Delayed Microvascular Shear Adaptation in Pulmonary Arterial Hypertension

Role of Platelet Endothelial Cell Adhesion Molecule-1 Cleavage

Robert Szulcek^{1,2}, Chris M. Happé¹, Nina Rol^{1,2}, Ruud D. Fontijn³, Chris Dickhoff⁴, Koen J. Hartemink^{4*}, Katrien Grünberg^{5†}, Ly Tu^{6,7}, Wim Timens⁸, George D. Nossent⁹, Marinus A. Paul⁴, Thomas A. Leyen^{3§}, Anton J. Horrevoets³, Frances S. de Man¹, Christophe Guignabert^{6,7}, Paul B. Yu¹⁰, Anton Vonk-Noordegraaf¹, Geerten P. van Nieuw Amerongen², and Harm J. Bogaard¹

¹Department of Pulmonology, ²Department of Physiology, ³Department of Molecular Cell Biology and Immunology, ⁴Department of Surgery, and ⁵Department of Pathology, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, the Netherlands; ⁶INSERM UMR_S 999, LabEx LERMIT, Centre Chirurgical Marie Lannelongue, Le Plessis-Robinson, France; ⁷Université Paris-Sud, School of Médecine, Le Kremlin-Bicêtre, Paris, France; ⁸Department of Pathology and Medical Biology, and ⁹Department of Pulmonology, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands; and ¹⁰Cardiovascular Division, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts

Abstract

Rationale: Altered pulmonary hemodynamics and fluid flow–induced high shear stress (HSS) are characteristic hallmarks in the pathogenesis of pulmonary arterial hypertension (PAH). However, the contribution of HSS to cellular and vascular alterations in PAH is unclear.

Objectives: We hypothesize that failing shear adaptation is an essential part of the endothelial dysfunction in all forms of PAH and tested whether microvascular endothelial cells (MVECs) or pulmonary arterial endothelial cells (PAECs) from lungs of patients with PAH adapt to HSS and if the shear defect partakes in vascular remodeling *in vivo*.

Methods: PAH MVEC (n = 7) and PAH PAEC (n = 3) morphology, function, protein, and gene expressions were compared with control MVEC (n = 8) under static culture conditions and after 24, 72, and 120 hours of HSS.

Measurements and Main Results: PAH MVEC showed a significantly delayed morphological shear adaptation ($P = 0.03$) and evidence of cell injury at sites of nonuniform shear profiles that

are critical loci for vascular remodeling in PAH. In clear contrast, PAEC isolated from the same PAH lungs showed no impairments. PAH MVEC gene expression and transcriptional shear activation were not altered but showed significant decreased protein levels ($P = 0.02$) and disturbed interendothelial localization of the shear sensor platelet endothelial cell adhesion molecule-1 (PECAM-1). The decreased PECAM-1 levels were caused by caspase-mediated cytoplasmic cleavage but not increased cell apoptosis. Caspase blockade stabilized PECAM-1 levels, restored endothelial shear responsiveness *in vitro*, and attenuated occlusive vascular remodeling in chronically hypoxic Sugen5416-treated rats modeling severe PAH.

Conclusions: Delayed shear adaptation, which promotes shear-induced endothelial injury, is a newly identified dysfunction specific to the microvascular endothelium in PAH. The shear response is normalized on stabilization of PECAM-1, which reverses intimal remodeling *in vivo*.

Keywords: pulmonary arterial hypertension; endothelial cell; shear stress; microcirculation; molecular biology

(Received in original form June 27, 2015; accepted in final form January 7, 2016)

*Present address: Department of Surgical Oncology, Cancer Institute, Antoni van Leeuwenhoek Hospital, Amsterdam, the Netherlands.

†Present address: Department of Pathology, Radboud University Medical Centre (Radboudumc), Nijmegen, the Netherlands.

§Present address: Sanquin Research and Landsteiner Laboratory, Amsterdam, the Netherlands.

Supported by the Dutch Lung Foundation (Longfonds) grant number 33.12.036 (H.J.B.), the Foundation Leducq (P.B.Y.), and the Netherlands CardioVascular Research Initiative grant number 2012-08 awarded to the Phaedra consortium (www.phaedraresearch.nl).

Author Contributions: Obtained funding: H.J.B., A.V.-N., R.S., G.P.v.N.A., and A.J.H.; conception and design: R.S., C.M.H., H.J.B., T.A.L., and G.P.v.N.A.; sample collection and performing experiments: R.S., C.M.H., N.R., R.D.F., C.D., K.J.H., K.G., L.T., W.T., G.D.N., M.A.P., and T.A.L.; analysis and interpretation: R.S., C.M.H., H.J.B., G.P.v.N.A., and F.S.d.M.; figure preparation: R.S., C.M.H., and G.P.v.N.A.; and writing: R.S., C.M.H., H.J.B., C.G., and P.B.Y.

Correspondence and requests for reprints should be addressed to Harm J. Bogaard, M.D., Ph.D., Department of Pulmonology, VU University Medical Center, De Boelelaan 1118, 1081 HV Amsterdam, the Netherlands. E-mail: hj.bogaard@vumc.nl

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 193, Iss 12, pp 1410–1420, Jun 15, 2016

Copyright © 2016 by the American Thoracic Society

Originally Published in Press as DOI: 10.1164/rccm.201506-1231OC on January 13, 2016

Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the

Subject: Pulmonary arterial hypertension (PAH) comprises a group of deadly lung diseases with different etiologies. Altered hemodynamics and increased fluid shear stress are recognized risk factors for all forms of PAH. Yet, the effect of high shear stress on pulmonary endothelial cells from patients with PAH has never been tested.

What This Study Adds to the

Field: We identified a novel dysfunction specific to the microvascular lung endothelium of patients with PAH that facilitates susceptibility to shear-induced endothelial injury. The endothelial shear response can be restored pharmacologically in cells derived from patients with idiopathic PAH, familial, and associated PAH. Treatment reverses occlusive remodeling in a rat model resembling the vasculopathy of PAH. Therefore, restoration of endothelial shear responses should be considered a novel treatment target in PAH.

