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## The mechanobiology of pulmonary vascular remodeling in the congenital absence of eNOS

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**Abstract** Primary pulmonary hypertension is a rare but deadly disease. Lungs extracted from PPH patients are deficient in endothelial nitric oxide synthase (eNOS), making the eNOS-null mouse a potentially useful model of the disease. To better understand the progression of pulmonary vascular remodeling in the congenital absence of eNOS, we induced pulmonary hypertension in eNOS-null mice using hypobaric hypoxia, and then quantified large artery structure and function in contralateral vessels. In particular, to assess structure we quantified diameter, wall thickness, and collagen, elastin and smooth muscle cell content; to quantify function we performed pressure-diameter tests. After remodeling, the pulmonary arteries had increased wall, collagen and elastin thicknesses compared to controls ( $P < 0.05$ ). The remodeled pulmonary arteries also had increased elastic moduli at low and high strains compared to controls ( $P < 0.05$ ). The increases in moduli at low and high strain correlated with increases in elastin and collagen thickness, respectively ( $P < 0.05$ ). These results provide insight into the mechanobiology of pulmonary vascular remodeling in the congenital absence of eNOS, and the coupled nature of these changes.

### 1 Introduction

Primary pulmonary hypertension (PPH) is a rare but deadly disease, principally claiming the lives of young and middle-aged women, often in 2–3 years from onset of symptoms (Rubin 1997). Recently, mutations associated with bone morphogenetic peptides have been identified in approximately 25% of cases of familial pulmonary arterial hypertension and 25% of cases of sporadic pulmonary hypertension (Lane

et al. 2000; Thomson et al. 2000). For the majority of familial and sporadic cases, however, no genetic abnormalities have been identified. Since lungs extracted from PPH patients are deficient in endothelial nitric oxide synthase (eNOS) (Giaid and Saleh 1995), mice genetically-engineered to be deficient in eNOS (eNOS-null mice) are a potentially useful model for studying the disease and its progression. eNOS is one of three enzymes that produce nitric oxide (NO), which is a constitutively produced, potent vasodilator (Persson et al. 1990; Leeman et al. 1994; Blitzer 1996). Not coincidentally, inhaled nitric oxide is one treatment for persistent pulmonary hypertension of the newborn that has had some success in human trials (Abman 1999; Clark et al. 2000).

Perhaps like PPH patients in the early stages of the disease, eNOS-null mice have mild pulmonary hypertension (Fagan et al. 1999). To investigate the progression of the disease, these mice have been exposed to severe hypoxia (such that partial pressure of oxygen is reduced by 50%), which increased pulmonary vascular resistance (Steudel et al. 1997), pulmonary artery pressures, and the number of muscularized arterioles (Fagan et al. 1999). However, the remodeling of large arteries, which can affect the pulse wave energy transmission in the cardiopulmonary system and thus ventricular function (O'Rourke 1982), was not investigated. The effects of severe hypoxia, which causes severe pulmonary hypertension, on the mechanical and biological properties of large, conduit arteries in this animal model of PPH remain unknown.

The goal of this study was to quantify the mechanical and biological aspects of hypertension-induced pulmonary vascular remodeling of large pulmonary arteries in this animal model. More precisely, we investigated the effects of hypoxia-induced pulmonary hypertension on the collagen and elastin content, and elasticity and damping coefficient of large pulmonary arteries of eNOS-null mice and the correlations between these measured biological and mechanical changes.

We hypothesized that in response to severe hypoxia, collagen and elastin would accumulate in the large pulmonary arteries, and that the tissue elastic modulus would increase at

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low strain due to the increased elastin content and increase at high strain due to the increased collagen content. We tested these hypotheses with quantitative histology on right main pulmonary arteries of eNOS-null mice exposed to 0 (control), 10 and 15 days of severe hypobaric hypoxia, and isolated vessel experiments on left main pulmonary arteries. Once the sequence of mechanical and biological events in the progression of pulmonary vascular remodeling in pulmonary hypertension are better understood, novel treatment options to stop or reverse these coupled changes can be designed.

## 2 Methods

### 2.1 Animal handling

Twenty-seven male and female eNOS-null mice (B6.129P2-Nos3<sup>tm1Unc</sup>/J, Jackson Laboratory, Bar Harbor, ME, USA) were divided into three equal groups: 0-day control, 10-day hypoxic, and 15-day hypoxic (five males and four females each group). All mice were between 6 and 8 weeks of age at the end of the 0-, 10- or 15-day experimental protocol. The mice in the 10- and 15-day hypoxic groups were exposed to hypobaric hypoxia in a small altitude (SALT) chamber at the University of Wisconsin Biotron facility. By reducing the barometric pressure from that at sea level (760 mmHg) to that at approximately 17,000 ft elevation (380 mmHg), the partial pressure of O<sub>2</sub> was reduced from approximately 160 mmHg (= 21% × 760 mmHg) to 80 mmHg (= 21% × 380 mmHg). Barometric pressure returned to sea level conditions for no more than 30 min once per day for regular animal care and maintenance. Mice were weighed at the end of the exposure period (see Table 2) and euthanized by intraperitoneal injection of 150 mg/kg pentobarbital solution. All protocols and procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee.

Left and right pulmonary arteries (PAs) were excised between the first and second pulmonary artery bifurcations and placed in fresh Dulbecco's phosphate buffered saline solution (PBS, without calcium chloride or magnesium chloride, Sigma Chemical Co., St. Louis, MO, USA). The right PA was preserved for histology in frozen sections without pressurization or fixing as done previously (Kobs et al. 2005). The left PA was mounted in an arteriograph system for mechanical testing.

