PECAM-1 and caveolae form the mechanosensing complex necessary for NOX2 activation and angiogenic signaling with stopped flow in pulmonary endothelium

John Noel,1 Hui Wang,1 Nankang Hong,1 Jian-Qin Tao,1 Kevin Yu,1 Elena M. Sorokina,1 Kristine DeBolt,1 Michelle Heayn,2 Victor Rizzo,2 Horace Delisser,3 Aron B. Fisher,1 and Shampa Chatterjee1

1Institute for Environmental Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania; 2Anatomy and Cell Biology, Temple University School of Medicine, Philadelphia, Pennsylvania; and 3Perelman Center for Advanced Medicine and Department of Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania

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Abstract

We have previously studied endothelial mechanotransduction using in situ (isolated lungs and aorta) as well as in vitro (flow-adapted cells) systems. We showed that endothelial cells respond to abrupt cessation of flow with a signaling cascade characterized by the generation of reactive oxygen species (ROS; Refs. 1, 5, 8, 31, 46). The response to loss of shear required a preceding period of exposure to flow to reach a flow adapted state. Statically cultured cells kept for short periods under flow did not show any response to stop of flow (5, 8, 35). Lack or removal of flow in this model did not compromise oxygen delivery to the lung tissues or cells as normal oxygen tension was maintained during stop of flow in vivo (ventilated lung) and in vitro (continuous flow of medium through side ports) (1, 5, 30, 46). We identified that the earliest change that occurred with cessation of flow was endothelial cell membrane depolarization [via deactivation of a K+ (KATP) channel] leading to NADPH oxidase 2 (NOX2) assembly.

What are the elements on the endothelium that sense the altered flow stimulus and activate a signaling cascade that leads to K+ channel closure and NADPH oxidase activation? We have observed that statically cultured endothelial cells demonstrate low KATP channel expression and activity that are increased upon exposure to prolonged periods of flow (5). This indicated that the channel is induced by flow, pointing to the presence of an upstream shear sensor or a primary transducer on the endothelium that “senses” flow and leads to increased channel expression. A cell surface entity would ideally be well suited to be a primary transducer. Based on previous studies on transduction of mechanical forces including shear stress by a mechanosensory complex comprising of platelet endothelial cell adhesion molecule 1 (PECAM-1; Ref. 42) and on reports of PECAM-1 activation (tyrosine phosphorylation) upon flow (9), we investigated whether PECAM-1, by virtue of its junc-tional location and cytoskeletal linkage, might serve as a

vascular physiology (such as embryonic morphogenesis and organization of the vascular tree) while irregular or abnormal shear can lead to vascular dysfunction and disease (19, 27). Thus the mechanosignaling that accompanies various shear profiles and patterns, regular or aberrant, governs susceptibility to atherosclerosis, by inducing athero-protective or athero-prone phenotypes in endothelial cells (10, 22). It thus becomes important to understand the link among the mechanical force, the shear sensing machinery and biochemical signaling within the cell. Despite recent advances in this field, relatively little is known about the sensing apparatus or machinery in cells that transduces the physical forces associated with changes in blood flow into signaling moieties.

Cells sense the physical stimulus in their environment and translate these physical forces into biochemical signals (20, 37). Sensing and responding to a physical force require specialized structures and machinery that can engage in signal transduction (12, 23, 42).

In the vascular system, with a highly distributed network of blood vessels, mechanical forces arising from blood flow initiate signaling that helps maintain vascular structure and function. Indeed, shear associated with blood flow is sensed by the endothelium and the resultant signaling regulates normal
mechanosensor for the loss of blood flow. Our earlier studies on mechanotransduction showed that lack of caveolin-1 (and thus caveolae) prevented the pulmonary endothelial ROS production with cessation of flow (33, 45). We thus investigated the role of PECAM-1 in mechanosensing and the association (if any) of PECAM-1 and caveolin-1 (and thus caveolae) in the pulmonary endothelium.

Based on our results, we conclude that PECAM-1 is an important component of the mechanosensing machinery in the pulmonary endothelium and conversely that the biochemical and cellular responses to altered flow are compromised in the absence of PECAM-1.

MATERIALS AND METHODS

Reagents and Antibodies

Dihydroethidium (DHE), carboxy-H$_2$DFFDA, and acetylated low-density lipoprotein (AcLDL) coupled to Alexa 594 and Amplex Red were purchased from Life Technologies (Carlsbad, CA). Acetinoliteirine (HPLC grade) was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies to PECAM-1 (M20) were from Santa Cruz Biotechnology (Santa Cruz, CA), and antibodies to VEcadherin were from Abcam (Cambridge, MA). Anti-caveolin-1 was either from Abcam (Cambridge, MA) or Transduction Laboratories (San Jose, CA). Polyclonal anti-VE-cadherin-FITC was from eBiosciences (San Diego, CA). Anti-β-actin and nonimmune IgG (mouse and rabbit) were from Sigma-Aldrich. Antibody to PECAM that binds to IgD1 in PECAM was obtained from Centocor (Malvern, PA; Ref. 38a). The phospho-PECAM (p-Tyr713PECAM) antibody was from Assay Biotechnology (Sunnyvale, CA). Anti-flotillin 1 was from BD Transduction Laboratories (Lexington, KY). Anti-mouse IgG-coated magnetic beads were from Dynal Biotech (Life Technologies, Grand Island, NY). The 25-micrometer green fluorescent microspheres (Fluoro-Max) were purchased from Thermoscientific (Lafayette, CO). The Duolink kit was from Olink (Uppsala, Sweden).