The term “pulmonary arterial hypertension” (PAH) comprises a highly heterogeneous group of deadly lung diseases that occur in idiopathic and heritable forms but are more frequently associated with connective tissue disease, congenital heart disease, drugs, and toxins (1). Exuberant cellular growth in PAH culminates in characteristic occlusive pulmonary vascular remodeling as well as an increase in mean pulmonary artery pressure and pulmonary vascular resistance that eventually lead to right heart failure and death (2). The mechanisms that give rise to PAH are poorly understood but believed to entail the combination of multiple risk factors or “hits” involving increased vulnerability to vascular injury or defective vascular repair (3, 4). Together, these multiple hits facilitate the selective outgrowth of abnormal pulmonary vascular cells, including endothelial cells (ECs) that resemble several hallmarks of cancer (5, 6). Despite recent advances in preclinical models, the trigger for the vascular remodeling remains elusive.

Altered hemodynamics in the pulmonary vasculature, particularly in

patients with congenital post-tricuspid systemic-to-pulmonary shunts, was early on recognized as a risk factor for pulmonary hypertension associated with occlusive vascular remodeling (7). Later, it was demonstrated that pulmonary hypertension in these patients is accompanied by an abnormal EC phenotype, suggestive for an altered endothelial adaptation to high fluid shear stress (8). Yet, high shear stress (HSS) by itself appeared insufficient to initiate severe PAH-like remodeling experimentally and clinically (1, 9); therefore, HSS is seen as a predisposition that must synergize with some form of EC dysfunction or injury to cause remodeling (10, 11). Although the exact nature of this “second hit” remains elusive, experimentally, blockade of vascular endothelial growth factor receptor 2 (VEGFR2) has been frequently used (12, 13). VEGFR2, mainly known for its function in vascular development (14), forms a shear-sensor complex with the two endothelial junction proteins vascular endothelial (VE)-cadherin and platelet endothelial cell adhesion molecule-1 (PECAM-1) (15). Thereby, VEGFR2 blockade may cause dysfunction of EC shear sensing, leaving the cells unable to adapt morphology and function to the increased mechanical force. Although there is little evidence for the direct involvement of VEGFR2 inhibition in clinical PAH (16), it is conceivable that other mechanisms, such as intrinsic defects, toxins, and perhaps even genetic mutations, interfere with EC shear responses, which could be the pivotal source for endothelial injury and

subsequent vascular remodeling in PAH (8). Yet, the effect of HSS on pulmonary EC from patients with PAH has never been tested.

We hypothesize that a defective adaptation to HSS is part of the endothelial dysfunction in PAH, whereby enhancement of shear responsiveness will improve the vasculopathy of PAH. Hence, we tested whether microvascular ECs (MVECs) and pulmonary arterial ECs (PAECs) isolated from patient lungs with diverse etiologies of group I PAH adapt to HSS and if restoration of the EC shear response reverses vascular remodeling in the chronic hypoxia and Sugen5416 (SuHx) animal model for PAH.

Some of the results of this study have been previously reported in the form of an abstract (17).

Methods

Primary Cell Isolation

Lobectomy tissue was used for control MVEC isolations. Pulmonary artery rings and peripheral microvascular tissue for the isolation of PAH EC were obtained from patients with clinically well-characterized PAH group I (familial, associated, and idiopathic PAH [iPAH] cases) (Table 1). Primary lung EC showed typical growth patterns emerging into cobblestone monolayers and were positive for endothelial markers (see Figure E1 in the online supplement). Cell isolation was based on the previously published protocol (18) and modified as specified in the online supplement. The study was approved by the institutional review board of the VU University Medical Center (VUMc,

Table 1. Characteristics of Patients with Pulmonary Arterial Hypertension for the Isolation of Pulmonary Microvascular Endothelial Cells and Pulmonary Arterial Endothelial Cells

No.	mPAP (mm Hg)	Etiology	Treatment	Sex	Age (yr)	Surgery
1	69	Assoc. PAH	PDE5-I, ERA	F	59	Obd
2*	N.A.	iPAH	PDE5-I	M	9	Ltx
3	65	iPAH	PDE5-I, ERA, PGI2	F	54	Obd
4*	N.A.	Assoc. PAH	None	M	32	Obd
5*	64	fPAH	PDE5-I, ERA, PGI2	F	40	Ltx
6	43	iPAH	PDE5-I, PGI2	F	42	Ltx
7	89	iPAH	PDE5-I, ERA, PGI2	F	22	Ltx

Definition of abbreviations: Assoc. PAH = pulmonary arterial hypertension associated with other disease; ERA = endothelin receptor antagonist; fPAH = familial pulmonary arterial hypertension; iPAH = idiopathic pulmonary arterial hypertension; Ltx = lung transplantation; mPAP = mean pulmonary artery pressure; MVEC = microvascular endothelial cell; N.A. = not available; Obd = obduction; PAEC = pulmonary arterial endothelial cell; PAH = pulmonary arterial hypertension; PDE5-I = phosphodiesterase type 5 inhibitor; PGI2 = prostacyclin.

*MVEC and PAEC isolated from the same patient lung.

Amsterdam, the Netherlands), and consent was given.

Animals

Sugen (SU5416, Tocris Bioscience, Bristol, UK) and hypoxia mediated PAH-like vascular remodeling was induced as described previously (19). The treatment group received an intraperitoneal bolus injection of the pan-caspase inhibitor Z-Asp-2,6-dichlorobenzoyloxymethylketone (2 mg/rat; Z-Asp, ALX-260-029; Enzo Life Science, Farmingdale, NY) three times a week for 2 weeks starting at the normoxic period (20). The study was approved by the local animal welfare committee (VU-FYS 13-01, VUmc). For details refer to the online supplement.

Shear Stress

Ibidi μ -slides (Integrated BioDiagnostics, Munich, Germany) with varying channel geometries were used (Figure E2A) (21).

