Lung heparan sulfates modulate $K_{fc}$ during increased vascular pressure: evidence for glycoalyx-mediated mechanotransduction

Randal O. Dull,1,2,3,4 Mark Cluff,1 Joseph Kingston,1 Denzil Hill,1 Haivan Chen,1 Soeren Hoehne,1 Daniel T. Malleske,4 and Rajwinderjit Kaur1

1Department of Anesthesiology, Lung Vascular Biology Laboratory, 2Department of Bioengineering, 3Department of Pharmaceutical Chemistry, and 4Department of Pediatrics, Division of Neonatology, University of Utah School of Medicine, Salt Lake City, Utah

Submitted 14 March 2011; accepted in final form 5 December 2011

Dull RO, Cluff M, Kingston J, Hill D, Chen H, Hoehne S, Malleske DT, Kaur R. Lung heparan sulfates modulate $K_{fc}$ during increased vascular pressure: evidence for glycoalyx-mediated mechanotransduction. Am J Physiol Lung Cell Mol Physiol 302: L816–L828, 2012. First published December 9, 2011; doi:10.1152/ajplung.00080.2011.—Lung endothelial cells respond to changes in vascular pressure through mechanotransduction pathways that alter barrier function via non-Starling mechanism(s). Components of the endothelial glycoalyx have been shown to participate in mechanotransduction in vitro and in systemic vessels, but the glycoalyx’s role in mechanosensing and pulmonary barrier function has not been characterized. Mechanotransduction pathways may represent novel targets for therapeutic intervention during states of elevated pulmonary pressure such as acute heart failure, fluid overload, and mechanical ventilation. Our objective was to assess the effects of increasing vascular pressure on whole lung filtration coefficient ($K_{fc}$) and characterize the role of endothelial heparan sulfates in mediating mechanotransduction and associated increases in $K_{fc}$. Isolated perfused rat lung preparation was used to measure $K_{fc}$ in response to changes in vascular pressure in combination with superimposed changes in airway pressure. The roles of heparan sulfates, nitric oxide, and reactive oxygen species were investigated. Increases in capillary pressure altered $K_{fc}$ in a nonlinear relationship, suggesting non-Starling mechanism(s), nitrite-arginine methyl ester and heparanase III attenuated the effects of increased capillary pressure on $K_{fc}$, demonstrating active mechanotransduction leading to barrier dysfunction. The nitric oxide (NO) donor S-nitrosothioglutathione exacerbated pressure-mediated increase in $K_{fc}$. Ventilation strategies altered lung NO concentration and the $K_{fc}$ response to increases in vascular pressure. This is the first study to demonstrate a role for the glycoalyx in whole lung mechanotransduction and has important implications in understanding the regulation of vascular permeability in the context of vascular pressure, fluid status, and ventilation strategies. Endothelium; pulmonary edema; permeability

THE ENDOTHelial GLYCOALYX has been hypothesized to play a role in vascular barrier regulation through both passive and active mechanisms. Passive properties include the formation of a molecular filter overlying the cell-junction limits that water flux (2, 14) and protein flux (3, 38, 41) into the cell junction. Active barrier regulation occurs through mechanotransduction that alters junctional integrity via nitric oxide (NO) and reactive oxygen species (ROS) (15, 16, 18). Observations regarding glycoalyx-mediated signal transduction, however, have come from cultured endothelial cells and not from in vivo or ex vivo whole organ studies that would validate a physiological role for the glycoalyx in a more complex as well as clinically relevant model.

Recently, we reported that lung capillary endothelial cells, in vitro, respond to increases in hydrostatic pressure by production of intracellular NO and ROS that mediate barrier dysfunction manifested by an increase in hydraulic conductivity (15, 16, 18). Pressure-induced production of NO is a response characteristic of whole lung microvessels (24), clearly demonstrating that cultured endothelial cells and in situ pulmonary vessels share common responses to hemodynamic forces. We demonstrated that heparan sulfates (HS) were crucial for both the activation of endothelial nitric oxide synthase (eNOS) and pressure-induced increase in permeability. The present study extends our observations from cell culture models (15, 16, 18, 24) to a physiologically and clinically relevant model; our results provide the first evidence for a role of the glycoalyx, specifically HS, in mechanotransduction and barrier regulation in the intact lung.

METHODS

Ex Vivo Lung Preparation

All animal experiments were approved by the University of Utah’s Institutional Animal Care and Use Committee and in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council). Adult male Sprague-Dawley rats (300–400 g) were anesthetized with ketamine-xylazine (90:10 mg/kg), a tracheotomy was performed and they were mechanically ventilated with a pressure-controlled ventilator (Kent Scientific, Torrington, CT) at a respiratory rate of 60/min, FIO2 = 0.21 and positive end-expiratory pressure (PEEP) of 3.0 cmH2O. The chest and pericardial sac were sequentially opened and ligatures were placed around the aorta and pulmonary artery. Heparin (200 U) was injected into the pulmonary artery and allowed to circulate for 2 min. The rat was exsanguinated via transection of the abdominal aorta; the left atrium was cannulated via a left ventriculostomy. The pulmonary artery was cannulated via the right ventricle and lungs were perfused with Krebs-Ringer-bicarbonate solution containing 3% bovine serum albumin.

Pulmonary arterial (Ppa) and left atrial pressures (P(LA)) were measured continuously via in-line pressure transducers (P-75, Harvard Apparatus, Natick, MA) connected to an analog-to-digital board. Solenoids (20 PSI, 12 V DC, Cole Parmer; Mount Vernon, IL) were placed in-line on both the arterial and venous tubing and could be closed simultaneously for measuring double-occlusion pressures (Pdo). An in-line ultrasonic flow probe (Transonic, Ithaca, NY) was placed in the pulmonary artery cannula and flow data was recorded in real time. Lungs were suspended from a force transducer (Radnoti, Monrovia, CA) and lung weight was zeroed; lung weight was zeroed; lung weight, vascular pressures and flow were recorded using a custom-written program (LabVIEW, National Instruments, Austin, TX).
Reagents

Heparanase III (E.C. 4.2.2.8) was from Ibex Technologies (Montreal, QC, Canada); Krebs-Ringer buffer, nitro-l-arginine methyl ester (l-NAME), nitro-d-arginine methyl ester (d-NAME), S-nitrosoglutathione (GSNO), and diethyl pyrocarbonate (DEPC) were purchased from Sigma Chemical (St. Louis, MO); Mn(III)-tetra(4-benzoic acid) porphyrin chloride (TBAP) was purchased from Biomol (Plymouth Meeting, PA). Bovine serum albumin fraction V was purchased from Proliant (Ankeny, IA). Anti-nitrotyrosine was from Abcam (Cambridge, MA) and HSS-1 and 3G10 from US Biological (Swampscott, MA).

Calculation of $K_f$

The change in lung weight expressed as milliliters of filtered fluid during the time interval from 18 to 20 min was divided by time (2 min) and then by the capillary pressure (Ppc), where Ppc = (Ppa + Pla)/2, yielding milliliters per minute per centimeter H2O; this value was normalized to 100 g of predicted lung weight (PLW), which was calculated from the equation PLW = 0.0053 (rat weight) - 0.48 (32).