### 2.2 Histology and quantitative image analysis

Histological staining and measurement of wall thickness, collagen, elastin and smooth muscle cell (SMC) content in isolated right main pulmonary arteries were performed as previously described (Kobs et al. 2005). Briefly, each vessel was slow frozen in tissue freezing medium surrounded by 2-methyl butane cooled by liquid nitrogen without pressurization or chemical fixation. At least two 5 μm sections were cut at -20°C on a cryostat from the proximal and distal ends of each vessel for each assay. These sections were

stained with Verhoff Van Geisen (VVG) solution to identify elastin, picro-sirius red (SR) to identify collagen, and hematoxylin to measure intimal-to-adventitial wall thickness. For the VVG and SR stains, two representative fields of view (FOV) were chosen for each location (proximal or distal) by a single observer blinded to the experimental condition. The area positive for protein was identified by color thresholding in the FOV and compared to the total tissue area to produce a percent protein in the artery wall. The percent of SMC in the wall was indirectly quantified as the difference between total tissue and percent elastin plus percent collagen. That is, %SMC = 100% - (%elastin + %collagen). Wall thickness was measured with line measurement tools by averaging ten equally spaced positions around the whole vessel circumference for each location (proximal or distal). These measurements were also taken by a single individual blinded to the experimental condition.

For the collagen, elastin and SMC percentages and wall thickness measurements, no significant differences were found between the proximal and distal locations. Therefore, proximal and distal data for each vessel were pooled to generate a length-wise average for each measurement. Collagen, elastin or SMC thickness or content were computed as the percent collagen, elastin or SMC respectively, multiplied by the wall thickness for each vessel.

### 2.3 Mechanical testing

Left main pulmonary arteries were mounted in an arteriograph chamber in which vessels can be stretch to in vivo length, pressurized, and visualized by transillumination microscopy (Chesler et al. 2004). PBS was used to fill the arteriograph bath, buffered to maintain a constant pH of 7.4 and heated to 37°C. Calcium free PBS was used to prevent SMC contraction during testing. Pressure transducers were situated immediately up- and downstream of the vessel. An upstream in-line pressure servomechanism continually adjusted the computed average transmural pressure via computer control (LabVIEW, National Instruments, Austin, TX, USA).

Immediately after mounting, the left PA was pressurized to 5 mmHg and its unstretched length was measured suture-to-suture. The vessel was then stretched 140% to approximate in vivo axial length as measured by microcomputed tomography in representative animals exposed to 0-, 10- and 15-days of hypoxia ( $n = 3$  at each timepoint, data not shown). The vessel was allowed to equilibrate in PBS at 37°C for 60 min, after which it was mechanically preconditioned by cycling the pressure five times from 5 to 60 mmHg at 0.014 Hz. The response was repeatable after the third cycle in general. The fifth cycle of data was used to measure mechanical properties under dynamic loading conditions: dynamic circumferential moduli at low and high strain and the pulse damping coefficient. To measure mechanical properties under static loading conditions, the vessel was subjected to seven pressure steps; these were from 5 to 10, 20, 30, 40, 50, 60, and again to 10 mmHg for 45 s each. Steps were separated by ten times

175 the step duration (450 s) of rest at 5 mmHg to allow tissue  
 176 recovery from previous steps (Lakes 1998). Inner diameter  
 177 and left and right wall thicknesses were continuously opti-  
 178 cally measured by a video dimension analyzer (Living Sys-  
 179 tems Instrumentation, Burlington, VT, USA); these data as  
 180 well as upstream and downstream pressures were sampled at  
 181 1 Hz and saved on a PC for further analysis.

## 182 2.4 Calculations

183 For the purposes of the mechanical analysis, the artery wall is  
 184 assumed to be incompressible and homogeneous. Using the  
 185 thin wall assumption, which is valid for radius to wall thick-  
 186 ness (WT) ratio greater than 10, circumferential wall stress  
 187 ( $\sigma$ ) was calculated according to:

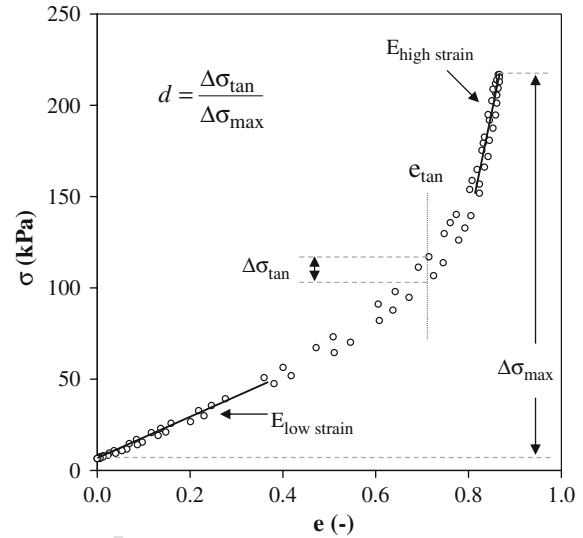
$$188 \sigma = \frac{ID}{2WT} P,$$

189 where  $P$  is the transmural pressure, ID is the inner diam-  
 190 eter and WT is based on the optically (not histologically)  
 191 measured value. Circumferential strain ( $e$ ) was calculated us-  
 192 ing Green's formulation for large deformations (Fung 1993)  
 193 based on circumferential stretch ( $\lambda$ ), which is the ratio of  
 194 pressure-dependent, deformed circumference ( $\pi$  OD) to cir-  
 195 cumference at the baseline pressure of 5 mmHg ( $\pi OD_5$ ):

$$196 e = \frac{1}{2}(\lambda^2 - 1), \text{ where } \lambda = \frac{\pi OD}{\pi OD_5} = \frac{OD}{OD_5}$$

197 Unlike thick-walled arteries, pulmonary arteries are prone  
 198 to collapse at zero transmural pressure; thus, a non-zero base-  
 199 line pressure is typically used (Faury et al. 1999). Collapse  
 200 is to be avoided since it is likely to damage endothelial cells.  
 201 Thus, the vessel state at 5 mmHg was used as an approxima-  
 202 tion of the unloaded state, which was used as the reference  
 203 state in all calculations.