Cells were seeded with 40,000 cells/cm² and allowed to attach. Thereafter, unidirectional, pulsatile shear stress was gradually increased (2.5, 15, and 21 dyn/cm²) in intervals of 24 hours (Figure E2B). Shear adaptation, based on cell morphology and orientation, was quantified from phase-contrast images using Photoshop CS6 (Adobe, San Jose, CA). Cells were defined as shear adapted when 75% or more of the cells elongated (twice as long as wide). Details are in the online supplement.

Reverse Transcriptase–Polymerase Chain Reaction, Immunofluorescence, Histology, Western Blots, Kits, and Reagents

For Western blot analysis, antibodies were used against PECAM-1 recognizing extracellular (MEM-05, Invitrogen, Carlsbad, CA) or intracellular epitopes (C-20, Santa Cruz Biotechnology, Dallas, TX) in human

samples and c-terminal epitopes in rats (Abbiotec, San Diego, CA). The Image-iT LIVE Green Caspase Detection Kit (Molecular Probes, Eugene, OR) and the DeadEnd Fluorometric TUNEL assay (Promega, Fitchburg, WI) were used in accordance with manufacturer's instructions. Z-Asp was applied *in vitro* in a final concentration of 20 μ M. Details are found in the online supplement.

Cell Transfection and PECAM-1 Silencing

A 20-pM small interfering RNA (siRNA) pool against PECAM-1 (Santa Cruz) or nontargeting scrambled RNA (Santa Cruz) was transfected by electroporation. See the online supplement for details.

Statistics

Results were confirmed in at least three donors. The exact number of samples is specified in the text. Statistics were performed

Figure 1. Morphological shear adaptation of pulmonary arterial hypertension (PAH) microvascular endothelial cells (MVECs) is delayed. Representative phase-contrast images (scale bar, 400 μ m) of control MVEC, PAH MVEC, and PAH pulmonary arterial endothelial cells (PAECs) after exposure to high shear stress for 24, 72, and 120 hours. Arrow indicates direction of flow. Inlays show 1.5 $\times$ magnification (scale bar, 200 μ m). To the right, quantifications of shear adaptation (control, n = 8; PAH, n = 7; PAEC, n = 3; one-way analysis of variance) presented as number of not shear-adapted cells. Nr. = number.

using GraphPad Prism 5 (GraphPad Software, San Diego, CA), and *P* values of 0.05 or less were considered significant. Data are presented as mean $\pm$ SEM.

Results

Morphological Shear Adaptation of MVEC, but Not PAEC, Is Delayed in PAH

Pulmonary EC from different vascular beds and different etiologies were exposed to HSS to test the hypothesis that PAH EC have a defective shear response (Figure 1). Control MVEC responded quickly to HSS, with $57.0 \pm 3.9\%$ of cells acquiring an elongated morphology after 24 hours and $35.6 \pm 6.0\%$ of the cells aligning within a close angle of 30° to the axis of

flow (Figure E3). Shear adaptation of PAH MVEC was diminished, as only $45.8 \pm 2.8\%$ of the cells elongated after the first 24 hours. The remaining PAH MVEC persisted in their nonadapted, cobblestone morphology, whereas PAH PAEC isolated from the same PAH lungs adapted with similar efficiency as control MVEC and thereby significantly faster ($P = 0.02$) than their microvascular counterparts.

After 72 hours, control MVEC and PAH PAEC had reached full shear adaptation, with 75% or more of all cells elongated. In clear contrast, only $60.4 \pm 1.8\%$ of the PAH MVEC had elongated at this point in time and did not reach full shear adaptation until 120 hours after shear onset. Taken together, MVEC from patients with iPAH, familial, and associated group I PAH presented with

a delayed EC shear adaptation, with most pronounced morphological differences at 72 hours after shear onset.

PECAM-1 Protein Levels Are Decreased in PAH MVEC

To gain mechanistic insight in the delayed shear adaptation of PAH MVEC, protein expression of the known shear sensors VE-cadherin, PECAM-1, and VEGFR2 (15) was quantified in static cultures (Figure 2A). Of the three proteins, PECAM-1 was significantly decreased ($P = 0.02$) in PAH MVEC, whereas VE-cadherin and VEGFR2 levels were similar to control MVEC. To put the decreased PECAM-1 protein levels into context, expression and activation of PECAM-1 signaling mediators were investigated (Figure E4A). Phosphorylation of

Figure 2. Platelet endothelial cell adhesion molecule-1 (PECAM-1) protein expression and interendothelial localization are disturbed in pulmonary arterial hypertension (PAH) microvascular endothelial cells (MVECs). (A) Protein expression of the shear sensors vascular endothelial (VE)-cadherin, PECAM-1, and vascular endothelial growth factor receptor 2 (VEGFR2) in static control versus PAH MVEC (control, $n = 6$; PAH, $n = 7$; unpaired Student's *t* test). Representative Western blots are shown. Data normalized to total ERK1/2. (B) Relative (rel.) mRNA expression of control and PAH MVEC under static culture conditions and after 72 hours of high shear stress (control, $n = 3$; PAH, $n = 3$; two-way analysis of variance). (C) Representative PECAM-1 and VE-cadherin immunostaining of control and PAH MVEC under static culture conditions, and (D) 72 hours after high shear stress (scale bars, 50 μ m). Arrowheads highlight areas of low peripheral PECAM-1. Nuclei were counterstained with Hoechst. Image intensities are not equal. Arrow represents direction of flow. a.u. = arbitrary units.

ERK1/2 was significantly increased, and phosphorylation of SRC and caveolin-1 levels were significantly decreased, as reported previously (22, 23). In clear contrast, the PECAM-1 independent shear-responsive 5' adenosine monophosphate-activated protein kinase (AMPK α) and protein kinase B (AKT) were not differentially expressed in PAH MVEC; therefore, we concluded that specifically PECAM-1-dependent signaling was altered.