Pressure-Volume Curves

Tidal volumes (VT) in isolated perfused rat lungs during pressure-controlled ventilation were measured with a differential pressure transducer (DP-45, Validyne, Northridge, CA), driven by a Carrier Frequency Bridge Amplifier (type 677, Hugo Sachs Elektronik-Harvard Apparatus). Airflow (ml/s) was recorded with a custom-written program (LabVIEW 8.5, National Instruments, Austin, TX) at 200 Hz sampling rate, and each respiratory cycle was integrated to obtain VT. A calibration curve was created by applying gas flow (air) (air pressure = 1 bar) from negative 16.16 ml/s to positive flow at 16.16 ml/s, through the DP-45 and recording the corresponding voltage output. This flow was regulated with a calibrated flowmeter (Dwyer, model UA1049) (accuracy ±2%) connected to a pressure regulator. The calibration curve was entered in a custom scale before recording of VT. The lungs were ventilated with different peak inspiratory pressures (PIP) and positive end-expiratory pressures (PEEP) ($ΔP = PIP - PEEP$) to record the resulting VT values. Flow/VT was recorded for 1 min for each $ΔP$. We used inflation pressures that produced VTs identical to previous literature values (20, 33) during standard tidal volume (ST VT). Low tidal volume ventilation (Low VT) inflation pressures were chosen to recreate clinically relevant lung-protective strategies.

Tissue Nitrate/Nitrite Concentrations

Whole-lung protein preparations were assayed in triplicate for concentrations of nitrate/nitrite (NO$_x$) using a chemiluminescence analyzer for NO detection (NOA 280i; Sievers Instruments, Boulder, CO) as previously described (27). Briefly, 50 μl of sample was injected into the reaction chamber containing a saturated solution of 0.05 M vanadium (III) chloride in 1 M HCl heated to 95°C to reduce NO$_x$ to NO. The liberated NO was carried in gas phase into the analyzer by a constant flow of helium gas. The analyzer was calibrated by using a standard curve derived from serial dilutions of a 0.1 M stock solution of sodium nitrite (NaNO$_2$). Individual results for tissue NO$_x$ are expressed as micromoles of NO per micrograms total protein per Δ wet weight; the normalization to Δ wet weight is to correct for the dilution of NO$_x$ by increased extravascular lung water.

Immunohistochemistry

Lungs were perfused with neutral-buffered formalin via the pulmonary artery while Pla was held at 5 cmH$_2$O; formalin was also instilled into the trachea at a pressure of 25 cmH$_2$O. Lungs were processed by standard histological methods. Anti-NOS-tyrosine, HSS-1, and 3G10 antibodies were conjugated to a biotin-labeled secondary antibody (IgM) and then incubated with streptavidin-horseradish peroxidase.

Experimental Protocols

Protocol 1: Low VT + double pressure step. Lungs were ventilated at Low VT and perfused for 20 min at isogravimetric pressure. Baseline $K_{fc}$ ($K_{fc1}$) was determined by increasing left atrial pressure (Pla$_1$) to 7.5 cmH$_2$O for 20 min followed by a return to isogravimetric conditions for 20 min. A second pressure step (Pla$_2$) to 7.5, 10, 12, and 15 cmH$_2$O was performed for 20 min and $K_{fc2}$ was measured. The ratio $K_{fc2}/K_{fc1}$ was used to assess the influence of Pla on the filtration coefficient. Double-occlusion pressures (Pdo$_1$, Pdo$_2$, Pdo$_3$) were measured before and after each pressure step to ensure stability of the individual preparation. The general scheme for the double-step protocol is shown in Fig. 1A, top and middle.

The double pressure step protocol was used for the following groups: control, l-NAME, TBAP, heparanase III, and GSNO. Test reagents were instilled into the pulmonary artery beginning 10 min into the second isogravimetric period and were present in the recirculating media for the remainder of the experiment. Test reagents were used at the following concentrations: l-NAME (200 μM), TBAP (200 μM), heparanase III (75 and 150 μIU/ml), and GSNO (500 μM).

Protocol 2: ST VT + double pressure step protocol. Lungs were ventilated with ST VT, and $K_{fc}$ was measured at baseline Pla = 7.5 cmH$_2$O and after a second step of Pla to 15 or 17 cmH$_2$O, as described above. Reagents were added to the perfusate beginning 10 min into isogravimetric period 2 and were present in the circulating media for the remainder of the experiment.

Protocol 3: low VT + single-step protocols. To examine the influence of the baseline $K_{fc}$ measurement (initial pressure pulse) on subsequent $K_{fc}$ measurements, we evaluated $K_{fc}$ following a step from isogravimetric conditions to Pla = 7.5 or 15 cmH$_2$O. Pdo values were measured before and after each pressure step. The $K_{fc}$ obtained from $Δ$Pla = 0 to 15 cmH$_2$O was compared with $K_{fc2}$ obtained from the double pressure step protocol when Pla = 15 cmH$_2$O. l-NAME (final concentration, 200 μM) was added to the media 10 min into the isogravimetric period and was present for the step increase in Pla to either 7.5 or 15 cmH$_2$O. The general scheme for the single-step protocol is shown in Fig. 1A, bottom.

Statistics

All data are presented as box plots showing sample minimum and maximum, 25th and 75th percentile, and median. Differences between groups were assessed by ANOVA followed by either Tukey’s honestly significant difference or Scheffé’s post hoc test.

All statistical analyses were performed with Kaleidagraph for Windows (version 4.0.3), Synergy Software (Reading, PA); probability levels ($P < 0.05$) were taken to indicate statistical significance.
Fig. 1. Experimental design. A: protocols 1 and 2 = low and standard (ST) tidal volume (VT) ventilation, respectively, with 2 increases in left atrial pressure (Pla). Protocol 3 = Low VT ventilation with a single increase in Pla. IG1 and IG2 = isogravimetric periods 1 and 2, respectively. Pdo, double-occlusion pressure; Kfc, filtration coefficient. Baseline Kfc1 was determined during step 1 at Pla, 7.5 cmH2O in protocols 1 and 2. During step 2, Pla2 was increased to 7.5, 10, 12, 15, or 17 cmH2O and Kfc2 was measured. The ratio of Kfc2/Kfc1 was used to assess the effect of increasing Pla on whole lung permeability. In protocol 3, lungs were exposed to a single increase in Pla from IG1 to Pla = 7.5 or 15 cmH2O. B: pressure-volume curves. Lungs were ventilated with either Low peak inspiratory pressure (PIP) [7–8 cmH2O, positive end-expiratory pressure (PEEP) = 3 cmH2O] or ST PIP (10–12 cmH2O, PEEP = 3 cmH2O); airflow vs. time was integrated to derive VT, which was normalized to rat body weight (kg). Pair, air pressure.
RESULTS

Pulmonary Hemodynamics

We assessed pulmonary hemodynamics in all lungs (Table 1). As can be seen in Table 1, at any given Pla, measured Ppa, Ppc, and Pdo did not vary between groups. As expected, Ppa increased linearly as a function of elevated Pla. Ppa was not altered by l-NNAME, d-NNAME, TBAP, or heparan III at any given Pla. Pulmonary capillary pressure, Ppc, [Ppc = (Ppa + Pla)/2], was not different across treatment groups when Pla = 15 or 17 cmH2O. Pulmonary artery Pdo1, Pdo2, and Pdo3 were not significantly different across groups at Pla = 15 or 17 cmH2O.