204 The static stretch response of the vessel was analyzed  
 205 isochronally, 15 s into the pressure step. Two dynamic cir-  
 206 cumferential elastic moduli were calculated from the aver-  
 207 age inflation/deflation slope of the stress-strain curve: the  
 208 low strain tangent ( $E_{\text{low strain}}$ ) and the high strain tangent  
 209 ( $E_{\text{high strain}}$ ) (see Fig. 1). The damping coefficient was also  
 210 calculated from the dynamic-loading stress-strain relation-  
 211 ship. First, the strain ( $e_{\text{tan}}$ ) at the point in the stress-strain  
 212 curve where the rate of change of the slope was a max-  
 213 imum was identified. Then, the stress difference between  
 214 inflation and deflation at this point ( $\Delta\sigma_{\text{tan}}$ ) was calculated.  
 215 The stress difference between the maximum and minimum  
 216 stress values ( $\Delta\sigma_{\text{max}}$ ) was also calculated for each curve. Fi-  
 217 nally, the damping coefficient  $d$  (see Fig. 1) was computed  
 218 as  $\Delta\sigma_{\text{tan}}/\Delta\sigma_{\text{max}}$  as in (Kobs et al. 2005). For all calculations,  
 219 the pressure-dependent wall thickness values WT were de-  
 220 rived from conservation of mass assuming no axial extension  
 221 and the optically measured wall thickness value at 60 mmHg  
 222 (Faury et al. 1999).



**Fig. 1** Representative PA dynamic stress-strain curve (15-day) showing elastic moduli ( $E_{\text{low strain}}$  and  $E_{\text{high strain}}$ ) and damping coefficient ( $d$ ). See text for details

## 2.5 Statistics

ANOVA models were used to examine differences in colla-  
 gen, elastin, SMC and wall thicknesses and vessel dynamic  
 behavior between the experimental groups (0-, 10- and  
 15-day). Repeated measures ANOVA models were used to  
 examine differences in collagen, elastin and SMC percent  
 and vessel static behavior between the experimental groups.  
 All results are presented as mean  $\pm$  standard deviation.

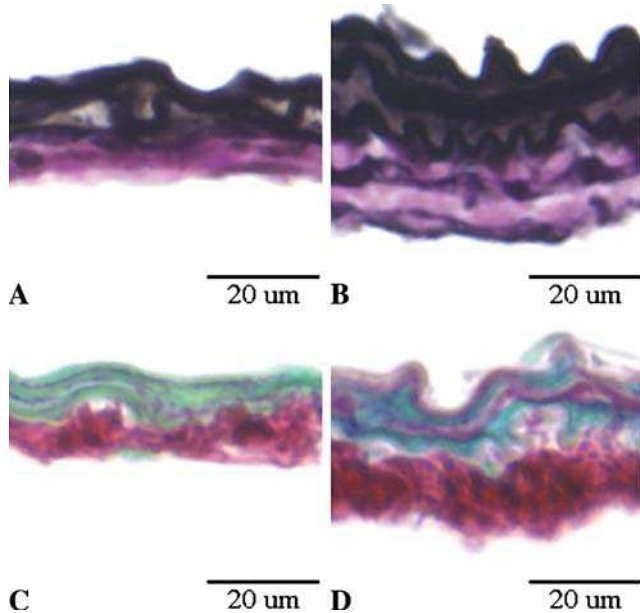
Analyses of correlation between the mechanical and bio-  
 logical endpoints were performed using non-parametric  
 Spearman's rank correlation coefficient. The regression anal-  
 ysis coefficient of determination ( $R^2$ ) is reported to reflect the  
 linearity of the correlative relationships; Spearman's correla-  
 tion coefficient ( $r_s$ ) and its associated  $P$  value (if significant)  
 is reported to reflect the trends in the data. For each signifi-  
 cant test, a general two-sided significance level of 5% was  
 applied. All statistical analyses were performed using SAS  
 software (SAS Institute Inc., Cary, NC, USA) version 8.1.

## 3 Results

### 3.1 Histology and quantitative image analysis

Representative images of control and 10-day hypoxic vessels  
 stained for VVG and SR are shown in Fig. 2. Average percent-  
 ages of collagen, elastin and SMC in the artery wall of the 0-,  
 10- and 15-day groups are given in Table 1. While the percent  
 elastin in the arterial wall did not significantly change with  
 hypoxia, the percent collagen did ( $P < 0.05$  for both 10-day  
 versus control and 15-day versus control). Percent SMC at  
 10 days was significantly lower than at 0 days ( $P < 0.05$ ).

Collagen, elastin, and SMC thicknesses (percent protein  
 content multiplied by wall thickness for a given vessel) also



**Fig. 2** Representative images of elastin and collagen staining in PAs of eNOS-null mice. **a, b** VVG staining for elastin, which appears black; **c, d** SR staining for collagen, which appears red. **a, c** 0-day control; **b, d** 10-day hypoxia vessels. Scalebar is 20  $\mu\text{m}$  for all images

**Table 1** Quantitative histology results for right pulmonary arteries of eNOS-null mice exposed to 0-, 10- and 15-days of hypobaric hypoxia