Animal Use

Animal use was reviewed and approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Male C57BL/6, caveolin-1$^{-/-}$, and NOX2$^{-/-}$ mice weighing ~20 g were obtained from Jackson Laboratories (Bar Harbor, ME). Breeding pairs of PECAM-1$^{-/-}$ mice originally created by Tak W. Mak (13) were provided to us by Dr. Joseph Madri (Yale University). These mice were backcrossed for over 10 generations into the C57BL/B6 background. All protocols were approved by the University of Pennsylvania Animal Care and Use Committee with the exception that lungs were not moved to the imaging platform. Perfusion was maintained at 2 ml/min with a peristaltic pump.

Intravital Microscopy for ROS Detection in the Intact Lung

Imaging of ROS using a difluorofluorescein probe [5- and 6-carboxy-2', 7'-difluorodihydrofluorescein diacetate (CMH$_2$DFFDA)] was based on reports previously described (47, 48). After isolation and perfusion of the lung as described above, the lung was allowed to equilibrate in KRB buffer for 5 min. Next, endothelial marker Alexa 594 acetylated low density lipoprotein (Alexa594-AcLDL) was added (final concentration of 0.5 μg/ml) to the perfusate followed by 10 μM H$_2$DFFDA. After an additional 15 min of perfusion to facilitate loading of the dyes in the pulmonary endothelium, the lung was removed from the chamber and immediately positioned on the stage of a confocal microscope while perfusion and ventilation were maintained. Stop of flow was attained by turning off the perfusion pump, but ventilation continued throughout the duration of the lung imaging. ROS production in isolated lungs was monitored in the presence of oxidized DHE, was adapted from previous reports (51, 52). Lung isolation and perfusion were carried out as above, and the lung was perfused for 30 min with 50 μM DHE. Experiments were carried out in a similar fashion to the imaging study as described earlier with the exception that lungs were not moved to the imaging platform. The flow of perfusate was stopped for 1 h by stopping the perfusion by switching off the pump. Immediately after the completion of the stop of flow period, the DHE and its oxidation products were extracted from tissue pieces of the lung as previously described (52). The pieces were frozen in liquid nitrogen and individually ground to a powder using a mortar and pestle resting in a bath of liquid nitrogen. The ground tissue was resuspended in 500 μl acetonitrile, sonicated at 4 – 6 watts for three cycles of 15 s each, and centrifuged for 10 min at 12,000 g. After the supernatant was removed to a separate tube, the pellet was retained for protein quantitation. The supernatant was concentrated by evaporation of acetonitrile in a SpeedVac in dark. HPLC analysis of the supernatant showed the ROS production in isolated lungs was monitored in the presence of oxidized DHE products, we chose a long duration of stop of flow period (1 h) to enable greater 2-OHE accumulation in lungs.

Detection of 2-hydroxyethidium (2-OHE), the superoxide-specific oxidation product of DHE, was adapted from previous reports (51, 52). Lung isolation and perfusion were carried out as above, and the lung was perfused for 30 min with 50 μM DHE. Experiments were carried out in a similar fashion to the imaging study as described earlier with the exception that lungs were not moved to the imaging platform. The flow of perfusate was stopped for 1 h by stopping the perfusion by switching off the pump. Immediately after the completion of the stop of flow period, the DHE and its oxidation products were extracted from tissue pieces of the lung as previously described (52). The pieces were frozen in liquid nitrogen and individually ground to a powder using a mortar and pestle resting in a bath of liquid nitrogen. The ground tissue was resuspended in 500 μl acetonitrile, sonicated at 4 – 6 watts for three cycles of 15 s each, and centrifuged for 10 min at 12,000 g. After the supernatant was removed to a separate tube, the pellet was retained for protein quantitation. The supernatant was concentrated by evaporation of acetonitrile in a SpeedVac in dark. HPLC analysis of the supernatant showed the 2-OHE peak; this was normalized to the total ethidium to quantitate ROS production in isolated lungs.

Flow Adaptation of Endothelial Cells and Cessation of Flow In Vitro

The artificial capillary system (FiberCell Systems, Frederick, MD) for flow adapting PMVEC has been described previously (5, 6, 8). Stop of flow was achieved by turning off the peristaltic pump to stop flow of culture medium over the cells; the cells, however, were fed by oxygenated medium from the side ports to ensure that lack of flow did not compromise oxygen and nutrient supply. Cells were subjected to 24 h of flow adaptation followed by stop of flow and removed from the capillary chambers or kept under statically cultured conditions.

Lung Isolation

Mouse lung isolation and perfusion were carried out as described previously (6, 8, 49, 50). Briefly, mice were anesthetized, the trachea was cannulated, and the lungs were ventilated with 5% CO$_2$ in air. The chest was opened, the pulmonary artery was cannulated, and the lungs were cleared of blood by gravity-driven flow of Krebs-Ringer bicarbonate solution (KRB; in mM: 118 NaCl, 4.7 KCl, 1.2 MgSO$_4$·7H$_2$O, 1.2 KH$_2$PO$_4$, and 24.9 NaHCO$_3$) supplemented with 10 mM glucose and 5% wt/vol dextran. The lung was dissected free and hung in an environmental chamber maintained at 37°C for dye loading. Perfusion was maintained at 2 ml/min with a peristaltic pump.