Lung Pressure-Volume Measurements

Pressure-controlled ventilation was used in the present experimental protocols; to quantify Vt delivered at net inflation pressure, we built a custom-made system to integrated airflow per unit time to derive Vt. The resulting pressure-Vt curve presents ΔP (PIP – PEEP) vs. Vt, normalized to body weight (kg) (Fig. 1B). In our Low Vt group, average Vts were in the range of 2.0–2.5 ml/breath; in the ST Vt group, Vt were 3.0–3.5 ml/breath. The Low Vt group was ventilated with 7–8 cmH2O providing a Vt per body weight of ~4–6 ml/kg. The ST Vt groups were ventilated with 10–12 cmH2O yielding Vt per body weight of ~6–8 ml/kg.

Low Vt Studies

Baseline Kfc. All lungs in the double-step groups had a baseline measurement (Kfc1) at Pla1 = 7.5 cmH2O; thus Kfc1 for all groups was pooled and yielded an average Kfc1 = 0.081 ± 0.04 ml·min⁻¹·cmH2O⁻¹ per 100 g (n = 112). Subgroup analysis of baseline Kfc1 was compared between all control groups and no differences were noted. This rules out time- and technique-dependent factors that may have confounded subsequent Kfc comparisons. Pulmonary artery pressure (Ppa1), double-occlusion pressure (Pdo1), and capillary pressure (Ppc1) are denoted by a subscript “1” to indicate the baseline pressure step from which the measurement was derived.

Protocol 1: double pressure step. The second step increase in Pla is designated Pla2, and the measured Kfc is denoted as Kfc2. Pulmonary artery pressure (Ppa2), double-occlusion pressure (Pdo2), and capillary pressure (Ppc2) are denoted by a subscript “2” to indicate the second pressure step from which the measurement was derived. The ratio of Kfc2/Kfc1 derived when Pla = 7.5 cmH2O for both pressure steps (C7–7) is considered “control” for the remainder of the studies. The control Kfc2/Kfc1 = 1.43 ± 0.78 (n = 8).

To characterize the influence of increased capillary pressure on Kfc, lungs were exposed to a second step increase in Pla2 to 10, 12, 15, or 17 cmH2O for 20 min. There was no significant difference in Kfc1, Kfc2, or Kfc2/Kfc1 when Pla = 7.5, 10, or 12 cmH2O. When Pla2 = 15 or 17 cmH2O, Kfc2 increased significantly compared with the baseline (Fig. 2A). At Pla2 = 15 cmH2O, Kfc2 = 0.65 ± 0.35 ml·min⁻¹·cmH2O⁻¹ per 100 g and the Kfc2/Kfc1 ratio increased to 7.9 ± 3.27 (P < 0.01). At Pla = 17 cmH2O, Kfc2/Kfc1 = 0.96 ± 1.50 ml·min⁻¹·cmH2O⁻¹ per 100 g. The Kfc2/Kfc1 vs. Pla relationship is presented in Fig. 2A and shows nonlinear dynamics of the pressure vs. permeability relationship that cannot be explained by a simple Starling mechanism.

l-NNAME. Inhibition of endothelial NO synthase with l-NNAME (200 μM) had no effect on pulmonary hemodynamics (Table 1) or on baseline Kfc2/Kfc1 (i.e., when Pla2 = 7.5 cmH2O) where l-NNAME-treated lungs had a Kfc ratio of 1.68 ± 1.35 ml·min⁻¹·cmH2O⁻¹ per 100 g. l-NNAME attenuated the in-

<p>| Table 1. Rat lung pulmonary hemodynamics |
|-----------------|----------------|------|------|--------|------|------|-------|--------|</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Pla1</th>
<th>Ppa1</th>
<th>Pce1</th>
<th>Pdo1</th>
<th>Pla2</th>
<th>Ppa2</th>
<th>Pce2</th>
<th>Pdo2</th>
<th>Pdo3</th>
</tr>
</thead>
<tbody>
<tr>
<td>C7–7</td>
<td>8</td>
<td>8.50±1.17</td>
<td>11.30±1.15</td>
<td>9.90±0.82</td>
<td>4.70±1.44</td>
<td>8.70±1.05</td>
<td>11.60±1.07</td>
<td>10.13±0.77</td>
<td>4.41±1.45</td>
<td>3.31±0.64</td>
</tr>
<tr>
<td>C7–10</td>
<td>8</td>
<td>8.88±1.08</td>
<td>10.85±1.66</td>
<td>9.86±1.30</td>
<td>4.05±1.91</td>
<td>11.72±1.21</td>
<td>13.58±1.90</td>
<td>12.65±1.48</td>
<td>3.72±2.06</td>
<td>3.04±0.48</td>
</tr>
<tr>
<td>C7–12</td>
<td>13</td>
<td>8.33±0.71</td>
<td>10.89±1.14</td>
<td>9.61±0.90</td>
<td>4.02±0.93</td>
<td>13.19±0.88</td>
<td>15.59±1.16</td>
<td>14.39±0.99</td>
<td>3.46±1.16</td>
<td>2.95±0.27</td>
</tr>
<tr>
<td>C7–15</td>
<td>11</td>
<td>9.27±0.12</td>
<td>12.33±0.52</td>
<td>10.80±0.29</td>
<td>4.12±0.43</td>
<td>16.58±0.16</td>
<td>19.32±0.60</td>
<td>17.95±0.35</td>
<td>3.57±0.38</td>
<td>3.04±0.48</td>
</tr>
<tr>
<td>C7–17</td>
<td>5</td>
<td>8.07±0.49</td>
<td>11.67±1.28</td>
<td>9.87±0.88</td>
<td>6.73±2.19</td>
<td>19.35±1.03</td>
<td>21.71±1.00</td>
<td>20.53±1.00</td>
<td>6.03±2.45</td>
<td>6.01±2.09</td>
</tr>
<tr>
<td>LN7–7</td>
<td>8</td>
<td>8.85±0.18</td>
<td>11.67±0.69</td>
<td>10.26±0.39</td>
<td>3.96±0.73</td>
<td>8.77±0.43</td>
<td>11.78±0.83</td>
<td>10.33±0.48</td>
<td>3.42±0.44</td>
<td>3.03±0.30</td>
</tr>
<tr>
<td>LN7–10</td>
<td>6</td>
<td>9.03±0.266</td>
<td>11.54±0.10</td>
<td>10.29±0.16</td>
<td>4.13±0.22</td>
<td>11.98±0.19</td>
<td>13.98±0.18</td>
<td>12.98±0.16</td>
<td>3.27±0.41</td>
<td>2.86±0.47</td>
</tr>
<tr>
<td>LN7–12</td>
<td>10</td>
<td>8.97±0.30</td>
<td>11.68±0.80</td>
<td>10.33±0.43</td>
<td>4.10±0.53</td>
<td>14.25±0.23</td>
<td>16.24±0.77</td>
<td>15.25±0.43</td>
<td>3.23±0.50</td>
<td>2.96±0.50</td>
</tr>
<tr>
<td>LN7–15</td>
<td>9</td>
<td>9.23±0.19</td>
<td>12.40±0.46</td>
<td>10.82±0.30</td>
<td>4.35±0.46</td>
<td>16.62±0.15</td>
<td>19.40±0.52</td>
<td>18.01±0.32</td>
<td>3.58±0.45</td>
<td>3.14±0.57</td>
</tr>
<tr>
<td>LN7–17</td>
<td>5</td>
<td>8.79±0.78</td>
<td>11.45±0.74</td>
<td>10.12±0.58</td>
<td>5.87±1.57</td>
<td>20.39±1.23</td>
<td>22.04±1.08</td>
<td>21.21±1.04</td>
<td>5.43±1.62</td>
<td>4.78±1.25</td>
</tr>
<tr>
<td>HEP7–7</td>
<td>15</td>
<td>8.90±0.17</td>
<td>11.83±0.26</td>
<td>10.36±0.16</td>
<td>4.29±0.32</td>
<td>8.84±0.17</td>
<td>11.71±0.23</td>
<td>10.28±0.15</td>
<td>3.82±0.33</td>
<td>3.20±0.23</td>
</tr>
<tr>
<td>HEP7–15</td>
<td>8</td>
<td>9.27±0.11</td>
<td>11.78±0.30</td>
<td>10.52±0.13</td>
<td>4.04±0.46</td>
<td>16.43±0.24</td>
<td>18.47±0.21</td>
<td>17.45±0.11</td>
<td>3.24±0.26</td>
<td>2.83±0.24</td>
</tr>
<tr>
<td>DN7–15</td>
<td>16</td>
<td>9.23±0.140</td>
<td>11.86±0.27</td>
<td>10.54±0.16</td>
<td>4.41±0.31</td>
<td>16.67±0.55</td>
<td>18.77±0.50</td>
<td>17.70±0.51</td>
<td>3.72±0.30</td>
<td>3.22±0.33</td>
</tr>
<tr>
<td>TBAP7–15</td>
<td>9</td>
<td>9.24±0.23</td>
<td>12.77±0.55</td>
<td>10.32±0.51</td>
<td>4.51±0.43</td>
<td>16.53±0.26</td>
<td>19.80±0.50</td>
<td>18.17±0.30</td>
<td>3.90±0.83</td>
<td>3.38±0.72</td>
</tr>
</tbody>
</table>

