Lysyl Oxidases Play a Causal Role in Vascular Remodeling in Clinical and Experimental Pulmonary Arterial Hypertension

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Objective—Pulmonary vascular remodeling, the pathological hallmark of pulmonary arterial hypertension, is attributed to proliferation, apoptosis resistance, and migration of vascular cells. A role of dysregulated matrix cross-linking and stability as a pathogenic mechanism has received little attention. We aimed to assess whether matrix cross-linking enzymes played a causal role in experimental pulmonary hypertension (PH).

Approach and Results—All 5 lysyl oxidases were detected in concentric and plexiform vascular lesions of patients with idiopathic pulmonary arterial hypertension. Lox, LoxL1, LoxL2, and LoxL4 expression was elevated in lungs of patients with idiopathic pulmonary arterial hypertension, whereas LoxL2 and LoxL3 expression was elevated in laser-capture microdissected vascular lesions. Lox expression was hypoxia-responsive in pulmonary artery smooth muscle cells and adventitial fibroblasts, whereas LoxL1 and LoxL2 expression was hypoxia-responsive in adventitial fibroblasts. Lox expression was increased in lungs from hypoxia-exposed mice and in lungs and pulmonary artery smooth muscle cells of monocrotaline-treated rats, which developed PH. Pulmonary hypertensive mice exhibited increased muscularization and perturbed matrix structures in vessel walls of small pulmonary arteries. Hypoxia exposure led to increased collagen cross-linking, by dihydroxylysinonorleucine and hydroxylysinonorleucine cross-links. Administration of the lysyl oxidase inhibitor β-aminopropionitrile attenuated the effect of hypoxia, limiting perturbations to right ventricular systolic pressure, right ventricular hypertrophy, and vessel muscularization and normalizing collagen cross-linking and vessel matrix architecture.

Conclusions—Lysyl oxidases are dysregulated in clinical and experimental PH. Lysyl oxidases play a causal role in experimental PH and represent a candidate therapeutic target. Our proof-of-principle study demonstrated that modulation of lung matrix cross-linking can affect pulmonary vascular remodeling associated with PH. (Arterioscler Thromb Vase Biol. 2014;34:1446-1458.)

Key Words: anoxia • extracellular matrix • hypertension, pulmonary • muscle, smooth • protein-lysine 6-oxidase

Pulmonary arterial hypertension (PAH) is a life-threatening disease characterized by elevated pulmonary artery pressure caused by increased pulmonary vascular resistance, ultimately leading to right heart failure and death.1 A combination of vasoconstriction, and vascular wall remodeling, which includes excessive extracellular matrix (ECM) deposition, and smooth muscle cell hyperplasia leads to occlusion of the small pulmonary arteries.2 Therapeutic options for the clinical management of PAH are limited, and the disease remains essentially untreatable, underscoring the need for a better understanding of the pathogenic mechanisms at play that give rise to increased pulmonary vascular resistance.3 In this regard, 1 key area of interest is to clarify the causes and nature of the pulmonary vascular remodeling associated with PAH.

To date, most studies that have explored vascular remodeling in pulmonary hypertension (PH) have focused on increased cell proliferation and apoptosis resistance as an underlying cause of medial hypertrophy and neointimal formation. Regulation of pulmonary artery smooth muscle cell (PASMC) proliferation, apoptosis, and contraction has received much attention, with potassium and calcium channels, growth factors (including transforming growth factor-β,
Nonstandard Abbreviation and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CTEPH</td>
<td>chronic thromboembolic PH</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>IPAH</td>
<td>idiopathic pulmonary arterial hypertension</td>
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<tr>
<td>PASMC</td>
<td>pulmonary artery smooth muscle cell</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PH</td>
<td>pulmonary hypertension</td>
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<tr>
<td>VSD</td>
<td>ventricular septal defect</td>
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the bone morphogenetic proteins, and platelet-derived growth factor [PDGF]), and soluble mediators, such as endothelin and serotonin being attributed key roles in promoting increased PASMC mass. Many of these mediators are thought to be produced by other vascular cell types, including adventitial fibroblasts and endothelial cells, which exert paracrine control of PASMC growth. Aberrant endothelial cell growth also contributes to the formation of obstructive plexiform lesions.

Comparatively, fewer studies (such as pioneering work by Kerr et al) have addressed a role for perturbed ECM metabolism in pulmonary vascular remodeling although ECM constituent proteins, such as collagen, elastin, fibronectin, and proteoglycans, play a pivotal role in cell migration, proliferation, and differentiation, which are likely to affect vessel wall remodeling. It has been well established that gene expression, deposition, and localization of collagen and elastin in the pulmonary vasculature are altered in PAH. In addition, modulating proteolytic processes, which can affect vascular matrix turnover, can attenuate the course of experimental PAH.

However, to date, few studies have examined a role for the ECM assembly machinery in the development of PAH. To this end, we have examined the expression and function of the lysyl oxidase family of ECM cross-linking enzymes in clinical and experimental PH.

Lysyl oxidases are copper-dependent amine oxidases, which catalyze the oxidative deamination of lysine and hydroxylysine residues, generating reactive semialdehydes that condense to form intramolecular and intermolecular covalent cross-links in elastin and collagen molecules. This process drives fiber maturation, imparting structural stability to the ECM. Five lysyl oxidases have been described to date, including the archetypical member, Lox, as well as 4 closely related, or Lox-like (LoxL) members, named LoxL1 to LoxL4, and all lysyl oxidases are capable of initiating collagen and elastin cross-linking. In this study, we describe the dysregulated expression of lysyl oxidases in multiple forms of clinical PH, including idiopathic PAH (IPAH), as well as chronic thromboembolic PH (CTEPH), and PAH caused by ventricular septal defect (VSD). We further establish a causal role for lysyl oxidases in the development of hypoxia-induced PH and hypoxia-associated pulmonary vascular remodeling. These studies thus highlight an important role—that can be pharmacologically manipulated—for perturbed ECM assembly in the aberrant vascular remodeling associated with PH.

Methods

Materials and Methods are available in the online-only Supplement.

Results

Lysyl Oxidase Expression Is Dysregulated in All Forms of PH

The expression of all 5 lysyl oxidases was assessed in lung tissues from apparently healthy human lung donors, as well as in lung tissue from patients with various forms of PH, including IPAH, CTEPH, and PAH caused by VSD (Table 1). Relative mRNA levels, as assessed by real-time reverse transcriptase-polymerase chain reaction, revealed increased expression of the LOX (ΔΔCt = 3.18, thus ≈ 9.2-fold increase; Figure 1A), LOXL1 (ΔΔCt = 1.95, thus ≈ 3.9-fold increase; Figure 1B), LOXL2 (ΔΔCt = 1.47, thus ≈ 2.8-fold increase; Figure 1C), and LOXL4 (ΔΔCt = 1.13, thus ≈ 2.2-fold increase; Figure 1D) genes in IPAH lung tissue when compared with control donor tissue. No changes were observed for LOXL3 gene expression (Figure II in the online-only Data Supplement) or for LOXL3 protein expression (Figure IIB and IIC in the online-only Data Supplement). Immunoblot analysis confirmed an increase in the LOX and LOXL2 protein expression in IPAH lung tissue (Figure 1E, quantified densitometrically in Figure 1F).