Superoxide Production as Monitored by HPLC Detection of 2-Hydroxyethidium

Detection of 2-hydroxyethidium (2-OHE), the superoxide-specific oxidation product of DHE, was adapted from previous reports (51, 52). The pieces were frozen in liquid nitrogen and individually ground to a powder using a mortar and pestle resting in a bath of liquid nitrogen. The ground tissue was resuspended in 500 μl acetonitrile, sonicated at 4 – 6 watts for three cycles of 15 s each, and centrifuged for 10 min at 12,000 g. After the supernatant was removed to a separate tube, the pellet was retained for protein quantitation. The supernatant was concentrated by evaporation of acetonitrile in a SpeedVac in dark. HPLC analysis of the supernatant showed the 2-OHE peak; this was normalized to the total ethidium to quantitate superoxide production. A control experiment used a superoxide generating system xanthine/xanthine oxidase (X/XO) in the lung (in the presence of 400 U/ml catalase) to validate the detection of 2-OHE by HPLC. Since the 2-OHE product had to be separated from other oxidized DHE products, we chose a long duration of stop of flow period (1 h) to enable greater 2-OHE accumulation in lungs.
plus horseradish peroxidase (HRP; 50 μg/ml) was added to the perfusate; this fluorophore does not permeate the cell membrane and thus detects extracellular H₂O₂. ANG II (50 μM) was added to the lung perfusate, aliquots of the perfusate were removed during 15- to 30-min intervals, fluorescence intensity of the samples was measured (excitation/emission, 545/5610) using a spectrofluorimeter (Photon Technology, Birmingham, NJ), and H₂O₂ concentration was expressed as arbitrary fluorescence units (7, 26). ROS generation was measured with WT and PECAM-1⁻/⁻ lungs; NOX2⁻/⁻ lungs were used as a negative (no ROS) control.

Association of PECAM-1 and Caveolin

Immunolabeling. Lung sections were treated with anti-caveolin-1 and anti-PECAM and visualized by fluorescence microscopy using a Zeiss LSM 510 Meta microscope coupled to a Chameleon Ultra mode-locked femtosecond pulse laser (set at 720 nm for 2-photon excitation of DAPI) and He-Ne and Ar lasers. Colocalization analysis was performed by the ImageJ program using colocalization plugin JACoP to calculate the standard colocalization coefficients (14). The extent of colocalization was measured by Pearson's coefficients, Overlap coefficient, and Manders' coefficients (28, 29).

Colocalization of PECAM-1 and caveolin-1 by Duolink proximal ligation assay. This assay allows for visualization of protein-protein colocalization by fluorescence microscopy (Duolink; Olink, Uppsala, Sweden) using primary antibodies for each protein obtained from different species (7), in this case a polyclonal anti-caveolin antibody generated in rabbits and a monoclonal antibody to PECAM-1 generated in mice. Lungs isolated from WT mice were perfused to clear the tissue of blood, inflated and fixed with methanol-acetone, and treated with a blocking reagent followed by labeling of the endothelial cell membrane by an antibody that is expressed in endothelial cells [anti-VE-cadherin (VE-cadherin) conjugated to a FITC label (anti-VE-cadherin-FITC); 1:500]. After several washes, the primary antibodies to PECAM-1 and caveolin-1 were added. The secondary antibodies to rabbit and mouse IgG are each attached to a unique synthetic oligonucleotide; ligation causes the oligonucleotides on the two secondary antibodies to hybridize only if the epitopes of the two target proteins are in proximity (<40 nm). Following hybridization, amplification and labeling of the product by detection protocol allow for the visualization of the interaction by fluorescence microscopy. In this case, a red signal indicated the interaction of caveolin-1 and PECAM-1. Duolink controls used to check nonspecific signal were 1) anti-PECAM-1/rabbit IgG, 2) anti-caveolin/mouse IgG, and 3) anti-caveolin/anti-PECAM-IgG1D1. The lung sections were also counterstained with a nuclear marker (DAPI) to enable visualization of endothelial cells in the sections.

Membrane fractionation of PMVEC. Membranes were isolated from endothelial cells using previously described methods (4). For each experiment, WT mouse PMVEC were grown to confluence (in 150-mm cell culture dishes) removed, pooled, and lysed. The plasma membrane was isolated from postnuclear homogenate using a Percoll gradient (established by mixing the homogenate with 30% Percoll in tricine, followed by centrifugation at 77,000 g for 25 min). After centrifugation, the plasma membrane was clearly visible in the ultra-centrifuge tube floating approximately one-half centimeter from the top of the tube. This band was collected, and subcellular fractionation on a sucrose gradient was carried out by loading this band onto a sucrose step gradient for overnight centrifugation at 87,400 g. Fractions were collected every 400 μl (11 fractions were collected), and protein was precipitated with 0.1% wt/vol deoxycholic acid in 100% wt/vol trichloroacetic acid. Proteins were run on a SDS-PAGE gel and immunoblotted for PECAM-1, caveolin-1, and the membrane marker flotillin using Odyssey Western blot analysis technique (Li-Cor, Omaha, NE). Secondary antibodies were IRDyeTM 800 goat anti-rabbit for the green channel and IRDyeTM 680 goat anti-mouse for the red channel. Blots were scanned by placing the membrane on the Odyssey color scanner, and the scanned images were converted to grayscale. All manipulations of contrast were done for the entire gel.

Caveolae immunoaffinity isolation. Caveolae were isolated as described in our past reports (41a). Briefly, endothelial cells were scraped into ice-cold, detergent-free Tricine buffer (250 mM sucrose, 1 mM EDTA, and 20 mM Tricine pH 7.4) and centrifuged to precipitate nuclear material. The resulting supernatant was mixed with 30% Percoll in Tricine buffer and subjected to ultracentrifugation for 25 min (Beckman ML850 rotor; 77,000 g, 4°C). The separated plasma membranes were collected, sonicated (3 × 30 s bursts), and incubated with anti-caveolin-1 conjugated goat anti-mouse IgG-coated magnetic beads for 1 h at 4°C. Bound material, representative of caveolea vesicles, was separated magnetically from unbound, noncaveolar membranes, subjected to SDS-PAGE and immunoblotted using indicated antibodies.

Functional interaction of PECAM-1 and caveolin-1. This was assessed by monitoring phosphorylation of PECAM in caveolin-1⁻/⁻ cells with stop of flow. PMVEC from WT and caveolin-1 null lungs were either statically cultured (S) or kept for 24 h under flow. Flow was stopped for 1 h (stop of flow), cells were removed and lysed, and cell lysates were immunoblotted using an antibody to phosphorylated PECAM-1. PMVEC from lungs of PECAM-1⁻/⁻ mice were used as controls. The phoshoTy713PECAM antibody recognizes PECAM-1 phosphorylation at the Tyr713 phosphorylation site and detects the phosphorylated PECAM band at approximately 130–140 kDa. β-Actin was used as a protein loading control. Immunoblots were quantitated using National Institutes of Health ImageJ software.