Values are means ± SD; N is sample size. Subscripts 1 and 2 denote first or second pressure step, respectively, from which the value is derived. Pla, left atrial pressure; Ppa, pulmonary artery pressure; Pce, pulmonary capillary pressure; Pdo, double occlusion pressure. All pressures were measured in cmH2O.
crease in $K_{fc}$ when $Pla_2 = 15$ and $17$ cmH$_2$O (Fig. 2B). The $K_{fc2}/K_{fc1}$ ratio at $Pla_2 = 15$ cmH$_2$O was $3.49 \pm 1.40$; when $Pla = 17$ cmH$_2$O, $K_{fc2}/K_{fc1}$ was $3.70 \pm 0.88$. These values are significantly different than untreated control ($P < 0.05$) as shown in Figs. 2 and 3. d-NAME (200 μM), the inactive enantiomer of l-NAME, had no effect on pressure-induced increase in $K_{fc2}$ (data not shown). GSNO increased the mean $K_{fc2}/K_{fc1}$ ratio when $Pla = 15$ cmH$_2$O to 9.1 ($P < 0.0001$) (Fig. 3).

**HEPARANASE III.** We tested two concentrations of heparanase III on pressure-mediated increases in $K_{fc}$. Heparanase III at 75 mLU/ml had no effect on $K_{fc}$ relative to controls (data not shown). At a concentration of 150 mLU/ml, heparanase III had no effect on $K_{fc2}$ when $Pla = 7.5$ cmH$_2$O, indicating that the enzyme did not alter baseline permeability, but heparanase III significantly attenuated the increase in $K_{fc2}$ when $Pla_2$ was increased to 15 cmH$_2$O (Hep7–15; Fig. 3). Heparanase III significantly reduced $K_{fc2}$ at $Pla_2 = 15$ cmH$_2$O to 0.134 ± 0.065 vs. 0.65 ± 0.36 in untreated lungs; the $K_{fc2}/K_{fc1}$ ratio for heparanase III-treated lung was 3.10 ± 1.59 vs. 7.91 ± 3.28 in untreated lungs ($n = 8$, $P < 0.0001$).

**ZONAL CHARACTERISTICS.** $K_{fc}$ measurements are dependent on vascular surface area that can be increased by vascular recruitment; zonal characteristics (ZCs) were derived from $\Delta P_{pa}/\Delta Pla$ as described by Brower et al. (10, 11) and Angland et al. (7) and indicate the percentage of zone 2 and 3 conditions at each $Pla$. In control lungs, ZC significantly increased with the increase in $Pla$ from 7.5 to 10 cmH$_2$O (0.44 vs. 0.58; $P < 0.05$); additional increases in $Pla$ above 12 cmH$_2$O did not result in statistically significant changes in ZC (Fig. 4A).

ZCs were derived for l-NAME-treated groups at $Pla_2$ at 7.5, 10, 12, 15, and 17 cmH$_2$O (Fig. 4A); increases in $Pla$ from 7.5 to 10 cmH$_2$O resulted in the only statistically difference in ZC. A comparison of ZC for control, heparanase III-treated, and l-NAME-treated lung at $Pla_2 = 15$ cmH$_2$O is shown in Fig. 4B. There were no significant differences in ZC between treatment groups at $Pla = 15$ cmH$_2$O, suggesting that recruitment was not responsible for observed differences in $K_{fc}$ when $Pla_2 = 15$ cmH$_2$O with heparanase and l-NAME.

**INTERSTITIAL VOLUME.** To assess the effect of increased $Pla_2$ and treatments (heparanase, l-NAME) on interstitial volume, we measured the retained lung weight after the second pressure steps ($\Delta V_i$) and normalized this value to predicted lung weight (PLW). A plot of $\Delta V_i/PLW$ for each group demonstrates that heparanase III and l-NAME significantly reduced interstitial volume, in direct correlation with their effects the $K_{fc2}/K_{fc1}$ ratio (Fig. 5).

