Collagen and elastin cross-linking is altered during aberrant late lung development associated with hyperoxia

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POSTNATAL LUNG DEVELOPMENT in mice is characterized by a period of intensive growth and remodeling of the lung parenchyma, which rapidly (over a period of days) generates a large number of small alveoli and thus maximizes the alveolar surface area over which gas exchange can take place. Secondary septation, the generation of new septa from preexisting septa, which then divide the air spaces, peaks in mice between 4 and 7 days after birth and is largely complete 21 days after birth. In humans, secondary septation is already well underway during late stages of pregnancy, and thus occurs in utero, and continues several years into postnatal life. Disturbances to the formation of secondary septa, such as the arrested secondary septation associated with bronchopulmonary dysplasia (BPD) in humans, leads to malformed lungs with fewer and larger alveoli. This common complication of premature birth is associated with significant morbidity and mortality.

The molecular mechanisms underlying the generation of secondary septa remain poorly understood, but most likely rely on the coordinated action of transcription factors, growth factor signaling, extracellular matrix (ECM) deposition and remodeling, and physical forces, such as breathing motions (41, 42). The generation and shaping of the lung ECM has received much attention in the context of normal and aberrant lung alveolarization (20, 38, 45). Infants with BPD exhibit increased collagen abundance in the lung (14), and collagen fibers and the lung collagen scaffold are malformed (60). There is also evidence of altered elastin turnover, as assessed by urinary excretion of the desmosine elastin cross-link, in infants with BPD (12). These data point to disturbances to ECM production and processing in BPD, which may be mimicked in animal models, employing either hyperoxia, caloric restriction, mechanical ventilation, or preterm delivery as an injurious stimulus in rats, mice, lambs, and baboons (6, 13, 49, 66). The lung histopathology that results is reminiscent of that of BPD in humans, with evident air space enlargement, septal wall thickening, and a reduced number of alveoli, as well as perturbations to ECM structures. Increased collagen levels have been reported in the lungs of hyperoxia-exposed adult rats (44). Elastin has received more attention, since normal elastin production and deposition are critical for proper development of alveoli (43, 57, 58). In hyperoxia-exposed or calorie-restricted mice, late lung development is impeded, and elastic fibers are described to be “irregularly distributed, tortuous, and abruptly terminating” (11). Similarly, in mechanically ventilated preterm lambs, late lung development is also impeded, and excessive production of elastin and accumulation of short, brush-like elastin fibers in the developing lung has been noted (2, 52).

To date, the underlying reasons for these perturbations to ECM structures have remained elusive. Excessive elastase activity has been considered as a mediator of pathological elastin degradation, which might underlie the paucity of elastin and bizarre elastin fiber patterns seen in affected lungs (21–23, 36). Additionally, the altered expression of proteins required for proper elastin fiber assembly has also been suggested to...
uncouple elastin synthesis and the assembly of elastin fibers (7–9). Several recent studies have revealed that the expression and activity of several ECM maturation systems, including collagen and elastin cross-linking enzymes of the lysyl oxidase (33), lysyl hydroxylase (65), and transglutaminase (64) families, are deregulated in both animal models of BPD and in tissues from BPD patients. These observations suggest that improper ECM cross-linking in the developing lung may contribute to disturbed alveolarization. This idea is supported by a recent report that lysyl oxidases play a causal role in the remodeling of the pulmonary vasculature that is associated with pulmonary arterial hypertension (48). Thus it was hypothesized and tested in this study that collagen and elastin cross-linking is perturbed during the arrested alveolarization that is associated with the exposure of mouse neonates to a hyperoxic environment. Additionally, a causal role for lysyl oxidases in generating perturbed ECM cross-linking was explored.

**MATERIAL AND METHODS**

**Animal studies.** All animal procedures were approved by the local authorities, the Regierungspräsidium Darmstadt (approvals B2/329 for pilot and P9.5 studies, and B2/1029 for P19.5 studies). For studies using the broad-spectrum lysyl oxidase inhibitor, b-aminopropionitrile (BAPN; A3134, Sigma, St. Louis, MO), a pilot study was first performed with a dose of 15 and 150 mg·kg⁻¹·day⁻¹ (three animals per group, observed for 5 days) to assess BAPN tolerance. The upper limit of this dose was based on previous studies with BAPN in adult mice (48). After the pilot study, BAPN was then applied at 15 mg·kg⁻¹·day⁻¹ for either 9 days [postnatal day (P) 9.5] or 19 days (P19.5). To minimize oxygen toxicity and to avoid potential confounders of milk production caused by changes in inspired oxygen levels, nursing dams were rotated every 24 h between normoxia and hyperoxia. Dams and pups received food ad libitum and were main-
col employed for the design-based stereomorphometric analysis of neonatal mouse lungs has been described in the detail previously (39), based on state-of-the-art methodology (47, 51, 56). Briefly, mouse pup lungs were instillation-fixed through a tracheal cannula at a hydrostatic pressure of 20 cmH2O with 1.5% (wt/vol) paraformaldehyde, 1.5% (wt/vol) glutaraldehyde in 150 mM HEPES, pH 7.4, for 24 h at 4°C (31). Lung tissue blocks were then collected according to systematic uniform random sampling for stereological analysis (50, 62). The total volume of the lungs was measured by Cavalieri’s principle (61). Lungs were embedded in toto in agar and cut into slices of 2-mm thickness. Lungs were treated with sodium cacodylate, osmium tetroxide, and uranyl acetate and embedded in glycol methacrylate (Technovit 7100, Heareus Kulzer, 64709003). Each tissue block was cut into sections of 2 µm, and every first and third section of a consecutive series of sections was stained with Richardson’s stain. All slides were scanned using a NanoZoomer-XR C12000 Digital slide scanner (Hamamatsu). Analyses were made using the Visiopharm NewCast computer-assisted stereology system (VIS 4.5.3). Analyses included the determination of mean linear intercept (MLI), alveolar septal wall thickness, total alveolar septal volume and surface area, as well as alveolar number, as described previously (39). Approximately 60 tissue section were evaluated per animal for all parameters, except the determination of alveolar number, in which case ~30 sections per animal were evaluated. In each case, 2–3% of each section was analyzed. The coefficient of error (CE), the coefficient of variation (CV), and the squared ratio between both (CE2/CV2) were measured for each stereological parameter, and the quotient threshold was set at <0.5 to validate the precision of the measurements.

