte specific with heterogeneous upregulation of endothelial P-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 (17). However, few studies have focused specifically on the systemic circulation that is embedded within the airway wall, and likewise exposed to the mechanical stresses of ventilation. Using intravital microscopy, we previously showed a stimulus-dependent increase in leukocyte adhesion in tracheal postcapillary venules in both mice and rats after the application of PEEP (12, 31). In the present study, our choice of 8 cmH₂O PEEP was based largely on our previous results. In addition, however, we selected it because of the clinical observation that within the ARDS network trial, the median set PEEP, using current standards of care, low tidal volume ventilation, was 8 cmH₂O (10). Thus it is a clinically relevant level of airway distension.

Predominantly polymorphonuclear leukocytes were associated with the tracheal airways after distension (12, 31). A pan-selectin inhibitor blocked the increased adhesion observed with the application of PEEP (12), as did distension in P-
selectin-deficient mice (31). P-selectin is stored in endothelial cell Weibel-Palade bodies, which provide storage for a number of proteins involved in inflammation, hemostasis, angiogenesis, and vasoregulation. There is general agreement that there is heterogeneity in the proteins contained within Weibel-Palade bodies, the vascular endothelium overall exhibits heterogeneity in their density and distribution, and the protein contents are exocytosed when these endothelial-specific storage granules are activated by both Ca$^{2+}$ and/or cAMP-dependent mechanisms (13, 15, 21). Proinflammatory agonists such as thrombin, histamine, and leukotrienes all depend on increased intracellular Ca$^{2+}$ to result in Weibel-Palade body exocytosis. Based on the proinflammatory effects of airway distension in this model, we predicted that the regulation of P-selectin would be Ca$^{2+}$ dependent.

Control of systemic endothelial cell intracellular Ca$^{2+}$ has been studied extensively. However, characterization of the Ca$^{2+}$ channels specifically expressed in systemic postcapillary venular endothelium remains incomplete. In general, both L-type (37) and T-type (7) voltage-gated channels and receptor-operated (11) and store-operated (8) Ca$^{2+}$ channels have been identified in systemic endothelium. However, the abundance and importance of each channel within different endothelial cell subtypes remains controversial (30, 35). Because of the work of Zhou and colleagues (40) showing inhibition of Weibel-Palade body exocytosis in pulmonary microvascular endothelium with blockade of T-type Ca$^{2+}$ channels, we first evaluated the effects of superfusion of inhibitors of voltage-gated channels on leukocyte adhesion after airway distension. Results confirmed the effectiveness of T-type channel inhibition on leukocyte adhesion (Fig. 1). L-type channel inhibition did not alter in vivo adhesion. Because Nebe and colleagues (19) had shown that mibebradil but not verapamil attenuated granulocyte and lymphocyte adhesion molecule expression in vitro, our in vivo result could have been related to the effects of superfused inhibitor on circulating cells. Consequently, we moved to an in vitro system to confirm that distension alters endothelial cell P-selectin expression. This approach presented the following two experimental challenges: 1) because of the difficulty of dissecting and culturing tracheal postcapillary venular endothelium, a relevant mouse endothelial cell subtype was needed, and 2) because of the complex geometry of postcapillary venules embedded within the airway wall, the longitudinal stresses imposed were impossible to directly parallel in vitro. We addressed the first issue by using mouse jugular VEC to ensure a uniform, systemic endothelial subtype for most experiments. These more accessible cells were used to develop the in vitro model and test several hypotheses with regard to the mechanisms of elongation-induced P-selectin expression. We confirmed our final observations in TvEC, which were more difficult to isolate because of the need for labeling and cell sorting before culture. However, we confirmed that our observations in jugular VEC with regard to P-selectin expression after cell elongation were in fact representative of tracheal, venular endothelial cells.

The second limitation, that of establishing a perfect parallel between in vivo and in vitro stresses, could not be resolved. We studied the complex stresses imposed by the application of PEEP in vivo in mice and explored the potential mechanisms of response as far as the in vivo model could take us. We next evaluated the effects of cyclic elongation on endothelial cell P-selectin expression, acknowledging that the distension parameters and results may not represent the exact in vivo situation of PEEP-induced distension. Several preliminary experiments were performed to establish the in vitro distension parameters. The magnitude of distension was modeled after the work of Tschumperlin (28) and Birukov (1) and colleagues who demonstrated that 18–20% elongation was equivalent to a pathological degree of endothelial cell distension, whereas 5% elongation was equivalent to a physiological degree of distension with ventilation. Although attempts were made to apply cyclic elongation at a frequency equivalent to the ventilatory frequency of the mouse (120 breaths/min), endothelial cells did not remain firmly attached to the silicone membrane being stretched during these rapid oscillations. In addition, the time course of P-selectin expression was evaluated over the course of 60 min. Because of the known rapid expression followed by endocytosis and restoring P-selectin to the Golgi within 20 min, we fully expected the time course of P-selectin expression to be a fairly rapid response. This expectation was met with in vitro P-selectin expression in jugular VEC exposed to 20% elongation increasing markedly after 5 min of cyclic elongation (Fig. 2). Furthermore, these results obtained in jugular VEC were confirmed in TvEC, although the response was less robust (Fig. 4D).

