Hyperoxia modulates TGF-β/BMP signaling in a mouse model of bronchopulmonary dysplasia

Miguel A. Alejandre-Alcazar,1 Grazyna Kwapiszewska,2 Irwin Reiss,3 Oana V. Amarie,1 Leigh M. Marsh,1 Julia Sevilla-Pérez,1 Malgorzata Wygrecka,4 Bastian Eul,1 Silke Köbrich,1 Mareike Hesse,1 Ralph T. Schermuly,1 Werner Seeger,1 Oliver Eickelberg,1 and Rory E. Morty1

Departments of 1Internal Medicine, 2Pathology, 3Paediatrics, and 4Biochemistry,
University of Giessen Lung Center, Justus Liebig University, Giessen, Germany

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Abstract

Bronchopulmonary dysplasia (BPD) was first described by Northway et al. (35) as a disease of preterm infants that received prolonged mechanical ventilation and oxygen supplementation for acute respiratory distress. The impact of oxygen toxicity, barotrauma, volutrauma, and infection on the developing lung resulted in “classic BPD,” histopathologically characterized by airway epithelial metaplasia, peribronchial fibrosis, and vascular smooth muscle hypertrophy (36). Advances in surfactant and steroid therapy and ventilation strategies have changed the pathological picture from “classic BPD” to “new BPD,” also called chronic lung disease of early infancy (CLD), which is characterized more by alveolar and capillary hypoplasia and less by fibroproliferative airway damage and parenchymal fibrosis (2). Impaired alveolus formation is observed in both forms of BPD and leads to a long-term reduction in total number of alveoli, and hence reduces the surface area available for gas exchange (3, 18, 19).

Whereas the pathogenesis of new BPD is largely unknown (3), hyperoxic injury is thought to disrupt critical signaling pathways that direct lung development, including branching and septation (46, 56). Many signaling pathways critical to these processes have been described (41, 46), notable among them, signaling by the transforming growth factor (TGF)-β superfamily, encompassing the TGF-β and bone morphogenetic protein (BMP) families (17, 41, 53–55).

TGF-β signaling is initiated by binding of TGF-β to the type II TGF-β receptor (TβRII), which subsequently complexes with the type I receptor (ALK-1 or ALK-5). The type I receptor transmits signals within the cell via second-messenger Smad proteins, namely Smads 1–4, or by Smad-independent pathways (27). TGF-β signaling can negatively regulate the branching (17, 41, 54) and septation (15, 52) phases of lung development. In the case of the latter, adenoviral-mediated transfer of TGF-β1 to the neonatal rat lung (15) or overexpression of TGF-β1 between postnatal days P7 and P14 in the mouse (52) both induced histological changes analogous to those seen in BPD. Surprisingly, however, Smad3 knockout mice exhibited retarded alveolarization between days P7 and P28 (6), suggesting that TGF-β also acts as a positive regulator of septation. This apparent paradox indicates that TGF-β signaling plays a critical and finely tuned role in alveolarization. Consistent with these data, Smad3 deficiency in adult mice caused air space enlargement and centrilobular emphysema in late life (4, 6, 33), suggesting a key role for TGF-β signaling in both the formation of alveoli and the maintenance of alveolar structure.

In contrast to TGF-β, BMP ligands bind their type I receptors ALK-3 (BMPRIa) or ALK-6 (BMPRIb), which activate the type II receptor (BMPRII), thereby initiating a signaling cascade primarily via Smads 1 and 4 (31). BMPs have been accredited with key roles in early lung development, particularly lung branching (9, 17, 53, 54). However, BMP-4 expres-

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sion declines prior to the saccular stage, suggesting that BMP-4 is not required for septation (41).

Signaling by the TGF-β/BMP superfamily clearly plays a key role in lung development. It has also been reported that chronic hypoxia can influence TGF-β ligand and receptor expression (51), and that very low birth weight infants receiving oxygen therapy exhibit elevated TGF-β levels in endotracheal aspirates (24). Thus we suspected that oxygen supplementation may influence TGF-β/BMP signaling in the neonatal lung.

Such perturbations could underlie the arrested alveolar development associated with BPD. Indeed, the identification of differential gene expression in lungs that develop BPD has been identified as a “research priority” in BPD by a recent National Institute of Child Health and Human Development/ National Heart, Lung, and Blood Institute and Office of Rare Diseases joint workshop (18). Therefore, the object of this study was to investigate the effects of hyperoxia on TGF-β/BMP superfamily signaling in the lung.