Shear-Dependent Gene Regulation Is Functional in PAH MVECs

To elucidate whether the decreased PECAM-1 protein expression is caused by a transcriptional defect, gene expression of the three shear-sensor genes VE-cadherin, PECAM-1, and VEGFR2 was quantified (Figure 2B). Both PAH and control MVEC exhibited similar mRNA expression levels with no differences under static culture conditions. Furthermore, gene expression of the candidate shear-responsive genes was compared after 72 hours of HSS to test whether defective shear-dependent activation is causative for the delayed shear response. Also here, PAH and control MVEC showed a similar increase in mRNA levels, indicating an intact transcriptional shear response. Furthermore, increased levels of bone morphogenetic protein receptor type 2 (BMPR2) and mothers against decapentaplegic homolog 6 (SMAD6) in control and PAH MVEC, suggested functional shear-induced BMP signaling (Figure E4B). Interestingly, transforming growth factor (TGF)- β_1 expression in PAH MVEC was increased by twofold, which, taken together with the slightly increased levels of SMAD7 and PAI1, suggested excessive TGF- β signaling in PAH MVEC after HSS challenge; this is in line with the current understanding of imbalanced TGF/BMP signaling in PAH (24).

PECAM-1 Interendothelial Localization Is Disturbed in PAH MVECs

The tested genes did not explain the decreased PECAM-1 protein levels or the altered PAH MVEC shear response. Therefore, subcellular localization of PECAM-1 was assessed, as it is essential for its function (25) and to rule out post-transcriptional effects. Under static as well as HSS conditions (Figures 2C and 2D), PAH MVEC showed an intermittent distribution of PECAM-1 with areas

entirely lacking the junctional protein, whereas control MVEC exhibited a uniform peripheral PECAM-1 staining in both culture conditions. Interestingly, VE-cadherin was found unaltered under the tested conditions, which showed that loss of cell material does not contribute to the diminished PECAM-1 levels in PAH MVEC. The side-by-side comparison further underlined the differences in morphological shear adaptation, and the summarized changes in PECAM-1 protein expression and localization suggested a central role for PECAM-1 in the delayed PAH shear adaptation.

PAH MVECs Are Susceptible to Injury at Sites of Nonuniform Shear Profiles

Vascular branch points, which are characterized by nonuniform shear profiles, are critical loci for the vascular remodeling in PAH (26). We therefore hypothesized that the delayed PAH MVEC shear adaptation has important implications at these sites (Figure 3). PAH MVEC showed severe cell loss when subjected to

nonuniform flow, especially between 48 to 72 hours after shear onset, when morphological shear adaptation was incomplete. Immunostaining revealed that PECAM-1 was partly lacking from these areas and specifically from sites of interendothelial gaps. PAH MVEC monolayers in areas characterized by laminar flow remained intact but showed a non-shear-adapted morphology and patchy distribution of PECAM-1. On the contrary, control MVEC adjusted their morphology to the different flow profiles and presented with an intact EC monolayer and homogenous interendothelial distribution of PECAM-1. Thus, EC with a delayed adaptation to shear are prone to injury induced by excessive shear rates and nonuniform shear profiles.

Silencing of PECAM-1 Resembles Delayed PAH MVEC Shear Adaptation

To determine whether reduced PECAM-1 protein expression is sufficient to delay EC shear adaptation similar to PAH MVEC, PECAM-1 was silenced in control MVEC

Figure 3. The delayed pulmonary arterial hypertension (PAH) microvascular endothelial cell (MVEC) shear adaptation facilitates endothelial injury at sites of nonuniform flow profiles. Representative side-by-side comparison of control and PAH MVEC shear adaptation 48 hours after application of uniform (laminar, inner branch) and nonuniform (bifurcation, outer branch) shear profiles (scale bars, 400 μ m). White areas indicate sites of severe cell loss. Arrows present general direction of flow. Platelet endothelial cell adhesion molecule-1 (green) and nuclei (blue) were stained (scale bars, 50 μ m). Arrowheads indicate interendothelial gaps. Owing to the channel geometry, some autofluorescence and light scattering is recognizable.

Figure 4. Platelet endothelial cell adhesion molecule-1 (PECAM-1) silencing resembles delayed shear adaptation, whereas stabilization of PECAM-1 normalizes shear responsiveness in all forms of pulmonary arterial hypertension (PAH). (A) Representative phase-contrast images (scale bar, 400 μ m) of control microvascular endothelial cell (MVEC) treated with either scrambled RNA (scMVEC) or small interfering RNA against PECAM-1 (siPECAM-1) at 72 hours after high shear stress (HSS). Insets show 1.5 $\times$ magnifications (scale bar, 200 μ m). Arrow indicates direction of flow. To the right, representative

(Figure 4A). PECAM-1 siRNA stably reduced protein expression by 50 to 70% to levels of PAH MVEC during the course of the experiment. PECAM-1–silenced MVEC exhibited a delayed shear adaptation closely resembling PAH MVEC. The siRNA-treated control MVEC did not reach full shear adaptation until 120 hours after shear onset, whereas scrambled controls showed full shear adaptation at 72 hours, indicating that the decrease in PECAM-1 is causative for the altered shear response.

Inhibition of Caspase-mediated PECAM-1 Cleavage Stabilizes Protein Levels and Restores PAH MVEC Shear Responsiveness *In Vitro*

A previous study showed that cytosolic PECAM-1 cleavage is caspase mediated; however, the functional consequence remained widely unclear (27). PAH MVEC exhibited increased levels of active caspase compared with controls (Figure 4B), which is supported by a recent report showing increased caspase activity in human samples and an animal model of PAH (28). Interestingly, the high levels of active caspase were not related to increased apoptosis (Figure 4C). Therefore, caspase inhibition was tested as a means to stabilize PECAM-1 protein levels and thereby improve shear responsiveness (Figure 4D). PAH MVEC treated with the pan-caspase inhibitor Z-Asp showed an increase in full-length PECAM-1 (130 kD) and decreased levels of its truncated cytoplasmic 28 kD fragment, whereas PECAM-1 levels in control MVEC remained unaffected, and their shear response was diminished (Figure E5). On the contrary, shear responses of Z-Asp–treated PAH MVEC were normalized to untreated control levels, reaching full shear adaptation 72 hours after shear onset (Figures 4D and 4E), whereas nontreated PAH MVEC showed the typical delay in adaptation. Importantly, the effect of Z-Asp was independent from the etiology of PAH and restored shear responsiveness in all samples.