In addition, LOX gene expression was increased in lung tissues from patients with CTEPH (ΔΔCt = 2.68, thus ≈ 6.4-fold increase; Figure IIIA in the online-only Data Supplement), whereas LOXL4 gene expression was decreased in lung tissue from patients with VSD (ΔΔCt = 1.25, thus ≈ 2.4-fold decrease; Figure III in the online-only Data Supplement). Taken together, these data indicate that the expression of lysyl oxidases is dysregulated in multiple forms of clinical PAH. It should be kept in mind that the effect of chronic pharmacological therapy on gene expression levels has not been assessed in this study.

Lysyl Oxidase Expression Is Dysregulated in the Pulmonary Vasculature of Patients With IPAH

Both plexiform and concentric lesions were identified in sections of lung tissue from patients with IPAH, where increased smooth muscle cell mass and perturbed internal elastic laminae were evident by α-smooth muscle actin and elastin staining, respectively, when compared with nonremodeled vessels in healthy control tissue (Figure 2). Pulmonary arteries from healthy lung donors and both plexiform and concentric vascular lesions were then screened by immunohistochemistry for protein localization of all 5 lysyl oxidases. The expression of all lysyl oxidases was detected in donor vessels and in both plexiform and concentric lesions from patients with IPAH (Figure 2). Pulmonary vessels (outer diameter, ≤ 150 μm) were laser-capture microdissected from donor and from lungs of patients with IPAH (Figure 3A–3D), and mRNA pools were screened for relative gene expression levels by quantitative real-time reverse transcriptase-polymerase chain reaction. Expression levels of the LOXL2 (Figure 3G) and LOXL3 (Figure 3H) genes were increased in the pulmonary vasculature of lungs of patients with IPAH, and expression levels of LOX also tended toward increased expression (Figure 3E). LOXL4 could not be detected in microdissected samples. These data indicate that the expression of lysyl oxidases is upregulated in remodeled vascular lesions associated with IPAH.
Table 1. Clinical Parameters of Human Subjects Reported in This Study

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<td>34</td>
<td>54</td>
<td>M</td>
<td>...</td>
<td>III–IV</td>
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* indicates data not available; CTEPH, chronic thromboembolic pulmonary arterial hypertension; F, female; IPAH, idiopathic pulmonary arterial hypertension; M, male; n/a, not applicable; n/m, not measured (organ donor, dead on arrival, or donation of body to science); NYHA, New York Heart Association; PAP, pulmonary arterial pressure; and VSD, ventricular septal defect.

†Systolic PAP determined by Doppler echocardiography.

‡Mean PAP determined invasively (by pulmonary artery catheter).

Lysyl Oxidase Expression Is Responsive to Low Oxygen Tension

To assess precisely where in the vascular lesions lysyl oxidase expression may be relevant to pathological vascular remodeling, PASMCs and adventitial fibroblasts were isolated from donor and from lungs of patients with IPAH and screened for lysyl oxidase gene expression under normoxic (21% O2 in incubator headspace gas) or moderate hypoxic (10% O2 in incubator headspace gas) conditions for 24 hours. LOX exhibited the highest baseline expression levels under normoxic conditions of all lysyl oxidases in PASMC (ΔΔCt ≈7; Figure 3I) and adventitial fibroblasts (ΔΔCt ≈7; Figure 3I). The expression of LOX was hypoxia-responsive in both PASMC (Figure 3I) and adventitial fibroblasts (Figure 3J) from both donor and patient lungs, with a dramatic ΔΔCt of ≈3.5 (thus ≈11.3-fold increase). In addition, the hypoxia responsiveness of LOX in adventitial fibroblasts was more pronounced in fibroblasts isolated from lungs of patients with IPAH than from donor lungs (Figure 3J). Both LOXL1 (Figure 3L) and LOXL2 (Figure 3N) were also hypoxia-responsive in adventitial fibroblasts. Baseline LOXL4 levels were elevated in PASMC from patients with IPAH under normoxic conditions when compared with PASMC from donors (Figure 3Q). Together, these data indicate that lysyl oxidases are expressed in the constituent vascular cell types of the pulmonary vasculature, that lysyl oxidase expression can be regulated by low oxygen tensions, and that lysyl oxidase expression is dysregulated in cells from patients with IPAH when compared with cells harvested from healthy lung donors.

Lung Lysyl Oxidase Expression Is Upregulated in Animal Models of PAH

The regulation of lysyl oxidase expression by hypoxia in isolated cells in vitro prompted us to explore lysyl oxidase expression in a well-established hypoxia-based model of PH. Mice exposed to normobaric hypoxia (10% O2 in inspired gas) exhibited a rapid (within 3 hours) and dramatic increase in lox (ΔΔCt =2.61, thus =6.1-fold increase), loxl2 (ΔΔCt =1.38, thus =2.6-fold increase), and loxl4 (ΔΔCt =0.69, thus =1.6-fold increase) gene expression in lung tissue (Figure 4A) when compared with normoxia-treated controls. This trend was largely preserved after a 24-hour exposure to hypoxia; lox (ΔΔCt =2.64, thus =6.2-fold increase) and loxl2 (ΔΔCt =1.00, thus =2.0-fold increase; Figure 4B). The increase in lox gene expression persisted over the entire time-course of hypoxic exposure, up to day 21, when the experiment was terminated (ΔΔCt =1.88, thus =3.7-fold increase; Figure 4C). These data confirm that, in addition to lung tissue from patients with IPAH, the expression of lysyl oxidases was also dysregulated—and followed similar trends—in the lungs of mice with hypoxia-induced PH. Interestingly, no changes in lysyl oxidase expression were observed in the microdissected lung parenchyma of hypoxia-exposed mice, revealing that vascular (and maybe airway) perturbations to lysyl oxidase expression did not carry across to the lung parenchyma (Figure IV in the online-only Data Supplement).

The expression of lysyl oxidases was also assessed in lung homogenates and isolated PASMC from rats in which PH...
had been induced with monocrotaline. A dramatic increase in lox expression was noted in both rat lung homogenates (ΔΔCt = 1.3, thus ≈ 2.5-fold increase; Figure VA in the online-only Data Supplement) and rat lung PASMC (ΔΔCt = 0.87, thus ≈ 1.3-fold increase; Figure VIA in the online-only Data Supplement). Changes in lysyl oxidase expression in monocrotaline-treated rats were not restricted to lox because downregulated expression of loxl3 and loxl4 was seen in rat lung homogenates (Figure VD and VE in the online-only Data Supplement), and upregulated expression of loxl2 was also seen in rat lung PASMC (Figure VIC in the online-only Data Supplement). Thus, increased pulmonary lox expression is a feature of 2 models of PAH, as well as in clinical PAH, lending credence to the idea that lox may represent a pathogenic factor in PAH. In further support of this idea, there was a significant correlation (Pearson coefficient, 0.92) between pulmonary lox mRNA levels and right ventricular systolic pressure (RVSP; Figure VIIA in the online-only Data Supplement), as well as a significant correlation (Pearson coefficient, 0.95) between pulmonary lox mRNA levels and Fulton Index (Figure VIIIB in the online-only Data Supplement).

