Mechanotransduction in the Lung
Pressure-induced endothelial Ca\(^{2+}\) oscillations in lung capillaries

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Kuebler, Wolfgang M., Xiaoyou Ying, and Jahar Bhattacharya. Pressure-induced endothelial Ca\(^{2+}\) oscillations in lung capillaries. Am J Physiol Lung Cell Mol Physiol 282: L917–L923, 2002; 10.1152/ajplung.00275.2001.—Endothelial second messenger responses may contribute to the pathology of high vascular pressure but remain poorly understood because of the lack of direct in situ quantification. In lung venular capillaries, we determined endothelial cytosolic Ca\(^{2+}\) concentration \([\text{Ca}^{2+}]_i\) by the fura 2 ratioing method. Pressure elevation increased mean endothelial \([\text{Ca}^{2+}]_i\), by Ca\(^{2+}\) influx through gadolinium-inhibitable channels and amplified \([\text{Ca}^{2+}]_i\) oscillations by Ca\(^{2+}\) release from intracellular stores. Endothelial \([\text{Ca}^{2+}]_i\) transients were induced by pressure elevations as little as 5 cmH\(_2\)O and increased linearly with higher pressures. Heptanol inhibition of \([\text{Ca}^{2+}]_i\), oscillations in a subset of endothelial cells indicated that oscillations originated from pacemaker endothelial cells and were propagated to adjacent nonpacemaker cells by gap junctional communication. Our findings indicate the presence of a sensitive, active endothelial response to pressure challenge in lung venular capillaries that may be relevant in the pathogenesis of pressure-induced lung microvascular injury.

ELEVATION OF LUNG microvascular pressures increases lung fluid leak and thereby predisposes the lung to pulmonary edema, a condition associated with high morbidity and mortality. Although this pathology has been traditionally attributed to the passive effects of high pressure (13, 33), growing evidence implicates inflammation as an active response of the pressure-challenged capillary. This is supported by the presence of inflammatory cells in the bronchoalveolar lavage in hydrostatic and high-altitude pulmonary edema (10, 17, 18, 26) and by our previous finding that pressure induces proinflammatory responses in the lung capillary, as reflected in the expression of leukocyte adhesion receptors (21). These considerations indicate that the lung capillary response to pressure challenge is more complex than previously believed and must be better understood.

Here we consider the question in the context of the endothelial cytosolic Ca\(^{2+}\) concentration \([\text{Ca}^{2+}]_i\). We have shown that increases of the endothelial cell (EC) \([\text{Ca}^{2+}]_i\), determine P-selectin expression in the lung capillary (21). However, since low-frequency \([\text{Ca}^{2+}]_i\), oscillations are constitutive in lung capillaries (35), it is important to understand the extent to which the oscillations form a part of the lung response to pathologic stimuli. Pacemaker EC located at capillary branch points generate \([\text{Ca}^{2+}]_i\), oscillations that propagate along the capillary wall as intercellular Ca\(^{2+}\) waves (35). Because \([\text{Ca}^{2+}]_i\); oscillations have come under increased scrutiny as inducers of cell signaling pathways (1, 11, 12), we considered the possibility that enhancement of \([\text{Ca}^{2+}]_i\), oscillations in the capillary wall may constitute a feature of the lung capillary response to pressure elevation.

MATERIALS AND METHODS

Fluorescent probes and drugs. Fura 2-AM (5 μM; Molecular Probes, Eugene, OR), gadolinium (GdCl\(_3\), 10 μM) and heptanol (3 mM; Sigma, St. Louis, MO) were diluted in 2% dextran (70 kDa; Pharmacia Biotech, Piscataway, NJ)-1% fetal bovine serum (Gemini Bio-Products, Calabasas, CA) HEPES solution containing 150 mmol/l Na\(^+\), 5 mmol/l K\(^+\), 1.5 mmol/l Ca\(^{2+}\), and 20 mmol/l HEPES (Calbiochem-Novabiochem, La Jolla, CA).

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biochem, San Diego, CA) at pH 7.4 and osmolarity of 295 mosM.

**Lung preparation.** Experimental procedures and setup have previously been described (21, 35). In brief, lungs excised from anesthetized Sprague-Dawley rats were continuously pumped perfused with 14 ml/min autologous rat blood at 37°C. Lungs were constantly inflates with positive airway pressure of 5 cmH2O. At baseline, pulmonary artery pressure and left atrial pressure (P_LA) were adjusted to 10 and 5 cmH2O, respectively. A microcatheter (PE-10; Baxter Diagnostics, McGaw Park, IL) was advanced through the left atrium and wedged in a pulmonary vein, allowing for local infusion of solutions (20, 35). Lungs were positioned on a vibration-free air table. To prevent drying, the surface was superfused with normal saline at 37°C.

