

Associated inflammation or increased flow-mediated shear stress, but not pressure alone, disrupts endothelial caveolin-1 in infants with pulmonary hypertension

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ABSTRACT

Endothelial caveolin-1 loss is an important feature of pulmonary hypertension (PH); the rescue of caveolin-1 abrogates experimental PH. Recent studies in human PH suggest that the endothelial caveolin-1 loss is followed by an enhanced expression of caveolin-1 in smooth muscle cells (SMC) with subsequent neointima formation. In order to evaluate caveolin-1 expression in infants with PH, we examined the available clinical histories, hemodynamic data, and the expression of caveolin-1, PECAM-1, vWF, and smooth muscle α -actin in the lung biopsy/autopsy specimens obtained from infants with congenital heart disease (CHD, n = 8) and lung disease (n = 9). In CHD group, PH associated with increased pulmonary blood flow exhibited loss of endothelial caveolin-1 and PECAM-1 in pulmonary arteries; additional vWF loss was associated with enhanced expression of caveolin-1 in SMC. In the absence of PH, increased or decreased pulmonary blood flow did not disrupt endothelial caveolin-1, PECAM-1, or vWF; nor was there any enhanced expression of caveolin-1 in SMC. In Lung Disease + PH group, caveolin-1, PECAM-1, and vWF were well preserved in seven infants, and importantly, SMC in these arteries did not exhibit enhanced caveolin-1 expression. Two infants with associated inflammatory disease exhibited loss of endothelial caveolin-1 and PECAM-1; additional loss of vWF was accompanied by enhanced expression of caveolin-1 in SMC. Thus, associated flow-induced shear stress or inflammation, but not elevated pulmonary artery pressure alone, disrupts endothelial caveolin-1. Subsequent vWF loss, indicative of extensive endothelial damage is associated with enhanced expression of caveolin-1 in SMC, which may worsen the disease.

Key Words: congenital heart defect, endothelial cells, lung disease, smooth muscle cells

Pulmonary hypertension (PH) is a progressive disease characterized by endothelial dysfunction, impaired vascular relaxation, vascular remodeling resulting in progressive increase in vascular resistance and pressure, and right ventricular (RV) hypertrophy, ultimately leading to RV failure and death. The main causes of PH during infancy are congenital heart disease (CHD), persistent PH of the newborn, and lung diseases such as respiratory distress syndrome (RDS), bronchopulmonary dysplasia (BPD), and congenital defects associated with hypoplasia of the lungs.^[1] Pulmonary circulation undergoes a profound change at birth, from high resistance/low flow to low resistance/

high flow system; furthermore, the pulmonary vascular development continues after birth. Cardiac anomalies overloading the pulmonary circulation during fetal life can lead to endothelial damage and abnormal pulmonary vascular development.^[2,3] Inhibition of angiogenesis adversely affects alveolization in the developing lungs, thus, blood vessels actively promote alveolar development.^[4] Preterm delivery disrupts normal pulmonary vascular and broncho-alveolar development and the resulting reduction

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in cross sectional area of the pulmonary vasculature leads to increased pulmonary vascular resistance and PH.^[5]

Experimental and clinical studies indicate that endothelial dysfunction/disruption may be a critical factor in initiating the cascade of events leading to PH. Impaired endothelium-dependent vascular relaxation indicative of eNOS dysfunction has been reported in PH.^[1,6-8] For optimum activation, eNOS is targeted to endothelial caveolae, the omega shaped invaginations (50-100 nm) in plasma membrane of a variety of cells including endothelial cells (EC) and smooth muscle cells (SMC). Under basal conditions, caveolin-1, a major resident scaffolding protein of caveolae, inhibits eNOS via its interaction; however, it regulates Ca²⁺ entry required for the eNOS activation. Caveolin-1 promotes agonist-stimulated eNOS activity, and regulates eNOS-mediated angiogenesis.^[9,10] It interacts with several transducing molecules, stabilizes them in an inactive conformation within caveolae, and blocks signaling events involved in cell proliferation. Caveolin-1 inhibits the activity of PDGF, RhoA, PY-STAT3, and its downstream effectors Bcl-xL, cyclin D1, and survivin, all implicated in PH. Caveolin-1 also modulates inflammatory response and maintains the function and integrity of caveolae,^[10-17] thus playing a key role in pulmonary vascular health.

In monocrotaline (MCT)-induced PH, the progressive loss of endothelial caveolin-1 and reciprocal activation of PY-STAT3 (proliferative transcription factor) and Bcl-xL (anti-apoptotic factor) occurs; the rescue of caveolin-1 inhibits proliferative pathways and attenuates PH.^[18-20] The importance of caveolin-1 in PH is further supported by a recent report of a child who presented with severe PH two years after having made a complete clinical recovery from acute respiratory distress syndrome. Lung biopsy revealed endothelial caveolin-1 loss, and the arteries with additional loss of von Willebrand Factor (vWF) exhibited robust expression of caveolin-1 in SMC, ultimately leading to neointima formation.^[21] Endothelial caveolin-1 loss associated with enhanced expression of caveolin-1 in SMC has also been reported in idiopathic PH. SMC with enhanced caveolin-1 expression have increased cytosolic Ca²⁺, and a capacity to participate in DNA synthesis and cell proliferation.^[22] Thus, caveolin-1 has a cell-specific dual role in the context of the disease stage of PH.^[10] The aim of this study was to examine the expression of caveolin-1 in infants with PH associated with CHD and RDS/BPD.