**Statistical analysis.** All statistical data presented in graphs are indicated as means ± SD. Data presented in Tables 2–5 are indicated as means ± SE. Differences between groups were compared by determining P values by one-way ANOVA with Tukey’s post hoc test for all experiments, except for a unpaired Student’s t-test analysis used for the RT-PCR analysis (Fig. 1). The P values < 0.05 were regarded as significant. All statistical analyses were performed with GraphPad Prism 6.0. Statistical outliers were identified by Grubb’s test.

**RESULTS**

Exposure of immature mouse lungs to 85% O2 drives aberrant lysyl oxidase expression. Previous studies on lysyl oxidase expression in the developing mouse lung using semiquantitative RT-PCR have suggested changes in lysyl oxidase expression over the course of postnatal lung maturation in the mouse, Fig. 1. Lysyl oxidase expression is deregulated during aberrant late lung development. Lysyl oxidase mRNA levels were assessed in lung homogenates from mouse pups exposed to 21% O2 (●) or 85% O2 (○) from the day of birth, at postnatal day (P) 9.5 (A) and P19.5 (B), by real-time RT-PCR. Values are means ± SD (n = 3–5 per group). The P values were determined by unpaired Student’s t-test. C: protein expression levels for all five lysyl oxidases were assessed in lung homogenates from mouse pups exposed to 21% O2 or 85% O2 from the day of birth, by immunoblot at P9.5 (left side of gel) and P19.5 (right side of gel). For lysyl oxidases and the upper β-actin sample, 50 µg of protein were loaded, which yielded overloaded β-actin bands. Hence, a second loading control, in which 10 µg of protein were loaded, is also provided for β-actin. Additionally, the Ponceau S-stained immunoblot membrane is included for the gel run for the 50-µg β-actin immunoblot, to confirm loading equivalence. Three mice per experimental group were evaluated. D: densitometric analysis of the data presented in C for mice at P19.5, where the pixel densities for lysyl oxidase bands were normalized for the pixel density of the β-actin bands from the same sample. Values are means ± SD. The P values were determined by unpaired Student’s t-test.
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Fig. 2. Administration of β-aminopropionitrile (BAPN) impacted mouse pup viability. A pilot study was conducted where solvent vehicle alone was applied daily (intraperitoneal), or BAPN was applied (intraperitoneal) at dose of 15 or 150 mg·kg\(^{-1}\)·day\(^{-1}\) (three animals per group, observed for 5 days). A: body mass measured at day 5 for the three groups. Values are means ± SD (n = 3 per group). The P values were determined by one-way ANOVA with Tukey’s post hoc test. B: images of representative mice from each of the three groups. The milk spot is indicated by an arrow, when present. C: the experimental protocol applied for subsequent studies. This figure is supported by Supplemental Video S1, in the online supplement.

during both normal lung development, as well as arrested late lung development associated with postnatal exposure to hyperoxia (33). Exposure of mouse pups to 85% O\(_2\) from birth resulted in persistently upregulated expression of *lox* at both P9.5, by 1.8-fold (Fig. 1A), and P19.5, by 5.0-fold (Fig. 1B). Additionally, an upregulation in mRNA expression of *lox2* in response to hyperoxia was also noted at P9.5 (2.1-fold; Fig. 1A) and P19.5 (6.6-fold; Fig. 1B) While the changes on Lox2 expression were not evident by immunoblot, the elevated expression of Lox was evident by immunoblot at P9.5 and P19.5 (Fig. 1C). Additionally, elevated protein levels of LoxL1 and LoxL4 were evident in the lungs of mouse pups exposed to 85% O\(_2\) from the day of birth, at P19.5 (Fig. 1C).

Administration of a lysyl oxidase inhibitor can normalize lysyl oxidase activity in the lungs of mice exposed to 85% O\(_2\). Administration of the broad-spectrum lysyl oxidase inhibitor BAPN at a dose of 150 mg·kg\(^{-1}\)·day\(^{-1}\) was toxic to developing mouse pups and results in impaired development evident from reduced body mass of 1.7 ± 0.1 g at P5.5 (Fig. 2A), reduced milk intake (note missing milk spot in the mouse pup documented in Fig. 2B), and reduced mobility (as evident in Supplemental Video S1; the online version of this article contains supplemental data). In contrast, mice receiving a dose one-log lower, at 15 mg·kg\(^{-1}\)·day\(^{-1}\), exhibited normal body mass (Fig. 2A), normal milk intake (Fig. 2B), and normal mobility (see Supplemental Video S1 in the online supplement). For this reason, BAPN was applied at a dose of 15 mg·kg\(^{-1}\)·day\(^{-1}\) for further studies, with mouse lungs harvested at P9.5 and P19.5 for analysis (Fig. 2C). The dose of 15 mg·kg\(^{-1}\)·day\(^{-1}\) was sufficient to normalize lysyl oxidase activity in developing lungs from mice exposed to 85% O\(_2\), as documented in Fig. 3.

