CHAPTER

Delayed microvascular shear adaptation in Pulmonary Arterial Hypertension: role of Platelet Endothelial Cell Adhesion Molecule-1 cleavage

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This article has an online data supplement, which is accessible from this issue’s table of content online at www.atsjournals.org
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Shear Stress Maladaptation in Pulmonary Arterial Hypertension
An Ageless Concept

Despite significant improvement in our understanding of mechanisms involved in pulmonary artery remodeling in pulmonary arterial hypertension (PAH), the actual course of events leading to this devastating disease remains enigmatic. Indeed, several conditions have been shown to predispose to PAH, including epigenetic modifications (1, 2), genetic mutations, autoimmune and inflammatory conditions, drugs and toxins, and cardiac defects associated with increased pulmonary blood flow and/or pressure. Importantly, none of them appears to be sufficient on its own to lead to overt PAH, commanding the “multiple hits” theory, triggering and perpetuating the intense pulmonary vasculopathy leading to PAH. Intriguingly, despite highly heterogeneous initial triggers, pulmonary vessels and arteries very stereotypically in a manner similar to acute respiratory distress syndrome, in which variable initial lung injuries ultimately lead to strikingly homogeneous lung pathology (3). This stereotypic vascular response in PAH includes the formation of complex cellular and fibrotic intimal lesions, smooth muscle cells’ proliferation, and apoptosis resistance, as well as adventitial infiltration and thickening (4). During the last decades, our understanding of the mechanisms involved in the endothelial dysfunction and the cancer-like angioproliferation in PAH has increased substantially from genetic, epigenetic, metabolic, and inflammatory abnormalities that contribute to this pulmonary vascular remodeling (5). Interestingly, the role of disturbed pulmonary blood flow and the concomitant shear stress, recognized early on as a risk factor for pulmonary vascular injury and remodeling (6), has now been reacknowledged.

In this issue of the Journal, Szulc and colleagues (pp. 1410–1420) demonstrate that human pulmonary artery endothelial cells (PAEC) isolated from subpleural lung microcirculation of patients with PAH at the time of transplantation or necropsy and submitted to ex vivo high shear stress exhibited delayed morphological adaptation compared with control PAEC (7). Interestingly, proximal PAH-PAEC exhibited normal shear stress–related adaptation, confirming the phenotypic heterogeneity of PAEC within the pulmonary vascular bed in PAH (8). At the microcirculation level, platelet endothelial cell adhesion molecule (PECAM)-1 expression and activation of its signaling mediators (extracellular signal–regulated kinases, SRC, and caveolin 1) were significantly decreased in PAH. PECAM-1 expression was not only reduced but was distributed unevenly, with areas entirely lacking the functional protein, especially in areas of nonuniform flow. Conversely, PECAM-1 independent shear-responsive kinases and PECAM-1 mRNA were normally expressed in PAEC, suggesting intact shear stress sensing, but specific alterations in PECAM-1 signaling post-transcriptionally presumably related to changes in protein expression or activity (9). The aberrant morphological adaptation to shear stress was reproduced in control PAEC by silencing of PECAM-1, whereas inhibiting caspases restored a normal phenotype in PAH-PAEC. Finally, in vivo treatment with a pan-caspase inhibitor slightly improved pulmonary hemodynamics and attenuated the intimal occlusive remodeling in the Sugen-induced PAH rat model.

The article from Szulc and colleagues (7) brings perhaps as many questions as answers. Are these phenotypic abnormalities observed within subpleural microcirculation (that includes capillaries and venules) truly representative of PAEC originating from distal pulmonary arteries, where the most striking remodeling process is expected to occur? What are the expected consequences of a short-term delay in PAEC shear stress adaptation and reduced PECAM-1 expression? Is the normal delayed PECAM-1 expression and the partial improvement of intimal remodeling after treatment with pan-caspase inhibition truly related to PECAM-1 restoration, rather than off-target effects? Finally, what is the contribution of the shear stress maladaptive response on skeletal muscle and right ventricle endothelial cells that are also affected in PAH (9, 10)?