Inhibition of Lysyl Oxidase Attenuates Hypoxia-Induced PH

The rapid (within 3 hours), dramatic (3.7- to 6.2-fold), and persistent (21 days) increase in lysyl oxidase expression in the lungs of mice exposed to hypoxia suggested that lysyl oxidases might play a causal role in the development or persistence of PH. For this reason, the broad-spectrum inhibitor of lysyl oxidases, β-aminopropionitrile, was administered to mice concomitantly with hypoxia exposure. Mice exposed to hypoxia developed PH, as evident by an increased RVSP
(Figure 4D) from 20.9±1.2 mmHg in normoxia-exposed mice to 32.3±5.7 mmHg in hypoxia-exposed mice. In mice that were treated daily with β-aminopropionitrile during hypoxia exposure at a dose of 150 mg β-aminopropionitrile/kg body mass, the RVSP at 21 days was partially normalized, at 26.5±3.3 mmHg. The increased RVSP was accompanied by a significant increase in the Fulton Index, as an indicator of right ventricular hypertrophy (Table 2). Treatment of

Figure 2. Lysyl oxidases are expressed in plexiform and concentric lesions of patients with idiopathic pulmonary arterial hypertension (IPAH). Representative serial sections (for LOX, LOXL1, LOXL2, LOXL3, and LOXL4; the elastin sections are not serial) illustrating elastin distribution (Hart stain) and immunohistochemical staining for α-smooth muscle actin (ACTA2) and lysyl oxidase family members (LOX–LOXL4) in the walls of small pulmonary arteries of a healthy human lung donor (Donor; Table 1, donor 2) and a patient with IPAH (Table 1, patient 19). Antibody staining specificity is demonstrated in Figure IB to IF in the online-only Data Supplement.
mice with β-aminopropionitrile for the 21-day course of hypoxia exposure attenuated the right ventricular hypertrophy driven by hypoxia, significantly reducing the Fulton Index (Figure 4E). In contrast, there was no effect of hypoxia or β-aminopropionitrile on systemic arterial pressure (not shown). An increase in hematocrit was consistent with chronic exposure to hypoxia (Figure 4F). Thus, β-aminopropionitrile administration was able to reduce the effect of a hypoxic environment on RVSP appreciably, demonstrating a causal role for lysyl oxidase activity in the development of PH in response to chronic hypoxia. It is likely that β-aminopropionitrile functions by inhibiting lysyl oxidase activity. However, β-aminopropionitrile has been accredited with transcriptional regulatory roles, which pointed us to examine the gene expression levels of lysyl oxidases in β-aminopropionitrile–treated mice. Remarkably and in line with previous studies, β-aminopropionitrile administration led to depleted lox mRNA levels in β-aminopropionitrile–treated mice (Figure VIII in the online-only Data Supplement). Thus, the ability of β-aminopropionitrile administered to mice to limit collagen cross-linking induced by hypoxia may be attributed either to direct enzymatic inhibition of lysyl oxidase activity or to reduce transcription of lysyl oxidase–encoding genes, or a combination of both (Table 2).

Inhibition of Lysyl Oxidase Attenuates Hypoxia-Induced Pulmonary Vascular Remodeling

Increased pulmonary vascular resistance as a consequence of pathological vascular remodeling underlies the development of elevated pulmonary artery pressure in PH. For this reason, the effect of β-aminopropionitrile on small pulmonary artery muscularization was assessed. After 21 days, hypoxia-exposed mice exhibited increased remodeling of small (≤70 μm) intra-acinar pulmonary arteries when compared with normoxia-exposed mice (compare Figure 5A and 5B). These changes were largely absent

Figure 3. Expression of lysyl oxidases in the vasculature and vascular cells of human patients with idiopathic pulmonary arterial hypertension (IPAH). A–H, Expression of lysyl oxidases is upregulated in the pulmonary vasculature of patients with IPAH. Small pulmonary arteries were microdissected from donor lung tissue section (A and B) and lung tissue section of patients with IPAH (C and D). E–H, The mRNA from microdissected pulmonary arteries was screened by quantitative real-time reverse transcriptase-polymerase chain reaction for the relative expression levels of LOX, LOXL1, LOXL2, and LOXL3 (n=8–12, per group). Values represent mean ΔCt±SD, referenced to the β2-microglobulin (B2M) gene. LOXL4 could not be detected in microdissected samples. I–R, Expression of some lysyl oxidases was responsive to hypoxia in vascular smooth muscle cells and adventitial fibroblasts, and this response was enhanced in fibroblasts from patients with IPAH. Pulmonary artery smooth muscle cells (PASMC) and adventitial fibroblasts (Adv. Fib.) were isolated from explanted lungs from 3 apparently healthy lung donors (open bars; donors 1, 2, and 3 in Table 1) and 3 patients with IPAH (closed bars; patients 13, 14, and 15 in Table 1) maintained in culture in a 21% O2 or 10% O2 headspace gas for 24 hours and screened for relative mRNA expression levels of lysyl oxidase family members by quantitative real-time reverse transcriptase-polymerase chain reaction. Values represent mean ΔCt±SD, referenced to the hydroxymethylbilane synthase (HMBS) gene. Two-group comparisons (donor vs patient) were made with an unpaired Student t test, whereas comparisons of multiple groups were made by 1-way ANOVA with Tukey post hoc test.
Expression of lysyl oxidases is rapidly increased in the lungs of mice exposed to normobaric hypoxia. Mice were maintained in a normoxic (21% O₂; open bars) or hypoxic (10% O₂; closed bars) environment and were euthanized after (A) 3 hours, (B) 24 hours, and (C) 21 days, after which relative mRNA expression levels of lysyl oxidase family members were assessed in lung tissues by quantitative real-time reverse transcriptase-polymerase chain reaction. Values represent mean ΔCt±SD, referenced to the hydroxymethylbilane synthase (hmbs) gene (n=6, per group). D–F, Inhibition of lysyl oxidases attenuates hypoxia-induced pulmonary arterial hypertension in mice. D, The right ventricular systolic pressure (RVSP), (E) the Fulton Index (ratio of the mass of the right ventricle:the mass of the left ventricle plus septum), and (F) hematocrit were assessed in mice, 21 days after continuous exposure to normoxia or hypoxia. Mice were treated either with placebo (0.9% [m/v] isotonic saline) administered as a 100-μL intraperitoneal bolus, daily, or with the lysyl oxidase inhibitor β-aminopropionitrile (BAPN) at a dose of 150 mg/kg daily, also administered as a 100-μL intraperitoneal bolus in 0.9% (m/v) isotonic saline. Values represent mean ΔCt±SD (n=6, per group). Comparisons between groups were made by 1-way ANOVA with Tukey post hoc test.

Table 2. Heart Remodeling Parameters and Hematocrit Levels for Mice Used in this Study

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<td>(LV+S)/BW, mg/g</td>
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<td>2.87±0.61</td>
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BW indicates body weight; LV+S, left ventricle plus septum; and RV, right ventricle.
Inhibition of Lysyl Oxidase Attenuated Hypoxia-Driven Perturbations to Collagen Cross-Linking