Both exposure to hyperoxia and treatment with BAPN impacted collagen cross-linking in developing mouse lungs. Exposure of developing mouse lungs to 85% O\(_2\) from the day of birth did not appreciably impact the noncollagen, nonelastin protein levels in the lungs (Table 2). However, exposure of developing mouse lungs to 85% O\(_2\) from the day of birth increased collagen protein levels in the developing lungs. Increased collagen abundance was evident when collagen was assessed as total collagen per lung (63% increase; Fig. 4A) and collagen content per total protein (42% increase; Fig. 4B). Additionally, exposure of developing mouse lungs to 85% O\(_2\) from the day of birth increased collagen cross-linking, as assessed by the increased abundance of the (DHLNL) collagen cross-link (by 11%; Fig. 4C), although levels of the HP (Fig. 4D) and HLNL (Fig. 4E) cross-links were not impacted. However, the DHLNL-to-HLNL ratio (DHLNL/HLNL), which reflects the lysyl hydroxylation status in collagen cross-links (54) and is an index of the fibrotic status of tissue (63), was also increased (by 18%; Fig. 4F). Administration of BAPN to normally developing (exposed to 21% O\(_2\)) mice had no impact on collagen levels in the lung (Fig. 4, A and B); however, it did lead to decreased (by 31%; Fig. 4D) and increased (by 15%; Fig. 4E) HP and HLNL levels, respectively, in normally developing lungs. When administered concomitantly with hyperoxia exposure, BAPN reduced collagen abundance when collagen was assessed as total collagen per lung (24% decrease; Fig. 4A) and collagen content per total protein (12% decrease; Fig. 4B). Furthermore, BAPN reversed the impact of 85% O\(_2\) exposure on the DHLNL/HLNL, which...
was normalized (was not significantly different from the 21% 
O₂-exposed, vehicle-treated mouse pups; Fig. 4
F). A particu-
larly striking effect of the exposure to 85% O₂ was a pro-
nounced shift in the collagen-to-elastin ratio, which was three-
fold increased by 85% O₂ exposure (Table 2).

Both exposure to hyperoxia and treatment with BAPN im-
pacted elastin cross-linking in developing mouse lungs. In 
stark contrast to the impact of inspired oxygen levels on 
collagen abundance in developing lungs, exposure of develop-
ning mouse lungs to 85% O₂ from the day of birth decreased 
insoluble elastin protein levels in the developing lungs [35% 
decrease assessed as total insoluble elastin percentage of total 
protein (Fig. 5A), or 38% decrease assessed as total insoluble 
elastin per lung (Table 3)]. Additionally, exposure of develop-
ning mouse lungs to 85% O₂ from the day of birth reduced the 
ratio of the desmosine to the isodesmosine elastin crosslinks

<table>
<thead>
<tr>
<th>Parameter</th>
<th>21% O₂ (Mean ± SE)</th>
<th>85% O₂ (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC-NE protein/lung, µg</td>
<td>2,387 ± 128.50</td>
<td>2,784 ± 140.30</td>
</tr>
<tr>
<td>Collagen/elastin, %</td>
<td>66.29 ± 5.19</td>
<td>180.40 ± 12.18</td>
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</table>

Values are means ± SE; n = 6–7 lungs per group. Additional cross-link data are present in Fig. 4. BAPN, β-aminopropionitrile; NC-NE protein, 
noncollagen-nonelastin protein. P values were determined by one-way ANOVA with Tukey’s post hoc analysis.

Fig. 4. Levels of total collagen and of colla-
gen cross-links in the lungs of developing 
mouse pups were altered by hyperoxia ex-
posure and BAPN administration. The abun-
dance of collagen per lung (A), collagen per 
unit total protein (B), as well as the collagen 
cross-links dihydroxylysinonorleucine (DHLNL; 
C), hydroxylysylpyridinoline (HP; D), and hy-
droxylysinonorleucine (HLNL; E), were as-
essed in the lungs from solvent vehicle-
treated and BAPN-treated mouse pups con-
comitantly exposed either to 21% O₂ or 85% 
O₂, at P19.5. F: additionally, the DHLNL-
to-HLNL ratio is presented. Values are 
means ± SD (n = 6–7 per group). The P 
values were determined by one-way ANOVA 
with a Tukey’s post hoc test. n.s., P > 0.05.
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Fig. 5. Levels of total insoluble elastin and of cross-links in insoluble elastin in the lungs of developing mouse pups were altered by hyperoxia exposure and BAPN administration. The abundance of insoluble elastin per unit total protein (A), as well as the ratio of the elastin cross-links desmosine/isodesmosine (B), and the levels of desmosine (C), and isodesmosine (D) in insoluble elastin, were assessed in the lungs from solvent vehicle-treated and BAPN-treated mouse pups concomitantly exposed either to 21% O2 or 85% O2, at P19.5. Values are means ± SD (n = 6–7 per group). The P values were determined by one-way ANOVA with Tukey’s post hoc test.