**Protocol 2: Standard VT studies (ST VT).** In this series of experiments, lungs were ventilated with PIP = 10–12 cmH$_2$O, an increase in ~30–40% increase relative to Low VT experiments; the effect of $Pla_2$ and test reagents (heparanase III, TBAP). The intracellular superoxide dismutase mimic, TBAP (200 μM), had no effect on pressure-induced increase in $K_{fc}$ ratio when assessed at $Pla = 15$ cmH$_2$O. TBAP at 200 μM is a high concentration and the duration of exposure was appropriate based on similar uses (25, 30). $K_{fc2}$ after a step to $Pla_2 = 15$ cmH$_2$O in TBAP-treated lungs was 0.63 ± 0.19 vs. control $K_{fc2} = 0.65 ± 0.36$ ml·min$^{-1}$·cmH$_2$O$^{-1}$ per 100 g; $K_{fc2}/K_{fc1} = 6.74 ± 1.58$ was not significantly different than untreated lungs at $Pla_2 = 15$ cmH$_2$O ($n = 9$; $p > 0.05$) (Fig. 3).
heparanase III + DEPC, l-NAME, p-NAME, GSNO) on $K_{fc}$ were measured at Pla = 7.5, 15, and 17 cmH$_2$O.

**DOUBLE PRESSURE STEP.** All lungs in this group had baseline $K_{fc}$ measured at Pla = 7.5 cmH$_2$O, followed by a second step increase to Pla = 15 or 17 cmH$_2$O. At ST VT baseline $K_{fc1}$ values were 0.078 ml·min$^{-1}$·cmH$_2$O$^{-1}$·per 100 g, which was not different from $K_{fc1}$ during Low VT (Fig. 6A). At Pla = 15 cmH$_2$O, the $K_{fc2}/K_{fc1}$ ratio was 7.1 ± 2.71 ($n$ = 12), which is significantly higher than the ratio obtained during Low VT and Pla = 15 cmH$_2$O. When Pla = 17 cmH$_2$O, $K_{fc2}/K_{fc1}$ = 15.80 ± 1.20.

HEPARANASE III. Heparanase III (150 mIU/ml) significantly reduced $K_{fc2}$ when Pla = 15 cmH$_2$O; mean $K_{fc2}$ was 0.35 ± 0.08 ml·min$^{-1}$·cmH$_2$O$^{-1}$·per 100 g, which was not different from $K_{fc1}$ during Low VT (Fig. 6A).

**L-NAME** reduced $K_{fc2}$ by almost 50% during ST VT and Pla = 15 cmH$_2$O, but the decrease did not reach statistical significance. GSNO significantly increased $K_{fc2}$ when Pla = 15 cmH$_2$O.

**C:** Increases in Pla to 17 cmH$_2$O result in marked pulmonary edema that was significantly reduced by heparanase (Hep7–17) and l-NAME (LN7–17).

values were 0.078 ± 0.03 ml·min$^{-1}$·cmH$_2$O$^{-1}$ per 100 g, which was not different from $K_{fc1}$ during Low VT (Fig. 6A). At Pla = 15 cmH$_2$O, the $K_{fc2}/K_{fc1}$ ratio was 7.1 ± 2.71 ($n$ = 12), which is significantly higher than the ratio obtained during Low VT and Pla = 15 cmH$_2$O. When Pla = 17 cmH$_2$O, $K_{fc2}/K_{fc1}$ = 15.80 ± 1.20.

HEPARANASE III. Heparanase III (150 mIU/ml) significantly reduced $K_{fc2}$ when Pla = 15 cmH$_2$O; mean $K_{fc2}$ was 0.35 ±
0.21 and $K_{fc2}/K_{fc1} = 3.12 \pm 1.93$ ($n = 12$) (Fig. 6B). DEPC inhibits heparanase by binding to the histidine residue within the active site and renders the enzyme inactive (35a). This approach mitigates changes in enzyme structure that would be altered by boiling as a means of inactivation. Heparanase III was incubated with DEPC (100 nM; 20 min), then added to the circulating perfusate. In contrast to active enzyme, heparanase-DEPC had no effect on $K_{fc}$ (Fig. 6B).

When Pla was increased to 17 cmH$_2$O, $K_{fc2}/K_{fc1}$ increased to 15.8 from 4.08 (Fig. 6C).

Heparanase III significantly attenuated the increase in $K_{fc}$ at Pla = 17 cmH$_2$O (P < 0.01) (Fig. 6C).

L-NAME had no effect on $K_{fc}$ at Pla = 17 cmH$_2$O. L-NAME significantly decreased ZC at Pla = 15 cmH$_2$O. L-NAME had no effect on ZC (Fig. 7).

Fig. 7. ZC after elevated Pla with ST VT. $\Delta V/\Delta P L W$ closely parallel the relationship of $K_{fc2}/K_{fc1}$ ratio between groups. There were no significant differences when Pla$_2$ = 15 cmH$_2$O between control, heparanase III-treated, or L-NAME-treated lungs (Fig. 8A). When Pla$_2$ = 17 cmH$_2$O, heparanase III and L-NAME significantly reduced $\Delta V/\Delta P L W$ (Fig. 8B).

INTERSTITIAL MECHANICS. To assess the effect of increased Pla$_2$ and treatments (heparanase III, L-NAME) on interstitial mechanics, we measured the retained lung weight ($\Delta V$) after the second pressure step and normalized this value to PLW. Plots of $\Delta V/\Delta P L W$ closely parallel the relationship of $K_{fc2}/K_{fc1}$ ratio between groups. There were no significant differences when Pla$_2$ = 15 cmH$_2$O between control, heparanase III-treated, or L-NAME-treated lungs (Fig. 8A). When Pla$_2$ = 17 cmH$_2$O, heparanase III and L-NAME significantly reduced $\Delta V/\Delta P L W$ (Fig. 8B).

Protocol 3: single pressure step. To assess the effect of the baseline pressure step (Pla$_1$) on subsequent $K_{fc}$ measurements (i.e., priming effect), we performed a single step from isogravimetric pressure ($0–2$ cmH$_2$O) to Pla$_2$ = 15 cmH$_2$O. This group of experiments was performed in Low VT lungs only. $K_{fc}$ during the single pressure step to Pla$_2$ = 15 cmH$_2$O was $0.33 \pm 0.16$ ml·min$^{-1}$·cmH$_2$O$^{-1}$ per 100 g and was significantly less than $K_{fc2}$ at Pla$_2$ = 15 cmH$_2$O in the double pressure pulse experiments ($0.34 \pm 0.16$ vs. $0.65 \pm 0.35$ ml·min$^{-1}$·cmH$_2$O$^{-1}$ per 100 g, Fig. 9).

Heparanase III reduced $K_{fc}$ during the single pressure step to $0.29 \pm 0.15$ ml·min$^{-1}$·cmH$_2$O$^{-1}$ per 100 g, compared with controls ($0.33 \pm 0.16$ ml·min$^{-1}$·cmH$_2$O$^{-1}$ per 100 g), although the effect did not reach statistical significance (Fig. 9).

L-NAME lowered $K_{fc}$ by 50%, compared with controls (in the single pressure step protocol. $0.13 \pm 0.09$ vs. $0.33 \pm 0.16$ ml·min$^{-1}$·cmH$_2$O$^{-1}$ per 100 g; $p = 0.11$) (Fig. 9). In fact, $K_{fc}$
measured from L-NAME-treated lungs using the single pressure protocol at Pla2/H11005 15 cmH2O, was reduced by almost 50% compared with the corresponding Kfc2 from L-NAME-treated lungs during the double pressure pulse protocol (0.13/H11006 0.09 vs. 0.23/H11006 0.12, ml·min/H11002 1·cmH2O/H11002 1 per 100 g), respectively.

