Heparan sulfates mediate pressure-induced increase in lung endothelial hydraulic conductivity via nitric oxide/reactive oxygen species

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Dull RO, Mecham I, McJames S. Heparan sulfates mediate pressure-induced increase in lung endothelial hydraulic conductivity via nitric oxide/reactive oxygen species. Am J Physiol Lung Cell Mol Physiol 292: L1452–L1458, 2007. First published March 9, 2007; doi:10.1152/ajplung.00376.2006.—We investigated the nonlinear dynamics of the pressure vs. hydraulic conductivity ($L_p$) relationship in lung microvascular endothelial cells and demonstrate that heparan sulfates, an important component of the endothelial glycocalyx, participate in pressure-sensitive mechanotransduction that results in barrier dysfunction. The pressure vs. $L_p$ relationship was complex, possessing both time- and pressure-dependent components. Pretreatment of lung capillary endothelial cells with heparanase III completely abolished the pressure-induced increase in $L_p$. This extends our (7) previous observation regarding heparan sulfates as mechanotransducers for shear stress. Inhibition of nitric oxide (NO) synthase completely abolished the pressure-induced increase in $L_p$. We hypothesized that heparan sulfates and perhaps other components of the glycocalyx participate in mechanotransduction. We show that lung microvascular endothelial cells in vitro maintain many functional similarities with in situ and intact lung preparations and retain their mechanosensing pathway(s). Herein, we provide a detailed characterization of the nonlinear dynamics of the pressure vs. $L_p$ relationship and show that heparan sulfates, NO, and ROS actively participate in pressure-mediated barrier dysfunction.

MATERIALS AND METHODS

Cell Culture

Bovine lung microvascular endothelial cells (BLMVEC) were purchased from Vec Technologies (Rensselaer, NY) and grown in MCDB-131 Complete (Vec Technologies). Cells were subcultured onto Costar Snapwell chambers (polycarbonate membranes, 0.4 $\mu$m pore size, 12-mm diameter). Before seeding the cells onto the membrane, the Snapwell chambers were gelatinized with 0.4% bovine fibronectin for 1 h. Cells were plated at a density of $1.25 \times 10^5$ cells/cm$^2$, and all $L_p$ measurements were conducted 9–10 days after plating.

$L_p$ Measurements

The system used to measure the volumetric flux rate ($J_v$) and $L_p$ has been described in detail (8). Briefly, the Snapwell culture unit was inserted into a custom-made permeability chamber that consisted of two pieces of transparent polycarbonate. The bottom half contained a small reservoir and a rubber O-ring that fit snugly under the rim of the Snapwell. The top half of the chamber was designed with another O-ring that sealed against the top rim of the Snapwell. Two spring loaded latches on the outside of the chamber pulled the two halves together to compress the O-rings and formed a watertight seal both above and below the Snapwell. To measure $L_p$, the inflow port of the cell culture chamber was connected by Silastic tubing to a pressure manifold filled with MCDB-131 (1% BSA, 25 mM HEPES, 1% cmH$_2$O) evoking calcium oscillations (14), generate reactive oxygen species (ROS; Ref. 11), and promote an inflammatory phenotype in capillary endothelial cells (10, 15). Increases in intra-alveolar pressures can induce alterations in capillary permeability (e.g., ventilator-induced lung injury) leading to the clinical observation that low-tidal volume ventilation improves survival in the acute respiratory distress syndrome (17A).

Therefore, understanding how lung endothelial cells sense mechanical forces may lead to clinically relevant interventions in conditions where pulmonary capillary pressure is elevated. In this report, we examine the complex, nonlinear dynamics between transendothelial hydrostatic pressure and hydraulic conductivity ($L_p$) as a means to investigate the mechanism(s) of pressure-induced mechanotransduction. We show that lung microvascular endothelial cells in vivo maintain many functional similarities with in situ and intact lung preparations and retain their mechanosensing pathway(s). Herein, we provide a detailed characterization of the nonlinear dynamics of the pressure vs. $L_p$ relationship and show that heparan sulfates, NO, and ROS actively participate in pressure-mediated barrier dysfunction.