Exposure of mouse pups to 85% O2 from the day of birth resulted in an apparent arrest of alveolarization, evident by fewer and larger air spaces, at P9.5 (Fig. 6B). Exposure of developing mouse lungs to 85% O2 from the day of birth reduced the apparent abundance of focal structures in the parenchyma of developing lungs at P9.5, where elastin fibers appeared more disorganized, by visual inspection. The administration of BAPN to normally developing (exposed to 21% O2) mouse pups did not have any apparent impact on the elastin structures in the lung (Fig. 6C) at P9.5, compared with mouse pups that received solvent vehicle alone (Fig. 6A) at P9.5, by visual inspection. Lungs harvested at P19.5 revealed punctate elastin foci in the septa in the lungs of mice that were exposed to 21% O2 from the day of birth, irrespective of whether solvent vehicle alone (Fig. 6E) or BAPN (Fig. 6G) was administered. Exposure of mouse pups to 85% O2 from the day of birth resulted in a pronounced arrest of alveolarization, evident by fewer and larger air spaces, and also thickened septa at P19.5. These parameters are quantified in the next section. By visual inspection, the elastin fibers in the septa were largely devoid of punctate foci and were disorganized and “feathery” in appearance (Fig. 6F). Treatment of mouse pups from the day of birth with BAPN appeared to partially restore punctate

(by 11%; Fig. 5B). Furthermore, it decreased elastin crosslinking, as assessed by the decreased abundance of the desmosine elastin cross-link (by 30%; expressed as desmosine per unit insoluble elastin; Fig. 5C), and by the decreased abundance of the isodesmosine elastin cross-link (by 21%; expressed as isodesmosine per unit insoluble elastin; Fig. 5D). Administration of BAPN to normally developing (exposed to 21% O2) mouse pups also reduced total insoluble elastin levels in the lung (by 10% Fig. 5A). Furthermore, BAPN administration to normally developing lungs increased the ratio of the desmosine to the isodesmosine elastin cross-links (by 9%; Fig. 5B) and increased the abundance of desmosine (Fig. 5C) and isodesmosine (Fig. 5D) cross-links in insoluble elastin by 14 and 5%, respectively. When administered concomitantly with hyperoxia exposure, BAPN administration did not impact total elastin levels (Fig. 5A); however, BAPN administration did partially restore the desmosine-to-isodesmosine ratio (Fig. 5B) and partially restored desmosine (Fig. 5C) and isodesmosine (Fig. 5D) abundance in insoluble elastin.

Both exposure to hyperoxia and treatment with BAPN impacted elastin deposition in the parenchyma of developing mouse lungs. Elastin presents as focal structures (“dots”) in the tips of developing septa in the immature lung (Fig. 6A).

Table 3. Effect of hyperoxia with or without BAPN administration on formation of elastin cross-links in developing mouse lung at postnatal day 19.5

<table>
<thead>
<tr>
<th>Parameter</th>
<th>21% O2 Vehicle</th>
<th>21% O2 BAPN</th>
<th>85% O2 Vehicle</th>
<th>85% O2 BAPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elastin/lung, µg</td>
<td>582.7 ± 16.00</td>
<td>569.1 ± 10.24</td>
<td>359.7 ± 14.75</td>
<td>350.9 ± 23.14</td>
</tr>
<tr>
<td>(Des + Isodes)/elastin, pmol/µg</td>
<td>14.14 ± 0.364</td>
<td>15.45 ± 0.317</td>
<td>10.55 ± 0.383</td>
<td>12.28 ± 0.276</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6–7 lungs per group. Additional cross-link data are present in Fig. 5. Des, desmosine; Isodes, isodesmosine. P values were determined by one-way ANOVA with Tukey’s post hoc analysis.
elastin foci to the septa (Fig. 6H), although septa remained clearly thickened and hypercellular, compared with septa in the 21% O2-treated group.