**Fluorescence microscopy.** Our methods for intravital microscopy have been reported (21, 35). Fluorophores were excited by mercury lamp illumination directed through a filter wheel and shutter (Lambda-10; Sutter Instrument, Novato, CA) equipped with filters appropriate for excitation at 340 and 380 nm (340HT15 and 380HT15; Omega Optical, Brattleboro, VT). Fluorescence emission was collected through an ultraviolet-compatible objective (Wplan FL, Brattleboro, VT). Fluorescence emission was collected at 340 and 380 nm (340HT15 and 380HT15; Omega Optical, VT). Fluorescence emission was collected through an ultraviolet-compatible objective (Wplan FL ×40 UV; Olympus America, Melville, NY), dichroic and emission filters (400 DCLPO2 and 510WB40; Omega Optical) by image intensifier (K13818; Video Scope International, Sterling, VA), and video camera (CCD-72; Dage-MIT, Michigan City, IN), then subjected to digital image analysis (MCID-M4; Imaging Research, St. Catharine’s, ON, Canada). Single venular capillaries were viewed at a focal plane corresponding to maximum diameter (12–20 μm).

[Ca2+]i imaging and analysis. EC [Ca2+]i, in lung venular capillaries was quantified using our previously described fura 2 ratiometric imaging technique (35). Membrane-permeant fura 2-AM, which deesterifies intracellularly to impermeant fura 2, was infused continuously for 20 min into pulmonary venular capillaries using the venous microcatheter. Fluorescence images were obtained at 10-s intervals. For each image, four sets of frame-averaged exposures were obtained that alternated every 132 ms between excitations of 340 and 380 nm. EC [Ca2+]i was determined from the computer-generated 340/380 ratio based on a K_d of 224 nmol/l and appropriate calibration parameters (14, 35). EC [Ca2+]i oscillations were analyzed for amplitude and frequency using fast Fourier transformation (FFT; Origin 3.54, Microlcm Software, Northampton, MA) (7). Data presented refer to the dominant wave component, i.e., the wave with highest amplitude, which we had previously identified to occur at the low-frequency end of the amplitude frequency spectrum (35). FFT analyses of imaging noise in our recording system, determined by recording fura 2 fluorescence 1 in unstimulated cultured bovine pulmonary artery EC (35), 2 in alveolar epithelial cells in situ, which do not exhibit spontaneous [Ca2+]i oscillations (1), or 3 in EC pretreated with the intracellular Ca2+ chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-AM, revealed wave components with amplitudes of <10 nmol/l. Hence, to exclude contamination from noise data, in the experimental FFT analyses, we rejected all wave components with an amplitude of <15 nmol/l. Capillary diameters were determined in triplicate from the 380-nm images as the perpendicular distance between longitudinal lines drawn through the endothelial mid-axis.

**Experimental protocols.** 1) Pressure elevation: baseline recordings were obtained at P_LA of 5 cmH2O. P_LA was raised to 10, 15, or 20 cmH2O, respectively, for 30 min by adjusting the height of the venous outflow. 2) Inhibition of external Ca2+ entry; the trivalent lanthanide gadolinium (10 μM), which effectively blocks stretch-activated Ca2+ influx (16), was added to the perfusing blood 10 min before P_LA elevation. 3) Gap junction uncoupling: heptanol (3 mM), the gap junctional uncoupler (9), was added to the perfusing blood 10 min before P_LA elevation. 4) Combined inhibition of external Ca2+ entry and gap junction uncoupling: both gadolinium (10 μM) and heptanol (3 mM) were added to the perfusing blood 10 min before P_LA elevation. 5) Ca2+-free conditions: infusion of nominally Ca2+-free HEPES solution containing 2% dextran and EGTA (0.5 mmol/l) through the venous microcatheter was started 10 min before P_LA elevation.

**Statistics.** All data are means ± SE. Values of several groups were compared by Wilcoxon and Friedman tests for dependent groups and by Kruskal-Wallis and Mann-Whitney U-tests for independent groups. Spearman’s coefficient of correlation, r_s, was calculated to test correlation between parameters, and linear regression analysis was performed (SigmaPlot; Jandel Scientific, San Rafael, CA). Statistical significance was assumed at P < 0.05.