	0-day	10-day	15-day
Percent elastin	31 $\pm$ 2%	31 $\pm$ 3 %	29 $\pm$ 3 %
Percent collagen	48 $\pm$ 6%	56 $\pm$ 5%*	55 $\pm$ 2%*
Percent SMC	20 $\pm$ 6%	13 $\pm$ 5%*	16 $\pm$ 4%
Elastin thickness ( $\mu\text{m}$ )	5.6 $\pm$ 0.7	9.4 $\pm$ 1.5**	9.4 $\pm$ 0.9**
Collagen thickness ( $\mu\text{m}$ )	8.7 $\pm$ 1.6	17.3 $\pm$ 2.4**	17.8 $\pm$ 1.6**
SMC thickness ( $\mu\text{m}$ )	3.7 $\pm$ 1.4	3.9 $\pm$ 1.4	5.0 $\pm$ 1.5
Wall thickness ( $\mu\text{m}$ )	18 $\pm$ 3	31 $\pm$ 3**	32 $\pm$ 2**

Data are means  $\pm$  standard deviation

\*  $P < 0.05$  versus 0-day

\*\*  $P < 0.001$  versus 0-day

253 were analyzed for changes with hypoxia. Elastin and colla-  
 254 gen thicknesses increased after 10 and 15 days ( $P < 0.001$   
 255 versus control for both, Table 1) whereas SMC thickness did  
 256 not. Neither elastin nor collagen thickness increased between  
 257 10 and 15 days.

## 258 3.2 Mechanical testing

### 259 3.2.1 Thin wall assumption

260 The thin wall assumption was found to be valid under most  
 261 conditions and pressures for the eNOS-null mice, with excep-  
 262 tions at 5 mmHg for 10- and 15-day hypoxic vessels. In par-  
 263 ticular, at 5 mmHg, the ratios of inner radius to wall thickness  
 264 for 0-, 10-, and 15-day vessels were  $12.6 \pm 1.8$ ,  $7.8 \pm 1.3$ ,  
 265 and  $7.5 \pm 0.7$ , respectively. As the pressure increased, this  
 266 ratio increased, validating this assumption above 10 mmHg

**Table 2** Mouse body weights, pulmonary artery (PA) axial lengths and optically-measured PA OD and ID ( $\mu\text{m}$ ) for the 0-, 10- and 15-day groups taken 15 s after each static pressure step and at the initial 5 mmHg resting pressure

	0-day	10-day	15-day
Body weight (g)			
Female	16 $\pm$ 0.4	14 $\pm$ 0.8*	15 $\pm$ 0.3
Male	21 $\pm$ 2.0	18 $\pm$ 2.1	20 $\pm$ 1.5
Axial Length (mm)	2.3 $\pm$ 0.2	2.8 $\pm$ 0.3**	3.0 $\pm$ 0.2**
5 mmHg			
OD	491 $\pm$ 21	527 $\pm$ 38*	534 $\pm$ 33*
ID	445 $\pm$ 21	473 $\pm$ 41	477 $\pm$ 34
10 mmHg			
OD	547 $\pm$ 24	572 $\pm$ 40	577 $\pm$ 35
ID	506 $\pm$ 23	525 $\pm$ 41	528 $\pm$ 37
20 mmHg			
OD	778 $\pm$ 58	687 $\pm$ 48**	679 $\pm$ 38**
ID	740 $\pm$ 61	646 $\pm$ 51**	637 $\pm$ 38**
30 mmHg			
OD	1033 $\pm$ 64	814 $\pm$ 55**	802 $\pm$ 46**
ID	1001 $\pm$ 66	774 $\pm$ 56**	763 $\pm$ 47**
40 mmHg			
OD	1113 $\pm$ 58	875 $\pm$ 74**	879 $\pm$ 59**
ID	1085 $\pm$ 59	837 $\pm$ 73**	844 $\pm$ 60**
50 mmHg			
OD	1141 $\pm$ 60	892 $\pm$ 76**	901 $\pm$ 65**
ID	1115 $\pm$ 60	857 $\pm$ 76**	864 $\pm$ 65**
60 mmHg			
OD	1159 $\pm$ 63	903 $\pm$ 76**	912 $\pm$ 67**
ID	1131 $\pm$ 62	869 $\pm$ 77**	877 $\pm$ 67**
10 mmHg			
OD	546 $\pm$ 25	587 $\pm$ 37**	595 $\pm$ 36**
ID	504 $\pm$ 27	539 $\pm$ 37**	548 $\pm$ 37**

The final step to 10 mmHg (last two rows) was performed to verify the absence of plastic deformation due to the mechanical testing protocol. Data are means  $\pm$  standard deviation

\*  $P < 0.05$  versus 0-day

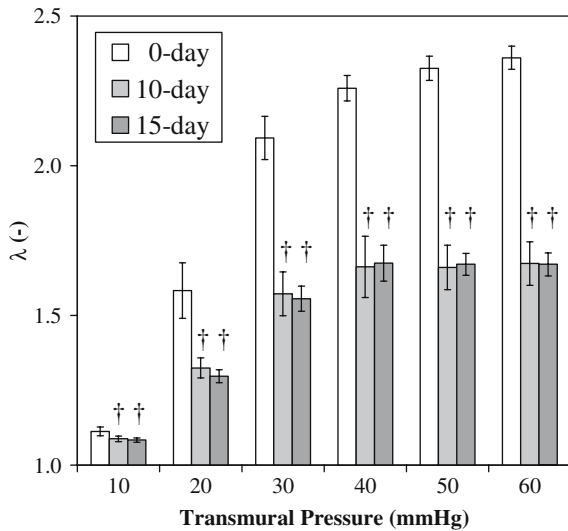
\*\*  $P < 0.001$  versus 0-day

for both hypoxic groups. At 60 mmHg the ratios for 0-, 10-, 267  
 and 15-day vessels were  $43.1 \pm 3.1$ ,  $25.5 \pm 3.3$ , and  $24.3 \pm 2.8$ , 268  
 respectively (see Table 2 for average ID and OD). 269

### 3.2.2 Static behavior 270

271 Optically-measured main pulmonary artery OD increased  
 272 significantly with hypoxia and imposed transmural pressure  
 273 (Table 2). At the baseline pressure of 5 mmHg, the 10- and  
 274 15-day hypoxic vessels were 7 and 9% larger in OD than the  
 275 0-day vessels, respectively. At the highest transmural pres-  
 276 sure of 60 mmHg, the 10- and 15-day hypoxic vessels were  
 277 22 and 21% smaller than the 0-day vessels, respectively. The  
 278 OD measured during a final step to 10 mmHg (the penulti-  
 279 mate row in Table 2) were 2% different on average from the  
 280 OD measured during the first step to 10 mmHg, indicating  
 281 the absence of plastic deformation during static loading.