As a result, the exact contribution of shear stress–dependent endothelial abnormalities within the complex pathobiology of PAH remains unknown. Nonetheless, in addition to its important role in blood clotting, inflammation, vascular tone, metabolism, angiogenesis, and repair, the endothelium is a complex mechanical signal-transduction interface that serves an important barrier function between flowing blood and its soluble plasma factors and the underlying vessel walls. Shear stress imposed upon the endothelium, defined as the tangential force per unit area caused by flowing blood (11), has been shown to modulate the endothelium phenotype, including its barrier function (12). There is also a large body of evidence from experimental and clinical studies suggesting that shear stress is clinically important in the pathophysiology of PAH. Indeed, intense shear stress has been shown to be sufficient to cause severe pulmonary vascular injury and remodeling in large animal studies in which complex neointimal lesions comparable with human disease develop when the lung circulation is directly connected to the systemic circulation (6), or in patients with untreated nonrestrictive, post-tricuspid congenital shunts whom majoritarily develop advanced neointimal lesions and PAH (13). Recent data also suggest that concurrent shear stress is required for the development and maintenance of severe occlusive vascular lesions after Sugen-induced pulmonary vascular injury (14). This is also supported by pathological reports of a complete reversal of pulmonary remodeling within the native PAH lung after prolonged normalization of pulmonary hemodynamics after contralateral single lung transplantation (15).

Taken together, shear stress and potentially shear stress–related PAEC maladaptation undoubtedly contribute to the development, maintenance or exacerbation of the stereotypic pulmonary vascular injury and remodeling in PAH. This behavior shares numerous similarities with acute respiratory distress syndrome, in which distinct initial triggers lead to comparable mechanical stress (e.g., volutrauma) that exacerbates functional and structural alterations in this stereotypic lung injury (3). Importantly, minimizing this...
subsequent mechanical stress with protective ventilation improves survival and is now the cornerstone supportive therapy in acute respiratory distress syndrome. Hence, there is an urgent need to improve our understanding of the molecular relevance and consequences of these shear stress–related abnormalities in PAH and how shear stress–related interventions, in addition to marked improvements in pulmonary hemodynamics, may affect disease progression. The data presented by Szulcek and colleagues (7) rehighlight that PAEC and shear stress–mediated abnormalities likely play a key role in the actual course of events culminating in PAH. They also rehighlight the fact that apoptosis is timely regulated in PAH, suggesting that drugs aimed at blocking apoptosis of distal PAEC might prevent the development vascular remodelling in PAH, whereas promoting apoptosis in end-stage PAH might improve it. This could explain the authors’ limited effects on established vascular lesion when they blocked apoptosis. Although many questions on the role of shear stress remained to be answered, the paper by Szulcek and colleagues represents an important step forward in our understanding of the complex pathobiology of PAH.

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

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References

Diagnosis of Latent Tuberculosis Infection in HIV-infected Pregnant Women
“Baby Steps” toward Better Tuberculosis Control in Pregnancy

Despite significant reductions in incidence, the global burden of tuberculosis (TB) is immense. In 2014, there were an estimated 9.6 million new cases, accounting for 1.1 million deaths, with an estimated 1.2 million (12.5%) of these new cases being in patients infected with HIV (1). Although TB is more common among men, globally TB is a leading cause of death among women of childbearing age (1). The higher risk for TB during pregnancy, as well as adverse outcomes for mother and child, have been previously recognized (2–5) and are potentially related to the physiological partial inhibition of the cellular immune system necessary to tolerate the fetus (6, 7).

There is evidence of treatment delays for TB in pregnancy, possibly related to diagnostic difficulties because of the symptom masking by the pregnancy and greater reluctance for diagnostic tests, particularly imaging, as well as general lack of awareness (4, 5).
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 (HSS) on pulmonary endothelial cells from PAH patients has never been tested.

What This Study Adds to the Field: We identified a novel dysfunction specific to the microvascular lung endothelium of PAH patients, which facilitates susceptibility to shear induced endothelial injury. The endothelial shear response can be restored pharmacologically in cells derived from idiopathic PAH, familial and associated PAH patients. 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.