Angiogenic Potential of PMVEC

The Matrigel plug assay is used as a test for angiogenesis in vivo. In this assay, PMVEC are introduced into cold liquid Matrigel and injected as a plug into nude mice. The solid gel permits penetration of host endothelium to form new blood vessels inside the plug. PMVEC (1 × 10⁶), obtained after 72 h flow adaptation followed by 1 h of stopped flow, were mixed with 0.5 ml of growth factor-reduced Matrigel and injected subcutaneously into nude mice, using a B-D 26G1/2 needle so that the entire content can be delivered to form a single plug under the skin. After 5 days, the animals were killed; the plug was extracted from under the skin and washed in cold PBS to remove blood and cells sticking to the plug surface. The plugs were then analyzed for vessel formation by two methods. In one method, small pieces of the plug were stained with Alexa594-AcLDL that labels vascular structures. For the other method, the plugs were cryosectioned and stained with endothelial marker VE-cadherin (using anti-VE cadherin Ab) to outline endothelial structures. Five to eight sections were made from each plug. The vascularization within the plug reflects the extent to which blood vessels from the host entered the plug and is an index of the angiogenic signal emanating from the plug, i.e., from the endothelial cells initially present in the plug (3a).

Cessation or Stop of Pulmonary Blood Flow In Vivo in Live Mice

To attain stop of flow in vivo, 25-μm green fluorescent polystyrene microspheres were injected into the lung through the jugular vein. Twenty-four hours later the mice were killed and lungs sectioned and stained with hematoxylin and eosin. Sections were imaged at different magnifications.

VEGF Expression In Vivo

In separate experiments, animals were killed at 1, 4 and 7 days poststop of flow (as induced by beads) in the lung vessels. Lungs were removed and dehydrated by sequential sucrose treatment (10, 20 and 30% sucrose). The tissue was embedded in OCT blocks and longitudinal sections were immunostained for VEGF using anti-VEGF165 antibody (red fluorescence) and compared with nonspecific IgG.
Utrent lungs from age matched mice were used as controls. All sections were imaged by confocal microscopy (Bioradiance 2000); images were acquired and quantified for VEGF fluorescence by ImageJ software. Briefly, images were split into red (VEGF) and green (microtubule) channels; the green channel was masked and the red used for quantitation (after threshold is set to measure only regions that were stained).

**Electron Microscopy (Immunogold Staining)**

Lungs from WT or PECAM-1/−/− mice were isolated, perfused with 0.1 M Na cacodylate buffer, and fixed with 4% paraformaldehyde for 10 min. These were cut into pieces, and two tissue blocks (1 × 1 × 3 mm) were sampled from each lobe. The tissue blocks were further fixed by immersion in 4% paraformaldehyde for 4 h on ice, followed by a thorough wash with 0.1 M Na cacodylate buffer, permeabilized with 0.02% Triton-X-100 in PBS, and then blocked with 50 mM glycine in PBS and a mixture of 5% normal goat serum, 0.1% cool water fish skin gelatin, and 2% BSA in PBS. Following this, the tissue blocks were incubated overnight at 4°C with mouse anti caveolin primary antibody (1:50 dilution) and then incubated with an Ultra Small-Grade (0.8 nm) gold-labeled goat anti mouse secondary antibody (Electron Microscopy Sciences, Hatfield, PA) for 1 h. This was followed by fixation with 2% glutaraldehyde in Na cacodylate buffer for 5 min and incubation with Aurion R-Gent SE-EM silver enhancement solution (Electron Microscopy Sciences) for 25 min. After further fixations in 1% osmium tetroxide for 15 min and then 2% uranyl acetate for 10 min, the tissue blocks were dehydrated with graded acetone, embedded with Epon, and polymerized at 6°C for 24 h. The 85-um thick ultrathin sections were prepared with Leica Ultracut machine and imaged in JEM-100-CX electron microscope operating at 60 kV. Around 5–10 images were captured from each tissue block; caveolae were identified by their structure and counted for each cell. Data were presented as the total number of the caveolar structures per field. The density of caveolae was determined by counting the number of caveolae on the P face in at least three different fields. The number of caveolae was expressed per square micrometer. Statistical analysis was performed using the two-tailed paired t-test.

**Statistical Analysis**

Values are means ± SD. Data sets were compared using a Student’s t-test or ANOVA in SigmaStat (SPSS, San Jose, CA). Results were considered statistically significant at P < 0.05.

**RESULTS**

**Endothelial Mechanosignaling-Induced ROS Production with Stopped Flow Is Compromised in Pulmonary Endothelium of PECAM-1/−/− Lungs**

The isolated lung in situ model allows for monitoring changes to the endothelium upon removal of shear, i.e., stop of flow; that ventilation is continued throughout the experiment ensures that the oxygen tension is unaffected so that the changes observed reflect the effects of the loss of flow component. ROS production (as monitored by DFF fluorescence) with stopped flow is observed in WT lungs but it is absent in lungs from NOX2−/− mice. This is consistent with our previous reports using other fluorescent probes and methods (31, 34, 50). Lungs from PECAM-1/−/− mice showed lower ROS production compared with WT lungs (Fig. 1A). For imaging the DFF fluorescence in the lungs, focusing and image capture were carried out in reference to an independently captured image using an endothelial marker Alexa 594-conjugated AcLDL (data not shown). We attempted to limit the quantification (of fluorescence intensity of H2DFFDA) to the signal arising from subpleural microvasculature. As is well known, AcLDL also labels certain nonendothelial cell types such as macrophages, leukocytes, etc.; however, in isolated perfused lungs completely cleared of blood this possibility is somewhat low. Image analysis of the intensity of the fluorescence signal with stopped flow showed that the relative increase in ROS in WT lungs was 40% over baseline, while in lungs from PECAM-1/−/− mice it was ~20% over baseline (Fig. 1B). A time course of images was captured in a single region of the lung to allow for measurement of ROS increase in the same area as a function of time.