IMMUNOHISTOCHEMISTRY. To validate the effects of heparanase III on removing lung HS, anti-HS antibody (HSS-1) was used to stain for HS. Control lungs demonstrate substantial capillary staining for HS within the alveolar septa (Fig. 10 A); heparanase III completely abolished HS staining (Fig. 10 B). An alternative approach to validate the efficacy of heparanase III was achieved by using 3G10, an antibody that recognizes the neoepitope on the HS chain generated by the activity of heparanase III. Control lungs showed no 3G10 staining whereas heparanase III-treated lungs showed significant staining (Fig. 10, C and D). Collectively, these two complementary approaches demonstrate significant removal of HS from the lung vasculature.

Staining for nitrotyrosine was increased in lungs exposed to increased vascular pressure (Pla2 7.5 vs. 15 cmH2O) as shown in Fig. 11, A vs. B, respectively. During low vascular pressure (Pla2 = 7.5 cmH2O), small regions of positive staining are seen at low power (×20) and shown as a close-up (inset, ×60) (Fig. 11A). Following exposure to Pla2 = 15 cmH2O, an increase in nitrotyrosine staining is observed in both low- and high-powered images (Fig. 11B). Heparanase-treatment before increasing vascular pressure significantly reduced nitrotyrosine staining (Fig. 11C).

TISSUE NOx. We directly measured lung tissue NOx concentrations. The relationship between NO concentration vs. Kfc2/Kfc1 for pooled Low VT and ST VT are presented in Fig. 12 A. The Low VT group shows a statistically significant linear correlation between NOx vs. Kfc2/Kfc1 (r2 = 0.59, n = 29) and the Low VT group is left shifted compared with the ST VT group (inset). The combination of increased vascular pressure and ST VT resulted in significantly higher tissue NOx concentrations; the NO vs. Kfc2/Kfc1 relationship was fitted to an exponential curve (r2 = 0.92); the permeability response was flat above an NOx concentration of ~4 μmol.

Tissue NOx levels are presented in Fig. 12, B and C. During Low VT and baseline Pla (7.5 cmH2O), tissue NOx was low (0.028 ± 0.026 μmol) and increased almost 12-fold when vascular pressure was increased to 15 cmH2O (C7–15; mean NOx = 0.35 μmol; P < 0.01). GSNO increased tissue NOx levels to 0.70 μmol. Heparanase III treatment had no effect on NOx levels during baseline Pla (Hep7–7); during elevated vascular pressure, heparanase (Hep7–15) decreased the tissue NOx from 0.35 to 0.23 μmol (P > 0.05). NOx could not be...
measured in the L-NAME groups because L-NAME liberates NOx, which interferes with the assay.

During ST VT, increasing vascular pressure resulted in an exponential increase in tissue NOx. Baseline NOx at Pla = 7.5 cmH2O (C7–7) was 0.03 μmol, which is identical to that of the Low VT group. However, the combination of higher VT and increasing in vascular pressure to 15 and 17 cmH2O resulted in marked increased NOx concentrations to 0.7 and 8.4 μmol, respectively (Fig. 12C). GSNO was synergistic with increasing Pla and increased NOx levels to 2.6 μmol. Heparanase III significantly attenuated the increase in NOx when Pla = 15 cmH2O to 0.5 μmol (P < 0.01, Fig. 12C).

DISCUSSION

The major findings of this study were 1) $K_{fc}$ increased in a nonlinear relationship to vascular pressure, over the range of 7.5 to 17 cmH2O; 2) heparanase III significantly attenuated the NOx concentrations.

![Figure 11. Immunohistochemistry for nitrotyrosine. A: control lungs at Pla = 7.5 cmH2O showed patchy staining. B: when lungs were exposed to Pla = 15 cmH2O, marked nitrotyrosine staining was evident. C: heparanase III treatment prior to increasing Pla attenuated nitrotyrosine staining.](image)

![Figure 12. Lung nitrate/nitrite (NOx) concentrations. NOx was measured in lung tissue following increased Pla. A: relationship of NOx concentration vs. $K_{fc2}/K_{fc1}$ ratio for pooled Low VT (LTV) and ST VT (STV) data. Inset shows close up of relationship at low NOx concentrations; note linear correlation of Low VT group vs. NOx and left shift of Low VT vs. ST VT group. B: during Low VT ventilation, an increase in Pla from 7 to 15 cmH2O results in a 4-fold increase in tissue NOx. GSNO further increased tissue NOx levels when Pla = 15 cmH2O. Heparanase treatment at low Pla had no effect on NOx (Hep7–7) but significantly reduced NOx concentrations at Pla = 15 cmH2O (Hep7–15). C: during ST VT increases in Pla to 15 cmH2O result in large increases in NOx. GSNO at Pla = 15 cmH2O further increased NOx to 2.60. When Pla = 17 cmH2O, NOx increased to 8.41; heparanase significantly reduced the NOx to 0.54, when Pla = 15 cmH2O (n = 4–6). m1–m4, fit parameters. Chisq, chi squared.](image)
ated the pressure-induced increase in $K_{fc2}$ and inactivation of heparanase III by DEPC abolished its inhibitory effect; 3) 1-NAME (200 μM) significantly attenuated the pressure-induced increase in $K_{fc2}$, whereas the NO donor GSNO increased permeability; 4) TBAP had no effect on pressure-induced increase in $K_{fc2}$; and, finally, 5) the first step increase in Pla increased the sensitivity of the vasculature to the second step in Pla, a phenomenon we call pressure conditioning.

**Nonlinear Relationship of $K_{fc}$ vs. Pla**

For our initial studies, we performed a detailed characterization of increasing Pla on $K_{fc}$. In these studies, $K_{fc1}$ was measured in all lungs at Pla$_1$ = 7.5 cmH$_2$O. After a second isovolumetric period, Pla$_2$ was increased to 7.5, 10, 12, 15, or 17 cmH$_2$O and $K_{fc2}$ was derived. In each set of experiments, the ratio of $K_{fc2}/K_{fc1}$ was calculated to establish the influence of Pla$_2$ on whole lung permeability; the $K_{fc2}/K_{fc1}$ ratio did not significantly change until Pla$_2$ = 15 cmH$_2$O. The $K_{fc2}/K_{fc1}$ ratio following double pressure steps of 7.5/7.5, 7.5/10, 7.5/12, 7.5/15, and 7.5/17 was 1.43, 1.43, 2.83, 7.5, and 9.86, respectively. The nonlinear dynamics of the pressure vs. permeability relationship mimics what was observed in a cell culture model using lung capillary endothelial cells (15) and suggests a non-Starling mechanism. $K_{fc}$ is the transvascular water flux normalized to capillary pressure and, therefore, assuming vascular permeability remained unaltered by pressure, the $K_{fc}$ vs. Pla relationship should have a slope of zero. The nonlinear relationship, which has been reported in vitro, and now in a whole organ model, suggests a non-Starling mechanism consistent with active vascular mechanotransduction.