Results of P-selectin expression using the voltage-gated Ca$^{2+}$ channel inhibitor mibebradil paralleled in vivo results of leukocyte adhesion after airway distension with this inhibitor. A substantial reduction in P-selectin expression was observed (Fig. 3). However, the L-type channel inhibitor verapamil, although ineffective in vivo at blocking leukocyte adhesion, showed a small inhibitory effect of P-selectin expression in vitro. Because verapamil has been shown to have some T-type channel inhibitory effects at this concentration (1 μM), we suggest that this partial inhibition might be related to the lack of specificity of verapamil (5). Experiments performed when intracellular and extracellular Ca$^{2+}$ were reduced demonstrate the essential nature of extracellular Ca$^{2+}$ for maximum P-selectin expression after cyclic elongation.

Our experiments using the pharmacological inhibitor mibebradil, a benzimidazolyl-substituted tetraline derivative, demonstrated the importance of the T-type Ca$^{2+}$ channel, since mibebradil is considered to be one of the most potent T-type Ca$^{2+}$ channel blockers (14, 16). However, three subtypes of the T-type Ca$^{2+}$ channels exist, including Ca$^{3}, 3.1$, Ca$^{3}, 3.2$, and Ca$^{3}, 3.3$ (39). Therefore, we determined the expression of each in mouse jugular VEC and in tracheal venular endothelium by quantitative real-time PCR. Compared with brain tissue, which is known to express all three T-type Ca$^{2+}$ channels (39), both types of endothelial cells expressed Ca$^{3}, 3.1$ almost exclusively (Table 2). Interestingly, Zhou and colleagues (40) demonstrated that, in lung microvascular endothelial cells exposed to thrombin, the Ca$^{3}, 3.1$ T-type Ca$^{2+}$ channel played a critical role in controlling Weibel-Palade body exocytosis and consequent von Willebrand factor release.

To confirm the importance of Ca$^{3}, 3.1$ T-type Ca$^{2+}$ channel specifically in distension-induced P-selectin expression, we reduced expression of this channel using specific siRNA in jugular VEC and TvEC. Our results demonstrated that the reduction in expression of Ca$^{3}, 3.1$ resulted in a significant decrease in distension-induced P-selectin in both cell types. In jugular VEC, these results were confirmed by ELISA and
compared with the known Ca,3.1 T-type Ca\textsuperscript{2+}-dependent effects of thrombin on Weibel-Palade body activation (Fig. 4C and Ref. 40). Thus our results are consistent with placing excessive endothelial cell elongation in the same category of Ca\textsuperscript{2+}-dependent proinflammatory modulators of Weibel-Palade body activation. However, it remains unclear why excessive elongation of endothelial cells leads to increased flux of Ca\textsuperscript{2+} through voltage-gated channels. In lung microvascular endothelial cells, thrombin has been shown to depolarize the membrane potential into a window-current range sufficient to activate Ca\textsubscript{v} 3.1 (36). Wu and colleagues (36) showed that the T-type window range is close to the resting membrane potential of many endothelial cells. Thus only small depolarizing currents are needed to open T-type Ca\textsubscript{v} 3.1 channels. It is possible that repeated stimulation with excessive cyclic stretch causes slow and/or moderate changes in membrane potential that ultimately promote channel opening. The specific changes in electrostatic forces during cyclic endothelial cell elongation require further study.

In summary, we have established an in vitro model of systemic venous endothelial cell activation by mechanical distension. Furthermore, we have confirmed the calcium dependence of P-selectin expression after endothelial cell distension and the importance of the voltage-gated Ca\textsubscript{v} 3.1 T-type Ca\textsuperscript{2+} channel. Our in vitro results are consistent with our in vivo observations demonstrating that excessive mechanical distension of the airway is proinflammatory.

GRANTS
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