MATERIALS AND METHODS

With the approval of our Institutional Review Board, we examined available clinical histories, hemodynamic data, and lung tissue obtained at autopsy/biopsy from infants (n = 20). Based on the underlying diagnosis, the patients

were divided into two groups: (1) CHD, n = 8, included in this group was a fetus; and (2) RDS/BPD, n = 9. Lung tissue from three additional infants (age: 3 hours to 58 days) who died of causes unrelated to cardiopulmonary diseases, and from one fetus, served as controls.

CHD group

Group 1A: CHD with increased pulmonary blood flow. This group included one fetus and four infants. Patient one: Because of prenatal diagnosis of Down syndrome and complete atrio-ventricular canal (AVC) defect, the pregnancy was terminated at 21 weeks gestation. Patient two: At age 23 days, cardiac catheterization in an infant revealed secundum atrial septal defect (ASD2°, Qp/Qs, 2.5:1), supra valvular aortic stenosis (AS), and pulmonary stenosis (PS; pressures in mmHg; LV, 154/20; Ao, 92/45 m62; RV, 73/8; proximal PA 47/16 m31; mean PA pressure of 23 in the distal PAs). After surgical correction of AS and PS, despite vasopressors, ventilator support, and inhaled nitric oxide (iNO), the infant continued to deteriorate with persistent low blood pressure, low cardiac output and gradually increasing PA pressure. The infant suffered pulmonary hypertensive crisis on post-op Day five, and was unresponsive to resuscitative measures. Patient three: An infant with ASD2° and patent ductus arteriosus (PDA) was treated with diuretics for heart failure soon after birth. At 10 months, he had mild pulmonary venous desaturation (93%), PH (in mmHg; PA 62/31, m46; aortic pressure 72/37, m53), patent foramen ovale (PFO), and a small PDA which was coiled. Because of the unusual presentation, a lung biopsy was done at the age of one year. He is currently doing well on sildenafil. Patient four: An infant with Shone's complex (coarctation of aorta, hypoplastic aortic arch, mitral and aortic valve stenosis), underwent coarctation and the aortic arch repair during the neonatal period. Cardiac catheterization at the age of eight months revealed AS (Ao pressure 79/49, m64; LV 108/8 mmHg), moderate PH (58/30, m44 mmHg), elevated pulmonary wedge (21 mmHg), left atrial pressures (m22 mmHg), and a mean gradient of 10 mmHg across the mitral valve. At the age of 13 months he underwent supra-annular mitral valve replacement. Following surgery, he acquired a large left ventricle to right atrial shunt (LV to RA) at a level just below the replaced mitral valve. A repeat cardiac catheterization revealed a large LV to RA shunt (Qp/Qs, 1.9:1), severe PH (89/47, m62 mmHg), pulmonary wedge pressure (21 mmHg), AS (LV 125/15; Ao 78/54, m64 mmHg), and mitral stenosis and insufficiency. Despite intensive medical management he continued to deteriorate and expired at the age of 16 months. Patient five: An infant with Down syndrome and a complete AVC defect underwent intracardiac repair at the age of four months. At age one year, he was diagnosed to have asthma, requiring four hospital admissions during the year. He

was not on any cardiac medication. At 23 months he was admitted with respiratory syncytial viral infection. After an initial improvement he died on the fifth day.

Group 1B: CHD with decreased pulmonary blood flow. Two infants with RV outflow obstruction died on Day two and seven, respectively. The third infant with pulmonary atresia (P At), small VSD, PFO, PDA, and small RV with minimal flow through tricuspid valve, developed cardiac arrest and died on Day 30 during an abdominal surgery for intestinal perforation.

Lung disease group

Group 2A: RDS/BPD. Five infants in this group with severe RDS and PH died within one to 20 days despite intensive therapy including ventilation support and iNO. The sixth infant born by c-section due to a prenatal diagnosis of non-immune hydrops, administered surfactant, and ventilated (high frequency oscillation and jet ventilators). Because of poor oxygenation he was started on iNO on Day 18. Echocardiography revealed PH. PH therapy (iNO, sildenafil, and iloprost) was not successful and the infant died on Day 53. The seventh infant had a large PDA ligated at 12 days age. He continued to have respiratory and nutritional problems, and was diagnosed to have PH at the age of four and a half months. Inhaled NO therapy was instituted for 12 days. At six months, he was assessed to have severe PH which was treated with iNO, sildenafil, and iloprost; the infant died 18 days later.

Group 2B: BPD associated with inflammation. Two premature infants with RDS had large PDA closed at two weeks with two courses of ibuprofen or indomethacin. One infant at three and a half months was diagnosed with PH, and managed for 15 days with iNO, and sildenafil for nine days to wean off iNO. At six months, sildenafil and iNO were restarted because of echocardiographic evidence of PH. In addition, the infant required multiple RBC and platelet transfusions for persistent thrombocytopenia. Two months before death he was diagnosed with hemophagocytic lympho-histiocytosis, a rare and severe inflammatory disease.^[23] The second infant was diagnosed with PH at two and a half months; iNO was started and subsequently, sildenafil, iloprost, and dipyridole were instituted without any improvement. In addition to PH, the infant had several bouts of infection, and blood and tracheal aspirates grew multiple organisms requiring successive therapy with several antibiotics.