1-NAME had no effect on baseline $K_{fc1}$ but significantly attenuated the increase in $K_{fc2}$ when Pla$_2$ = 15 and 17 cmH$_2$O. These findings are similar to results obtained from monolayers of bovine lung capillary endothelial cells (15, 19, 43). The increase in hydrostatic pressure appears to activate eNOS, leading to an increase in endothelial permeability. The role of hydrostatic pressure and increased transendothelial flow on NO production has been reported by Burns et al. (12), using human umbilical vein endothelial cells as a model system. Thus there is a consistent finding both in vitro and now in an ex vivo lung preparation where pressure-mediated mechanotransduction involves eNOS activation and subsequent barrier dysfunction. GSNO, an NO donor, exacerbated pressure induced increase in $K_{fc}$ during both Low VT and ST VT protocols, supporting the conclusion that NO is a mediator of endothelial barrier dysfunction.

**Heparanase Experiments**

This is the first study to demonstrate a role for vascular HS on mechanotransduction using a whole lung model. In the present study, heparanase III (E.C. 4.2.2.8) had no effect on baseline $K_{fc1}$ yet significantly attenuated the increase in $K_{fc2}$ when Pla$_2$ was increased to 15 and 17 cmH$_2$O (Figs. 3 and 6). These results are similar to our observations in vitro (15) strongly supporting the conclusion of glycoalyx-mediated mechanotransduction (26, 36, 42). We tested DEPC-inactivated heparanase III and found that it had no effect on reducing $K_{fc}$, during elevated vascular pressure; thus heparanase’s enzymatic activity was required to attenuate mechanotransduction. To validate the removed of HS by heparanase, we performed immunohistochemistry and demonstrated nearly complete removal of vascular heparan staining after heparanase treatment. Likewise, the staining of heparanase-treated lungs by 3G10, an antibody that recognizes the neoepitope created by heparanase, demonstrated robust staining.

To determine whether heparanase’s reduction in $K_{fc}$ was actually due to a reduction in permeability and not due to altered interstitial pressure, we present the following line of reasoning. Negrini et al. (28) used intravascular heparanase III to induce lung injury in rabbits and reported an increase in interstitial pressure due to the accumulation of interstitial fluid. In their experiments, heparanase III-treated lungs had an increase in wet-to-dry weight ratio consistent with an increase in interstitial fluid. As interstitial pressure increased, the gradient for fluid flux decreased and, therefore, could reduce the apparent value of $K_{fc}$. In the present study, the increase in lung weight following each pressure step was taken as a marker for $\Delta V_i$ (45); heparanase III-treated lungs had a significantly lower $\Delta V_i/PLW$ at Pla = 15 and 17 cmH$_2$O compared with controls (Figs. 5 and 8), consistent with a reduction in endothelial permeability and reduced interstitial edema. The significantly lower values for both $K_{fc}$ and $\Delta V_i/PLW$ in heparanase-treated lungs support the conclusion that mechanotransduction and increased permeability were attenuated by removal of vascular HS.

**TBAP**

TBAP is a cell-permeable superoxide dismutase mimetic that scavenges free radicals. We previously reported (15) that TBAP attenuated pressure-induced increases in endothelial permeability, suggesting that ROS were partially involved in barrier alterations. In the present study, TBAP had no effect on pressure-induced increase in $K_{fc}$. We conclude that ROS are not the cause of pressure-mediated barrier dysfunction in this ex vivo lung model.

**Technical Considerations**

The relationship between Pla, Ppc, and $K_{fc}$ is complex and requires assumptions that have been addressed elsewhere (9). To rule out factors other than mechanotransduction that could have influenced measured $K_{fc}$, we address the following technical issues.

**Pulmonary hemodynamics.** Ppa, pulmonary artery flow, and Pdo$_1$, Pdo$_2$, and Pdo$_3$ were measured throughout the experiment. There were no significant differences in pulmonary hemodynamic variable that could explain the observed differences in $K_{fc2}$ when Pla$_2$ = 15 or 17 cmH$_2$O (Table 1).

**Recruitment.** Fluid flux is dependent on vascular surface area; if step increases in Pla increased recruitment, our measured $K_{fc}$ values may reflect only the increasing surface area and not an actual change in endothelial permeability. Short et al. (40) reported that, in rat lungs, recruitment of subpleural capillaries accurately reflected recruitment of interior capillaries; using similar methods, Presson et al. (35) showed that lung capillary recruitment was 90% complete when capillary pressure equaled 10 cmH$_2$O and was nearly 100% complete when capillary pressure was 12 cmH$_2$O. Pulmonary artery pressure is an indicator of downstream resistance and should decrease with increasing vascular recruitment. In the present study Ppa...
was stable after 3–5 min following the step increase in Pla, suggesting that recruitment and downstream resistance was not changing.

Evaluation of ZC (ΔP/ΔPla) was undertaken to further evaluate the effects of increased Pla on vascular recruitment and to quantify the relative degree of Zone 2 and Zone 3 conditions throughout the experiments. During Low VT experiments when Pla2 = 7.5 cmH2O, all lungs were primarily in Zone 2 as evidenced by ZC = 0.44. Increases in Pla2 to 10, 12, 15, and 17 cmH2O increased ZC to 0.58, 0.64, 0.71, and 0.76, respectively. According to ZC analysis, recruitment was completed when Pla = 12 cmH2O consistent with the finding of Presson et al. (35). In summary, Kfc continued to increase after ZC had plateaued, suggesting that changes in endothelial permeability as the cause for increased Kfc.

**Mechanism of Pressure-Induced Increase in Kfc**

Our results, using an intact lung model, demonstrate that permeability changes as a function of vascular pressure and are similar to mechanotransduction pathways elucidated with use of cultured endothelial cells (12, 15, 16, 18, 31). These findings challenge the Starling principle and demonstrate non-Starling mechanism(s) to account for the development of pulmonary edema. Enhanced staining of NO-tyrosine during increased vascular pressure and the reduction of NO-tyrosine after heparanase treatment strongly supports our hypothesis for a HS-eNOS mechanotransduction pathway. In fact, direct measurement of tissue NOx showed increasing tissue NOx with increased vascular pressure. Heparanase III reduced tissue NOx concentration by 50% when Pla = 15 cmH2O in the Low VT groups and reduced NOx by eightfold, when Pla = 17 cmH2O, during ST VT. Lastly, GSNO increased tissue NOx and significantly increased Kfc during elevated vascular pressure. Taken together, these results support our conclusion that NOx is involved in barrier dysfunction. A schematic for the proposed mechanism of glycocalyx mechanotransduction is shown in Fig. 13.

Kuebler (24) has shown that an increase in Pla increases lung capillary NO formation, consistent with the findings of our study. In fact, high vascular pressure has been shown to activate cytochrome P-450 (CYP450)-dependent pathways in the lung resulting in increased Kfc and metabolites of the CYP450 pathway, including 20-HETE, can activate eNOS in pulmonary endothelial cells (13). Experiments to elucidate the mechanism(s) linking the glycocalyx to eNOS activation and alterations in barrier function are currently underway in our laboratory. Stretch-activated transient receptor potential (TRP) channels likely participate in lung vascular mechanotransduction and have been shown to modulate both CYP450 pathways and eNOS activation (5, 21, 34).