To identify the ROS species generated with stopped flow in the lung (since H2DFFDA is sensitive to H2O2 among other oxidants but not to O2−), we used the superoxide specific dye DHE. First, the specificity of the probe for superoxide was tested using the superoxide generating system (X/XO). Isolated perfused lungs were prelabeled with DHE and treated with X/XO in the presence of catalase; lung extracts were analyzed by HPLC to confirm the probe had sufficient capacity for oxidation. The ratio of the specific superoxide product 2-OHE to the unreacted DHE from the same extract gives an indication of the superoxide levels without artifacts from unequal probe loading or variations in sample tissue size. We observed 2-OHE production post-X/XO addition from the peak; this peak is abolished upon pretreatment of lungs with superoxide dismutase (Fig. 2A). After the specificity of DHE for superoxide was established in our model, lungs from WT and NOX2−/− mice were labeled with DHE and subjected to 1 h of stopped flow; these lungs were then excised and DHE and its oxidation products were extracted. Our results (Fig. 2B) show that superoxide produced with cessation of flow in WT mice is significantly higher than that generated in NOX2−/− lungs exposed to the same conditions. Superoxide production in PECAM-1/−/− lungs while significantly diminished compared with WT lungs is higher than that observed in NOX2−/− (a system that generates low superoxide). Sequential analysis of the superoxide-specific product 2-hydroxyethidium (2-OHE) and the unreacted DHE provided an intrinsic normalization of the amount of oxidation, avoiding artifacts from unequal probe loading or variation in tissue sample size. Analysis of the ratio of 2-OHE to DHE for at least three lungs indicated a significantly lower amount of superoxide in PECAM-1/−/− lungs (0.22) to that in the WT lungs (0.34; Fig. 2C). The NOX2−/− lungs had the lowest amount of superoxide activity (0.10), which was approximately the same as the signal detected in samples from naïve (untreated) lungs (dashed line). This implies that a small amount of basal superoxide is generated in naïve lungs or that nonspecific fluorescence is detected in all samples.

Effect of Loss of PECAM-1 on NOX2 Activation by ANG II (NOX2 Agonist)

We monitored NOX2 activation in lungs in situ in WT and PECAM-1/−/− mice by treating lungs with a NOX2 agonist ANG II. NOX2 activation by ANG II (50 μM) was assessed by ROS generation in lungs as evidenced by oxidation of membrane impermeable dye Amplex Red (+HRP) at 15, 30, 45, and 60 min of recirculating perfusion (Fig. 3). Aliquots were removed at different periods of agonist induced NOX2 activation making this a suitable assay for monitoring ROS under continued perfusion. There was no detectable ROS production.
in the absence of added agonist in the lungs studied (WT, PECAM-1−/−, and NOX2−/−). Upon addition of ANG II to the perfusate, there was a linear increase in oxidized Amplex Red that was 8- to 10-fold greater than the basal rate indicating a constant rate of H2O2 production. There was no significant difference in ANG II-induced ROS production by PECAM-1−/− lungs; however, the ANG II-stimulated rate of H2O2 production was markedly diminished in the NOX2−/− lungs (as compared with WT) although it was slightly greater than untreated WT lungs. These data suggest that the NOX2 activation machinery is intact in PECAM-1−/− lungs.

**Association of PECAM-1 with Caveolin-1**

**Colocalization.** We have previously reported that caveolin-1 (the major coat protein of caveolae) plays a role in signaling with stop of flow; we observed that lungs from caveolin-1−/− mice showed significantly lower ROS production compared with WT (33, 45). To investigate whether caveolin-1 is involved in PECAM-1 signaling with stop of flow, we examined the location of PECAM-1 on plasma membrane. We checked the distribution of PECAM-1 by dual labeling for PECAM-1 and caveolin-1 by immunofluorescence of fixed lung sections.
as well as by monitoring PECAM-1 expression in caveolin-rich cell membrane fractions. Visual inspection of the colocalized signal (yellow channel) reveals regions of strong overlap separated by areas of little apparent or no overlap (Fig. 4, A–C).

We carried out colocalization analysis by expressing the fraction of localization in each component of a dual color image by various colocalization coefficients. Pearson’s coefficient, a standard pattern recognition for matching one image with another, measures the degree of overlap between two patterns independent of the intensity of the signal. This involves subtracting the average values from the original values. Pearson’s coefficient indicates a 57% colocalization. The overlap coefficient, also independent of signal intensity caused by differential labeling of the fluorophores or photobleaching or different setting of the amplifiers, shows a 70% correlation between PECAM-1 and caveolin-1. The Mander’s coefficients are proportional to the amount of fluorescence of the colocalizing objects in each component of the image relative to the total fluorescence in that component. The Mander’s coefficients indicate that 71% of the PECAM-1 was colocalized with the caveolin-1, while 87% of the caveolin-1 was colocalized with the PECAM-1. Overall, the colocalization analysis shows a statistically significant correlation in the signals from caveolin-1 and PECAM-1 (Fig. 4D).

Next, we used the Duolink procedure to evaluate the interaction of caveolin-1 and PECAM-1. For the lung sections, we chose part of a pulmonary vessel. We counterstained the lung sections for endothelial cells using anti-VE-cadherin (green) and nuclei using nuclear marker DAPI (blue) to observe cells/endothelial cells in each field (Fig. 4E). Magnification of
the image shows that along the pulmonary vessel red fluorescent dots appear in cells that have green fluorescence (Fig. 4E). Each dot represents a single molecular interaction between the two proteins PECAM-1 and caveolin. For each experiment, the nonspecific Duolink signal was monitored by controls using anti-PECAM-1/rabbit IgG, anti-caveolin-1/mouse IgG, and anti-PECAM-IgG1/anti-caveolin. These showed no fluorescence dots thus ruling out any nonspecific interactions (data not shown).