Double immunofluorescence

Formaldehyde preserved lung tissue was processed for paraffin block. Five to six μm sections were cut, deparaffinized, rehydrated, and double immunofluorescence was performed as previously described.^[17,21] Primary antibodies and dilution used

were as follows: caveolin-1 α , 1:100; PECAM-1, 1:30, Santa Cruz biotechnologies Inc, Calif.; smooth muscle (SM) α -actin, 1:20, Sigma-Aldrich, Mo.; and vWF, 1:50, Dako, Carpinteria, Calif. Appropriate secondary antibodies Alexa 488 (donkey anti-rabbit, 1:300, green color, Molecular Probes, Eugene, Oreg.) and Alexa 594 (donkey anti-mouse, anti-goat, or anti-rabbit 1:200, red color) were used. The sections were examined with a laser scanning confocal fluorescence microscope (MRC 1000, Bio-Rad). The negative controls were run in the absence of primary antibodies. Arteries exhibiting autolysis and other postmortem changes were excluded from analysis.

Statistical analysis

The data are expressed as means \pm SE. For statistical analysis, we used student *t*-test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

The infants in the lung disease group were younger compared with the CHD group (age in months, 2.9 ± 1.3 vs. 6.7 ± 3.1 , $P = \text{ns}$). Gestational age in the RDS group was lower compared with the CHD group (in weeks, 26 ± 0.8 vs. 35 ± 1.8 , $P < 0.05$). Diagnosis, gestational age, age at autopsy/biopsy, and PA pressure are shown in Table 1.

Expression of caveolin-1 and smooth muscle α -actin

CHD with left-to-right shunt. SMC do contain caveolin-1, but EC have the highest amount of caveolin-1.^[24] Consistent with previous studies,^[16,18] caveolin-1 is not detected in normal pulmonary arterial SMC (Figures 1A and C, 2, 3A and B). Pulmonary arteries from patients with CHD and PH reveal loss of endothelial caveolin-1 and enhanced expression of caveolin-1 in SMC (Fig. 1A). It is noteworthy that not all arteries exhibit loss of endothelial caveolin-1, and, in the absence of endothelial caveolin-1 loss, enhanced expression of caveolin-1 in SMC does not occur (Fig. 1A, panels 2 and 3). Moderately large left-to-right shunt at the atrial septal level with normal pulmonary artery pressure neither disrupts endothelial caveolin-1 nor is there enhanced expression of caveolin-1 in SMC. Observed medial thickening in this patient may be related to the terminal event of pulmonary hypertensive crisis (Fig. 1B). Disrupted endothelial caveolin-1 associated with enhanced expression in SMC is seen also in a pulmonary artery from the fetus with AVC; but not in the fetus without CHD (Fig. 1C).

CHD with right-to-left shunt and obstructed pulmonary blood flow. Pulmonary arteries from infants with CHD with obstructed pulmonary blood flow and low pulmonary

Table 1: Age, diagnosis and mean PA or RV systolic Pressure

| Group | No | Gest age (Wks) | Age at autopsy/ Biopsy | Diagnosis | mPAP/RVSP (mmHg) | Increased medial wall thickness |
|----------------------------------|----|----------------|------------------------|----------------------------------|------------------|---------------------------------|
| I. CHD | | | | | | |
| IA: Increased PBF | 1 | 21 | Fetus | AVC & Down syndrome | NA | + |
| | 2 | 36 | 33d | PS, AS, ASD2° | RVSP 73, mPAP 23 | + |
| | 3 | 41 | 10 mo | ASD2°, PDA | mPAP 46 | + |
| | 4 | 30 | 16 mo | Shone's complex & LV to RA shunt | mPAP 62 | + |
| | 5 | 28 | 23 mo | S/P AVC repair & Down syndrome | Est. RVSP 79 | + |
| 1B: Decreased PBF | 1 | 37 | 2d | PS | RVSP 100 | - |
| | 2 | 37 | 7d | PS & Down syndrome | RVSP 54 | - |
| | 3 | 28 | 31d | PAt, VSD, PFO | NA | - |
| II. RDS/ BPD | | | | | | |
| IIA: Without Assoc. Inflammation | 1 | 24 | 1d | RDS | Est. RVSP 45 | + |
| | 2 | 27 | 1d | RDS | Est. RVSP 65 | + |
| | 3 | 23 | 2d | RDS | Est. RVSP 60 | + |
| | 4 | 26 | 4d | RDS | Est. RVSP 41 | + |
| | 5 | 28 | 20d | RDS | R→L (PDA, PFO) | + |
| | 6 | 30 | 53d | RDS | Est. RVSP 55 | + |
| | 7 | 28 | 6 mo | BPD | Est. RVSP 90 | + |
| IIB: Assoc. with Inflammation | 1 | 24 | 9 mo | BPD | Est. RVSP 86 | + |
| | 2 | 27 | 8 mo | BPD | Est. RVSP 70 | + |

Assoc: associated; **BPD:** bronchopulmonary dysplasia; **CHD:** congenital heart defect; **Est:** estimated; **Gest:** gestational; **LV:** left ventricle; **mPAP:** mean pulmonary artery pressure; **PBF:** pulmonary blood flow; **NA:** not available; **Qp/Qs:** pulmonary to systemic blood flow ratio; **R→L:** right to left shunt; **RA:** right atrium; **RDS:** respiratory distress syndrome; **RVSP:** right ventricular systolic pressure; **S/P:** status-post; **(d:** day/s; **wks:** weeks; **mo:** month/s)