**RESULTS**

We quantified EC [Ca2+]i in digitally imaged, single venular capillaries of the isolated blood-perfused rat lung. Baseline [Ca2+]i, was determined at P_LA of 5 cmH2O, which approximates physiological pressure in lung venular capillaries (5).

Increasing P_LA from 5 to 20 cmH2O increased mean EC [Ca2+]i and the amplitude of EC [Ca2+]i oscillations. Both increases were initiated after a lag of 3–5 min and continued progressively for the 30-min pressure elevation period (Fig. 1). The oscillation frequency did not change from baseline. Returning P_LA to 5 cmH2O reestablished baseline [Ca2+]i values in <2 min.

To determine the effects of different levels of pressure increase, we instituted P_LA elevations to 10, 15, or 20 cmH2O in random order in each of which we held baseline conditions for 15 min before pressure elevation. The resulting EC [Ca2+]i increases at the end of each 30-min pressure elevation period occurred in proportion to the imposed pressure increase (Fig. 2).

To determine the effect of external Ca2+ entry on these responses, we instituted P_LA elevations to 20 cmH2O while infusing capillaries with either gadolinium, the inhibitor of cell membrane calcium channels, or Ca2+-free HEPES. Both treatments inhibited the increase in mean [Ca2+]i, but not the increase in [Ca2+]i oscillations (Fig. 3), indicating that amplitude increases were independent of external Ca2+ entry.

Intercellularly propagating EC [Ca2+]i waves were evident in these capillaries both during baseline and pressure elevation (Fig. 4A). These [Ca2+]i waves emanated from a specific subset of EC located at the microvascular branch points, the “pacemakers,” and were propagated to adjacent EC. As previously reported (35), [Ca2+]i was 30% higher in pacemaker, compared with nonpacemaker, EC (Fig. 4B). This difference was also preserved when lungs were perfused with Ca2+-free HEPES (data not shown, n = 6).

To compare pressure responses of pacemaker vs. nonpacemaker EC, we determined the diameter in-
Fig. 1. Temporal profile of endothelial cell cytosolic Ca\(^{2+}\) concentration (EC [Ca\(^{2+}\)]\text{\textsubscript{i}}) response to increased pulmonary artery pressure (P\textsubscript{LA}). A: images are of the 340-/380-nm fluorescence ratio color-coded for [Ca\(^{2+}\)]\text{\textsubscript{i}}. Images were obtained at P\textsubscript{LA} (in cmH\textsubscript{2}O) of 5 (left) and after 30 min of P\textsubscript{LA} elevation to 20 (right). Note marked vasodilation after P\textsubscript{LA} elevation (dotted line). Vessel margins are depicted by line sketch, blood flow direction is by arrow. B: [Ca\(^{2+}\)]\text{\textsubscript{i}} profile in a single EC of lung venular capillary. [Ca\(^{2+}\)]\text{\textsubscript{i}} was determined continuously in 10-s intervals at baseline (P\textsubscript{LA}, 5 cmH\textsubscript{2}O), during 30 min of P\textsubscript{LA} elevation to 20 cmH\textsubscript{2}O, and for 5 min after return to baseline P\textsubscript{LA}. Arrowheads, baseline [Ca\(^{2+}\)]\text{\textsubscript{i}} oscillations; arrows, pressure-induced oscillations of increased amplitude. Arrowheads, baseline [Ca\(^{2+}\)]\text{\textsubscript{i}} oscillations; arrows, pressure-induced oscillations of increased amplitude. C: group data of mean EC [Ca\(^{2+}\)]\text{\textsubscript{i}} (top) and amplitude of [Ca\(^{2+}\)]\text{\textsubscript{i}} oscillations (bottom) are shown as 5-min averages at baseline (P\textsubscript{LA}, 5 cmH\textsubscript{2}O), during 30 min of P\textsubscript{LA} elevation to 20 cmH\textsubscript{2}O, and for 5 min after return to baseline P\textsubscript{LA}. Data from n = 8 capillaries, *P < 0.05 vs. baseline (P\textsubscript{LA}, 5 cmH\textsubscript{2}O).
creases at capillary branch-point and midsegmental locations. Pressure elevation from 5 to 20 cmH2O increased these diameters by 10.22% and 38.5%, respectively (Fig. 4B). This difference was statistically significant (P < 0.05), indicating that despite the equal pressure increase the increase of diameter was considerably less at branch points. Despite this difference, both [Ca2+]i oscillations and mean [Ca2+]i increased to the same extent at branch-point and midsegmental EC.