The functional interaction of PECAM-1 and caveolin-1 was evaluated by assessing phosphorylation of PECAM-1 in the presence and absence of caveolin-1, using an antibody against...
a peptide derived from PECAM-1. With this antibody, phosphorylated PECAM-1 (when phosphorylated at tyrosine713) is detected as a band at ~130 kDa. With mechanical stimuli, PECAM-1 has been reported to be phosphorylated (at Tyr 713) (9, 39). We observed a band at approximately 130–140kDa in WT cells that was not observed in PECAM-1−/− cell lysate. There was increase in phospho-PECAM in flow-adapted PMVEC subjected to stop of flow compared with static cells (Fig. 4F). This band was very weak in cells that lacked caveolin-1 (Fig. 4, F and G). Thus caveolin-1 (and caveolae) are required for the functional activation of PECAM-1.

To check of PECAM-1 was resident in caveolin-1-rich light density membrane fractions, we assessed PECAM-1 expression in plasma membrane fractions of PMVEC (Fig. 5A). Immunoblotting these membrane fractions show enrichment for caveolin-1 in fractions 5–8, consistent with previous reports of the distribution of caveolin-1 in endothelium (17); PECAM-1 expression was observed only in caveolin-1-rich fractions. The heavier fractions contain membranes in varying amounts as seen by the expression of plasma membrane marker flotillin. Next, we checked PECAM expression in caveolar vesicles isolated from plasma membranes of mouse PMVEC. Caveolae showed high PECAM-1 levels compared with noncaveolar membranes (i.e., membrane that is not bound to the caveolin-1-coated magnetic beads), which showed low to no PECAM expression (Fig. 5B). In our experiments, noncaveolar fraction showed a caveolin-1 signal, as larger vesicles with caveolin-1 are often not accessible to the immunoaffinity pull down assay and thus remain unbound to the magnetic beads. Because of this, there is also a faint PECAM-1 band in the noncaveolar fraction.

Effect of Loss of PECAM-1 on Caveolin-1 Expression

Since PECAM-1 and caveolin-1 were functionally associated, could lack of PECAM-1 affect caveolin-1 expression and caveolae? Our investigations revealed that there were no differences in WT or PECAM-1−/− mice in expression of caveolin-1 in mouse lung homogenates (Fig. 6, A and B). Ultrathin thawed cryosections were fixed, cryoprotected, and immunolabeled for caveolin-1. Transmission electron microscopy images showed caveolae as flask-shaped profiles located beneath the plasma membrane. The mean density of caveolae in WT lungs was not significantly different from PECAM-1−/− lungs. Thus the lack of PECAM-1 did not alter the number of caveolae or the expression of caveolin-1 in mouse lung endothelium (Fig. 6, C and D).

Mechanosignaling via PECAM-1 Correlates with Neovascularization In Situ

To understand the functional role for ROS produced with stop of flow, we assessed the angiogenic potential of cells subjected to stop of flow (72 h under flow followed by stop of flow). Cells were mixed in Matrigel and were injected as subcutaneous plugs into nude mice. Five days later plugs containing flow-adapted cells subjected to stop of flow or statically cultured cells were excised and treated with the endothelial marker Alexa594-AcLDL. Visible vessel formation was observed that was significantly greater in flow-adapted WT compared with PECAM-1−/− cells cultured under similar conditions (Fig. 7, A and B). Cryosections of the plugs showed staining with anti-VE cadherin indicating endothelium within the plugs (Fig. 7, C and D). Statically cultured cells from both WT and PECAM null lungs showed low neovascularization.

Lack of PECAM-1 and Neutrophil Recruitment and VEGF Expression with Stopped Flow in the Lung In Vivo

What are the consequences of mechanosignaling with stop of flow in the lung? To investigate this, we occluded lung vessels in vivo. This, of course, is not comparable to stop of flow in vitro or in situ and is closer to a microemboli model; nevertheless, it represents obstructed flow in an in vivo setting. We injected 25–μm beads into the lungs of WT, PECAM-1−/−, and NOX2−/− mice; 24 h later the mice were killed and the lungs were fixed and sectioned. Hematoxylin and eosin staining showed that the infiltration of polymorphonuclear neutrophils that occurs 24 h after obstruction of blood flow in WT mice is significantly compromised in the null mice (Fig. 8). The polymorphonuclear neutrophils were clustered in regions around the beads (Fig. 8). We also checked VEGF expression in WT lungs at 1–7 days poststop of blood flow. At day 4 poststopped flow, VEGF expression increased significantly compared with untreated lungs; this decreased by day 7 (data not shown). We thus monitored VEGF expression at day 4 across the three types of lungs, i.e., WT, PECAM-1−/−, and NOX2−/−. Lack of PECAM-1 and NOX2 (and thus mechanosignaling-induced ROS production) abolished the increase in VEGF expression that was observed in WT lungs pointing to a role for PECAM-1 induced NOX2 activation in triggering an angiogenic signal with obstruction of blood flow (Fig. 9, A and B).

DISCUSSION

Mechanosignaling associated with blood flow has hitherto involved studying cellular responses with onset of shear or start of flow. In a departure from this paradigm, we investigated shear from the point of removal of shear on endothelial cells,
a condition that has physiological similarity with vascular obstruction. In general, stop of flow involves 1) compromised oxygen delivery to tissue, and 2) alteration of the mechanical component of blood flow. We established models where stop of flow did not compromise oxygen delivery and nutrient supply; using these models we observed that abrupt cessation of flow causes ROS production via activation of endothelial cell NADPH oxidase (34, 35, 50). We discovered elements of the cascade, viz. K⁺ channel depolarization and phosphatidylinositol 3-kinase/AKT activation that participated in NOX2 assembly (6, 50). However, the mechanosensing elements on the endothelium that “sensed” the changes in tension on the membrane with stop of flow remained unknown.