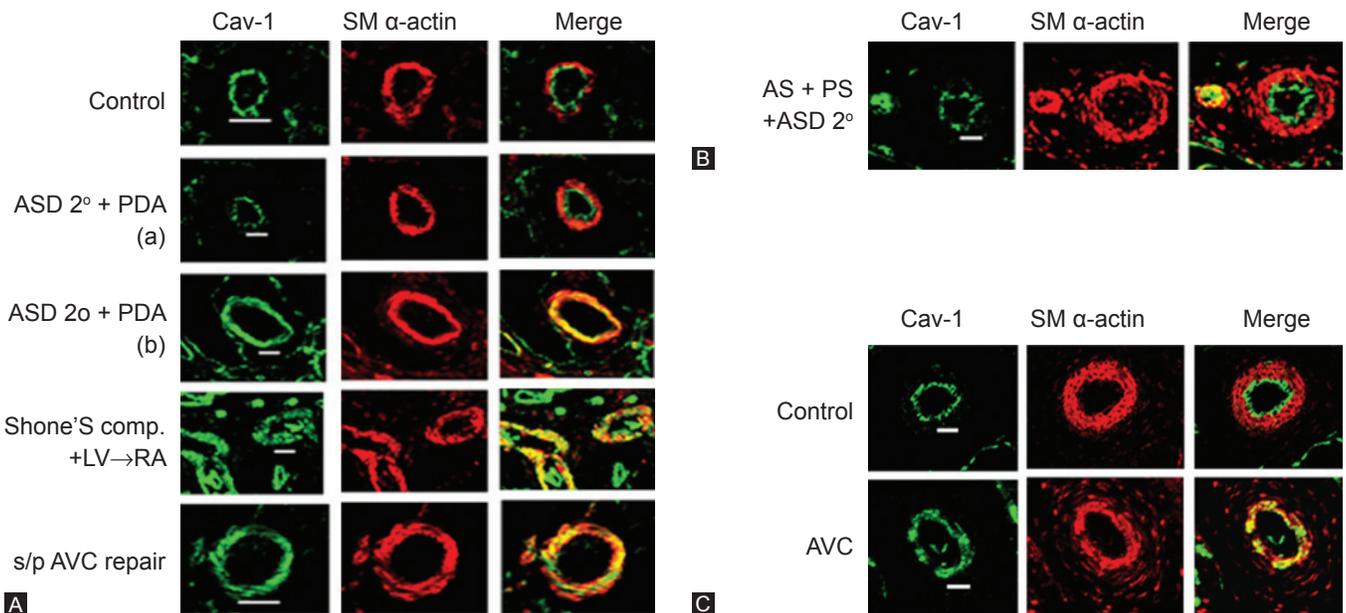


Figure 1: (A) There is enhanced expression of caveolin-1 in SMC accompanying the loss of endothelial caveolin-1 in arteries from patients with PH, CHD and increased left to right shunt. Bar = 25 μm. (B) Normal expression of endothelial caveolin-1 and SM α-actin in an artery from a patient with normal pulmonary artery pressure and increased left to right shunt. Bar = 25 μm. (C) Endothelial caveolin-1 loss with enhanced expression of caveolin-1 in SMC is seen in an artery from a fetus with AVC. Bar = 25 μm.

artery pressure, show well preserved endothelial caveolin-1 without any evidence of enhanced expression of caveolin-1 in SMC (Fig. 2).

RDS/BPD and PH. Figure 3A depicts pulmonary arteries from infants with RDS/BPD. Despite the presence of PH but with normal pulmonary blood flow, these arteries exhibit neither the loss of endothelial caveolin-1 nor enhanced caveolin-1 expression in SMC.

BPD and PH associated with inflammation. Figure 3B shows arteries from two different infants with PH associated with lung disease and an inflammatory process depict the progression of the disease. Artery in the middle panel shows significant reduction in the expression of endothelial caveolin-1 but without a breach in the endothelial layer, and importantly, without enhanced expression of caveolin-1 in SMC. In contrast, the artery in the bottom panel exhibits loss of endothelial caveolin-1

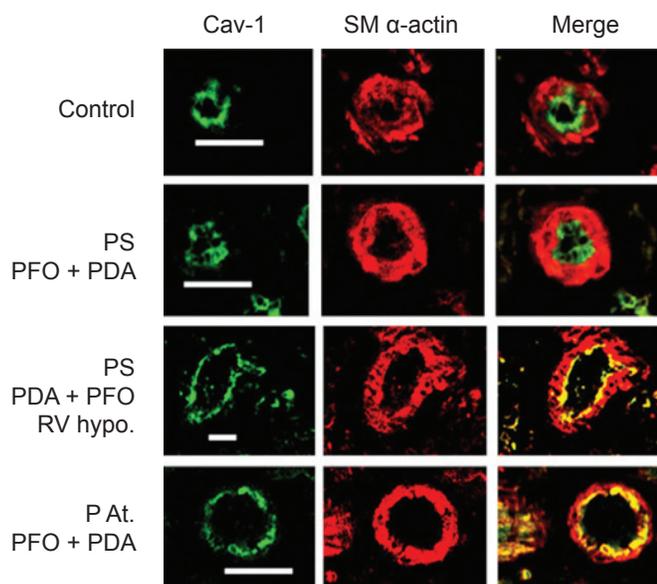


Figure 2: Arteries from infants with CHD with RV outflow obstruction show expression of caveolin-1 in EC and SM α -actin in SMC, without enhanced expression of caveolin-1 in SMC. RV hypo = RV hypoplasia, PAt. = Pulmonary Atresia. Bar = 25 μ m.

accompanied by enhanced expression of caveolin-1 in SMC.

Expression of caveolin-1 and PECAM-1

Caveolin-1 and PECAM-1 colocalize in EC (Fig. 4). Progressive nature of the disease can be appreciated in two different arteries from the same infant. One depicts marked reduction in the expression of endothelial caveolin-1 and PECAM-1 without enhanced expression of caveolin-1 in SMC (A). The other shows significant loss of endothelial caveolin-1 and PECAM-1 accompanied by robust expression of caveolin-1 in SMC (B).