Stabilization of PECAM-1 by Z-Asp Attenuates Occlusive Remodeling *In Vivo*

Based on the presented *in vitro* findings, Z-Asp was administered as an acute intervention to SuHx rats with established PAH to test PECAM-1 stabilization and restoration of the endothelial shear response as a possible treatment target (Figure 5A). Two weeks of repetitive treatment with Z-Asp significantly decreased cleaved caspase 3 isoforms (CC3, $P = 0.03$), as a general marker of proapoptotic signaling in the SuHx model (29) and enhanced c-terminal PECAM-1 protein levels ($P = 0.01$) (Figures 5B and 5C). The treatment reduced arterial elastance ($P = 0.03$) and total pulmonary resistance ($P = 0.007$) (Table 2), which was confirmed with histological staining showing a significantly decreased formation of occlusive lesions ($P = 0.04$, Figure 5D). The attenuated occlusive remodeling was caused by a specific and significant reduction in intimal thickness ($P < 0.0001$), whereas the media remained thickened, indicating endothelial-specific effect of caspase inhibition (Figure 5E). The *in vivo* experiments confirm the *in vitro* findings showing a positive effect of caspase blockage on PECAM-1 levels and reversal of intimal remodeling in PAH lungs.

Discussion

We identified a novel dysfunction specific to the pulmonary microvascular endothelium of patients with diverse PAH etiologies, which manifests as a delayed morphological adaptation to HSS, facilitates susceptibility to shear-induced endothelial injury, and is caused by caspase-mediated cleavage of the shear sensor PECAM-1 (Figure 6). Importantly, we demonstrated that stabilization of PECAM-1 by caspase inhibition restored shear responsiveness *in vitro* and attenuated occlusive remodeling *in vivo*. Our findings support the notion that dysfunctional shear adaptation, shear-induced injury, and vascular remodeling are

interrelated, making MVEC shear responsiveness a unifying determinant in several forms of PAH.

Pulmonary EC dysfunction is a critical element in the pathogenesis of PAH, characterized by loss of vasodilator responses due to a progressive imbalance in favor of endogenous vasoconstrictors, such as serotonin and endothelin-1, which in turn affect the function of various other vascular cells, including smooth muscle cells, fibroblasts, and pericytes (30). Next to dysfunctional vasoconstrictor and growth factor secretion, loss of barrier function is believed to be a feature of endothelial dysfunction in PAH (31). PECAM-1 is an endothelial junction molecule that contributes to overall barrier integrity via homotypic binding (32). Temporal changes in PECAM-1 gene expression, relative protein amounts, and peripheral localization can impair cell–cell cohesion and wound healing capabilities (25, 33). Our findings are in line with these reports showing reduced protein levels and disrupted junctional organization of PECAM-1 in PAH MVEC.

In addition to endothelial barrier regulation and maintenance, PECAM-1 also functions as a scaffold protein that tethers signaling molecules and coordinates signaling in positive and negative feedback loops (25). Although altered PECAM-1 signaling in PAH remains to be fully defined, our data strongly implicate a central role for PECAM-1 in the defective EC shear response, as PECAM-1 silencing fully resembled the PAH MVEC shear phenotype. This is in accordance with extensive evidence suggesting PECAM-1 as direct transducer of mechanical forces (15) that couples fast temporal shear changes into EC and thereby mediates timing of NO-dependent vasodilation (34). However, to our knowledge this report represents the first evidence directly linking defective EC shear responsiveness to a human disease.

Further evidence for a central role of PECAM-1 in pulmonary pathology comes

Figure 4. (Continued). Western blots for silencing efficiency. Blot intensities were quantified (*italic characters*). ERK1/2 was loading control. (B) Representative immunostaining (*scale bar*, 20 μ m) of active caspases (*green*) in control and PAH MVEC. Cells were partly treated with the caspase inhibitor Z-Asp-2,6-dichlorobenzoyloxymethylketone (Z-Asp). Nuclei (*blue*) were counterstained with Hoechst. (C) Representative terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling (*scale bar*, 20 μ m). (D) Representative phase-contrast images of PAH MVEC with and without Z-Asp treatment after 72 hours of HSS. *Arrow* indicates direction of flow. To the *right*, representative Western blots for full-length PECAM-1 (130 kD) and its truncated cytoplasmic fragment (28 kD). ERK1/2 and glyceraldehyde phosphate dehydrogenase (GAPDH) were loading controls. (E) Quantification of non-shear-adapted cell fractions after 24, 72, and 120 hours of HSS (control, $n = 3$; PAH, $n = 3$; unpaired Student's *t* test).

Figure 5. Caspase inhibition stabilizes platelet endothelial cell adhesion molecule-1 (PECAM-1) levels and attenuates intimal thickening in Sugen5416 and hypoxia (SuHx)-treated rats. (A) Study design. SuHx rats were treated with vehicle or Z-Asp-2,6-dichlorobenzoyloxymethylketone (Z-Asp) after the hypoxic period, and control animals, held under normoxic conditions, were vehicle treated (control, n = 5; SuHx, n = 8; Z-Asp, n = 12; one-way analysis of variance or unpaired Student's *t* test). (B) Relative protein expression of active caspase 3 as a general marker for proapoptotic signaling, and (C) C-terminal PECAM-1 in whole lung lysates. Representative blots are shown with β-actin as loading control. (D) Representative immunohistochemical staining for von Willebrand factor (green), α-smooth muscle actin (red), and nuclei (blue) on small peripheral lung vessels (outer diameter [OD] ≤ 60 μm; scale bar, 20 μm). To the right, corresponding quantification of vessels characterized by occlusive lesions. (E) Representative elastic van Gieson staining (scale bar, 20 μm) and associated quantifications of intimal and medial wall thickness. a.u. = arbitrary units; Cath = open chest right ventricular catheterization; Ctrl = control; Echo = echocardiography; rel. = relative.

from PECAM-1 knockout mice that possessed context-dependent protective or deteriorating effects in inflammatory as well as other vascular disorders (35) and

spontaneously developed lung disease resembling idiopathic pulmonary fibrosis (36), which in humans is associated with pulmonary hypertension (37).