**Airway Pressures and Kfc**

A major finding of the present study was the combined influence of airway pressure and VT on tissue NOx and Kfc when vascular pressure was increased. At ST VT, baseline Kfc was not different than baseline Kfc at Low VT (Fig. 6A), thus endothelial permeability is not altered by the changes in VT when Pla < 15 cmH2O.

---

**Fig. 13. Schematic of hypothesized role of glycocalyx in lung vascular mechanotransduction.** Left: during static conditions, the glycocalyx maintains barrier function over the intercellular junction. Right: during increased vascular pressure, the increased hydraulic flow through the glycocalyx deforms or stresses the glycosaminoglycan (GAG) fibers, which in turn activates endothelial nitric oxide synthase (eNOS) and leads to barrier dysfunction. ΔPc, change in capillary pressure; Q, flow; ZO-1 and ZO-2, zona occludens-1 and -2; vin, vinculin, VE-Cad, vascular endothelial cadherin; ECM, extracellular matrix.
When lungs were ventilated with ST Vr and Pla was elevated, $K_{ec}$ increased to a greater extent than when lungs were ventilated with Low Vr at elevated Pla. For example, at Low Vr and Pla = 17 cmH2O, $K_{ec2}$ increased ~5-fold (Fig. 2) whereas at ST Vr and Pla = 17 cmH2O, $K_{ec2}$ increased ~15-fold. Thus inflation pressure had a significant effect on the permeability response to increased vascular pressure.

Heparanase and L-NAME had a greater effect in the reduction of $K_{ec}$ during ST Vr compared with Low Vr. These results cannot be explained by changes in vascular recruitment, since ZC did not change with heparanase or L-NAME treatments.

Yin et al. (47) demonstrated that in statically inflated lungs NO participates in a negative feedback loop, via a cGMP mechanism, that attenuates hydrostatic pulmonary edema. They suggest that elevated vascular pressure activates stretch-induced calcium channel opening (presumably TRPV4) resulting in eNOS activation, NO-stimulated soluble guanylate cyclase activity, and cGMP-mediated inactivation of TRPV4 channels. In their model, L-NAME exacerbated pressure-induced $K_{ec}$ and NO donors attenuated pressure-induced increase in $K_{ec}$. The major difference that explains the results of Yin et al. Compared with the present work is the role of cyclic ventilation in maintaining endothelial cGMP levels. Lung cGMP levels are positively influenced by mechanical ventilation and nonventilated lungs show a significant reduction in cGMP levels (8, 22, 34). A complex interplay between endothelial cell cGMP concentrations and barrier function has recently been described by Pearson and colleagues (34, 37) and Kuebler (23), who demonstrated that cGMP concentrations increased in lung endothelial cells in a Vr- and time-dependent manner. This increase in cGMP stimulates the activity of PDE2A, an enzyme that degrades cyclic AMP, leading to barrier dysfunction and elevated $K_{ec}$. Support for this idea can be inferred from the findings of Parker and Ivey (32), who reported that high vascular pressure-induced permeability was attenuated with isoproterenol, an α-adrenergic agonist that increases intracellular cAMP.

Direct measurements of tissue NOx in lung samples from our study revealed differences in the NOx concentrations vs. $K_{ec2}/K_{ec1}$ relationship (Fig. 12) depending on Vr: the slope of NOx vs. $K_{ec2}/K_{ec1}$ was steeper and left shifted in the Low Vr group compared with the ST Vr group. This means that during low Vr, vascular permeability was greater at a given NOx concentration. During ST Vr there is a large increase in tissue NOx in response to elevated vascular pressure; this supports our conclusion that the combined effects of alveolar stretch and endothelial mechanotranstduction result in more severe edema development.

NO can contribute to barrier dysfunction through mechanisms other than altering cGMP-to-cAMP ratios. For example, NO and its reactive metabolites such as peroxynitrite can directly nitrosylate tyrosine residues on endothelial proteins, resulting in barrier dysfunction. NO* and ONOO*-induced nitrosylation of β-actin are principal mechanisms of TNF-induced barrier dysfunction (29). Our immunohistochemistry data demonstrated an increase in NO-tyrosine staining in lungs subjected to increased Pla and, conversely, a reduction in NO-tyrosine in heparanase-treated lungs. Superoxide anions, produced via the activation of NAD(P)H oxidase (17) and mitochondrial oxidases (4, 39), can react with endogenous NO to generate peroxynitrates, leading to oxidative barrier dysfunc-

tion. The lack of effect of TBAP, an intracellular superoxide dismutase mimetic, suggests that ROS are not the primary mechanism underlying pressure-induced increase in $K_{ec}$.

NO can also nitrosylate and activate conductance of the TRP-family ion channels that allow calcium influx and sustain the calcium-dependent activation of eNOS (48). Cytoplasmic cys553 and cys558 can be S-nitrosylated and appear to account for the direct NO sensitivity of TRPC5, TRPV1, TRPV3, and TRPV4 channel gating. Other TRP family members including TRPC1 and TRPC4 possess conserved cys residues on the putative pore region that predicts NO-sensitive activation. The balance between cGMP gating of stretch activated channels as proposed by Yin et al. (47) and NO activation of TRP channel (48) has yet to be reconciled in the lung vasculature. Nitrosylation of TRP channels and subsequent calcium influx may lead to continued activation of eNOS and resultant barrier dysfunction.

Clinical Relevance

The Acute Respiratory Distress Syndrome Network data has demonstrated that low-Vr ventilation improves outcomes in patients with existing lung injury (1, 6), whereas conservative fluid strategies improve pulmonary function and reduce ventilator-free days (46). From a clinical perspective, lung vascular mechanotransduction may contribute to worsening extravascular lung water resulting from overaggressive fluid resuscitation in combination with elevated airway pressure and may help explain the beneficial mechanism(s) of conservative fluid management and low-Vr ventilation.

ACKNOWLEDGMENTS

We are indebted to Ibex Technologies (Montreal, Quebec, Canada) for the generous gift of high-quality heparanase III for use in this study. A special thanks to Drs. Kurt Albertine, Robert Lane, and Lisa Joss-Moore for helpful discussions during this work and manuscript preparation.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant 5R01HL085255-04 and the Presidential Endowed Chair in Anesthesiology to R. O. Dull.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: R.O.D. and D.T.M. conception and design of research; R.O.D. performed experiments; R.O.D., M.C., J.K., D.H., H.C., S.H., D.T.M., and R.K. analyzed data; R.O.D. interpreted results of experiments; R.O.D. drafted manuscript; R.O.D. and J.K. edited and revised manuscript; R.O.D. approved final version of manuscript; M.C., J.K., D.H., H.C., S.H., D.T.M., and R.K. for helpful discussions during this work and manuscript preparation.

REFERENCES


HEPARAN SULFATE AND LUNG FILTRATION COEFFICIENT