The changes to vascular matrix structures provoked by hypoxia and partially reversed by β-aminopropionitrile administration (Figure 5E–5H) suggested that the inhibition of lysyl oxidases with β-aminopropionitrile may modulate the effect of hypoxia on collagen and elastin cross-linking. The abundance of both total collagen per lung (Figure 6A) and total elastin per lung (Figure 6B) was increased in the lungs of hypoxia-exposed mice, as was the abundance of total protein per lung (Figure 6C). Hypoxia drove an increase in the abundance of the sum of collagen cross-links isolated from the lungs of hypoxia-exposed mice (Figure 6D). A more detailed analysis of the collagen cross-link abundance revealed that these changes were limited to the rapidly (over hours) generated intermediate collagen cross-links, where the abundance of dihydroxylysinooyluronic acid was increased (Figure 6E) and the abundance of hydroxylysinonorleucine was not significantly changed (Figure 6F). There was no effect of hypoxia on the abundance of the slowly (over weeks) generated mature collagen cross-links, hydroxylysylpyridinoline and histidinohydroxyslysinonorleucine (Table 3). Administration of β-aminopropionitrile to mice during the 21-day course of exposure to hypoxia largely normalized the abundance of the sum of collagen cross-links isolated from the lungs of hypoxia-exposed mice (Figure 6D) and also largely normalized the abundance of dihydroxylysinoorleucine (Figure 6E) with the abundance of hydroxylysinoorleucine not significantly changed (Figure 6F). In contrast, β-aminopropionitrile was without the effect on the abundance on hydroxylysylpyridinoline and histidinohydroxylsinosinonorleucine (Table 3). There was no effect of hypoxia, or indeed of β-aminopropionitrile, on the abundance of the elastin cross-links, desmosine or isodesmosine (Table 4). Taken together, these data demonstrate that exposure of adult mice to hypoxia drives increased collagen abundance and collagen cross-link formation, notably of the rapidly generated cross-links, dihydroxylysinoorleucine and hydroxylysinosinonorleucine, but does not affect lysyl oxidase-mediated cross-linking of elastin. Furthermore, β-aminopropionitrile treatment can attenuate...
the effect of hypoxia on collagen deposition and collagen cross-linking. The nomenclature refers to the reduced variants of these intermediate cross-links.

**Discussion**

The data presented here demonstrate that lysyl oxidase expression was dysregulated in the lungs of patients with IPAH, CTEPH, and PAH caused by VSD. The expression of lysyl oxidases was also upregulated in vascular lesions associated with IPAH. As is evident from Table 1, all patients were chronically medicated, and we cannot rule out that the drugs administered to these patients may have also affected lysyl oxidase expression because the effect of the pharmacological agents on transition of lysyl oxidase–encoding genes has not been established. All 5 lysyl oxidases were expressed in PASMC and adventitial fibroblasts. In PASMC, LOX was induced by hypoxia; and in adventitial fibroblasts, LOX, LOXL1, and LOXL2 were induced by hypoxia. Interestingly, the induction of LOX expression by hypoxia was more pronounced in isolated adventitial fibroblasts from patients with IPAH when compared with those from lung donors. In addition, baseline levels of LOXL4 expression were higher in isolated PASMC from patients with IPAH than those from lung donors. These data point to intrinsic differences in the regulation of lysyl oxidases in patients with IPAH when compared with donors. These data promoted us to explore a possible causal role for lysyl oxidases in PH, and this study was undertaken in a mouse model of hypoxia-induced PH, where lysyl oxidase activity was blocked by the broad-spectrum lysyl oxidase inhibitor, β-aminopropionitrile.

Mice exposed to hypoxia exhibited a rapid and dramatic increase in the expression of lox and loxl2, as early as 3 hours after exposure. Increased lox and loxl2 expression persisted for 24 hours, and the elevated lox expression persisted over

![Figure 6](image-url)

**Figure 6.** Both exposure to hypoxia and β-aminopropionitrile (BAPN) administration can modulate the abundance of matrix molecules and the abundance of collagen cross-links in the lung. The abundance of (A) total collagen, (B) total elastin, (C) total protein, (D) total collagen cross-links (per units collagen), (E) the dihydroxylysinonorleucine (DHLNL) collagen cross-link (per unit collagen), and (F) the hydroxylysylpyridinoline (HNL) collagen cross-link (per unit collagen) assessed in mice, 21 days after continuous exposure to normoxia or hypoxia, with or without BAPN administration, as described in the legend of Figure 4. Comparisons between groups were made by 1-way ANOVA with Tukey post hoc test. Additional collagen and elastin cross-link data are presented in Tables 3 and 4, respectively.

Table 3. Effect of Hypoxia With and Without BAPN Administration on Lung Collagen Cross-Linking

<table>
<thead>
<tr>
<th></th>
<th>21% O₂</th>
<th>10% O₂</th>
<th>P Value vs 21% O₂</th>
<th>P Value vs 10% O₂</th>
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<tbody>
<tr>
<td>Placebo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HP/collagen, mol/mol</td>
<td>0.203±0.020</td>
<td>0.189±0.032</td>
<td>0.184±0.020</td>
<td>0.170±0.014</td>
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<td>0.011±0.002</td>
<td>0.008±0.001</td>
<td>0.009±0.005</td>
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<tr>
<td>HPL/collagen, mol/mol</td>
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<td>0.364±0.049</td>
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<td>HNL/collagen, mol/mol</td>
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<td>0.053±0.004</td>
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<td>DHLNL/HNL</td>
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<td>3.054±0.195</td>
<td>3.221±0.160</td>
<td>3.194±0.271</td>
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</tbody>
</table>

Additional cross-link data are presented in Figure 6. BAPN indicates β-aminopropionitrile; DHLNL, dihydroxylysinonorleucine; HNL, histidinohydroxylysinonorleucine; HNL, hydroxylysylpyridinoline; and HP, hydroxylysylpyridinoline.
the entire duration of the hypoxia exposure (21 days). Mice exposed to a hypoxic environment that were concomitantly treated with β-aminopropionitrile exhibited improved pulmonary hemodynamics and attenuated right ventricular hypertrophy, which developed as a consequence of perturbed pulmonary hemodynamics. In addition, β-aminopropionitrile administration to hypoxia-exposed mice reduced vascular remodeling and seemed to have reduced vascular matrix deposition when compared with placebo-treated controls. These data demonstrate that the (1) increased expression of lysyl oxidases in the lungs of hypoxia-exposed mice played a causal role in the pulmonary vascular remodeling associated with exposure to hypoxia and (2) dose of β-aminopropionitrile used was sufficient to interfere with, and indeed normalize, matrix cross-linking. Clearly, β-aminopropionitrile is a broad-spectrum lysyl oxidase inhibitor, and as such, no specific role can be ascribed to a particular lysyl oxidase in the pulmonary vascular remodeling associated with hypoxia exposure. Implication of particular lysyl oxidases in this process awaits the generation of transgenic animals or the development of enzyme-specific inhibitors.

Although the mouse model of hypoxia-induced PH has its limitations when extending observations to human disease, the increase in LOX and LOXL2 expression in lungs from patients with IPAH paralleled the trends observed with lox and loxl2 in hypoxia-exposed mice, lending credence to our suggestion that lysyl oxidases may participate in a vascular remodeling mechanism common to clinical and experimental PH. This idea is further supported by observations made in a second animal model of PH, the rat monocrotaline model, where increased lung expression of loxl2 was noted in the lungs and PASMC from rats that developed PH in response to monocrotaline administration.

Levels of lysyl oxidase are directly correlated with elastin and collagen cross-linking and elastin and collagen fiber structure and stability. For example, targeted deletion of the lox gene, which causes perinatal lethality, also causes extended fragmentation of elastic fibers and discontinuities of both internal elastic lamina and lamellae in lox–/– mice. In addition, lox–/– mice also exhibit disruption of vascular smooth muscle cell contact and detachment of endothelial cells from the basal lamina, as well as alterations to endothelial cell morphology. Therefore, it is not surprising that lox–/– mice have a high incidence of aortic aneurysms, aortic tortuosity, and aortic rupture. It follows that increased expression of lysyl oxidases, over and above normal expression levels, might have the opposite effect. Although vascular stiffening is often attributed to a paucity of vascular elastin, we propose here that an overabundance of ECM cross-linking enzymes may lead to increased cross-linking of matrix molecules aberrantly, such as the increased collagen cross-linking described in the present study, in lungs from hypoxia-exposed mice, perhaps resulting in a more dense fiber network (as is clearly evident in Figure 5E–5H). Fibers that are too dense may also result in well stiffening or rigidification of the vessel wall, attributable not to paucity, but rather to an over-cross-linked ECM. In support of such an idea, patients with chronic heart failure exhibit elevated lysyl oxidase levels, which correlate with both increased collagen cross-linking and increased left ventricular chamber stiffness. More recent studies have underscored the importance of lysyl oxidase–mediated collagen cross-linking in heart failure, by demonstrating that elevated filling pressures in patients with hypertension with stage C chronic heart failure correlate not with total collagen but rather with the degree of collagen cross-linking in the myocardium.