Expression of caveolin-1 and vWF

Arteries exhibiting endothelial caveolin-1 express vWF and both localize in the endothelial layer (Fig. 5). Loss of caveolin-1 and vWF in arteries from infants with PH associated with CHD and increased pulmonary blood flow, and PH associated with BPD and inflammation, exhibit enhanced expression of caveolin-1 in SMC. Loss of vWF, however, does not occur in arteries without the endothelial caveolin-1 disruption. Furthermore, consistent with reported observation in MCT-induced PH,^[25] not all arteries with vWF loss exhibit enhanced expression of caveolin-1 in SMC, but all arteries with enhanced caveolin-1 expression in SMC are accompanied by vWF loss. Elevated PA pressure without accompanying increased pulmonary flow or inflammation does not disrupt endothelial caveolin-1; additionally, and importantly, this state also does not result in vWF loss or enhanced expression of caveolin-1 in SMC. Thus, progressive disruption of endothelial cell membrane

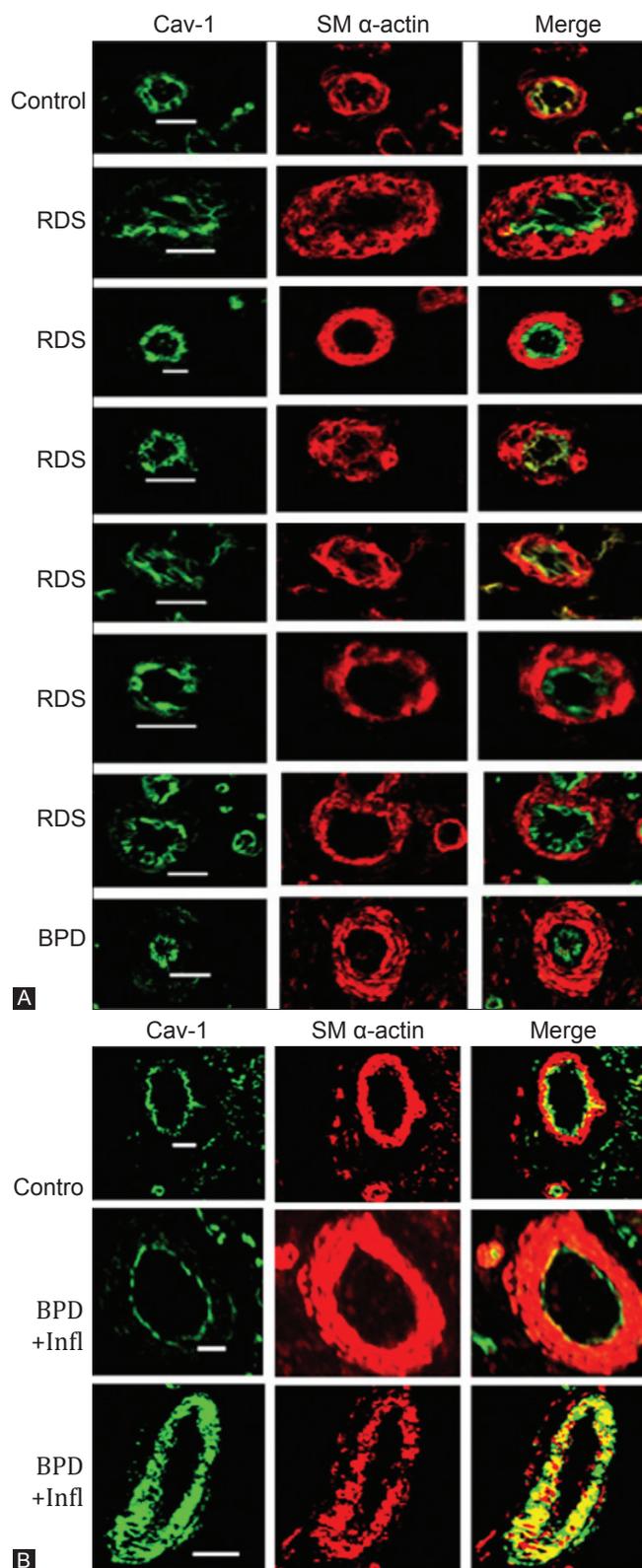


Figure 3: (A) Caveolin-1 expression in the endothelial layer, without enhanced expression of caveolin-1 in SMC in arteries seen in infants with PH and RDS/BPD. Bar = 25 μ m. (B) Significant reduction of endothelial caveolin-1 without enhanced expression of caveolin-1 in SMC (middle panel), and endothelial caveolin loss associated with enhanced expression of caveolin-1 in SMC (bottom panel). Bar = 25 μ m.

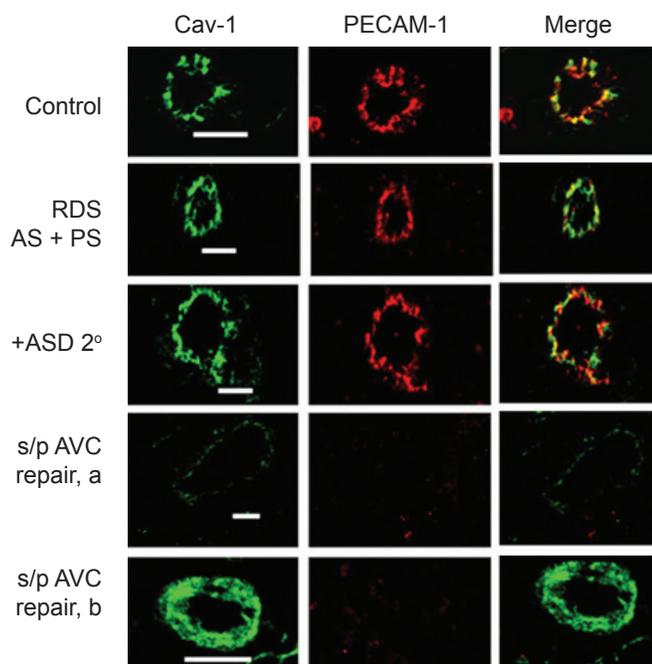


Figure 4: Caveolin-1 and PECAM-1 co-localize in EC. Arteries show significant loss of caveolin-1 and PECAM-1 without (a) and with enhanced expression of caveolin-1 in SMC (b) Bar = 25 μ m.