ABSTRACT

Altered pulmonary hemodynamics and high fluid 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.

We hypothesize that failing shear adaptation is an essential part of the endothelial dysfunction in all forms of PAH and tested whether microvascular (MVEC) respectively arterial endothelial cells (PAEC) from PAH patient lungs adapt to HSS and if the shear defect partakes in vascular remodeling in-vivo.

PAH MVEC (n=7) and PAH PAEC (n=3) morphology, function, protein and gene expressions were compared to control MVEC (n=8) under static culture conditions and after 24, 72, and 120 hours HSS. PAH MVEC showed a significantly delayed morphological shear adaptation (p=0.03) and evidence of cell injury at sites of non-uniform 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 inter-endothelial localization of the shear sensor 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 (SuHx) rats modeling severe PAH.

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 upon stabilization of PECAM-1, which reverses intimal remodeling in-vivo.

INTRODUCTION

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 thought 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 (EC) 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), wherefore HSS is seen as a pre-disposition that must synergize with some form of EC dysfunction or injury to cause remodeling (10, 11). While 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 VE-cadherin and 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. While there is little evidence for the direct involvement of VEGFR2 inhibition in clinical PAH (16), it is conceivable that other mechanisms like 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 PAH patients 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 (MVEC) and arterial endothelial cells (PAEC) 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 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 clinically well characterized PAH group I patients (familial, associated and idiopathic PAH cases) (Table 1). Primary lung EC showed typical growth patterns emerging into cobblestone monolayers and were positive for endothelial markers (Supplemental Figure E1). Cell isolation was based on the previously published protocol (18) and modified as specified in the online supplement. The study was approved by the IRB of the VU University Medical Center (VUmc, 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 injections of the pan-caspase inhibitor Z-Asp-2,6-dichlorobenzoxyloxymethylketone (2 mg/rat, Z-Asp, ALX-260-029, Enzo Life Science, Farmingdale, NY, USA) 3 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 (Supplemental 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; 21 dyn/cm²) in intervals of 24 h (Supplemental Figure E2B).

Table 1: PAH patient characteristics for the isolation of pulmonary microvascular endothelial cells (MVEC) and pulmonary arterial endothelial cells (PAEC).

<table>
<thead>
<tr>
<th>No.</th>
<th>mPAP (mmHg)</th>
<th>Etiology</th>
<th>Treatment</th>
<th>Gender</th>
<th>Age (yr)</th>
<th>Surgery</th>
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<td>F</td>
<td>59</td>
<td>Obd</td>
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<tr>
<td>2*</td>
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<td>PDE5-I</td>
<td>M</td>
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<td>Ltx</td>
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<td>PDE5-I, ERA, PGI2</td>
<td>F</td>
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<td>Obd</td>
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<tr>
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<td>M</td>
<td>32</td>
<td>Obd</td>
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<tr>
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<td>40</td>
<td>Ltx</td>
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<td>F</td>
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<tr>
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<td>iPAH</td>
<td>PDE5-I, ERA, PGI2</td>
<td>F</td>
<td>22</td>
<td>Ltx</td>
</tr>
</tbody>
</table>

Abbreviations: PAH = Pulmonary Arterial Hypertension; iPAH = idiopathic PAH; fPAH = familial PAH; assoc. PAH = PAH associated with other disease; mPAP = mean pulmonary artery pressure; Obd = obduction; Ltx = lung transplantation; N. A. = not available; PDE5-I = phosphodiesterase type 5 inhibitor; ERA = endothelin receptor antagonist; PGI2 = prostacyclin; *MVEC and PAEC isolated from the same patient lung.
Shear adaptation, based on cell morphology and orientation, was quantified from phase-contrast images using Photoshop CS6 (Adobe, San Jose, CA, USA). Cells were defined shear adapted when ≥75% of the cells elongated (twice as long as wide). Details in the online supplement.