**Treatment of hyperoxia-exposed mouse pups with BAPN did not restore normal lung architecture.** The changes in lung architecture seen by visual inspection of lung sections alluded to in the preceding paragraph were quantified by a designed-based stereomorphometric approach. The full spectrum of stereomorphometric data is provided for lungs from mice at P9.5 in Table 4, and for lungs from mice at P19.5 in Table 5. Key elements of the stereomorphometric analyses are provided for lungs from mice at P9.5 in Fig. 7, and for lungs from mice at P19.5 in Fig. 8. In plastic-embedded lungs that were processed for stereomorphometric analysis by treatment with uranyl acetate and osmium tetroxide, visual inspection suggested that BAPN treatment generated a more complex lung than did solvent vehicle-treated lungs in pups exposed to 21% O2 at P9.5 (Fig. 7, compare A and C). Exposure of mouse pups to 85% O2 generated less complex lungs, with fewer, larger air spaces, evident by visual inspection (Fig. 7, compare A and B). Treatment of 85% O2-exposed mouse pups with BAPN did not visibly impact lung complexity compared with lungs from solvent vehicle-treated mouse pups exposed to 85% O2 (Fig. 7, compare B and D). Indeed, a stereological analysis revealed
that treatment of mouse pups from the day of birth with BAPN under 21% O₂ conditions did not appreciably alter lung structure, with the alveolar surface area [91.98 ± 27.77 cm² (solvent vehicle) vs. 101.40 ± 16.43 cm² (BAPN); Fig. 7E], septal thickness [11.02 ± 1.79 μm (solvent vehicle) vs. 12.06 ± 1.28 μm (BAPN); Fig. 7F], MLI [39.27 ± 7.51 μm (solvent vehicle) vs. 38.77 ± 2.70 μm (BAPN); Fig. 7G], and number of alveoli [1.75 ± 0.45 × 10⁶ (solvent vehicle) vs. 1.96 ± 0.5402 × 10⁶ (BAPN); Fig. 7H] unchanged at P9.5. Exposure of mouse pups from birth to 85% O₂ did dramatically impact alveolar surface area [91.98 ± 27.77 cm² (21% O₂) vs. 57.89 ± 7.26 cm² (85% O₂); Fig. 7E], MLI [39.27 ± 7.51 μm (21% O₂) vs. 65.39 ± 6.547 μm (85% O₂); Fig. 7G], and number of alveoli [1.746 ± 0.4495 × 10⁶ (21% O₂) vs. 1.11 ± 0.27 × 10⁶ (85% O₂); Fig. 7H] at P9.5. Treatment of 85% O₂-exposed mouse pups with BAPN did not appreciably alter any of these three parameters compared with solvent vehicle-treated pups exposed to 85% O₂ (Fig. 7, E, G, and H). There appeared to be a trend toward increased alveolar surface area (Fig. 7E), decreased septal thickness (Fig. 7F) and MLI (Fig. 7G), and increased alveoli number (Fig. 7H) when comparing the mean values of the solvent vehicle- vs. BAPN-treated groups within the hyperoxia-exposed arm of the study; however, none of these changes was statistically significant. For this reason, it was speculated that a longer exposure time may highlight any corrective effects of BAPN administration to hyperoxia-exposed mouse pups. Therefore, the study was repeated for double the duration, and mouse lungs were harvested for stereomorphometric analysis at P19.5 (Fig. 8).

Visual inspection suggested that BAPN treatment did not generate a more complex lung than did solvent vehicle-treated lungs in pups exposed to 21% O₂ at P19.5 (Fig. 8, compare A and C). Exposure of mouse pups to 85% O₂ generated less complex lungs, with fewer, larger air spaces and thickened septa evident by visual inspection (Fig. 8, compare A and B). Treatment of 85% O₂-exposed mouse pups with BAPN did not visibly impact lung complexity compared with lungs from solvent vehicle-treated mouse pups exposed to 85% O₂ (Fig. 8, compared B and D). Furthermore, by visual inspection, within the 85% O₂-exposed group, BAPN treatment appeared to drive septal thickening (compare BAPN- and solvent vehicle-treated groups in Fig. 8E). Stereological analysis revealed that treatment of mouse pups from the day of birth with BAPN under 21% O₂ conditions did not appreciably impact alveolar surface

Table 4. Structural parameters of developing mouse lungs exposed to hyperoxia with or without BAPN administration at postnatal day 9.5 as assessed by stereological analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>21% O₂</th>
<th>85% O₂</th>
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<tbody>
<tr>
<td>V (lung), cm³</td>
<td>Vehicle (Mean ± SE)</td>
<td>BAPN (Mean ± SE)</td>
</tr>
<tr>
<td>V (lung), cm³</td>
<td>0.16 ± 0.011</td>
<td>0.15 ± 0.006</td>
</tr>
<tr>
<td>CV V (lung)</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>Vₚ (par/lung), %</td>
<td>88.81 ± 2.06</td>
<td>88.19 ± 1.84</td>
</tr>
<tr>
<td>Vₚ (nonpar/lung), %</td>
<td>11.19 ± 2.06</td>
<td>11.81 ± 1.84</td>
</tr>
<tr>
<td>Nₖ (alv/par) 10⁶, cm⁻¹</td>
<td>1.25 ± 0.07</td>
<td>0.84 ± 0.06</td>
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<tr>
<td>Sᵥ, cm⁻¹</td>
<td>651.46 ± 22.8</td>
<td>438.51 ± 22.05</td>
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<td>Vₚ (alv air/par), %</td>
<td>62.99 ± 2.67</td>
<td>71.41 ± 1.60</td>
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<tr>
<td>V (alv air), cm³</td>
<td>0.087 ± 0.006</td>
<td>0.094 ± 0.005</td>
</tr>
<tr>
<td>CV V (alv air, lung)</td>
<td>0.22</td>
<td>0.17</td>
</tr>
<tr>
<td>V (sep), cm²</td>
<td>0.051 ± 0.007</td>
<td>0.013 ± 0.001</td>
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</tbody>
</table>

Values are means ± SE; n = 7–8 lungs per group. P values were determined by one-way ANOVA with Tukey’s post hoc analysis.