The data we report here demonstrate that in the hypoxia model of PH, exposure of mice to hypoxia drives the increased cross-linking of collagen by the rapidly synthesized bifunctional collagen cross-links, dihydroxylysinonorleucine and hydroxylysylonorleucine. These cross-links are generated within hours and typically reflect newly synthesized collagen. These cross-links serve as precursors for the mature trifunctional collagen cross-links, hydroxylysylpyridinoline (from dihydroxylysylnorleucine) and histidinohydroxylysylnorleucine (from hydroxylysylnorleucine) that are generated slowly (after weeks) and represent collagen maturation. The observed increase in dihydroxylysylnorleucine and hydroxylysylnorleucine in response to hypoxia is consistent with these bifunctional cross-links being generated during a short (3 weeks) time-course, as is the reduction in the abundance of these cross-links after a 3-week β-aminopropionitrile administration regimen. It is evident from Table 3 that the abundance of the mature trifunctional hydroxylysylpyridinoline and histidinohydroxylysylnorleucine cross-links (measured per unit collagen) after 3 weeks of hypoxia exposure was unchanged. This most likely reflects the generation of new collagen, stimulated by hypoxia exposure, that would not yet have formed the slowly generated trifunctional hydroxylysylpyridinoline and histidinohydroxylysylnorleucine cross-links.

Neither hypoxia nor β-aminopropionitrile seemed to affect the abundance of desmosine or isodesmosine cross-links in lung elastin (Table 4) although hypoxia did drive

| Table 4. Effect of Hypoxia With and Without BAPN Administration on Lung Elastin Cross-Linking |
|---------------------------------|-----------------|----------------|------------------|-----------------|-----------------|------------------------|-----------------|----------------|------------------|
|                                | 21% O2 Placebo | 21% O2 BAPN   | 10% O2 Placebo | 10% O2 BAPN    | P Value vs 21% O2 Placebo | P Value vs 10% O2 Placebo |
| Des/elastin, mol/mol           | 7.145±0.661    | 7.151±0.684   | 6.558±0.656    | 6.819±0.381    | 0.338            | 0.881                   |
| Iso/elastin, mol/mol           | 6.783±0.595    | 6.813±0.648   | 6.239±0.553    | 6.387±0.299    | 0.308            | 0.966                   |
| (Des+iso)/elastin, mol/mol     | 13.928±1.256   | 13.964±1.331  | 12.797±1.208   | 13.206±0.678   | 0.323            | 0.928                   |
| Des/iso                        | 1.053±0.007    | 1.050±0.008   | 1.050±0.014    | 1.067±0.011    | 0.941            | 0.031                   |

BAPN indicates β-aminopropionitrile; des, desmosine; and iso, isodesmosine.
increased elastin production per se. These data may suggest that under normoxic condition, the elastin is already maximally cross-linked; however, these data do not rule out additional cross-linking by other matrix cross-linking systems, such as the transglutaminases, which have not been addressed in this study.

In addition to an effect of vessel stiffening or rigidification, an abnormally stabilized (through abnormally high cross-linking) ECM may also generate matrix fibers that are resistant to proteolysis, and thus, resistant to normal vascular remodeling and turnover processes. Indeed, hyper-cross-linking of collagen fibers in tissue-engineered arteries through stimulation of lysyl oxidase activity increased the stability of collagen fibers to proteolysis. It is well recognized that a delicate balance exists among matrix production, deposition, and degradation, in response to vascular injury, and a remodeling-resistant matrix may contribute to the persistence of aberrant matrix structures in the vascular wall. It is also worth noting that matrix remodeling events regulate the behavior of cells in the local environment, by releasing matrix bound growth factors, and proteolytically released fragments of matrix molecules, such as endostatin, which can inhibit local endothelial cell proliferation and migration. Protease-resistant matrix components would disturb these processes. Although the increased collagen cross-linking reported here may seem moderate (=20% over normal), it should be borne in mind that values reflected abnormal cross-linking has developed ≥1 month after vessel injury. Indeed, it was proposed that therapeutic strategies to overexpress lysyl oxidase (by lysyl oxidase overexpression) were proposed with the aim of increasing vessel stiffness or rigidification.

Although lysyl oxidase inhibition seems to be beneficial in the context of hypoxia-induced pulmonary vascular remodeling, β-aminopropionitrile has also been reported to worsen certain types of cardiovascular disease (eg, in an experimental animal model of abdominal aortic aneurysm). In that study, lysyl oxidases were thought to provide a protective or compensatory mechanism against aneurysm formation, and indeed, it was proposed that therapeutic strategies to overexpress support the cross-linking of soluble matrix proteins (by lysyl oxidase overexpression) were proposed with the aim of stabilizing the aneurysm.

A spectrum of matrix-independent roles has also been suggested for lysyl oxidases in the cardiovascular system, which might be relevant to the onset or progression of PH. LOX is a potent chemomatoctant for both vascular smooth muscle cells and fibroblasts. LOX activity also modulates the sensitivity of smooth muscle cells and fibroblasts to other chemomatoctants, such as PDGF-BB, by oxidizing cell surface receptors, including PDGF receptor-β. Thus, local production of lysyl oxidases by constituent cells of the vascular wall may drive neointimal formation or medial hypertrophy by direct recruitment of vascular smooth muscle cells. This would be consistent with the LOX-dependent vessel wall muscularization we report in this study. Along these lines, LOX is also potently chemomatoctic for monocytes, which are emerging as important contributors to pulmonary vascular remodeling associated with hypoxia, together with other inflammatory processes relevant to pulmonary vascular remodeling, which may also be affected by the chemomatoctic properties of lysyl oxidases.

Lysyl oxidases can act as transcriptional regulators, having been identified in the nucleus of fibrogenic cells, and LOX can directly activate both the collagen III and the elastin promoters. As discussed above, increased abundance of ECM in vessel walls is known to increase pulmonary activity, as demonstrated for extralobar pulmonary artery stiffening in hypoxia-induced PH. These observations, combined with the trends in lysyl oxidase expression we report here, suggest that upregulation of lysyl oxidase expression in the pulmonary vasculature may drive increased collagen and elastin production, and thereby contribute to increased vessel stiffening. In support of this idea,lox mRNA expression correlated with RVSP in hypoxia-exposed mice. Furthermore, concomitant treatment of hypoxia-exposed mice with β-aminopropionitrile treatment normalized collagen cross-linking and RVSP and also reduced right heart hypertrophy associated with elevated RVSP.

How lysyl oxidase expression is regulated in the pulmonary vasculature remains to be clarified. We demonstrate here that LOX is hypoxia-inducible in PASM and that LOX, LOXL1, and LOXL2 are hypoxia-inducible in adventitial fibroblasts (Figure 3). However, in the absence of hypoxic stimuli, other regulatory mechanisms may drive lysyl oxidase expression in the pulmonary vasculature of patients with PAH. PDGF, a pathogenic factor and a validated therapeutic target in IPAH, potently upregulated LOX expression in aortic smooth muscle cells. The expression of lysyl oxidases is also regulated by transforming growth factor-β and dysregulated by transforming growth factor-β signaling has been causally implicated in experimental PH. Thus, the increased expression of lysyl oxidases in lung tissues and vessels of patients with PAH may result from increased PDGF or transforming growth factor-β signaling in these patients.