In accordance with our findings, an abnormal EC phenotype suggestive for disturbed endothelial shear responses was early on identified in patients with

Table 2. Characterization of Rats Treated with Sugen5416 and Housed under Chronic Hypoxia

Parameter	SuHx + Vehicle	SuHx + Z-Asp	P Value
Lungs			
TPR	0.8 ± 0.2	0.4 ± 0.1	0.007
Lung mass (corr. TL)	50.0 ± 5.0	50.0 ± 8.0	n.s.
RV			
RVSP, mm Hg	67.0 ± 13.0	61.0 ± 18.0	n.s.
Ees, mm Hg/ml	129.8 ± 47.8	114.2 ± 60.2	n.s.
Ea, mm Hg/ml	348.7 ± 88.3	253.3 ± 26.9	0.03
Ees/Ea	3.2 ± 1.8	3.9 ± 1.8	n.s.
RV mass, corr. TL	12.0 ± 2.5	11.0 ± 2.0	n.s.
Fulton, RV/(LV + S)	0.5 ± 0.1	0.5 ± 0.1	n.s.

Definition of abbreviations: corr. TL = corrected for tibia length; Ea = arterial elastance; Ees = end systolic elastance; LV + S = left ventricle and septum; n.s. = not significant; RV = right ventricle; RVSP = right ventricular systolic pressure; SuHx = chronic hypoxia and Sugen5416; TPR = total pulmonary resistance; Z-Asp = Z-Asp-2,6-dichlorobenzoyloxymethylketone.

congenital heart disease who developed PAH with severe vascular remodeling, but the exact role of this misadaptation remained unknown (8). Recent mathematical models have postulated that vascular remodeling in the lung and the increase in shear stress are interdependent and originate from small distal arteries and arterioles (38). By demonstrating that

MVEC but not PAEC derived from the same PAH lung, exhibit marked defects in shear adaptation, we confirmed these data and highlight the importance of phenotypic endothelial heterogeneity in PAH (39). Yet, it remains impossible to determine whether delayed shear adaptation and diminished PECAM-1 levels are early contributors or later consequences in PAH, because the

tested cells were derived from patients with end-stage disease.

Regardless of whether defective shear adaptation and PECAM-1 expression are early or late developments in PAH, our data suggest that these defects are common to all patients with PAH with the tested etiologies. Therefore, high blood flow velocity might not only be a necessary inducer of endothelial injury and trigger of pulmonary vascular remodeling but furthermore could maintain the vascular pathology. This notion is supported by reports of reversal of occlusive remodeling after normalization of pulmonary blood flow by single lung transplantation (40) and by animal studies showing that hemodynamic alteration by pneumonectomy or hypoxia alone are insufficient to induce occlusive pulmonary vascular remodeling (11, 19). However, at this point it is unknown whether PECAM-1-deficient animals exhibit a greater propensity for PAH when subjected to hypoxia, pneumonectomy, left-to-right shunts, or other PAH risk factors that alter pulmonary hemodynamics.

The finding that gene expression and shear-dependent transcriptional regulation of PECAM-1 was normal, combined with the increased caspase activity in PAH MVEC, led us to the assumption that cleavage might cause the decreased PECAM-1 protein levels, as caspases get activated through post-translational modification via proteolysis. Our assumption was supported by previous findings of caspase-mediated cleavage of PECAM-1 (27) and increased caspase activity in human samples and animal models of PAH (28, 29). Here, onset of PAH-like vascular remodeling in SuHx rats was prevented by caspase inhibition, which was supposedly mediated by apoptosis blockade (12). By using caspase inhibition to restore PECAM-1 levels and thereby shear responsiveness *in vitro* and reverse established, occlusive remodeling in progressive PAH *in vivo*, we extended the previous observations and provide an alternative explanation. Furthermore, we showed that the proapoptotic signaling and increased caspase activity led to functional alterations in PAH MVEC but did not cause cell death. This is supported by data showing no causal relation between CC3 and cell apoptosis in the SuHx model (29). Mechanistically, the truncated part of PECAM-1 has been proven to exhibit enhanced binding affinity to γ -catenin

Figure 6. Microvascular endothelial cell (MVEC) dysfunction contributes to pulmonary arterial hypertension (PAH) via defective shear responses and consequent shear-induced injury. The defective endothelial response to shear is an intrinsic dysfunction of the microvascular endothelium, wherefore high shear stress might be both a trigger and a maintenance factor for the vascular remodeling in PAH. We propose exuberant proapoptotic signaling and caspase-mediated cytoplasmic cleavage of platelet endothelial cell adhesion molecule-1 (PECAM-1) as cause for the disturbed signaling and delayed shear response. The endothelial shear adaptation can be normalized by prevention of PECAM-1 cleavage through caspase blockage with Z-Asp-2,6-dichlorobenzoyloxymethylketone (Z-Asp). Binding sites of the used PECAM-1 antibodies are depicted in blue.

and SHP-2 and thereby possibly cause competitive inhibition of PECAM-1 signaling (27). Interestingly, drugs like phosphodiesterase inhibitors that are clinically used for the treatment of PAH are also known to prevent cleavage of molecules like VE-cadherin (41). In summary, PAH MVEC function is impeded by caspase-mediated protein alteration, truncation, and disruption of signaling. However, the cells seem to escape cell death by excessive proliferative signaling, which challenges the idea of an antiapoptotic EC phenotype in end-stage PAH (31).