Here we investigate the initial element(s) that participate in sensing shear in the pulmonary endothelium. Several reports show a role for PECAM-1 in sensing physical force (9, 39, 42). By virtue of its localization at cell-cell junctions and due to its cytoskeletal linkage (downstream of PECAM-1 expression is the α₃β₃ integrin signal), PECAM-1 on the pulmonary endothelium can be hypothesized to be part of a sensory complex that senses stop of flow.

In this study, mechanosensing and the attendant signaling were monitored via ROS production; we were thus judicious in the selection of dyes to be used. Instead of the more extensively used chlorinated fluorescein derivatives such as H₂DCFDA (which are notorious for photo enhancement of the fluorescence signal), we used the fluoro compound H₂DFFDA. This fluorinated derivative exhibits improved photostability, and the carboxy group in H₂DFFDA improves cell retention. As H₂DFFDA oxidation occurs predominantly via H₂O₂, we used in separate experiments, a second dye DHE, to identify if superoxide was produced with stop of flow. DHE is reported to react with superoxide to form 2-OHE. It also forms other products, but 2-OHE is the superoxide specific adduct. As 2-OHE had to be obtained from the lung extract by HPLC, its separation from other DHE products and subsequent detection necessitated exposing the lungs to longer periods of stopped flow (1 h) to enable greater 2-OHE accumulation in lungs. In contrast, confocal imaging of DFF in intact lungs could detect small changes in fluorescence due to the sensitivity of microscopy. In addition, visualization was aided by pseudocolor and thus DFF fluorescence could be seen within 1–5 min of stopped flow. Despite differences in the period of stopped flow in the lungs, both these dyes showed that lack of PECAM-1 reduced the response to stop of flow (in terms of ROS production) by the pulmonary endothelium. Lungs from mice with knockout of PECAM-1 produced ROS that was intermediate between WT and NOX2⁻/⁻ lungs. This indicates that PECAM-1 is possibly part of a complex or part of a family of sensors that participates in signaling with loss of flow and that its loss abrogates but does not completely abolish the signaling with stop of flow. This signaling involves the eventual activation of NOX2; thus lack of NOX2 causes very low to no ROS production as we have shown here and reported earlier (6, 50).

NOX2 activation involves assembly of its cytosolic and membrane components; incomplete assembly can alter ROS production. We thus checked NOX2 activation in WT and PECAM-1⁻/⁻ lungs treated with ANG II. For this we used another H₂O₂-sensitive dye Amplex Red that differs from DFF.
in that it monitors ROS (H$_2$O$_2$) production only in the vessel lumen (as it a membrane impermeable dye). The advantage of this assay is that NOX2 activation (and ROS) can be assessed over multiple time points from the same lung by removing aliquots of the perfusate at different time points postagonist stimulation (7). This assay is suitable for studying the total H$_2$O$_2$ produced in whole lungs under continuous perfusate flow but not under stop of flow. For stop of flow, the pump would have to be turned off; perfusate retrieval would necessitate restart of flow (reperfusion), thus introducing the confounding effects of reperfusion induced ROS into the measurement. We observed that ANG II-treated PECAM-1$^{-/-}$ lungs showed similar ANG II-induced NOX2 activation (ROS production) comparable with WT lungs. Thus NOX2 activation machinery is intact in PECAM-1$^{-/-}$ lungs. The diminution of ROS in PECAM-1 null lungs as observed in Figs. 1 and 2 is thus specific to stop of flow induced ROS production and is not a generalized negative effect on NOX2 activation in PECAM-1$^{-/-}$ mice.

Our earlier studies on stop of flow in lungs in situ had shown a role for caveolin-1 in mechanosignaling (33, 45). Caveolae are flask-like invaginations of the plasma membrane; these invaginations are lipid-rich cellular domains that concentrate plasma membrane proteins and lipids. Caveolae serve as signaling platforms for receptors, kinases, and several proteins all of which recognize external stimuli and transduce signals to modulate cellular activity. Among the proteins reportedly located within caveolae are NOX proteins and endothelial nitric oxide synthase (16, 18, 21, 53). Our colocalization studies indicate that most of cellular PECAM-1 was located in caveolae. This was also reported in mouse lung development studies where patterns of caveolin-1 expression overlapped with PECAM-1 expression (40). How does caveolin-1 status affect mechanosignaling that occurs via PECAM-1? We reported earlier that mice with knockout of caveolin-1 (i.e., lacking caveolin-1 and thus caveolae) showed a complete abolition of ROS with stop of flow in lungs in situ (33); however, PECAM-1$^{-/-}$ lungs show a diminution in ROS pro-
duction. Thus lack of caveolae (and therefore a presumable loss of a signaling scaffold or platform comprising PECAM-1 and NOX2) completely prevented mechanosignaling (33), while lack of PECAM-1 merely reduced it. We also observed phosphorylation of PECAM-1 in (flow adapted) WT cells upon stop of flow. This did not occur in caveolin-1 null cells, indicating the requirement of caveolin-1 (and caveolae) in PECAM-1 activation. Studies elsewhere have also shown that PECAM-1 and caveolin-1 are functionally linked in several signaling processes including those participating in vasculogenesis (40).

Does loss of PECAM-1 affect caveolin-1 expression and caveolar number? In other words, does the diminution in mechanosignaling in PECAM-1−/− arise from altered caveolar expression? Our investigations revealed that caveolin-1 expression or numbers of caveolae are not significantly reduced in PECAM-1−/− lungs compared with WT, indicating that PECAM-1 (as part of the caveolar complex) functions as a mechanosensing platform that initiates signaling with loss of shear. Of course, PECAM-1 is known to be distributed heavily at cell-cell contacts while caveolae are found both at the luminal surface and cell borders. Presumably, the subset of PECAM-1 (and caveolae) that is on the luminal surface would play a major role in shear sensing compared with that at the cell contacts.