In another recent report, copper chelation was demonstrated to dampen endothelial cell proliferation in the context of PAH. Copper chelation would also inhibit lysyl oxidases, suggesting that lysyl oxidases may directly affect the proliferation of the constituent cells of the vessel wall; however, a specific role for lysyl oxidases in endothelial cell proliferation was not addressed in that study.

We propose that the elevated lysyl oxidase activity in the pulmonary vasculature contributes to the persistence and over-abundance of ECM components (such as collagen and elastin fibers), resulting in fibers that are improperly remodeled (over–cross-linked), thereby contributing to vascular disease. These fibers are also most likely resistant to proteolytic degradation, which disturbs the delicate balance between matrix synthesis and degradation that accompany vascular injury. As such, this report introduces a new player in the molecular context of hypoxia-induced pulmonary vascular remodeling, which may also contribute to the persistence of aberrant matrix structures in the vascular wall. It is also worth noting that matrix remodeling events regulate the behavior of cells in the local environment, by releasing matrix bound growth factors, and proteolytically released fragments of matrix molecules, such as endostatin, which can inhibit local endothelial cell proliferation and migration. Protease-resistant matrix components would disturb these processes. Although the increased collagen cross-linking reported here may seem moderate (=20% over normal), it should be borne in mind that values reflected abnormal cross-linking has developed ≥1 month after vessel injury. Indeed, it was proposed that therapeutic strategies to overexpress support the cross-linking of soluble matrix proteins (by lysyl oxidase overexpression) were proposed with the aim of stabilizing the aneurysm.

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In this study, we hypothesized that lysyl oxidase expression and activity was dysregulated in PH. The data we report here indicate that the expression of lysyl oxidases is indeed dysregulated in both clinical PH (IPAH, CTEPH, and VSD) and hypoxia-induced PH in mice. Furthermore, a causal role for lysyl oxidase activity was confirmed in hypoxia-induced PH, and the lung matrix cross-linking systems have been validated as candidate targets for intervention with the aim of normalizing elevated pulmonary artery pressures.

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mechanisms underlying pulmonary vascular remodeling associated with PH. The ease of targeting lysyl oxidases pharmacologically makes this family of matrix remodeling enzymes particularly interesting candidates for further investigation in pulmonary vascular disease.

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Disclosures

None.

References

39. Ooi CY, Wang Z, Tabinia DM, Eckhoff JC, Chesler NC. The role of collagen in extralobar pulmonary artery stiffening in response to...


Significance

Pulmonary arterial hypertension is a fatal lung disease, unless patients undergo lung transplantation. The key pathological feature of pulmonary hypertension is the occlusion of small pulmonary arteries, which occurs by aberrant vascular remodeling. Although previously this vascular remodeling has been attributed to altered growth properties of constituent cells of the vessel wall, we demonstrate here that in patients and animal models of pulmonary hypertension, collagen cross-linking enzymes are dysregulated. Furthermore, in hypoxia-induced pulmonary hypertension in mice, hypoxia drives increased collagen cross-linking and perturbs matrix architecture in the lung vessel walls. Blocking lysyl oxidase activity limited the lung vessel and heart remodeling driven by hypoxia exposure, demonstrating a causal role for lysyl oxidases and perturbed collagen cross-linking in experimental pulmonary hypertension. Our proof-of-principle study demonstrated that modulation of lung matrix cross-linking can affect pulmonary vascular remodeling associated with PH.
Lysyl Oxidases Play a Causal Role in Vascular Remodeling in Clinical and Experimental Pulmonary Arterial Hypertension

Alexander H. Nave, Ivana Mizíková, Gero Niess, Heiko Steenbock, Frank Reichenberger, María L. Talavera, Florian Veit, Susanne Herold, Konstantin Mayer, István Vadász, Norbert Weissmann, Werner Seeger, Jürgen Brinckmann and Rory E. Morty

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Lysyl oxidases play a causal role in vascular remodeling in clinical and experimental pulmonary arterial hypertension

Alexander H. Nave; Ivana Mižíková; Gero Niess; Heiko Steenbock; Frank Reichenberger; María L. Talavera; Florian Veit; Susanne Herold; Konstantin Mayer; István Vadász; Norbert Weissmann; Werner Seeger; Jürgen Brinckmann; Rory E. Morty

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This online supplement consists of one supplementary table, and eight supplementary figures.
Supplementary Table 1. Primers employed in this study.

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FIGURE LEGENDS

Supplementary Figure I. Demonstration of the antibody specificity of the lysyl oxidase antibodies employed in this study. (A) Protein (25 µg) from adult human lung tissue samples was boiled in the presence of dithiothreitol and resolved by SDS-PAGE. Samples were blotted onto nitrocellulose membranes, which were then probed with the primary and secondary antibodies detailed in the Materials and Methods section. Additionally, staining specificity of antibodies was assessed by immunohistochemistry using competing peptides, as described in the Material and Methods, for (B) LOX, (C) LOXL1, (D) LOXL2, (E) LOXL3, and (F) LOXL4.

Supplementary Figure II. Expression of LOXL3 in the lungs of patients with idiopathic pulmonary arterial hypertension. The relative expression levels of LOXL3 mRNA in explanted lungs from nine patients with idiopathic pulmonary arterial hypertension (IPAH) and in explanted lungs from seven healthy lung donors (Donor) were assessed by quantitative real-time RT-PCR (A). Values represent mean ΔCt ± S.D., referenced to the hydroxymethylbilane synthase (HMBS) gene. (B) Protein expression was similarly assessed by immunoblot, where representative expression levels in five patients and four lung donors are presented. A densitometric analysis of the immunoblot data is provided in (C) comparing data from controls (open bars) and patients (closed bars). In all instances, a two-group comparison (donor versus patient) was made with an unpaired Student’s t-test. Patient clinical parameters are provided in Table 1.

Supplementary Figure III. Expression of lysyl oxidases is upregulated in the lungs of patients with chronic thromboembolic pulmonary hypertension and pulmonary hypertension secondary to ventricular septal defect. (A–E) The relative expression levels of mRNA encoding five lysyl oxidase family members in explanted lungs from eight patients with chronic thromboembolic pulmonary hypertension (CTEPH), four patients with pulmonary hypertension secondary to ventricular septal defect (VSD) and in explanted lungs from twelve healthy lung donors (Donor) were assessed by quantitative real-time RT-PCR. Values represent mean ΔCt ± S.D., referenced to the hydroxymethylbilane synthase (HMBS) gene. In all instances, a two-group comparison (donor versus patient) was made with an unpaired Student’s t-test. Patient clinical parameters are provided in Table 1.

Supplementary Figure IV. Expression of lysyl oxidases in the lung parenchyma of adult mice exposed to 21% O₂ or 10% O₂ for 21 days. (A–C) Representative illustration of the microdissection of lung parenchyma from cryosections of mouse lung tissue. (D) The relative expression levels of lysyl oxidase mRNA in microdissected mouse lung tissues were assessed by quantitative real-time RT-PCR, using lung tissue from 21% O₂-exposed (open bars) and 10% O₂-exposed (closed bars). Values represent mean ΔCt ± S.D., referenced to the hydroxymethylbilane synthase (hmbs) gene. In all instances, a two-group comparison (21% O₂ versus 10% O₂) was made with an unpaired Student’s t-test; and P values less than 0.1 are illustrated.