In conclusion, because of unknown systemic effects, we do not specifically recommend caspase inhibition as a new PAH treatment, although there have been clinical studies in which oral application of caspase inhibitors was well tolerated (42). To that end, our article is meant as a conceptual/mechanical study demonstrating that restoration of EC shear adaptation via stabilization of PECAM-1 attenuated intimal hyperplasia in PAH animals, which could embody an endothelium-specific treatment strategy for PAH. Additionally, we showed successful anticaspase application in cells

from iPAH, familial, and associated PAH cases, which indicated a final, common mechanism. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank the Dutch Lung Foundation, Netherlands CardioVascular Research Initiative, the Dutch Heart Foundation, Dutch Federation of University Medical Centers, The Netherlands Organization for Health Research and Development, and the Royal Netherlands Academy of Sciences. They also thank Jan van Bezu for technical support and Jeroen Kole for graphical input.

References

- Simonneau G, Gatzoulis MA, Adatia I, Celermajer D, Denton C, Ghofrani A, Gomez Sanchez MA, Krishna Kumar R, Landzberg M, Machado RF, *et al.* Updated clinical classification of pulmonary hypertension. *J Am Coll Cardiol* 2013;62:D34–D41.
- Voelkel NF, Gomez-Arroyo J, Abbate A, Bogaard HJ, Nicolls MR. Pathobiology of pulmonary arterial hypertension and right ventricular failure. *Eur Respir J* 2012;40:1555–1565.
- Botney MD. Role of hemodynamics in pulmonary vascular remodeling: implications for primary pulmonary hypertension. *Am J Respir Crit Care Med* 1999;159:361–364.
- Yuan JX, Rubin LJ. Pathogenesis of pulmonary arterial hypertension: the need for multiple hits. *Circulation* 2005;111:534–538.
- Rai PR, Cool CD, King JA, Stevens T, Burns N, Winn RA, Kasper M, Voelkel NF. The cancer paradigm of severe pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2008;178:558–564.
- Guignabert C, Tu L, Le Hir M, Ricard N, Sattler C, Seferian A, Huertas A, Humbert M, Montani D. Pathogenesis of pulmonary arterial hypertension: lessons from cancer. *Eur Respir Rev* 2013;22: 543–551.
- Heath D, Whitaker W. The small pulmonary blood vessels in atrial septal defect. *Br Heart J* 1957;19:327–332.
- Rabinovitch M, Bothwell T, Hayakawa BN, Williams WG, Trusler GA, Rowe RD, Olley PM, Cutz E. Pulmonary artery endothelial abnormalities in patients with congenital heart defects and pulmonary hypertension: a correlation of light with scanning electron microscopy and transmission electron microscopy. *Lab Invest* 1986; 55:632–653.
- Dickinson MG, Bartelds B, Borgdorff MA, Berger RM. The role of disturbed blood flow in the development of pulmonary arterial hypertension: lessons from preclinical animal models. *Am J Physiol Lung Cell Mol Physiol* 2013;305:L1–L14.
- Sakao S, Taraseviciene-Stewart L, Lee JD, Wood K, Cool CD, Voelkel NF. Initial apoptosis is followed by increased proliferation of apoptosis-resistant endothelial cells. *FASEB J* 2005;19:1178–1180.
- Okada K, Tanaka Y, Bernstein M, Zhang W, Patterson GA, Botney MD. Pulmonary hemodynamics modify the rat pulmonary artery response to injury: a neointimal model of pulmonary hypertension. *Am J Pathol* 1997;151:1019–1025.
- Taraseviciene-Stewart L, Kasahara Y, Alger L, Hirth P, Mc Mahon G, Waltenberger J, Voelkel NF, Tudor RM. Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. *FASEB J* 2001;15:427–438.
- Nicolls MR, Mizuno S, Taraseviciene-Stewart L, Farkas L, Drake JL, Al Hussein A, Gomez-Arroyo JG, Voelkel NF, Bogaard HJ. New models of pulmonary hypertension based on VEGF receptor blockade-induced endothelial cell apoptosis. *Pulm Circ* 2012;2: 434–442.
- Holmes K, Roberts OL, Thomas AM, Cross MJ. Vascular endothelial growth factor receptor-2: structure, function, intracellular signalling and therapeutic inhibition. *Cell Signal* 2007;19:2003–2012.
- Tzima E, Irani-Tehrani M, Kiosses WB, Dejana E, Schultz DA, Engelhardt B, Cao G, Delisser H, Schwartz MA. A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. *Nature* 2005;437:426–431.
- Voelkel NF, Gomez-Arroyo J. The role of vascular endothelial growth factor in pulmonary arterial hypertension: the angiogenesis paradox. *Am J Respir Cell Mol Biol* 2014;51:474–484.
- Szulcek R, Fontijn RD, Joemai S, Horrevoets AJ, Vonk-Noordegraaf A, Bogaard HJ, van Nieuw Amerongen GP. The micro- but not macrovascular lung endothelium of pulmonary arterial hypertension patients shows a defective adaptation to high levels of fluid shear stress [abstract]. *Am J Respir Crit Care Med* 2014; 189:A4807.
- van der Heijden M, van Nieuw Amerongen GP, van Bezu J, Paul MA, Groeneveld AB, van Hinsbergh VW. Opposing effects of the angiopoietins on the thrombin-induced permeability of human pulmonary microvascular endothelial cells. *Plos One* 2011;6: e23448.
- de Raaf MA, Schali J, Gomez-Arroyo J, Rol N, Happe C, de Man FS, Vonk-Noordegraaf A, Westerhof N, Voelkel NF, Bogaard HJ. SuHx rat model: partly reversible pulmonary hypertension and progressive intima obstruction. *Eur Respir J* 2014;44:160–168.
- Taraseviciene-Stewart L, Gera L, Hirth P, Voelkel NF, Tudor RM, Stewart JM. A bradykinin antagonist and a caspase inhibitor prevent severe pulmonary hypertension in a rat model. *Can J Physiol Pharmacol* 2002;80:269–274.
- Cicha I, Beronov K, Ramirez EL, Osterode K, Goppelt-Strube M, Raaz D, Yilmaz A, Daniel WG, Garlisch CD. Shear stress preconditioning modulates endothelial susceptibility to circulating TNF-alpha and monocytic cell recruitment in a simplified model of arterial bifurcations. *Atherosclerosis* 2009; 207:93–102.
- Bakhshi FR, Mao M, Shajahan AN, Piegeler T, Chen Z, Chernaya O, Sharma T, Elliott WM, Szulcek R, Bogaard HJ, *et al.* Nitrosation-dependent caveolin 1 phosphorylation, ubiquitination, and degradation and its association with idiopathic pulmonary arterial hypertension. *Pulm Circ* 2013;3:816–830.
- Tu L, Dewachter L, Gore B, Fadel E, Dartevelle P, Simonneau G, Humbert M, Eddahibi S, Guignabert C. Autocrine fibroblast growth factor-2 signaling contributes to altered endothelial phenotype in pulmonary hypertension. *Am J Respir Cell Mol Biol* 2011;45: 311–322.
- Upton PD, Morrell NW. TGF-beta and BMPR-II pharmacology: implications for pulmonary vascular diseases. *Curr Opin Pharmacol* 2009;9:274–280.
- Privratsky JR, Newman PJ. PECAM-1: regulator of endothelial junctional integrity. *Cell Tissue Res* 2014;355:607–619.