and caveolin-1, and subsequent loss of vWF, results in enhanced expression of caveolin-1 in SMC.

DISCUSSION

The major findings of our study are as follows:

1. PH associated with CHD and increased pulmonary blood flow leads to progressive loss of pulmonary endothelial proteins, caveolin-1, and PECAM-1. The additional loss of vWF is accompanied by an enhanced expression of caveolin-1 in SMC; these changes were also present in a fetus. In contrast, moderately large left-to-right shunt at the atrial level in the presence of normal pulmonary artery pressure, however, does not disrupt endothelial caveolin-1, nor is there enhanced expression of caveolin-1 in SMC. Similarly, in CHD with RV outflow tract obstruction group, there is no loss of endothelial caveolin-1, PECAM-1 or vWF, or enhanced expression of caveolin-1 in SMC.
2. In PH associated with RDS/BPD, the expressions of caveolin-1, PECAM-1, and vWF are well preserved without enhanced caveolin-1 expression in SMC; however, in two patients with PH associated with BPD and inflammation, pulmonary arteries exhibited varying degrees of endothelial caveolin-1 loss. In one infant, there was reduction in the endothelial caveolin-1 expression, but without the disruption of the endothelial layer as shown in Figure 3B, and importantly, there was no vWF loss (data not shown),

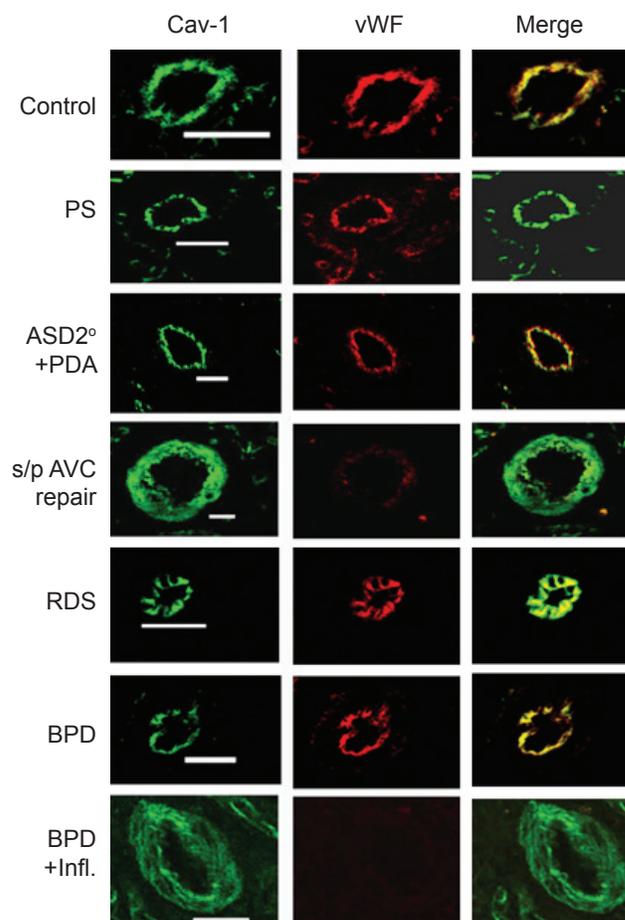


Figure 5: Both caveolin-1 and vWF are expressed in EC. Loss of endothelial caveolin-1 and vWF is accompanied by enhanced expression of caveolin-1 in SMC. Bar = 25 μ m.

nor was there enhanced expression of caveolin-1 in SMC. Interestingly, pulmonary arteries from the second infant revealed the absence of endothelial caveolin-1 accompanied by vWF loss (Fig. 5) and enhanced expression of caveolin-1 in SMC (Fig. 3B).

These two cases illustrate the progression of endothelial damage in PH associated with BPD and inflammation. Consistent with previous studies, the loss of vWF in addition to endothelial caveolin-1 loss is indicative in that extensive endothelial damage is followed by an enhanced expression of caveolin-1 in SMC.^[19,25]

Reduction in the expression of pulmonary endothelial caveolin-1 has been reported in clinical and experimental forms of PH.^[18-21,26-28] In MCT-induced PH, progressive loss of endothelial caveolin-1 with the activation of proliferative and antiapoptotic pathways occurs before the onset of PH. Loss of caveolin-1 is accompanied by the loss of other endothelial cell membrane proteins (PECAM-1, Tie2 and soluble guanylate cyclase) indicating a generalized endothelial cell membrane disruption. At two weeks

post-MCT, concomitant with PH, loss of cytosolic proteins (HSP90, Akt and $\text{I}\kappa\text{B-}\alpha$) occurs; however, at this stage, the expression of vWF is well preserved without any evidence of enhanced expression of caveolin-1 in SMC.^[18,28] At four weeks post-MCT, 29% of arteries exhibit vWF loss in addition to endothelial caveolin-1; additionally, 70% of these arteries exhibited enhanced expression of caveolin-1 in SMC, underscoring the progressive and heterogeneous nature of vascular damage in PH.^[25] vWF is a large multimeric glycoprotein synthesized by vascular EC and stored in Weibel Palade bodies. Plasma level of vWF is a marker for EC damage, and elevated plasma level of vWF in patients with PH portends poor prognosis.^[29-32] Furthermore, increased circulating level of angiotensin-2 is an independent predictor of poor outcome in patients with idiopathic PH;^[33] since both vWF and angiotensin-2 are stored in Weibel Palade bodies, their loss is indicative of extensive EC damage. This is further supported by recent studies showing increased circulating EC in patients with CHD and irreversible PH.^[34]