Supplementary Figure V. Expression of lysyl oxidases in lung tissue from rats in which pulmonary hypertension had been induced with monocrotaline. The relative expression levels of (A) lox, (B) loxl1, (C) loxl2 (D) loxl3, and (E) loxl4 mRNA were assessed in lung homogenates from rats in which pulmonary hypertension had been induced with monocrotaline. The mRNA levels were assessed by quantitative real-time RT-PCR. Values represent mean ΔCt ± S.D., referenced to the hydroxymethylbilane synthase (hmbs) gene. In
all instances, a two-group comparison (monocrotaline *versus* vehicle) was made with an unpaired Student’s *t*-test.

**Supplementary Figure VI.** Expression of lysyl oxidases in PASMC from the lungs of rats in which pulmonary hypertension had been induced with monocrotaline. The relative expression levels of (A) *lox*, (B) *loxl1*, (C) *loxl2* (D) *loxl3*, and (E) *loxl4* mRNA were assessed in mRNA pools from PASMC from rats in which pulmonary hypertension had been induced with monocrotaline. The mRNA levels were assessed by quantitative real-time RT-PCR. Values represent mean ΔCt ± S.D., referenced to the hydroxymethylbilane synthase (*hmbs*) gene. In all instances, a two-group comparison (monocrotaline *versus* vehicle) was made with an unpaired Student’s *t*-test.

**Supplementary Figure VII.** Correlation of *lox* mRNA levels with right ventricular systolic pressure and right ventricular hypertrophy. The relative expression levels of *lox* in mice after exposure to 21% O₂ or 10% O₂ were correlated with (A) right ventricular systolic pressure (RSVP) or (B) the Fulton Index, as a measure of right ventricular remodeling.

**Supplementary Figure VIII.** Impact of β-aminopropionitrile administration on the mRNA expression of lysyl oxidases in lung tissue of mice in which pulmonary hypertension had been induced with hypoxia. The relative expression levels of (A) *lox*, (B) *loxl1*, (C) *loxl2* (D) *loxl3*, and (E) *loxl4* mRNA were assessed in mice, 21 days after continuous exposure to 21% O₂ or 10% O₂, with or without β-aminopropionitrile (BAPN) administration, as described in the legend to Figure 4. The mRNA levels were assessed by quantitative real-time RT-PCR. Values represent mean ΔCt ± S.D., referenced to the hydroxymethylbilane synthase (*hmbs*) gene. Comparisons between groups were made by one-way ANOVA with Tukey’s *post hoc* test.
Online Supplementary Figure I

A

B  LOX

C  LOXL1

D  LOXL2

E  LOXL3

F  LOXL4

75 kDa  50 kDa  37 kDa

LOX  LOXL1  LOXL2  LOXL3  LOXL4
Online Supplementary Figure II

A

\[ \text{LOXL3 } (P=0.224) \]

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| Relative mRNA expression | ![Graph](image)

B

\[ \text{LOXL3} \]
\[ \text{α-tubulin} \]

Donor  
IPAH

C

Pixel density normalized to α-tubulin

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Online Supplementary Figure III

A

Relative mRNA expression \( \Delta Ct \)

P=0.003

LOX

B

Relative mRNA expression \( \Delta Ct \)

LOX1

C

Relative mRNA expression \( \Delta Ct \)

LOX2

D

Relative mRNA expression \( \Delta Ct \)

LOX3

E

Relative mRNA expression \( \Delta Ct \)

LOX4

P=0.041
Online Supplementary Figure IV

A

B

C

D

relative mRNA expression [ΔCt]

P=0.009

P=0.085

lox loxl1 loxl2 loxl3 loxl4

-4 -2 0 2 4 6

100 μm

100 μm

100 μm

100 μm

Online Supplementary Figure IV

A

B

C

D

relative mRNA expression [ΔCt]

P=0.009

P=0.085

lox loxl1 loxl2 loxl3 loxl4

-4 -2 0 2 4 6

100 μm

100 μm

100 μm

100 μm

Online Supplementary Figure IV

A

B

C

D

relative mRNA expression [ΔCt]

P=0.009

P=0.085

lox loxl1 loxl2 loxl3 loxl4

-4 -2 0 2 4 6

100 μm

100 μm

100 μm

100 μm
Online Supplementary Figure V

A  \( lox \)

B  \( loxl1 \)

C  \( loxl2 \)

D  \( loxl3 \)

E  \( loxl4 \)
Online Supplementary Figure VI

**A**

*Lox*

Relative mRNA expression ($\Delta Ct$) for CTRL and MCT treatments.

**P = 0.015**

**B**

*Lox1*

Relative mRNA expression ($\Delta Ct$) for CTRL and MCT treatments.

**P = 0.070**

**C**

*Lox2*

Relative mRNA expression ($\Delta Ct$) for CTRL and MCT treatments.

**P = 0.010**

**D**

*Lox3*

Relative mRNA expression ($\Delta Ct$) for CTRL and MCT treatments.

**P = 0.080**

**E**

*Lox4*

Relative mRNA expression ($\Delta Ct$) for CTRL and MCT treatments.

**P = 0.200**
Online Supplementary Figure VII

A

![Graph A: Scatter plot showing relative LOX mRNA expression [ΔCt] vs. RVSP (mmHg). Pearson coefficient: 0.92]

B

![Graph B: Scatter plot showing relative LOX mRNA expression [ΔCt] vs. RV/(LV+S). Pearson coefficient: 0.95]
Online Supplementary Figure VIII

**A**

\[ \Delta \text{Ct} \]

**B**

\[ \Delta \text{Ct} \]

**C**

\[ \Delta \text{Ct} \]

**D**

\[ \Delta \text{Ct} \]

**E**

\[ \Delta \text{Ct} \]
Materials and Methods

Human Tissues and Cells
Diseased lung tissue was obtained from lungs explanted from 12 patients with IPAH, six patients with CTEPH, and four patients with pulmonary hypertension due to VSD that underwent lung transplantation (Table 1). Control lung material was obtained from 12 apparently healthy organ donors. The study protocol was approved by the Ethics Committee of Justus Liebig University School of Medicine (Reference number AZ 31/93). Written informed consent was obtained from each subject or their next-of-kin. Material was harvested and processed for mRNA and protein isolation, laser-capture microdissection or immunohistochemistry exactly as described previously.1

Cell culture
Cell culture of primary lung fibroblasts and primary human pulmonary artery smooth muscle cells was undertaken exactly as described previously by our group.2, 3 Cells were exposed to a normoxic (21% O2 in incubator headspace) or a hypoxic (10% O2 in incubator headspace) environment for 24 h.

Laser-Capture Microdissection
Pulmonary arteries of outer diameter of ≤150 μm were laser-capture microdissected from human and mouse lung tissue, and microdissected material was processed for quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) exactly as described previously.4

Immunoblotting and Real-Time PCR
Immunoblotting and real-time RT-PCR were undertaken exactly as described previously.1 Mean ΔΔCt values were calculated from ΔCt(disease)−ΔCt(control), from which fold-changes were calculated by fold-change=2ΔΔCt.1 Primers for real-time RT-PCR are described in supplementary Table I, available in the online supplement. Antibodies for immunoblotting were as follows: (all from Santa Cruz, La Jolla, CA): goat anti-LOX (sc-32409; 1:750), mouse anti-LOXL1 (sc-166632; 1:750), goat anti-LOXL2 (sc-48723; 1:750), goat anti-LOXL3 (sc-48728; 1:750), and goat anti-LOXL4 (sc-48731; 1:750). Alpha-tubulin was detected with mouse anti-α-tubulin (Sigma Aldrich, St Louis, MO, T-5168; 1:1000) and was used to assess loading equivalence. The specificity of the lysyl oxidase antibodies employed is documented in Supplementary Figure I, available in the online supplement.