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penicillin-streptomycin). An air-bubble flow marker was tracked as it passed through an 18-cm length of clear borosilicate capillary tubing. A digital video camera was mounted on a stand overlooking the glass capillary tubes and streamed continuous digital images of the capillary tubes and air bubbles to the Data Acquisition virtual instrument written in LabVIEW (National Instruments, Austin, TX).

At the end of every experiment, each monolayer was assessed for confluency by fixation in 4% neutral-buffered formalin for 30 min followed by 15 min in Ladd multiple stain (Ladd Research Industries, Williston, VT). Monolayers were examined microscopically under high power for any evidence of nonconfluency or damage. All monolayers included in the data analysis were 100% confluent at the conclusion of the experiment.

**Pressure Protocols**

Three different protocols, based on the size of the pressure step (ΔP), were used to examine the pressure vs. Lp relationship. In Protocol 1, we tested step increases in ΔP = 5 cmH2O, using pressures of 10 to 15 to 20 cmH2O, with monolayers being exposed to each pressure for 1 h. In Protocol 2, we tested step increases in ΔP = 10 cmH2O (i.e., 10 to 20 to 30 cmH2O), and in Protocol 3, we tested step increases in ΔP = 20 cmH2O (10 to 30 cmH2O). In all experiments, monolayers were exposed to each pressure for 1 h.

**Heparanase**

Heparanase III (E.C.4.2.2.8) was dissolved in MCDB-131 (without serum) to a final concentration of 15 mU/ml. Cells were washed three times with plain MCDB-131, incubated with heparanase III (15 mU/ml) for 1 h, and finally rinsed twice with plain MCDB-131. Heparanase was not present in the media during the measurements of Lp.

**NO Synthase Inhibition**

L-NAME (Nω-nitro-L-arginine methyl ester HCl) and d-NAME (Nω-nitro-D-arginine methyl ester HCl; Sigma Chemical, St. Louis, MO) were dissolved in MCDB-131 (no serum) to a final concentration of 20 μM. Cells were incubated with L-NAME (10 μM) for 1 h before assembling the permeability chamber, and media with L-NAME (10 μM) were used to fill the permeability chamber such that L-NAME was present in the media during the experiment.

d-NAME was used as an inactive control for assessing the inhibition of NO synthase. Cells were incubated with d-NAME (10 μM) for 1 h before assembling the permeability chamber, and media with d-NAME (10 μM) were used to fill the permeability chamber such that d-NAME was present in the media during the experiment.

**Intracellular ROS Scavenging**

TBAP [tetakis-(4-benzoic acid) porphorin; Biomol, Plymouth Meeting, PA] was dissolved in 2% aqueous bicarbonate solution per manufacturer’s instruction to a final working concentration of 100 μM. Cells were incubated with TBAP (100 μM) for 1 h, and TBAP was present in the media during the experiments.

**Stretch-Activated Ion Channels**

Gadolinium (Gd3+ Cl-6-hydrate; Sigma Chemical) was dissolved in MCDB-131 to a final concentration of 20 μM, cells were incubated for 1 h, and gadolinium was present in the media during experiments.

**NO/ROS Fluorescence**

2′7′-Dichlorofluorescein diacetate (DCFA; 10 μM), a cell-permeable, ROS-sensitive dye, was added to cell culture media inside the permeability chamber and was present during the duration of selected experiments. At the end of an experiment, the Snapwell chamber was rinsed with PBS and fixed in 2% paraformaldehyde. The polycarbonate filter was cut off the Snapwell chamber and mounted on a slide using Fluoromount-G (Southern Biotechnology, Birmingham, AL) and covered with a glass coverslip. Fluorescent images were obtained with an Olympus microscope (model 1X70) equipped with a ×20 objective and a CCD camera (C4742m; Hamamatsu, Bridgewater, NJ). Images of multiple high-powered fields (hpf) containing ~200 cells/hpf were captured and analyzed for fluorescence intensity using IPLab imaging software (version 3.5.5; Scanalytics, Rockville, MD).

**Statistics**

Data are expressed as means ± SE with differences between groups and treatments assessed using ANOVA followed by Student’s t-test or multiple comparisons test(s) using JMP 5.0 statistical software (SAS, Cary, NC).