To determine the role of intercellular communication in these responses, we infused capillaries with the cell communication inhibitor heptanol. In the presence of heptanol, pressure-induced [Ca2+]i oscillations were blocked in midsegmental, but not in branch-point, EC (Fig. 4C), indicating that the oscillations originated in branch-point pacemaker EC and propagated to adjacent EC through gap junctions. However, heptanol did not block the pressure-induced increase of mean [Ca2+]i in midsegmental or branch-point EC. When capillaries were infused with both heptanol and gadolinium, the [Ca2+]i response in midsegmental EC was completely abrogated (Fig. 4D).

**DISCUSSION**

We show here that increasing lung vascular pressure enhanced the amplitude, but not frequency, of EC [Ca2+]i oscillations, while also increasing the mean EC [Ca2+]i. The enhanced oscillations were inhibited by heptanol in midsegmental but not in branch-point EC, indicating that the oscillations arose in branch-point pacemaker EC then propagated to adjacent EC by gap junctional communication. Importantly, neither gadolinium nor external Ca2+-free conditions blocked the enhanced oscillations in branch-point EC, indicating that the oscillations were driven by intracellular Ca2+ release and not by influx of external Ca2+. These findings indicate that, in EC in situ, pressure-induced cell stretch enhances Ca2+ oscillations.

**Temporal profile.** The ~5-min lag in the onset of these [Ca2+]i responses contrasts with the response in

![Fig. 2. Sensitivity of EC [Ca2+]i response to increased P_LA. After baseline measurements (P_LA, 5 cmH2O), P_LA of 10, 15, and 20 cmH2O was established in random order for 30 min. Every pressure elevation period was followed by 15 min at P_LA = 5 cmH2O, which reestablished baseline [Ca2+]i. Data were quantified in the last 5 min of each pressure elevation period. Linear regression lines depict pressure-dependent increase of mean EC [Ca2+]i, (top, r_s = 0.937, P < 0.001) and amplitude of [Ca2+]i oscillations (bottom, r_s = 0.765, P < 0.001). Data from n = 6 capillaries, *P < 0.05 vs. baseline (P_LA, 5 cmH2O).

![Fig. 3. Mechanotransduction of EC [Ca2+]i response to increased P_LA. Inhibition of external Ca2+ entry (n = 6) or Ca2+-free conditions (n = 6) was established by infusions of gadolinium or Ca2+-free dextran, respectively, started 10 min before recordings. A: [Ca2+]i profile in a single EC of lung venular capillary. [Ca2+]i was determined continuously during gadolinium infusion in 10-s intervals at baseline (P_LA, 5 cmH2O), during 30 min of P_LA elevation to 20 cmH2O, and for 5 min after return to baseline P_LA. Arrowheads, baseline [Ca2+]i; oscillations; arrows, pressure-induced oscillations of increased amplitude. B: group data of mean EC [Ca2+]i, (top) and amplitude of [Ca2+]i oscillations (bottom) during infusion of gadolinium (open bars) or Ca2+-free dextran (solid bars) are shown as 5-min averages at baseline (P_LA, 5 cmH2O), during 30 min of P_LA elevation to 20 cmH2O, and for 5 min after return to baseline P_LA. *P < 0.05 vs. baseline (P_LA, 5 cmH2O), #P < 0.05 vs. control data in identical lungs (not shown).
cultured EC in which mechanical stretch mobilizes Ca$^{2+}$ within seconds (27, 30, 34). Although mechanisms are unclear, this lag was not due to delayed EC distortion since pressure-induced changes of capillary diameter are complete in <15 s (32). The lag may be protective since lung capillary EC are normally exposed to pressure pulsations transmitted from the arterial segment (3) and cyclic distortional stress during
the breathing cycle (23). The lag exceeds the time course of these distortional fluctuations, thereby ensuring the absence of potentially deleterious EC (Ca^{2+}) increases during these physiological cycles. Processes that quickly restore baseline [Ca^{2+}], after relief of pressure stress may also be protective for similar reasons.

Sensitivity. Previous studies of lung hydrostatic stress indicate that pressure elevations of >50 cmH₂O deteriorate the microvascular barrier (2, 28, 33), whereas more intermediate elevations to 20–50 cmH₂O increase the capillary filtration coefficient (13, 29) and the transvascular protein flow (25). Here, we demonstrate that P_LA increases of as little as 5 cmH₂O are capable of generating second messenger responses in lung capillary EC. P_LA elevation from 5 to 10 cmH₂O dilates and, thereby, circumferentially stretches lung venular capillaries by ~10% (32). Our findings indicate that, in the lung, microvascular pressure elevations that were previously considered nonpathogenic (22, 29) activate an important endothelial signaling mechanism that is potentially proinflammatory.