EC play a pivotal role in maintaining vascular health. The apposition of EC and SMC facilitates crosstalk and coregulation. EC shield SMC from direct pressure and flow-induced shear stress. Vascular SMC proliferation plays an important role in the progression of vascular disease. Caveolin-1 modulates mitogenic signaling, and functions as an antiproliferative factor; it regulates Ca^{2+} entry in SMC and modulates vascular tone. Caveolin-1^{-/-} SMC exhibit increased proliferation and migration, and a sustained increase in Ca^{2+} when exposed to contractile agents such as endothelin.^[35,36] In response to cyclic strain, caveolin-1 has been shown to translocate from caveolae to noncaveolar sites within the plasma membrane in SMC. Furthermore, under increased mechanical stress/strain, translocated caveolin-1 can trigger cell cycle progression and cell proliferation. Within caveolae, caveolin-1 acts as a negative regulator of cell proliferative signaling molecules including extracellular signal-regulated kinase (ERK). In response to stretch, caveolin-1, translocated from caveolae, facilitates cell proliferation via ERK activation. Importantly, caveolin-1 antisense treatment abolishes stretch-induced cell proliferation in SMC.^[37-39] Extensive endothelial damage observed during the progression of PH leads to a breach in the endothelial layer, exposing SMC to high pressure and cyclic shear stress, leading possibly to translocation and enhanced expression of caveolin-1, thus facilitating further cell proliferation and migration. Enhanced caveolin-1 expression, altered Ca^{2+} handling, increased cytosolic $[\text{Ca}^{2+}]_i$, and increased DNA synthesis have been reported in SMC isolated from patients with idiopathic PH; furthermore, silencing caveolin-1 mRNA results in a decrease in capacitance Ca^{2+} entry and in DNA synthesis.^[22] Thus, SMC with increased expression of caveolin-1 switches from being an antiproliferative molecule to a proproliferative

one, and this may be one of the factors leading to SMC change from contractile to synthetic phenotype resulting in further cell proliferation, cell migration, and possibly neointima formation. This view is further supported by a case report of a child with PH showing the loss of endothelial caveolin-1 and vWF with subsequent enhanced expression of caveolin-1 in SMC in PH, followed by neointima formation and loss of response to therapy.^[21] Thus, enhanced expression of caveolin-1 in SMC, a sequence of events following extensive EC damage or loss, may facilitate cell proliferation and migration, and contribute to the progression of PH. Interestingly, in a carotid artery injury model, a significant correlation has been shown to exist between the degree of neointima formation and the propensity of vascular SMC proliferation.^[40]

In the present study, it is significant that infants with PH, associated CHD, and increased pulmonary blood flow exhibit both progressive disruption of the pulmonary endothelial layer, and loss of endothelial cell membrane proteins (caveolin-1 and PECAM-1). Enhanced expression of caveolin-1 in SMC was present only in the arteries with an additional loss of vWF. In contrast, increased pulmonary blood flow in the absence of PH does not disrupt endothelial caveolin-1, nor is there enhanced expression of caveolin-1 in SMC. Therefore, it is not surprising that CHD with RV obstruction and low pulmonary blood flow has no effect on the expression of caveolin-1, PECAM-1, or vWF in the pulmonary vasculature. Interestingly, loss of endothelial caveolin-1 and enhanced expression of caveolin-1 in SMC were also present in a fetus with Down syndrome and a complete AVC defect. Alterations in caveolin-1 expression in this fetus are not likely related to the chromosomal abnormality because another infant with Down syndrome in the low pulmonary flow group did not exhibit these alterations. Nor can these changes be attributed to the fetal stage, because an age-matched fetus without cardiopulmonary disease exhibited normal expression of endothelial caveolin-1 without enhanced expression of caveolin-1 in SMC. The role of caveolin-1 in pulmonary vascular development in fetus is not well understood. Caveolin-1 α is expressed in EC in vascular plexus. It is thought that caveolin-1 may facilitate vasculogenesis.^[41] Thus, the altered volumes of pulmonary blood flow, the altered levels of oxygen in blood delivered to the lungs,^[2] and the resulting disruption of endothelial caveolin-1 may have an impact on the developing fetal pulmonary vasculature. Furthermore, recent studies indicate that in fetus, elevated pressure and increased flow results in persistence of fetal pulmonary bifurcation phenotype which may facilitate endothelial injury.^[3] Despite the limitation of our study, it is persuasive to consider that the altered patterns of pulmonary blood flow and volume can affect endothelial function and integrity even in fetal life, especially in the presence of elevated pulmonary artery pressure.