**RESULTS**

While characterizing the permeability behavior of BLMVEC (8) over a range of physiological pressures, we observed a nonlinear relationship between the volumetric flux rate, Jv (cm/s), and hydrostatic pressure, ΔP (cmH2O). As the Starling equation predicts a linear response of Jv vs. ΔP, this nonlinear behavior could only be explained by an increase in Lp in direct response to ΔP. Therefore, we examined Lp as a function of increasing ΔP over a physiological range of pressures (10–30 cmH2O) and tested several hypotheses regarding the underlying mechanism(s).

BLMVEC were held at ΔP = 0 cmH2O for 1 h, and then ΔP was stepped to 10 cmH2O for 1 h. Lp was stable at ΔP = 10 cmH2O with an average Lp of 3.65 × 10−7 ± 6.52 × 10−8 cm·s−1·cmH2O−1 (n = 10). Lp at 10 cmH2O (Lp10cmH2O) will be considered “baseline” for further comparisons. Lp10cmH2O was stable for up to 4 h (data not shown). Data are presented as both absolute values (10−7 cm·s−1·cmH2O−1) or as “normalized Lp” derived by dividing Lp curves by the average Lp obtained during the last 5 min at 10 cmH2O (i.e., average Lp at time = 55–60 min).

**Effect of ΔP Step Size on Lp**

**Protocol 1.** ΔP was stepped in 5-cmH2O increments, from 10 to 15 cmH2O and then to 20 cmH2O, for 1 h at each pressure, and results demonstrate Lp increased in a nonlinear manner: Lp10cmH2O = 3.65 × 10−7 ± 6.52 × 10−8 cm·s−1·cmH2O−1, Lp15cmH2O = 6.31 × 10−7 ± 1.56 × 10−7 cm·s−1·cmH2O−1, and Lp20cmH2O = 1.51 × 10−6 ± 5.25 × 10−7 cm·s−1·cmH2O−1. This pressure-induced increase in Lp represents a 1.73- and 4.14-fold increase, respectively, above the baseline (Figs. 1 and 2). Lp20cmH2O is significantly different than both Lp10cmH2O and Lp15cmH2O (Fig. 2; P < 0.05, n = 10).

**Protocol 2.** Step increase of ΔP = 10 cmH2O on Lp was examined. In this series of experiments, BLMVEC were held at ΔP = 0 cmH2O for 1 h, and then stepped to 10 cmH2O for determination of baseline, after which ΔP was stepped to 20 and then 30 cmH2O. The step in ΔP from 10 to 20 cmH2O resulted in 2.53 ± 0.38-fold increase in Lp, and the step to ΔP = 30 cmH2O resulted in a 14.54 ± 2.71-fold increase in Lp (Lp30cmH2O is significantly different than both Lp10cmH2O and Lp20cmH2O; P < 0.05, n = 13).

**Protocol 3.** Pressure was increased in a step of ΔP = 20, from baseline of ΔP = 10 to a final pressure of 30 cmH2O. This step increase in pressure resulted in an 11.04 ± 3.8-fold increase in Lp (Fig. 2; P < 0.05, n = 9).
Effect of Heparanase On Pressure vs. Lp Response

Secondary mediators that promote barrier dysfunction. Time-Dependent Effect

Protocol 1 (monolayers exposed to 10 to 15 to 20 cmH2O) was tested. In Protocol 3, 86% of monolayers (7 of 9) showed an increase in Lp. For all statistical comparisons, nonresponders in each group were included in the analysis of that data set and account for the larger SE at higher pressures.

Time-Dependent Effect

Not all BLMVEC monolayers responded to changes in ΔP. In Protocol 1, ~80% (8 of 10) of monolayers displayed an increase in Lp. In Protocol 2, 100% (n = 13) of monolayers responded to the increased pressure with an increase in Lp. In Protocol 3, 86% of monolayers (7 of 9) showed an increase in Lp. For all statistical comparisons, nonresponders in each group were included in the analysis of that data set and account for the larger SE at higher pressures.

Effect of Heparanase On Pressure vs. Lp Response

Heparanase III (15 mU/ml) increased baseline Lp by 2.4-fold, from 3.91 × 10^{-7} to 9.56 × 10^{-7} cm·s^{-1}·cmH2O^{-1} (P < 0.05) (Fig. 3A). Importantly, however, heparanase pretreatment completely abolished the pressure-induced increase in normalized Lp (P < 0.05 for heparanase Lp at 15 and 20 cmH2O vs. control at 15 and 20 cmH2O; n = 6; Fig. 3B).