282 The static stretch response of the vessels to steps in pres-  
 283 sure measured isochronally was greater in control vessels  
 284 than hypoxic vessels at all pressures tested (Fig. 3). That is,  
 285 control vessels always stretched more circumferentially in  
 286 response to transmural pressure increases than hypoxic ves-  
 287 sels. Differences were increasingly significant as the pressure



**Fig. 3** Circumferential isochronal stretch ratio for each pressure step (15 s after step) for the 0-, 10- and 15-day groups. Bars represent mean  $\pm$  standard deviation. †  $P < 0.001$  versus 0-day

steps increased in magnitude, with the greatest difference at 60 mmHg ( $P < 0.001$ ). There was no significant difference or trend between the stretch responses of the two hypoxic groups. Hypoxic groups also showed maximum stretch at 40 mmHg whereas control vessels continued to increase stretch with increasing pressure up to 60 mmHg.

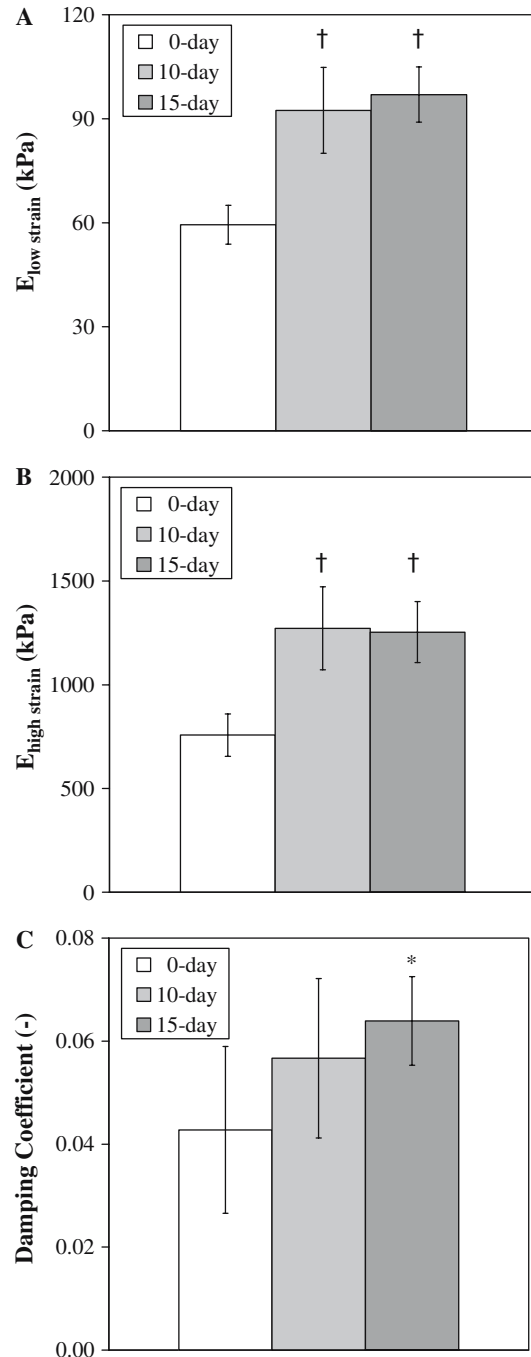
### 3.2.3 Dynamic behavior

Dynamic circumferential elastic moduli for the 0-, 10- and 15-day vessels were determined at low and high strains. While the two hypoxic groups were not significantly different from each other, both were significantly different from control ( $P < 0.001$ ; Fig. 4). Damping coefficient, which measures the pressure pulse damping capacity or tissue viscoelasticity, was greater in the 15-day group than in the 0-day group ( $P < 0.05$ ; Fig. 4), but no other differences were significant.

### 3.3 Mechanobiological correlations

To assess the presumptive correlative relationships between mechanical–functional and biological–structural indicators of remodeling,  $E_{\text{low strain}}$  was plotted versus elastin thickness and  $E_{\text{high strain}}$  was plotted versus collagen thickness (Fig. 5a, b). To assess the linearity of these relationships, the regression analysis coefficients of determination ( $R^2$ ) were measured, and to assess the trends in these relationships, the Spearman’s rank correlation coefficients ( $r_s$ ) were measured.