26. Cool CD, Stewart JS, Werahera P, Miller GJ, Williams RL, Voelkel NF, Tuder RM. Three-dimensional reconstruction of pulmonary arteries in plexiform pulmonary hypertension using cell-specific markers: evidence for a dynamic and heterogeneous process of pulmonary endothelial cell growth. *Am J Pathol* 1999;155:411–419.
27. Ilan N, Mohsenin A, Cheung L, Madri JA. PECAM-1 shedding during apoptosis generates a membrane-anchored truncated molecule with unique signaling characteristics. *FASEB J* 2001;15:362–372.
28. Diebold I, Hennigs JK, Miyagawa K, Li CG, Nickel NP, Kaschwich M, Cao A, Wang L, Reddy S, Chen PI, *et al*. BMPR2 preserves mitochondrial function and DNA during reoxygenation to promote endothelial cell survival and reverse pulmonary hypertension. *Cell Metab* 2015;21:596–608.
29. Bogaard HJ, Mizuno S, Guignabert C, Al Hussaini AA, Farkas D, Ruiter G, Kraskauskas D, Fadel E, Allegood JC, Humbert M, *et al*. Copper dependence of angioproliferation in pulmonary arterial hypertension in rats and humans. *Am J Respir Cell Mol Biol* 2012;46:582–591.
30. Rabinovitch M. Molecular pathogenesis of pulmonary arterial hypertension. *J Clin Invest* 2012;122:4306–4313.
31. Budhiraja R, Tuder RM, Hassoun PM. Endothelial dysfunction in pulmonary hypertension. *Circulation* 2004;109:159–165.
32. Privratsky JR, Paddock CM, Florey O, Newman DK, Muller WA, Newman PJ. Relative contribution of PECAM-1 adhesion and signaling to the maintenance of vascular integrity. *J Cell Sci* 2011;124:1477–1485.
33. Gratzinger D, Canosa S, Engelhardt B, Madri JA. Platelet endothelial cell adhesion molecule-1 modulates endothelial cell motility through the small G-protein Rho. *FASEB J* 2003;17:1458–1469.
34. Bagi Z, Frangos JA, Yeh JC, White CR, Kaley G, Koller A. PECAM-1 mediates NO-dependent dilation of arterioles to high temporal gradients of shear stress. *Arterioscler Thromb Vasc Biol* 2005;25:1590–1595.
35. Woodfin A, Voisin MB, Nourshargh S. PECAM-1: a multi-functional molecule in inflammation and vascular biology. *Arterioscler Thromb Vasc Biol* 2007;27:2514–2523.
36. Schenkel AR, Chew TW, Chlipala E, Harbord MW, Muller WA. Different susceptibilities of PECAM-deficient mouse strains to spontaneous idiopathic pneumonitis. *Exp Mol Pathol* 2006;81:23–30.
37. Smith JS, Gorbett D, Mueller J, Perez R, Daniels CJ. Pulmonary hypertension and idiopathic pulmonary fibrosis: a dastardly duo. *Am J Med Sci* 2013;346:221–225.
38. Postles A, Clark AR, Tawhai MH. Dynamic blood flow and wall shear stress in pulmonary hypertensive disease. *Conf Proc IEEE Eng Med Biol Soc* 2014;2014:5671–5674.
39. Aird WC. Endothelium in health and disease. *Pharmacol Rep* 2008;60:139–143.
40. Deb S, Yun J, Burton N, Omron E, Thurber J, Nathan SD. Reversal of idiopathic pulmonary arterial hypertension and allograft pneumonectomy after single lung transplantation. *Chest* 2006;130:214–217.
41. Flemming S, Burkard N, Renschler M, Vielmuth F, Meir M, Schick MA, Wunder C, Germer CT, Spindler V, Waschke J, *et al*. Soluble VE-cadherin is involved in endothelial barrier breakdown in systemic inflammation and sepsis. *Cardiovasc Res* 2015;107:32–44.
42. Pockros PJ, Schiff ER, Shiffman ML, McHutchison JG, Gish RG, Afdhal NH, Makhviladze M, Huyghe M, Hecht D, Oltersdorf T, *et al*. Oral IDN-6556, an antiapoptotic caspase inhibitor, may lower aminotransferase activity in patients with chronic hepatitis C. *Hepatology* 2007;46:324–329.