Table 5. Structural parameters of developing mouse lungs exposed to hyperoxia with or without BAPN administration at postnatal day 19.5 as assessed by stereological analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>21% O₂</th>
<th>85% O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>V (lung), cm³</td>
<td>Vehicle (Mean ± SE)</td>
<td>BAPN (Mean ± SE)</td>
</tr>
<tr>
<td>V (lung), cm³</td>
<td>0.26 ± 0.007</td>
<td>0.27 ± 0.014</td>
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<tr>
<td>CV V (lung)</td>
<td>0.07</td>
<td>0.12</td>
</tr>
<tr>
<td>Vₚ (par/lung), %</td>
<td>90.30 ± 0.969</td>
<td>91.24 ± 0.922</td>
</tr>
<tr>
<td>Vₚ (nonpar/lung), %</td>
<td>97.09 ± 0.969</td>
<td>87.86 ± 0.922</td>
</tr>
<tr>
<td>Nₖ (alv/par) 10⁶, cm⁻¹</td>
<td>3.03 ± 0.21</td>
<td>0.98 ± 0.10</td>
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<tr>
<td>Sᵥ, cm⁻¹</td>
<td>877.20 ± 13.17</td>
<td>476.40 ± 12.09</td>
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<tr>
<td>Vₚ (alv air/par), %</td>
<td>61.50 ± 0.55</td>
<td>68.52 ± 1.28</td>
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<tr>
<td>V (alv air), cm³</td>
<td>0.143 ± 0.004</td>
<td>0.167 ± 0.013</td>
</tr>
<tr>
<td>CV V (alv air, lung)</td>
<td>0.06</td>
<td>0.017</td>
</tr>
<tr>
<td>V (sep), cm²</td>
<td>0.090 ± 0.002</td>
<td>0.076 ± 0.003</td>
</tr>
<tr>
<td>CV V (sep, lung)</td>
<td>0.05</td>
<td>0.09</td>
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</table>

Values are means ± SE; n = 5 lungs per group. P values were determined by one-way ANOVA with Tukey’s post hoc analysis.

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area [203.80 ± 11.71 cm² (solvent vehicle) vs. 215.4 ± 13.31 cm² (BAPN); Fig. 8F], septal thickness [8.80 ± 0.52 μm (solvent vehicle) vs. 9.082 ± 0.90 μm (BAPN); Fig. 8G], or MLI [28.06 ± 0.69 μm (solvent vehicle) vs. 29.25 ± 1.98 μm (BAPN); Fig. 8H] at P19.5. However, BAPN treatment of 21% O₂-exposed mouse pups did yield a moderate increase in the total number of alveoli [7.05 ± 1.10 × 10⁶ (solvent vehicle) vs. 8.76 ± 0.33 × 10⁶ (BAPN); Fig. 8I]. Exposure of mouse pups from birth to 85% O₂ did dramatically impact alveolar surface area [203.80 ± 11.71 cm² (21% O₂) vs. 115.00 ± 11.36 cm² (85% O₂); Fig. 8F], septal thickness [8.80 ± 0.52 μm (21% O₂) vs. 13.20 ± 0.96 μm (85% O₂); Fig. 8G], MLI [28.06 ± 0.69 μm (21% O₂) vs. 57.75 ± 5.22 μm (85% O₂); Fig. 8H], and number of alveoli [7.05 ± 1.10 × 10⁶ (21% O₂) vs. 2.33 ± 0.43 × 10⁶ (85% O₂); Fig. 8I] at P19.5. Treatment of 85% O₂-exposed mouse pups with BAPN did not appreciably alter any of these parameters compared with solvent vehicle-treated pups exposed to 85% O₂, with the single exception of septal thickness, which was increased by BAPN treatment [13.20 ± 0.96 μm (solvent vehicle) vs. 15.18 ± 0.43 μm (BAPN); Fig. 8G], in line with the visibly increased septal thickness evident in Fig. 8E.

DISCUSSION

The aberrant late lung development associated with BPD is histopathologically characterized by a pronounced arrest of alveolarization, as a consequence of blunted secondary septation, leading to alveolar simplification, which is evident by a lower number of alveoli that are larger in size, in affected lungs
In addition to alveolar simplification, impacted lungs also exhibit pronounced perturbations to the ECM architecture, in particular, to the structure of collagen and elastin fibers in the parenchyma (59, 60). These changes, which include thickened and “twisted” parenchymal collagen fibers, as well as improperly deposited and remodeled elastin fibers, are observed in the lungs of human patients at risk for, or who have died with, BPD (11), as well as in the lungs of mice, rats, and other animal models. Figure 8 illustrates the effects of concomitant hyperoxia exposure and BAPN administration on lung architecture in developing mouse pups. Representative sections of plastic-embedded mouse lungs from solvent vehicle-treated (A and B) and BAPN-treated (C and D) mouse pups concomitantly exposed either to 21% O₂ (A and C) or 85% O₂ (B and D) at P19.5 are illustrated. E: higher magnification images for the solvent vehicle-treated and BAPN-treated groups exposed to 85% O₂ are also provided. Using a stereo-morphometric analysis, the alveolar surface area (F), septal thickness (G), mean linear intercept (H), and total number of alveoli in the lung (I) were determined. Values are means ± SD (n = 5 per group). The P values were determined by one-way ANOVA with Tukey’s post hoc test. Additional stereo-morphometric parameters are provided in Table 5.
baboons, and lambs, in which an arrest of alveolarization has been induced by exposure to elevated inspired oxygen concentrations, injurious mechanical ventilation, or combinations thereof (6, 13, 49, 66). Given the pivotal roles attributed to the ECM in directing both normal and aberrant late lung development, it has been speculated that these perturbations to ECM architecture may underlie the blunted secondary septation in affected lungs. However, to date, little is known about the molecular mechanisms that drive pathological changes to ECM architecture in impacted lungs: for example, what underlies the changes in abundance of ECM proteins, and why are collagen and elastin fibers malformed?