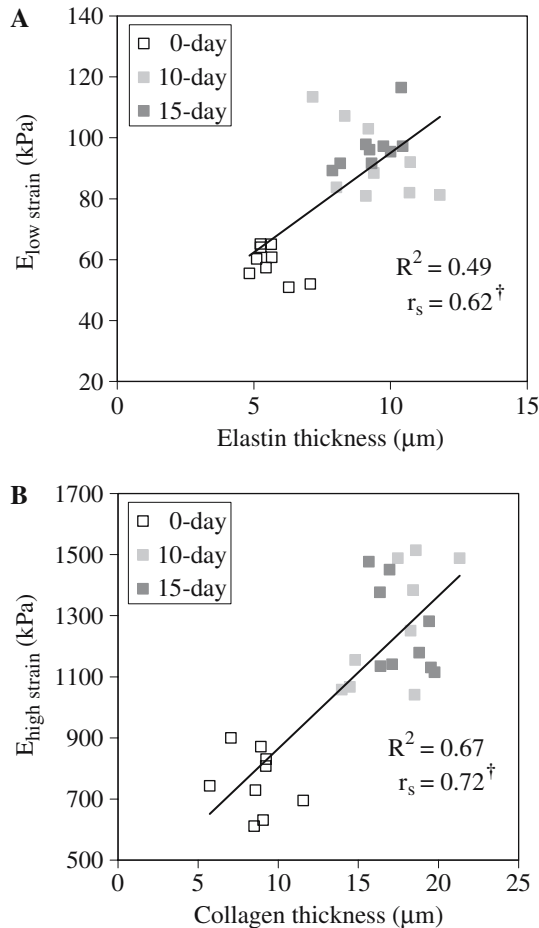
Between  $E_{\text{low strain}}$  and elastin thickness, there was a moderately linear relationship ( $R^2 = 0.49$ ) with a significant trend towards higher modulus with greater elastin thickness ( $r_s = 0.62$ ,  $P < 0.001$ ). Between  $E_{\text{high strain}}$  and collagen thickness, there was a stronger linearity ( $R^2 = 0.67$ ) with a significant trend towards higher modulus with greater collagen thickness ( $r_s = 0.72$ ,  $P < 0.001$ ).



**Fig. 4** Dynamic mechanical properties for the 0-, 10- and 15-day groups. **a** Elastic modulus at low strain, **b** elastic modulus at high strain, and **c** damping coefficient. Bars represent mean  $\pm$  standard deviation. \*  $P < 0.05$  versus 0-day; †  $P < 0.001$  versus 0-day

## 4 Discussion

Our results show that after 10 and 15 days of severe hypoxia, the main PAs of eNOS-null mice accumulate collagen and elastin, and have no change in SMC content. Mechanically, the PAs of eNOS-null mice exposed to hypoxia stretched



**Fig. 5** Correlations between **a** elastic modulus at low strain and elastin thickness and **b** elastic modulus at high strain and collagen thickness for all vessels.  $R^2$  and  $r_s$  values are provided in both panels.  $^\dagger P < 0.001$

much less in response to pressure increases, had higher circumferential elastic moduli at all strains, and had higher damping coefficients than control vessels. The increase in low strain elastic modulus correlated with increased elastin thickness; the increase in high strain elastic modulus correlated with increased collagen thickness; and the increase in damping coefficient did not correlate significantly with any histologically measured wall components. These results are discussed in more detail in the following sections.

#### 4.1 Histology and quantitative image analysis

As predicted, both collagen and elastin accumulated in response to severe hypertension in the eNOS-null mice. There was no appreciable change in SMC content despite an overall increase in wall thickness. Interestingly, while the amounts or thicknesses of collagen and elastin both increased with hypertension, only the percentage of collagen increased. The percent elastin in the wall remained relatively constant at 30%. Evidently, these structural changes occurred due to the increased solid wall stress created by the hypoxia-induced

hypertension. Whereas increased muscularization of small arteries likely accounts for increases in pulmonary vascular resistance with severe hypertension in the eNOS-null mice, increased ECM components in the large arteries may change the pressure pulse transmission characteristics of the vasculature, leading to increased right ventricular workload.

## 4.2 Mechanical properties

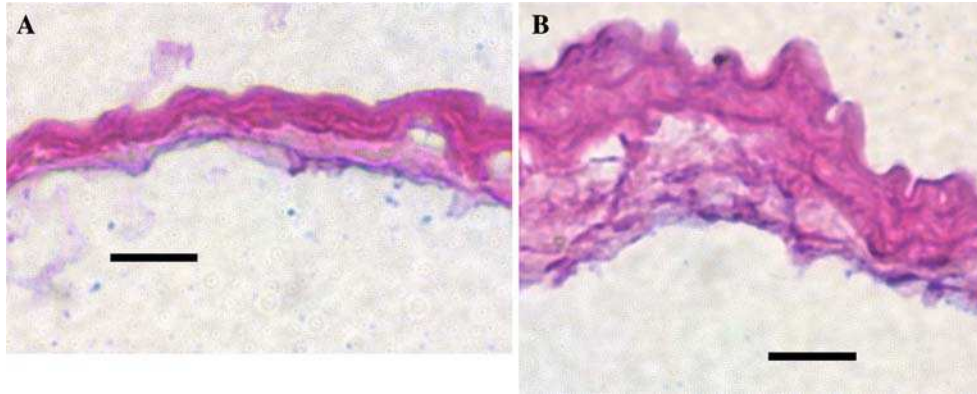
### 4.2.1 Static behavior

In all vessels, circumferential stretch increased with pressure for all pressures tested. Also, vessels exposed to hypertension stretched less at each pressure than those from normoxic controls. More interestingly, in vessels exposed to hypertension, the maximum circumferential stretch was reached at 40 mmHg whereas in normoxic controls circumferential stretch continued to significantly increase with pressure up to 60 mmHg. This finding is significant to vessel function since 40 mmHg is below the hypertensive systolic pressure of approximately 47 mmHg previously measured in eNOS-null mice exposed to two weeks of severe hypoxia (Fagan et al. 1999). A vessel that cannot stretch in response to a pulsatile pressure wave presents an increased resistance to flow (Zamir 2000). Therefore, these changes in circumferential stretch in eNOS-null mouse PAs in response to severe hypoxia may directly affect right ventricular energy requirements and contribute to right ventricular failure.