Mechanotransduction. Because pressure-induced [Ca^{2+}] oscillations occurred both under external Ca^{2+}-free conditions and in the presence of gadolinium, a nonspecific inhibitor of Ca^{2+} entry, we interpret the results to mean that the [Ca^{2+}] oscillations result from mobilization of intracellular Ca^{2+} stores, not entry of external Ca^{2+}. Ca^{2+} release from intracellular stores is mediated via two different receptor families, the inositol 1,4,5-triphosphate (IP₃) receptors and the ryanodine receptors. In addition, depletion of intracellular Ca^{2+} stores causes capacitative entry of extracellular Ca^{2+} through store-operated channels in the plasma membrane (4). Pacemaker-generated [Ca^{2+}] oscillations in lung capillaries are propagated as intercellular Ca^{2+} waves through gap junctions (35), a mechanism previously attributed to the generation and intercellular communication of IP₃ (6). Cultured EC subjected to cyclic stretch show increased phospholipase C activation leading to IP₃ generation (8, 31) and IP₃-mediated Ca^{2+} release from intracellular pools, which is not dependent on influx of extracellular Ca^{2+} (30). Hence, IP₃-mediated Ca^{2+} release may account for the pressure-induced generation of EC Ca^{2+} oscillations.

In heptanol-treated midsegmental EC, in which [Ca^{2+}], oscillations are absent, the pressure-induced increase of mean EC [Ca^{2+}] was not impaired. Hence, it may be independent from store-operated, capacitative Ca^{2+} influx. Because the increase of mean [Ca^{2+}] was inhibited by both gadolinium and external Ca^{2+}-free conditions, it resulted from entry of extracellular Ca^{2+}.

Pacemaker cells. Unexpectedly, diameter increases to pressure elevation varied regionally within the same capillary. Thus, for a pressure elevation from 5 to 20 cmH₂O, the diameter increase at capillary branch points was markedly less than that at midsegments, yet pressure-induced [Ca^{2+}]; transients were not different between these locations. These differential diameter responses suggest that pacemaker EC at branch points were stretched less than midsegmental EC. Nevertheless, they generated [Ca^{2+}]; transients that were similar, suggesting that pacemaker EC may be more mechanosensitive to stretch.

Mechanotransduction in the capillaries was heterogeneous in that, although gadolinium-inhibitable channels were present in all EC, pressure-induced [Ca^{2+}] oscillations were exclusively generated in pacemaker EC and propagated to adjacent nonpacemaker EC by intercellular communication. This pressure-induced response pattern is essentially similar to that induced pharmacologically in lung capillaries. Thus histamine induces Ca^{2+} entry in both pacemaker and nonpacemaker EC but generates [Ca^{2+}] oscillations only in pacemaker EC (35). The oscillations are then propagated to adjoining EC through gap-junctional communication. The unique ability of pacemaker EC to generate [Ca^{2+}], oscillations is not explained. A rheological explanation may be considered, in that shear forces acting on the vascular wall may differ between branch points and midsegments. However, baseline diameters do not differ between these locations, and low Reynolds number flows in lung capillaries make turbulence at branch points unlikely (19). The issue may be addressed by direct study of pacemaker EC. However, methods for isolating this specific subset of EC have not been established.

In conclusion, the present results indicate that increases of hydrostatic pressure evoke complex [Ca^{2+}] responses in lung capillary endothelium. Mean EC [Ca^{2+}] increases because of Ca^{2+} influx through membrane channels, whereas pacemaker-generated [Ca^{2+}] oscillations are induced presumably through store-release. Although the significance of this dual response remains unclear at present, we suggest that the rise of mean [Ca^{2+}] may provide the critical step that initiates secretion, for example, of P-selectin (21), or induces formation of nitric oxide by activating endothelial nitric oxide synthase (24). [Ca^{2+}] oscillations may activate mechanisms responsive to oscillation amplitude or frequency, as, for example, mitochondrial dehydrogenases (15), calmodulin-dependent protein kinases (11), or nuclear factor-κB-dependent gene transcription (12). Pacemaker EC may coordinate these mechanisms to regulate the overall response to pressure stress in individual capillary segments.

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