Recently, several studies have highlighted pathological changes in the expression and activity of several enzyme families in aberrantly developing lungs both in clinical BPD and in animal models of BPD. Among these enzyme families are the lysyl oxidases, lysyl hydroxylases, and transglutaminases, which drive the covalent intra- and intermolecular cross-linking of collagen and elastin fibers, and, as such, modulate the posttranslational maturation of the ECM architecture of the lungs. Although increased pulmonary expression and activity of ECM cross-linking enzymes have been noted in clinical and experimental BPD, to date, 1) no causal relationship has been established between elevated expression of ECM cross-linking enzymes and blunted alveolarization, and 2) no study has demonstrated that ECM cross-linking is perturbed when alveolarization is blunted. Hence, in this study, selecting the lysyl oxidase family of ECM cross-linking enzymes, it was hypothesized that increased lysyl oxidase expression contributed to disturbed alveolarization in a hyperoxia-based mouse model of BPD, and the disturbed ECM cross-linking was associated with perturbed alveolarization in this model. To test this idea, the abundance of lysyl oxidase-generated cross-links in elastin and collagen was assessed in the lungs of mice in which alveolarization was arrested by exposure of developing mouse pups to elevated inspired oxygen concentrations, and lysyl oxidase activity was inhibited using broad-spectrum small-molecule inhibitor, to assess a causal role for lysyl oxidases in aberrant alveolarization that is characteristic of this animal model of BPD.

The lysyl oxidase family of copper-dependent amine oxidases comprises five members, named Lox and LoxL1 to LoxL4. The expression of lysyl oxidases is elevated in the lung in clinical BPD (33), in clinical and experimental pulmonary arterial hypertension (48), and in clinical and experimental lung fibrosis (16). In a mouse model of ventilator-induced disturbances to alveolarization in mice, changes in lysyl oxidase expression have also been noted (7, 9, 23). In the present study, in a hyperoxia-based mouse model of BPD, the abundance of multiple lysyl oxidases was elevated in the lungs of mice exposed to elevated inspired oxygen levels. Given that lysyl oxidase expression was hyperoxia responsive, lysyl oxidase activity was neutralized in vivo, in a well-characterized mouse model of BPD, using the pan-lysyl oxidase inhibitor BAPN. Daily administration of BAPN at a dose of 150 mg·kg⁻¹·day⁻¹ was well tolerated, without any observable deleterious effects, and BAPN administration partially protected mice from hypoxia-driven increases in right ventricular systolic pressure. Additionally, BAPN administration helped preserve normal lung vascular matrix architecture, which is usually perturbed when pulmonary vessels are remodeled in response to hypoxia exposure (48). Thus these data underscore the importance of, and dangers of interfering with, ECM remodeling during organogenesis and development (37). A lower BAPN dose, reduced by one log, to 15 mg·kg⁻¹·day⁻¹, was well tolerated by developing mouse pups, with no apparent deleterious effects on mouse viability. This BAPN dose was also sufficient to normalize lysyl oxidase activity in lung homogenates.

Mouse pups exposed to 85% O₂ from the day of birth demonstrated a pronounced increase in the levels of total insoluble collagen in the lung at P19.5. The increase in collagen levels may reflect either increased collagen transcription or decreased collagen turnover, in response to hyperoxia. Along these lines, it is interesting to note that lysyl oxidases, which may act as scaffolding proteins in transcription factor complexes (26, 55), have been reported to drive transcription of the col3a1 gene, encoding the collagen III α1-subunit (18). As such, the elevated expression of lysyl oxidases may drive the elevated expression of collagens. However, other candidates also exist, such as transforming growth factor-β, since transforming growth factor-β signaling is both increased by exposure to hyperoxia and also drives collagen expression (3). Interestingly, concomitant treatment of hyperoxia-exposed mouse pups with BAPN dampened the ability of hyperoxia to drive increased collagen abundance in affected lungs. It may be speculated that 1) the BAPN inhibited the protranscriptional activity of lysyl oxidases on, for example, the col3a1 promoter; or 2) that decreased collagen cross-linking due to lysyl oxidase inhibition may have generated weakly cross-linked collagen peptides that were thus more amenable to proteolytic degradation, leading to less total collagen in the lungs of BAPN + hyperoxia-treated mice. At the same time, in contrast to the impact on collagen, exposure of newborn mouse pups to 85% O₂ depressed insoluble elastin levels in the lung. The collagen-to-elastin ratio of affected lungs was threefold increased, which would likely lead to a marked stiffening of the lung parenchyma. Given the importance of breathing motions and physical forces in driving alveolar development (41), the decreased lung compliance resulting from a shift in the collagen-to-elastin ratio almost certainly directly impacts the ability of the immature lung to develop properly (23).

Concerning the ECM cross-link status of mouse lungs following hyperoxia exposure, the abundance of the DHLNL collagen cross-link, as well as the DHLNL/HLNL, was increased in lungs from hyperoxia-exposed mice, suggestive of a profibrotic state (17, 63). The application of BAPN concomitantly with hyperoxia blunted this increase in lung collagen accumulation and partially normalized the DHLNL/HLNL. In summary, hyperoxia exposure drove alterations not only to
total insoluble collagen abundance in the lung, but also to collagen cross-linking in the lung, which could be partially reversed by BAPN administration, ostensibly by limiting the collagen cross-linking capacity of the increased lysyl oxidase activity observed in the lungs from hyperoxia-treated mice.