**RT-PCR, immunofluorescence, histology, Western blots, kits and reagents**

For Western blot analysis antibodies were used against PECAM-1 recognizing extracellular (MEM-05, Invitrogen, Carlsbad, CA, USA) or intracellular epitopes (C-20, Santa Cruz Biotechnology, Dallas, TX, USA) in human samples and c-terminal epitopes in rats (Abbiotec, San Diego, CA, USA). The Image-iT™ LIVE Green Caspase Detection Kit (Molecular Probes, Eugene, OR, USA) and the DeadEnd™ Fluorometric TUNEL assay (Promega, Fitchburg, WI, USA) were used in accordance with manufacturer’s instructions. Z-Asp was applied *in-vitro* in a final concentration of 20 µM. Details are made available in the online supplement.

**Cell transfection and PECAM-1 silencing**

20 pM siRNA pool against PECAM-1 (Santa Cruz) or non-targeting scRNA (scrambled, Santa Cruz) was transfected by electroporation. Refer to the online supplement for details.

**Statistics**

Results were confirmed in at least three donors. The exact number of samples (n) is specified in the text. Statistics were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) and p-values ≤0.05 were considered significant. Data are presented as mean ± 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±3.9% of cells acquiring an elongated morphology after 24 h and 35.6±6.0% of the cells aligning within a close angle of 30° to the axis of flow (Supplemental Figure E3). Shear adaptation of PAH MVEC was diminished, as only 45.8±2.8% of the cells elongated after the first 24 h. The remaining PAH MVEC persisted in their non-adapted, 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 h control MVEC and PAH PAEC had reached full shear adaptation with ≥75% of all cells elongated. In clear contrast, only 60.4±1.8% of the PAH MVEC had elongated at this point in time and did not reach full shear adaptation until 120 h after shear onset. Taken together, MVEC from idiopathic, familial and associated group I PAH patients
presented with a delayed EC shear adaptation with most pronounced morphological differences at 72 h 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 was investigated (supplemental Figure E4A). Phosphorylation of ERK1/2 was significantly increased, phosphorylation of SRC and caveolin-1 (CAV-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 (AMPKα) and protein kinase B (AKT) were not differentially expressed in PAH MVEC. We concluded that specifically PECAM-1 dependent signaling was altered.
Shear dependent gene regulation is functional in PAH MVEC

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 h 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. Further, increased levels of BMPR2 and SMAD6 in control and PAH MVEC, suggested functional shear induced BMP signaling (Supplemental Figure E4B). Interestingly, TGF-β1 expression in PAH MVEC was increased by 2-fold, which taken together with the slightly increased levels of SMAD7 and PAI1, suggested excessive TGF-β signaling in PAH MVEC after HSS challenge, which is in-line with the current understanding of imbalanced TGF/BMP-signaling in PAH (24).
PECAM-1 inter-endothelial localization is disturbed in PAH MVEC

The tested genes did neither explain the decreased PECAM-1 protein levels nor the altered PAH MVEC shear response. Therefore, sub-cellular 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 (Figure 2C and D) PAH MVEC showed an intermittent distribution of PECAM-1 with areas entirely lacking the junctional protein, while 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 MVEC are susceptible to injury at sites of non-uniform shear profiles

Vascular branch points, which are characterized by non-uniform 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 non-uniform flow, especially between 48 to 72 h 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 inter-endothelial 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 inter-endothelial distribution of PECAM-1. Thus, EC with a delayed adaptation to shear are prone to injury induced by excessive shear rates and non-uniform 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 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 h after shear onset, whereas scrambled controls showed full shear adaptation at 72 h 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 to 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 kDa) and decreased levels of its truncated cytoplasmic 28 kDa fragment, whereas PECAM-1 levels in control MVEC remained unaffected and their shear response was diminished (Supplemental Figure E5). On the contrary, shear responses of Z-Asp treated PAH MVEC were normalized to untreated control levels reaching full shear adaptation 72 h after shear onset (Figure 4D and E), whereas non-treated 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 tested samples.