The Hypoxia-Induced Pulmonary Hypertension Mouse Model
Samples from mice with hypoxia-induced pulmonary arterial remodeling and pulmonary hypertension were obtained exactly as described previously,5 after a 21-day exposure to 10% O2 or 21% O2 in the inspired air, for hypoxia and normoxia, respectively. Administration of 150 mg/kg (intraperitoneal) once daily β-aminopropionitrile (BAPN; in PBS as vehicle) to mice in the hypoxia model was approved by the appropriate government authority (Regierungspräsidium Gießen; approval number V54 – 19 c 20 15(1) GI 20/10 Nr. 85/2011). This dose was selected based on the published work of others, who employed the same dose in adult mice.5-9 BAPN administration did not appear to have any deleterious impact on mouse health, where no changes were observed in mouse body mass comparing, for example, hypoxia-exposed BAPN-treated mice versus hypoxia-exposed placebo-treated mice (Table 2). However, a single BAPN-treated mouse was euthanized after
developing blood in the urine (which was also detected in the peritoneal cavity upon autopsy).

Mice of mass approximately 22 g were employed in the study. Over the 21-day time course of the study, mice maintained under normoxic conditions gained approximately 2 g of mass, while mice maintained under hypoxia conditions lost approximately 2 g of mass. BAPN administration per se had no impact on weight loss. The heart rate of the mouse groups was always between 300 and 350 b.p.m., and did not differ significantly between the groups. Fulton Index data and hematocrit values for the experimental mouse groups are presented in Table 2.

Hemodynamic measurements were made exactly as described previously. Briefly: Animals were initially anesthetized intraperitoneally with ketamine (100 mg/kg body mass) and xylazine (12-15 mg/kg body mass). The left carotid artery was isolated and cannulated with a polyethylene cannula connected to a fluid-filled force transducer and the systemic arterial pressure (SAP) was measured. A catheter was inserted through the right jugular vein into the right ventricle for measurement of right ventricular systolic pressure (RVSP).

The Monocrotaline-Induced Pulmonary Hypertension Rat Model
Samples of lung tissue and isolated PASMC from rats with monocrotaline-induced pulmonary arterial remodeling and pulmonary hypertension were obtained exactly as described previously.

Morphometric Analysis
The degree of muscularization of small pulmonary arteries was assessed exactly as described previously. Briefly, 80-100 small vessels (outer diameter 20-70 \( \mu \)m) per section were counted at a 40× magnification and categorized either as non-muscularized, partially muscularized, or fully muscularized according to a smooth muscle content of <5, 5–75, or >75%, respectively. The proportion of pulmonary vessels in each muscularization class was assessed. Heart ratios for the determination of Fulton Index were assessed as described previously. Determinations were performed in a blinded fashion.

Immunohistochemistry and Elastin Staining
Immunohistochemical detection of lysyl oxidases and \( \alpha \)-smooth muscle actin was undertaken on 3-\( \mu \)m paraffin-embedded lung sections as described previously. Antibodies for immunohistochemistry were as follows (all from Santa Cruz, La Jolla, CA): goat anti-LOX (sc-32409; 1:200), goat anti-LOXL1 (sc-48720; 1:50), goat anti-LOXL2 (sc-48723; 1:50), goat anti-LOXL3 (sc-48728; 1:200), and goat anti-LOXL4 (sc-48731; 1:50). To demonstrate antibody specificity, competing peptides against all five LOX family members were employed: (all from Santa Cruz, La Jolla, CA): LOX peptide (sc-32409 P), LOXL1 peptide (sc-48720 P), LOXL2 peptide (sc-48723), LOXL3 peptide (sc-48728 P), and LOXL4 peptide (sc-48731 P), at a 1:100 antibody:peptide molar ratio. Antibodies and competing peptides were combined for 4 h at room temperature, prior to application to tissue sections. Alpha-smooth muscle actin was detected with mouse anti-\( \alpha \)-smooth muscle actin (Sigma Aldrich, St Louis, MO, A-2547; 1:1500). The specificity of the antibodies employed on human tissues has already been confirmed. Hart's elastica staining was undertaken exactly as described previously.
Analysis of Elastin and Collagen Crosslinks

For collagen cross-link analysis, specimens were cut into small pieces and reduced by sodium borohydride (Sigma, Germany; 25 mg NaBH₄/ml in 0.05 M NaH₂PO₄/0.15 M NaCl pH 7.4, 1 h on ice, 1.5 h at room temperature). Specimens were digested with high purity bacterial collagenase (C0773; Sigma, Germany; 50 U/ml, 37°C, 12 h). After centrifugation, the soluble fractions containing collagen cross-links were hydrolyzed in 6 N HCl at 110°C for 24 h. The hydrolysates were pre-cleared on CF-11 cellulose columns to remove the bulk of non-cross linked amino acids. Dried eluates were redissolved in sodium citrate loading buffer (pH 2.2) and analyzed on an amino acid analyzer (Biochrom 30, Biochrom, Great Britain) using a three buffer gradient system and post column ninhydrin derivatization. The column was eluted for 5 min (flow rate 15 ml/h) with sodium citrate buffer (pH 4.25), for 40 min with sodium citrate buffer (pH 5.35) and for 20 min with sodium citrate/borate buffer (pH 8.6) at 80° C. Retention times of individual cross-links were established with authentic cross-link compounds. Quantification was based on ninhydrin generated leucine equivalence factors (dihydroxylysinonorleucine (DHLNL), hydroxylysinoonorleucine (HLNL): 1.8; hydroxylysylpyridinoline (HP): 1.7; histidinoxylysinonorleucine (HHL): 1.97). The collagen content was analyzed in an aliquot of hydrolyzed specimens of the collagenase soluble fraction prior to CF-11 preclearance. For protein analysis specimens were cut into small pieces and digested with bacterial collagenase. After centrifugation, the soluble fraction containing collagen was subjected to hydrolysis and amino acid analysis according to standard protocol (Biochrome). Collagen content was calculated based on a content of 14 mg hydroxyproline in 100 mg collagen. The residual fraction was extracted by hot alkali (0.1 N NaOH, 95 °C, 45 min). After centrifugation the supernatant containing non-collagenous/nonelastin proteins and the insoluble residue containing insoluble elastin were subjected to hydrolysis and amino acid analysis. The content of elastin cross-links (isodesmosine (IDES) and desmosine (DES) were analyzed in an aliquot of the NaOH-insoluble fraction containing elastin after CF-11 preclearance by amino acid analysis described above (leucine equivalence factors IDES: 3.4; DES: 3.4).

Statistical analysis

Data are indicated as mean±S.D. For comparisons of two groups, statistical comparisons were evaluated with an unpaired Student’s t-test. For comparisons of three or more groups, comparisons were evaluated by one-way analysis of variance (ANOVA) with a Tukey’s post hoc test. Statistical outliers were identified by Grubb’s test, and in the case of an outlier, the sample/animal from which the outlier value was derived was completely excluded from all analyses. The normal (parametric) distribution of data was confirmed for all data sets with an Anderson-Darling test, applying correction for small sample sizes.

References