To determine if the heparanase-induced change in baseline Lp altered the ability of cells to respond to ΔP, we plotted baseline Lp for a wide range of measured values vs. the fold-increase in Lp at 20 cmH2O. As can be seen in Fig. 4, there was no relationship between baseline Lp and the pressure-induced Lp response. That is, the pressure-induced Lp response was remarkably consistent over a wide range of baseline Lp values. Thus the effect of heparanase on basal permeability appears quite distinct from its ability to attenuate the pressure-induced increase in Lp. These data suggest that heparanase directly altered the ability of the cells to respond to increases in hydrostatic pressure.

Effect of NO Synthase Inhibition On Pressure vs. Lp Response

Pretreatment of BLMVEC with l-NAME (1, 10, and 100 μM) to inhibit endothelial NO synthase had variable effects on Lp. l-NAME (1 μM) had no effect on baseline Lp and no effect on the pressure-induced increase in Lp (data not shown). l-NAME (10 μM) had no effect on baseline Lp at 10 cmH2O or on Lp at 20 cmH2O but significantly attenuated the pressure-induced increase at 30 cmH2O as shown in Fig. 5 (P < 0.05 at 30 cmH2O vs. control; n = 5). l-NAME (100 μM) made cells so leaky that Lp could not be measured. In contrast, d-NAME (10 μM), while having no effect on baseline permeability, had no effect on pressure-induced permeability results. d-NAME-treated cells were similar to controls at all pressures but significantly different than l-NAME treated cells at 30 cmH2O (Fig. 5).

Effect of Intracellular ROS Scavenging On Pressure vs. Lp Response

TBAP (100 μM), a cell-permeable SOD mimetic that scavenges both oxygen radicals and peroxynitrite, significantly attenuated the pressure-induced Lp at 30 cmH2O without altering baseline Lp (Fig. 6). Baseline Lp in these experiments were 1.51 × 10^{-7} ± 6.47 × 10^{-8} cm·H2O^{-1} for control and 1.27 × 10^{-7} ± 6.63 × 10^{-8} cm·H2O^{-1} for TBAP (P > 0.05; n = 6). Step increases in pressure increased Lp in the control group by 2.17 ± 0.61-fold at 20 cmH2O and by 1.42 ± 0.49-fold at 30 cmH2O (P < 0.05; n = 6). In the TBAP-treated group, Lp was stable at 20 cmH2O, remaining at 1.85 ± 0.41-fold above baseline (P > 0.05; n = 6) and at 30 cmH2O Lp increased to only 7.33 ± 1.67-fold above baseline (vs. controls = 14.42-fold increase at 30 cmH2O). TBAP significantly reduced the increase in Lp vs. control monolayers at 30 cmH2O (P < 0.05).

Fig. 1. Normalized hydraulic conductivity (Lp) vs. time (h) during step increases in lung capillary pressure (ΔP) to 10, 15, and 20 cmH2O. Lp was normalized to average value measured during the time interval from 55 to 60 min. Lp at 20 cmH2O was significantly greater than Lp at 10 cmH2O. The nonlinear increase in Lp cannot be explained by a simple Starling mechanism. Data are presented as normalized means ± SE, n = 10.

Fig. 2. Comparison of different pressure step protocols on Lp. Protocol 1 tests pressure increases of 5 cmH2O (n = 6). In the D-NAME-treated group, Lp at 20 cmH2O was significantly greater than Lp at 10 cmH2O. The nonlinear increase in Lp cannot be explained by a simple Starling mechanism. Data are presented as normalized means ± SE, n = 10.

Fig. 3. Comparison of variable effects of heparanase Lp at 10 and 20 cmH2O; *significantly different vs. Lp at 10 cmH2O; **significantly different vs. Lp at 10 cmH2O; ***significantly different vs. Lp at 10 cmH2O. P < 0.05 used for all comparisons.