In contrast to collagen cross-links, hyperoxia exposure reduced the levels of the elastin cross-links desmosine and isodesmosine per unit lung elastin in the lung, and this trend was reversed with concomitant BAPN treatment. It appears counterintuitive that, in mice that were treated with BAPN, more desmosine and isodesmosine elastin cross-links were observed. Indeed, since these cross-links are generated by lysyl oxidase, one might expect to see less desmosine or isodesmosine in the lungs of BAPN-treated mice. It is not immediately apparent why this is the case, but several possible explanations may be offered. As mentioned above, the lysyl oxidases have attracted recent attention as scaffolding molecules in transcription factor complexes (18), where LOX, LOXL1, and LOXL2 may regulate gene transcription. It may be that among the spectrum of genes regulated by lysyl oxidases that are acting as transcription factors are genes encoding enzymes or chaperones that limit elastin cross-linking. Inhibition of lysyl oxidases with BAPN may thus be able to impact the formation of elastin cross-links separately from the direct cross-linking activity of lysyl oxidases, hence the paradoxical situation where inhibition of lysyl oxidases by BAPN under hyperoxic conditions actually increased the abundance of desmosine and isodesmosine in insoluble lung elastin. This idea is supported by the observation that, under normoxic conditions, BAPN administration also drive increased desmosine abundance (compare the vehicle and BAPN groups under 21% O2 in Fig. 5C). Clearly, a lysyl oxidase-independent role for BAPN in the positive regulation of elastin cross-linking has not been ruled out. Alternatively, the dramatically shifted ratio of collagen to elastin may play a role in regulating lysyl oxidase activity on elastin. Lysyl oxidases physically associate with collagen fibers and other ECM structures, and the vastly increased abundance of collagen may have sequestered lysyl oxidases, making these enzymes less available to elastin, hence the reduced abundance of elastin cross-links in the hyperoxia groups. The latter idea gains credence from the observation in Fig. 4A (vehicle vs. BAPN groups within the 85% O2 arm), where BAPN administration under hyperoxic conditions reduced total lung collagen, which was associated with increased elastin cross-linking (Fig. 5C and D; vehicle vs. BAPN groups within the 85% O2 arm), perhaps due to less lysyl oxidase sequestering by less total collagen. Irrespective, an examination of the form and abundance of elastin foci in the developing lungs suggested that BAPN treatment under hyperoxic conditions generated more elastic foci in developing septa (“dots” indicated by arrows in Figs. 6, H vs. F) than were apparently present in vehicle-treated lungs under hyperoxic conditions.

Since BAPN administration under hyperoxic conditions partially normalized total lung collagen, the abundance of selected collagen and elastin cross-links, and elastin foci distribution in the developing septa, it was postulated that BAPN administration may improve lung architecture in mice exposed to hyperoxia during the neonatal period. Indeed, in mice exposed to hyperoxia for 9 days that concomitantly received BAPN, the mean values for surface area, septal thickness, MLI, and total number of alveoli in the lung all trended in an “improved” direction (i.e., toward the vehicle/21% O2 values), from the 85% O2/vehicle values, although no difference achieved a P value <0.05. For this reason, the study was extended for an additional 10 days. However, an additional 10 days of BAPN administration concomitant with hyperoxia exposure did not appreciably improve lung architecture compared with vehicle-treated, hyperoxia-exposed lungs, where the mean values for surface area, MLI, and total number of alveoli in the lung were unchanged between the 85% O2/BAPN and 85% O2/vehicle groups, and the septal thickness was markedly increased in the 85% O2/BAPN group compared with the 85% O2/vehicle group. This is not the first report on the effects of BAPN administration on late lung development. Indeed, Kida and Thurlbeck (29, 30) described the impact of BAPN administration on normal late lung development in healthy rat pups in 1980, where very high doses of BAPN were deleterious and impeded normal alveolarization. This is consistent with reports that targeted deletion of lysyl oxidases impacts the development of the cardiopulmonary system (24, 37, 40).

In interpreting these data, it is clearly important to remember that there is no good animal model of clinical BPD that recapitulates all pathological aspects of clinical BPD. The hyperoxia model largely recapitulates the blunted secondary septation seen in BPD. However, hyperoxia is a different injurious stimulus than is, for example, the inflammation seen in chorioamnionitis (32). Indeed, the role of antenatal inflammation (34) and the interaction of steroid signaling and inflammatory pathways (15, 35) have been demonstrated to modulate key pathogenic signaling pathways in a sheep model of BPD. This represents a key limitation of the hyperoxia model, and how lysyl oxidases impact ECM remodeling in other animal models of BPD has not been addressed in this study. Our study has also not addressed how vitamin A or steroids may alter ECM maturation, which is most likely a relevant line of investigation, given that both vitamin A (1) and steroids (53) are reported to modulate the formation of ECM structures in BPD animal models.

Thus, in summary, the exposure of immature mouse lungs to normobaric hyperoxia perturbed lung architecture increased the abundance of collagen and collagen cross-links and decreased the abundance of elastin and elastin cross-links. These perturbations to ECM cross-linking may underlie some of the aberrant ECM structures observed in affected lungs. These changes were also accompanied by increased expression of lysyl oxidases in the lung. However, antagonizing lysyl oxidase activity with the pan-lysyl oxidase inhibitor BAPN did not lead to any improvement in lung architecture, despite partially normalizing total collagen abundance, the abundance of some collagen and elastin cross-links, and elastin foci distribution in developing septa. These data highlight a possible role for perturbed ECM structures as an underlying cause of blunted alveolarization in response to hyperoxia exposure; however, these data do not support the antagonism of lysyl oxidases as an avenue to drive the normalization of alveolar development in oxygen-injured developing lungs.

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AUTHOR CONTRIBUTIONS


DISCLOSURES