Underdeveloped pulmonary vasculature and reduced cross sectional area of pulmonary vascular bed are major determinants of PH in premature infants. Ventilation injury and hyperoxia may further contribute to PH in this group.^[5] Importantly, PH itself has an adverse effect on lung growth.^[5,42] In our series of premature infants (seven of nine) with PH and associated RDS/BPD revealed well preserved endothelial caveolin-1, PECAM-1, and vWF, without any evidence of enhanced expression of caveolin-1 in SMC. It is significant that in this group, despite well preserved endothelial caveolin-1, elevated pulmonary pressure and vascular remodeling were present. Since caveolin-1 is an inhibitor of proliferative pathways, this observation suggests that endothelial caveolin-1 may be dysfunctional in this group. In the hypoxia model of PH, a tight coupling of caveolin-1 and eNOS occurs both at rest and during agonist-induced stimulation, resulting in eNOS dysfunction, and the disruption of caveolin-1 and eNOS coupling restored eNOS function,^[43] and possibly also caveolin-1 function. In *in vitro* studies, pulmonary arterial EC, on exposure to hypoxia, exhibit a tight coupling of eNOS and caveolin-1 and the activation of PY-STAT3.^[10] Interestingly, caveolin-1 is known to inhibit PY-STAT3 activation; therefore, its activation during hypoxia strongly suggests caveolin-1 dysfunction. Thus, the disruption as well as dysfunction of endothelial caveolin-1 may have an important role in the pathogenesis of PH.^[10,16] The pulmonary arterial medial wall thickening observed in RDS/BPD group may in part be related to (1) the maintenance of fetal pulmonary vascular phenotype, (2) hypoxia-induced vasoconstriction,

and (3) caveolin-1 dysfunction resulting in the loss of its inhibitory function on proliferative pathways.

Inflammation has been reported to play a significant role in PH.^[17] The presence of an inflammatory process is detrimental to patients with PH and associated BPD. In one infant, pulmonary arteries revealed significant reduction in endothelial caveolin-1 expression, but not a breach in the endothelial layer; vWF expression was preserved, and not surprisingly, SMC did not exhibit enhanced expression of caveolin-1. In contrast, the second infant with PH and associated BPD and inflammation revealed disruption of endothelial membrane proteins caveolin-1, PECAM-1, and vWF, and also enhanced expression of caveolin-1 in SMC. These two cases illustrate the progression of the disease in PH with associated BPD and inflammation. A breach in the endothelial layer exposes SMC to direct pressure and shear stress, which may facilitate translocation of caveolin-1 from caveolae and its enhanced expression in SMC. Thus, the endothelial disruption may influence the morbidity and mortality in these patients. Future studies will discern the proliferative pathways involved in the progression of PH with and without underlying endothelial disruption and endothelial caveolin-1 loss.

In summary, PH associated with CHD and increased pulmonary blood flow, or PH associated with BPD and inflammation leads to progressive disruption of endothelial membrane proteins, caveolin-1 and PECAM-1. As the disease progresses, vWF loss is followed by enhanced

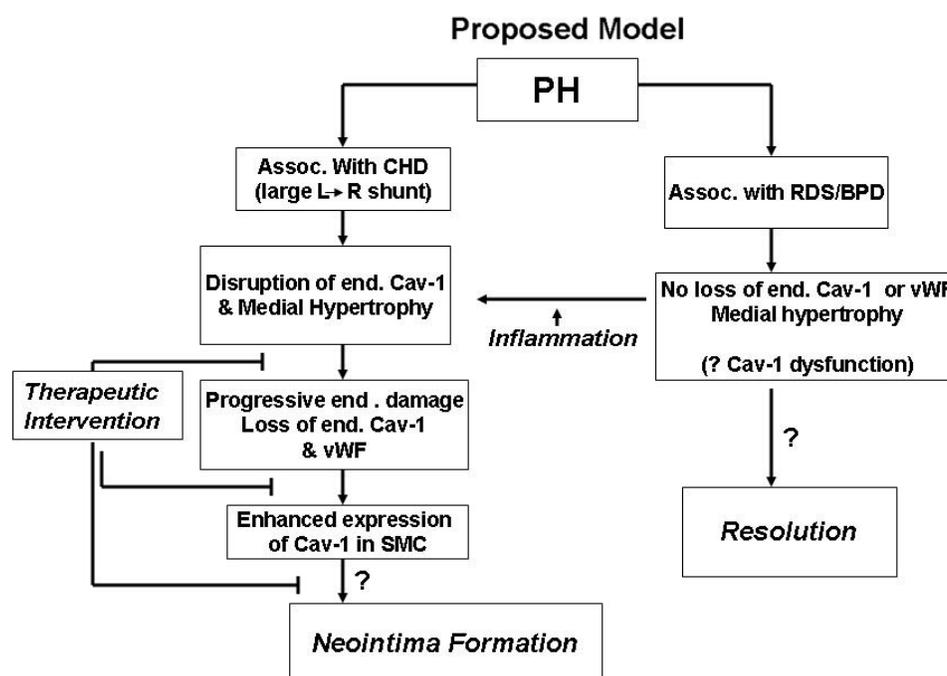


Figure 6: A proposed model for the progression of PH in infants is depicted in this figure. Assoc. = associated, Cav-1 = caveolin-1, end. = endothelial, L = left, R = right.

expression of caveolin-1 in SMC, which may further facilitate cell proliferation and migration, and possibly lead to neointima formation. In contrast, elevated pulmonary artery pressure alone does not appear to disrupt endothelial cell membrane integrity in infants, and the expression of endothelial proteins caveolin-1, PECAM-1, and vWF remains well preserved. Importantly, these arteries do not exhibit enhanced expression of caveolin-1 in SMC. Thus, the disruption or dysfunction of endothelial caveolin-1 may influence the course of PH in infants as proposed in Figure 6. Timing of therapeutic intervention may be crucial in preventing the progression of the disease.

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