Fig. 4. Effect of intracellular ROS scavenging on pressure vs. Lp relationship.
Effect of Interventions On NO/ROS Intracellular Fluorescence

Intracellular ROS were visualized using the fluorescent dye DCFA. Monolayers exposed to 10 cmH2O for 1 h had little or no measurable intracellular fluorescence (Fig. 7A; control 10 cmH2O). In contrast, cells exposed to ΔP = 30 cmH2O for 1 h had a 3.8-fold increase (P < 0.05 vs. control) in intracellular fluorescence (Fig. 7A; control 30 cmH2O). Monolayers pretreated with heparanase III and then exposed to ΔP = 30 cmH2O had no measurable increase in intracellular fluorescence (Fig. 7A; HEP 30 cmH2O). These data are also presented in Fig. 7B as a graph of intracellular fluorescence intensity in each group.

The effect of L-NAME on intracellular NO/ROS was assessed by measuring DCFA fluorescence in cells treated with L-NAME (10 μM) and then exposed to 30 cmH2O pressure for 1 h. L-NAME (10 μM) completely abolished the increase in DCFA fluorescence in cells exposed to 30 cmH2O (Fig. 7A; L-NAME 30 cmH2O). D-NAME (10 μM) had no effect on pressure-induced increase in DCFA fluorescence (Fig. 7A; D-NAME 30 cmH2O).

The effect of TBAP on ROS-induced intracellular fluorescence was examined in cells exposed to 30-cmH2O pressure for 1-h duration. TBAP (100 μM) significantly reduced the ROS-induced intracellular fluorescence after exposure to 30 cmH2O compared with control cells at 30 cmH2O (P < 0.05) as shown in Fig. 7A (TBAP, 30 cmH2O). Figure 7B presents means ± SE of DCFA fluorescence (n = 10–12 hpf for each group).
that vascular endothelial cells. In the present study, we observed may modulate the pressure-induced increases in lung microbarrier regulation (4) and hypothesized that heparan sulfates glycocalyx participate in mechanotransduction (7) and active permeability. We have shown recently that components of the law despite substantial evidence both in vivo (5, 6, 17, 18, 20, ally has been accepted that lung fluid balance obeys Starling's

tions. Lp was stable at pressures of 10 and 15 cmH2O for more than 2 h, and only after increasing pressure to 20 cmH2O did the mechanotransduction system become activated to a sufficient extent to increase Lp. Therefore, we believe that permeability studies conducted over shorter time periods may have been inadequate to achieve sufficient activation mechanotransduction pathway(s).

Our primary hypothesis was that heparan sulfates participate in pressure-induced mechanotransduction. Our results show a dual role for heparan sulfates in lung capillary barrier function: 1) as part of a passive, molecular filter, and 2) as an active signaling element. Heparanase increased baseline Lp10cmH2O demonstrating that heparan sulfates are part of the normal permeability barrier. To determine if the increase in baseline Lp altered the ability of cells to respond to increased pressure, we plotted baseline Lp vs. fold increase in Lp at 20 cmH2O (Fig. 4). When plotted this way, the pressure-induced increase in Lp was observed to be consistent across a wide range of baseline values. The range of baseline Lp in this plot includes the absolute values measured for the heparanase-treated group, suggesting that the mechanotransduction response is independent of baseline Lp. Therefore, we conclude that heparanase directly altered mechanotransduction on the cell surface.

We (7) have previously shown that heparan sulfates modulate mechanotransduction resulting in NO production and that NO participates in pressure-induced increase in Jv (23). Thus we had a mechanistic link to hypothesize that the heparan sulfates may also participate in a pressure-mediated increase in Lp. Therefore, we tested the NO inhibitor, L-NAME (10 μM), and found that it attenuated the pressure-induced increase in Lp by ~50%, while higher concentrations of L-NAME (100 μM) made the cells so permeable that Lp could not be measured. There were important differences in the response of aortic endothelial cells and lung microvascular endothelial cells to increased hydrostatic pressure and L-NAME. Based on data from Tarbell et al. (23), aortic endothelia were much less sensitive to changes in pressure and required a 10-fold higher concentration of L-NAME to achieve a similar attenuation of the Lp increase compared with lung microvascular endothelium in the present study.

As L-NAME only blocked 70% of the Lp response to pressure, we tested interventions aimed at other known mediators of mechanotransduction. Hwang et al. (9) and Ali et al. (2) reported that NAD(P)H oxidase-generated ROS are increased in endothelial cells exposed to shear stress and cyclic strain, respectively. To test whether ROS were generated by pressure in BLMVEC, we utilized the cell-permeable SOD mimetic, TBAP, and observed that it significantly attenuated the pressure-induced Lp response by ~60%. TBAP scavenges both ROS and peroxynitrite, and thus we do not know whether two separate pathways (NO synthase and NADPH oxidase) are activated in response to pressure or whether NO is simply converted to peroxynitrite, which is the primary barrier altering agent. Studies are underway to elucidate the role of NAD(P)H oxidase.