### 4.2.2 Dynamic behavior

As measured dynamically, the circumferential elastic moduli of eNOS-null mouse PAs at both low and high strains increased with hypertension. These results correlate with the finding of increased pulmonary vascular stiffness in patients with primary pulmonary hypertension (Laskey et al. 1993). The characterization of elastic moduli at two different positions in the stress-strain curve was an effective method of determining the dynamic mechanical properties of this non-linear material. It also allowed the correlation of these values with elastin and collagen content, which are primarily loaded under low strains and high strains, respectively. Incremental modulus also has been used to characterize vessel non-linear behavior by segmenting the stress-strain relationship into piece-wise linear relationships (Coulson 2002).

The PA damping coefficient calculated from the dynamic stress-strain curve increased approximately 50% from 0-day vessels to 10- and 15-day hypoxic vessels. Cox also observed damping behavior in canine PAs; qualitatively, damping increased with increasing frequency (from 0.01 to 1 Hz) and decreased with smooth muscle cell activation (Cox 1984). Functionally, higher damping values in the hypoxic vessels indicate more energy dissipation through inelastic mechanisms during systolic pressure loading. Thus, with increased damping, the work required of the right heart increases, adding to the long-term stress on the heart and risk of ischemia.



**Fig. 6** Representative images of proteoglycan staining of PAs from eNOS-null mice after **a** 0 and **b** 15 days of hypoxia. Alcian blue stains proteoglycans *blue*; other material stains *pink*. Scale bar is 20  $\mu$ m

394 Both damping and stiffness changes may adversely affect the  
395 efficiency of ventricular-vascular coupling.

#### 396 4.3 Mechanobiological correlations

397 One advantage of performing both mechanical–functional  
398 and biological–structural measurements on contralateral arter-  
399 ies during remodeling is the ability to correlate the mechan-  
400 ical changes with the biological changes. Our structure–func-  
401 tion correlations (Fig. 5) support the hypotheses that increased  
402 elastic modulus at low strains occurs due to elastin accumula-  
403 tion and that increased elastic modulus at high strains occurs  
404 due to collagen accumulation. However, we must add two  
405 notes of caution. First, while the structure-function data from  
406 the 0-, 10- and 15-day conditions can be fit by linear regres-  
407 sions, we do not have direct evidence that data from inter-  
408 mediate time points would have intermediate values. That is,  
409 after 5 days of hypoxia, it is possible that collagen increases  
410 significantly but high strain elastic modulus does not. More  
411 data from different conditions are required to conclude that  
412 the high strain modulus–collagen thickness and low strain  
413 modulus–elastin thickness relationships are linear. Second,  
414 we would note that evidence of correlation (even with sta-  
415 tistical significance) is not proof of causation. Since four  
416 of the six measured variables (low and high strain elastic  
417 moduli and collagen and elastin thicknesses) increased with  
418 hypoxia, correlations other than those for which we hypothe-  
419 sized causal relationships may have been significant. Exper-  
420 iments in which these mechanical properties are measured  
421 both before and after elastin or collagen degradation, such  
422 as have been performed in rabbit and dog arteries and veins  
423 (Dobrin and Canfield 1977; Kitoh et al. 1993), could address  
424 these issues.

425 Although SMC thickness increased as predicted, it did  
426 not correlate with the damping coefficient. Other important  
427 structural wall components and characteristics such as pro-  
428 teoglycans, ECM protein cross-linking, smooth muscle cell  
429 phenotype and vasoreactivity not measured in this study may  
430 be responsible for changes in damping coefficient. Proteogly-  
431 cans in particular have been shown to contribute to  
432 the viscous damping coefficient in articular cartilage (Bader

et al. 1992); however, their effects in vascular tissue are less  
clear. To assess the potential role of proteoglycans in vascular  
viscoelasticity, we performed a qualitative assay for proteo-  
glycan content with an Alcian blue histological stain, which  
identifies mucopolysaccharides or glycosaminoglycans. Five  
specimens in each group (0-, 10- or 15-day) were observed.  
As shown by representative images (Fig. 6), proteoglycan  
content is present mostly in the adventitia and did not increase  
appreciably with hypoxia. Thus, proteoglycan content is un-  
likely to account for the increase in vascular viscoelasticity  
that occurs with hypoxia-induced pulmonary hypertension in  
the PAs of eNOS-null mice.

Based on the correlation between collagen thickness and  
high strain modulus, therapies that slow collagen synthe-  
sis or lead to collagen breakdown – either at the gene- or  
protein-level – may be effective at decreasing vascular stiff-  
ness at hypertensive pressures. In the aorta, some recently  
synthesized thiazolium compounds that selectively break pro-  
tein cross-links and thereby reduce collagen stiffness, for  
example, have been found to decrease age-associated stiffen-  
ing in monkeys (Vaitkevicius et al. 2001) and diabetes-assoc-  
iated stiffening in rats (Wolffenbuttel et al. 1998). These treat-  
ments were also successful in improving ventricular-vascular  
coupling, which may reduce the risk of heart failure.

#### 427 4.4 Comparison to wild-type mice

428 The histological changes reported here in eNOS-null mice  
429 in response to hypoxia were comparable to those found in  
430 wild-type C57BL6 mice (Kobs et al. 2005), which are the  
431 genetic background for the eNOS-null strain. That is, in both  
432 mouse types, wall thickness, collagen thickness and elasti-  
433 n thickness increased with hypoxia-induced hypertension,  
434 and smooth muscle thickness was unchanged. An interest-  
435 ing difference is that in the congenital absence of eNOS, the  
436 average wall thickness was smaller at 0 days but not different  
437 after 10 or 15 days of hypoxia. Thus, the initial rate of change  
438 of wall thickness was faster in the absence of eNOS (from 0  
439 to 10 days) and similar thereafter (from 10 to 15 days).