Figure 3: The delayed PAH MVEC shear adaptation facilitates endothelial injury at sites of non-uniform flow profiles. Representative side-by-side comparison of control and PAH MVEC shear adaptation 48 h after application of uniform (laminar, inner branch) and non-uniform (bifurcation, outer branch) shear profiles (scale 400 µm). White areas indicate sites of severe cell loss. Arrows present general direction of flow. PECAM-1 (green) and nuclei (blue) were stained (scale 50 µm). Arrow heads indicate inter-endothelial gaps. Due to the channel geometry some auto-fluorescence and light scattering is recognizable.
Figure 4: PECAM-1 silencing resembles delayed shear adaptation, whereas stabilization of PECAM-1 normalizes shear responsiveness in all forms of PAH. (A) Representative phase-contrast images (scale 400 µm) of control MVEC treated with either scrambled RNA (scMVEC) or siRNA against PECAM-1 (siPECAM-1) at 72 h after HSS. Inlays show 1.5x magnifications (scale 200 µm). To the right, representative Western blots for silencing efficiency. Blot intensities were quantified (cursive characters). ERK1/2 was loading control. (B) Representative immunostaining (scale 20 µm) of active caspases (green) in control and PAH MVEC. Cells were partly treated with the caspase inhibitor Z-Asp. Nuclei (blue) were counter stained with Hoechst. (C) Representative TUNEL staining (scale 20 µm). (D) Representative phase-contrast images of PAH MVEC with and without Z-Asp treatment after 72 h HSS. To the right, representative Western blots for full length PECAM-1 (130 kDa) and its truncated cytoplasmic fragment (28 kDa). ERK1/2 and GAPDH were loading controls. (E) Quantification of non-shear adapted cell fractions after 24, 72 and 120 h HSS (n_{Ctrl}=3; n_{PAH}=3, unpaired student’s t-test).
Stabilization of PECAM-1 by Z-Asp attenuates occlusive remodeling \textit{in-vivo}

Based on the presented \textit{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 repetitive treatment with Z-Asp significantly decreased cleaved caspase 3 isoforms (CC3, \(p=0.03\)), as a general marker of pro-apoptotic signaling in the SuHx model (29), and enhanced c-terminal PECAM-1 protein levels (\(p=0.01\)) (Figure 5B and C). The treatment reduced arterial elastance (\(E_a, p=0.03\)) and total pulmonary resistance (\(TPR, p=0.007\)) (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 (\(p<0.0001\)), while the media remained thickened indicating endothelial-specific effect of caspase inhibition (Figure 5E). The \textit{in-vivo} experiments confirm the \textit{in-vitro} findings showing a positive effect of caspase blockage on PECAM-1 levels and reversal of intimal remodeling in PAH lungs.

<table>
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<th>Parameter</th>
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<td>0.4 ± 0.1</td>
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<td>Lung mass (corr. TL)</td>
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<td>RV mass (corr. TL)</td>
<td>12.0 ± 2.5</td>
<td>11.0 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Fulton (RV/(LV+S))</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

Abbreviations: TPR = total pulmonary resistance; corr. TL = corrected for tibia length; RVSP = right ventricular systolic pressure; Ees = end systolic elastance; Ea = arterial elastance; RV = right ventricle; LV+S = left ventricle and septum

\textbf{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 \textit{in-vitro} and attenuated occlusive remodeling \textit{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, fibroblast and pericytes (30). Next to dysfunctional vasoconstrictor and growth factor secretion, loss of barrier function
is long 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). While 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 from PECAM-1 knock-out 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 congenital heart disease that developed PAH with severe vascular remodeling but the exact role of this miss-adaptation 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, since the tested cells were derived from end-stage patients.

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 PAH patients 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 pro-apoptotic 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 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 pro-proliferative signaling, which challenges the idea of an anti-apoptotic 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, where 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 anti-caspase application in cells from idiopathic and familial and associated PAH cases, which indicated a final, common mechanism.

ACKNOWLEDGMENTS
We acknowledge the support from the Dutch Lung Foundation grant 33.12.036, Netherlands CardioVascular Research Initiative grant number 2012-08 awarded to the Phaedra consortium (www.phaedraresearch.nl): 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. Further we would like to thank Jan van Bezu for his technical support and Jeroen Kole for his graphical input.

DISCLOSURE
The authors have declared that no conflict of interest exists.
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