ROS generated with stop of flow in vitro has been reported by us to drive cell proliferation (33–35). To understand if this cell proliferation is random or leads to formation of new vessels, we investigated the angiogenic potential of flow-adapted cells subjected to stop of flow. The subcutaneous Matrigel plug model is a standard assay for detecting angiogenesis in situ. Matrigel matrix is used as this is similar to the natural environment of a basement membrane for endothelial cells and vascularization in the plugs (the plug itself is growth

Fig. 8. PECAM-1 signaling participates in neutrophil recruitment postocclusion in lungs in vivo; 25-μm microspheres were injected into the jugular vein of WT, PECAM-1−/−, and NOX2−/− mice. Mice were killed 24 h later, and lungs were sectioned and stained by hematoxylin and eosin. Images were acquired under ×10 and ×60 objectives. Bottom: magnification of boxed region.
factor free) is caused by angiogenic signals emanating from inside the plug (3a). We have reported earlier that when WT pulmonary endothelial cells subjected to flow adaptation and stop of flow are mixed with Matrigel and injected into the flanks of nude mice, vascularization within the plug was observed after 5 days. This was not observed with cells that did not generate ROS with stopped flow, i.e., NOX2/−/− cells (3a). This indicates that angiogenic signals emanating from the endothelial cells in the plug recruit endothelial cells from the host animal and form blood vessels within the plug (3a). Thus lack of vascularization indicated that the endothelial cells within the plug have low to no angiogenic potential. Vascularization within the plugs was assessed by staining pieces of the plug or selected regions of histological sections for vascular markers. We observed that flow-adapted WT cells subjected to stop of flow showed greater angiogenic potential compared with the statically cultured cells and to PECAM-1−/− cells (both flow adapted and static). In fact plugs with PECAM-1−/− cells did not shown neovessel structures within the plug as WT did, although they did show some staining for endothelial

Fig. 9. Expression of vascular endothelial growth factor (VEGF) in the lung is PECAM-1 and NOX2 dependent. A: 25-μm polystyrene beads (green fluorescent) were injected into the mice; mice were killed 4 days later, sectioned and stained for VEGF. B: quantitation of the fluorescence intensity from the microscopy images, using 8–10 fields per lung. Data are means ± SE from n = 3 independent experiments. Unt, untreated lungs; WT (IgG), IgG controls. *P < 0.05, compared with knockouts.
markers. Overall the Matrigel plug studies indicate that ROS generated with stop of flow modulates an angiogenic response.

Our data indicate that PECAM-1-induced mechanotransduction participates in vascularization in pulmonary endothelial cells; however, pulmonary obstruction in vivo (attained by ligation of the pulmonary artery) has been reported to lead to ROS-dependent systemic angiogenesis, with no vascularization within the pulmonary vasculature (36, 44). We reasoned that this was not due to the lack of an angiogenic signal in the pulmonary endothelial cells per se (as our data showed increased vascularization in plugs that was PECAM-1-dependent) but due to the unresponsiveness of the pulmonary artery. The pulmonary artery is not proangiogenic, except in rare cases where new vessels develop as a secondary source of perfusion in some lung carcinomas (32). We thus sought to achieve in vivo occlusion of smaller vessels by injecting 25-μm polystyrene beads via jugular vein. Hematoxylin and eosin staining of lung sections showed neutrophil accumulation in lungs of WT mice; that neutrophils were significantly lower in NOX2−/− pointed to a role for NOX2 activation in driving neutrophil influx. Low neutrophil infiltration in PECAM−1−/− lungs implies a role for PECAM mechanosignaling in neutrophil recruitment into the lungs. However, PECAM-1 is also a multifunctional protein that serves as an adhesion molecule to regulate leukocyte trafficking. Indeed a role for PECAM-1 in neutrophil recruitment with ischemic insult and other injury models has been reported earlier (3, 24, 43). Blockade of PECAM-1 inhibited neutrophil transmigration and significantly reduced necrosis in a feline model of myocardial ischemia reperfusion (38). However, these studies focused on transmigration of the available neutrophils, whereas our interest was to examine PECAM-1-induced signaling in recruiting neutrophils. Homing of neutrophils is a very complex process and is reported to occur via a signaling cascade in which adhesion molecules, chemokines, and chemotraffickers are expressed in a spatially and temporally controlled manner (2, 15, 41). Our data suggest a link between endothelial PECAM-1 and neutrophil recruitment: the lack of neutrophils in NOX2−/− mice point to a role for ROS (and mechanosignaling), although the trafficking effect of PECAM-1 possibly plays a role as well.

Is there an angiogenic signal postbead-induced stop of flow in the pulmonary vasculature in vivo? Also, what is the initiating signal for vascularization with the removal of flow stimulus? VEGF is well established to regulate angiogenesis; indeed, it is one of the most potent angiogenic factors identified so far that stimulates endothelial cell proliferation, migration, and tube formation. Our inspection of lungs postocclusion at 1, 4, and 7 days after bead insertion, revealed an increase in VEGF at day 4 in WT, which was reduced to the levels of untreated lungs by day 7. In both PECAM-1−/− and NOX2−/− lungs, there was no increase in VEGF, indicating that PECAM-1 and NOX2 mediate VEGF production.

Taken together our data indicate that PECAM-caveolae mechanosensing machinery on the endothelium is able to sense the changes in membrane tension with stop of flow; this triggers a signaling cascade that leads to ROS production. ROS propagates signaling that participate in neutrophil recruitment and angiogenic processes in the region of obstructed flow. We posit that these signals are for vascularization to restore the impeded blood flow.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES

PULMONARY ENDOTHELIAL MECHANOSENSING VIA PECAM-1