440 Analogous mechanical changes occurred in the two mouse  
441 types. In the wild-type strain, the tangent elastic modulus

increased with 10 and 15 days of exposure to hypoxia, and this increase correlated with the sum of elastin and collagen thickness (Kobs et al. 2005). Also, in the wild-type strain, the damping coefficient increased with hypoxia and did not correlate with smooth muscle cell content. The mechanical data are difficult to compare more precisely between mouse types since the previous and present studies were performed with different axial stretch ratios (1.12 vs. 1.4) and in different static and dynamic pressure ranges (5–25 mmHg vs. 5–60 mmHg). However, preliminary results in the wild-type strain with mechanical testing protocols identical to those used here suggest mechanical differences analogous to the biological ones: that in the congenital absence of eNOS, PAs stretch more at 0 days but are not different after 10 or 15 days of hypoxia (data not shown). Thus, the initial rate of change of static stiffness is faster in the absence of eNOS (from 0 to 10 days) and similar thereafter.

The somewhat confounding normalization of mechano-biological changes between 10 and 15 days may be explained by eNOS uncoupling. That is, under normal conditions, eNOS produces NO; however, under certain conditions, including hypertension (Takimoto et al. 2005) and possibly alveolar hypoxia (Archer et al. 1989, 1993; Chandel et al. 1998), eNOS uncoupling can occur such that eNOS produces more reactive oxygen species (ROS) than NO. In the absence of eNOS, this uncoupling reaction cannot occur, which may be protective of enhanced pathological vascular remodeling. Indeed, reducing ROS has been shown to normalize hypertension-induced remodeling in rat aortas (Pu et al. 2003) and in mice, the congenital absence of eNOS blunted systemic hypertension-induced left ventricular hypertrophy and dilation (Takimoto et al. 2005). In this case, the competing effects of increased vasoconstriction and decreased ROS production in the absence of eNOS may balance such that the chronic remodeling response is not different than in the presence of eNOS.

Future studies to quantify ROS generation in wild type and eNOS-null mouse lungs before and after chronic exposure to hypoxia are warranted. In addition, it is important to know whether the vascular mechanobiological states measured at 10 and 15 days are representative of those at even longer time points. Finally, these results do not answer the question of whether the eNOS-null mouse is an appropriate animal model of PPH. Our preliminary results suggest that it is not, but this suggestion must await a more quantitative comparison and possibly whole lung vascular mechanobiological studies.

#### 4.5 Experimental considerations

In these experiments, we did not measure the residual stress in these arteries or use the zero-stress state as the reference state in the strain calculations. Takamizawa and Hayashi (1987) found that residual stress equalized the radial distribution of wall stress in canine carotid arteries. In rats exposed to 10 days of hypoxia, residual stress measurements on main PAs have shown that the outer wall grew more circumferentially than the inner wall, which also had the effect of

equalizing the distribution of stress in the radial direction at the loaded state (Fung and Liu 1991). In these studies, the eNOS-null mouse PAs were thin enough that the radial distribution of stress could be ignored, but future studies to assess radial differences in growth rates in mouse PAs could yield further insight into the biomechanics of pulmonary vascular remodeling.

It was also assumed that mechanical end-effects were small. Since the average length-to-width ratio at 5 mmHg was 5.2:1 (the average suture-to-suture length was  $2.7 \pm 0.4$  mm; the average maximum diameter was  $0.52 \pm 0.04$  mm), it was reasonable to ignore end-effects at low pressures. However, this ratio decreased with increasing pressure as the diameter increased. At 60 mmHg, the average length-to-width ratio was 2.8:1, such that the vessels must be considered constrained by the end sutures. Given the short length and large diameter of the pulmonary artery in vivo, unconstrained test conditions for measuring tissue mechanical properties are not attainable even in principle. Nevertheless, there were no significant correlations or trends when axial length was compared with circumferential stretch within each group (data not shown). This finding suggests that shorter axial length had no significant effect on circumferential deformation or tissue mechanical properties measured in this study.

To perform the mechanical property calculations, we assumed the vessel walls were thin, incompressible, and homogeneous. The thin wall assumption was validated here for all 0-day vessels and 10- and 15-day vessels above 10 mmHg of pressure. The error incurred by using the thin wall stress formulation instead of the thick wall formulation (Timoshenko 1934) was less than 8% for all vessels at 5 mmHg transmural pressure ( $5.6 \pm 0.9\%$ ) and less than 2% for all vessels at 60 mmHg ( $1.7 \pm 0.4\%$ ). The incompressibility assumption of isolated mouse PAs has been shown to be valid elsewhere (Faury et al. 1999). Vasculature is inherently heterogeneous in the radial direction due to the layered nature of arteries and veins and often heterogeneous in the axial direction. However, the assumption of radial homogeneity is appropriate to a thin-wall analysis and insofar as we measured axial changes in biological properties, no differences were detected.

Finally, tissue injury or damage also may have affected our results. As shown by (von Maltzahn et al. 1984), in bovine carotid arteries, loss of adventitia will reduce the measured elastic modulus in isolated arteries. Extreme care was taken during excision to limit damage due to handling. More importantly, identical techniques were used to harvest right and left PAs; thus the absence of either intimal or adventitial damage that is evident histologically strongly suggests that the mechanically-tested vessels were similarly undamaged.

## 5 Conclusions

Hypoxia-induced pulmonary hypertension in the congenital absence of eNOS has been shown to induce significant pulmonary artery remodeling by collagen and elastin accumulation. Significant changes in large artery mechanical properties were also demonstrated, and these correlated

with measured histological changes. These results provide insight into the vascular structure, function, and structure–function relationships in large pulmonary arteries in this animal model. It remains unclear if the eNOS-null mouse is a better model of PPH than wild-type mice exposed to hypoxia. Elucidating the role of eNOS and endothelial-derived NO in large artery remodeling, pulmonary vascular function and right ventricular remodeling and dysfunction will yield insight into these complex processes.

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