To establish a mechanistic link between the ability of heparanase and NO/ROS inhibitors to attenuate the pressure-Lp response, we fluorescently quantified ROS levels after exposure to elevated hydrostatic pressure and after the combination of heparanase pretreatment and elevated hydrostatic pressure. Heparanase completely abolished the increase in intracellular ROS induced by pressure. Similarly, L-NAME and TBAP both attenuated a pressure-induced increase in Lp and attenuated pressure-induced ROS production. Taken together, these data
suggest that hydrostatic pressure activates the production of ROS via cell surface heparan sulfates and contributes to pressure-mediated barrier dysfunction.

Blockade of stretch-activated ion channels with gadolinium had no effect on the pressure-Lp response. In our system, cells are cultured on an inflexible polycarbonate membrane, and, therefore, cell stretch is unlikely to result when pressure is elevated. The effect of pressure on stretching polycarbonate filters was examined and found not to influence a pressure-induced increase in endothelial permeability (23). The lung capillary wall is compliant, and increases in hydrostatic pressure appear to distend the capillary wall and stretch endothelial cells enough to activate stretch-sensitive ion channels as evidenced by the inhibitory role of Gd3+ on both elevated vascular pressure- and high airway pressure-induced capillary leak (14, 19).

Fig. 7. A: intracellular ROS visualization using 2′,7′-dichlorofluorescin diacetate (DCFA). a: Control monolayers after 1 h at 10 cmH2O show little intracellular fluorescence. b: Control monolayers after exposure to 30 cmH2O; all cells show a significant increase in intracellular ROS fluorescence. c: Heparanase (HEP)-treated monolayers after exposure to 30 cmH2O; heparanase completely abolished the pressure-induced increase in intracellular ROS. d: 10 μM d-NNAME-treated monolayers after 1 h at 30 cmH2O shows a significant increase in fluorescence (no difference vs. controls). e: L-NNAME (10 μm) significantly attenuates pressure-induced fluorescence at 30 cmH2O. f: TBAP (100 μM) significantly attenuates pressure-induced fluorescence at 30 cmH2O. B: average fluorescence intensity plotted per group in cmH2O. *Significantly different than controls at 10 cmH2O (P < 0.05); **significantly different than controls at 30 cmH2O (P < 0.05). Fluorescence intensity derived from averages of n = 12 high-powered fields (hpf) containing ~200 cells/hpf. Data are presented as means ± SE.
There are many similarities between the cell culture model described here and the intact lung models. We measured baseline $L_p$ values that are identical to values measured directly from intact pulmonary capillaries, including $2.9 \times 10^{-7}$ (Ref. 3), $3.4 \times 10^{-7}$ (Ref. 12), and $6.6 \times 10^{-7}$ ml/cm²/s (Ref. 21). The magnitude of the pressure-induced increase in $L_p$ was very similar between our in vitro model and intact lungs. For example, in vitro, the increase in $L_p$ over the pressure range of 10–30 cmH₂O was ~20-fold, whereas the increase in the whole lung filtration coefficient ($K_{fc}$) was nearly similar at 5.10⁻³ (Ref. 2), 5.34 (Ref. 3), and 6.60⁻³ ml/cm²/s (Ref. 21). Similarly, small changes in hydrostatic pressure increased endothelial NO and ROS in intact lungs (11, 13), and this increase in ROS production resulted in an inflammatory phenotype, similar to our culture model. Collectively, our cell culture model maintains many characteristics of the in situ capillary.

In summary, we have shown that lung capillary endothelial cells possess an active mechanism to respond to increases in hydrostatic pressure, which involves NO/ROS and leads to increased $L_p$. This mechanism is dependent on heparan sulfates hydrostatic pressure, which involves NO/ROS and leads to increased $L_p$. This is the first report to demonstrate a role for heparan sulfates in pressure-mediated mechanotransduction, and this may have important clinical implications during conditions where pulmonary microvascular pressure is elevated.

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