MATERIALS AND METHODS

Chronic exposure to hyperoxia. The government of the State of Hessen approved all animal procedures [Regierungspärsidium Giessen II25.3–19c20 –15(1) GI20/10-Nr.22/2000]. Adult and neonatal C57BL/6J mice were housed in humidity- and temperature-controlled rooms on a 12:12-h light-dark cycle and were allowed food and water ad libitum. On day P1, pups from four to eight litters (born within 3 h of each other) were pooled and randomized to newly delivered dams. Pups from one half of the combined litters were maintained in 85% (vol/vol) O2, whereas pups from the other half remained in room air [21% (vol/vol) O2]. Nursing dams were rotated every 3 days between hermetically sealed chambers, continuously ventilated at a rate of 3.5 l/min. Oxygen levels were monitored with a Miniox II monitor (Catalyst Research, Owings Mills, MD). Dynamic compliance was assessed by the volume-pressure compliance method in anesthetized mice (26), where mice were mechanically ventilated with a tidal volume of 6 ml/kg.

Lung processing and morphometric analysis. Mice were killed by intraperitoneal injection of sodium pentobarbital and were exsanguinated by aortic transection. The heart and lungs were excised en bloc, and the lungs were pressure-fixed overnight at 20 cmH2O with 4% (mass/vol) paraformaldehyde in phosphate-buffered saline (PBS; 20 mM Tris-HCl, 137 mM NaCl, pH 7.6), as described previously (51). Paraffin sections (3 μm) were mounted on poly-l-lysine-coated glass slides, dewaxed with xylene (3 × 5 min), and rehydrated in a graduated series of ethanol solutions [100% (vol/vol), 95% (vol/vol), 70% (vol/vol), and finally PBS]. The mean linear intercept (MLI) and septal thickness were determined on sections stained for smooth muscle actin and counter-stained with hematoxylin and eosin, as described previously (51, 52).

Primary cell isolation and cell culture. Primary mouse alveolar type II (ATII) cells were isolated and passaged exactly as described previously (49). Primary human lung fibroblasts and primary human pulmonary artery smooth muscle cells (PASMC) were isolated from human donor lungs that had been rejected for transplantation, exactly as we have described previously (14, 37). The fibroblast-derived cell line NIH/3T3 and the mouse distal alveolar epithelial cell-derived MLE-12 cell line were maintained and passaged according to the recommendations of the American Type Culture Collection (ATCC, http://www.atcc.org). To investigate the effects of 85% O2 on the induction of apoptosis, proliferation, or synthesis of extracellular matrix components by TGF-β or BMP, cells were maintained under 21% O2 or 85% O2 for 24 h, prior to stimulation with TGF-β1 (2 ng/ml) or BMP-2, BMP-4, or BMP-7 (each at 100 ng/ml) for a further 48 h. Samples were analyzed at 24 and 48 h after application of TGF-β or BMP ligand.

Protein detection by immunoblot. Frozen, unfixed lung tissue was homogenized with a tissue grinder in lysis buffer [20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (vol/vol) Triton X-100] supplemented with Complete protease inhibitor cocktail (Merck Biosciences, Bad Soden, Germany). Lysates from cultured cells were similarly prepared using a cell scraper. Homogenates were clarified by centrifugation (10,000 g, 4°C, 10 min). Protein concentration was quantified with a Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Cell extracts (10 μg) resolved on a 10% reducing SDS-PAGE gel were transferred to a nitrocellulose membrane. Blots were probed with the following antibodies: rabbit anti-ALK-3 or anti-ALK-6 (1:2,000; R&D Systems, Wiesbaden, Germany); mouse anti-ALK-5 (1:1,500; Santa Cruz, San Francisco, CA); mouse anti-βTßRII (1:1,000; Santa Cruz); rabbit anti-Smad1; rabbit anti-phospho-Smad1 (Ser65/66/67); mouse anti-phospho-Smad2 (Ser465/467); and mouse anti-Smad3 (both at 1:1,000; Upstate, Charlottesville, VA); rabbit anti-Smad4 (1:1,000; Santa Cruz); and rabbit anti-Smad2/3 (Zymed, San Francisco, CA); whereas mouse α-tubulin (1:1,000; Santa Cruz) served as a loading control. Peroxidase-conjugated antimouse (1:1,000 to 1:2,000) and anti-rabbit (1:2,000 to 1:2,500) secondary antibodies were from R&D Systems. Densitometric analysis of protein bands was performed using a GS-800 model calibrated densitometer with Quantity One software (both from Bio-Rad Laboratories, Munich, Germany). Band intensities from samples were normalized for loading using the α-tubulin band from the same sample.

Expression ratios for immunoblot data reflect values normalized for tubulin: [band pixel density (PD) (85% O2)/tubulin PD (85% O2)]/[band PD (21% O2)/tubulin PD (21% O2)].

Total RNA isolation and semiquantitative and real-time RT-PCR. Total RNA was isolated from unfixed lung tissue or from cultured primary human lung fibroblasts using a Qiagen RNeasy kit (Qiagen, Hilden, Germany), followed by DNase treatment to remove any contaminating genomic DNA. Total RNA was screened for mRNA encoding ALK-1, BMPRII, connective tissue growth factor (CTGF), inhibitor of differentiation (Id) 1, Id2, Id3, and plasminogen activator inhibitor-1 (PAI-1) using the primers indicated in Table 1 and the hspa8 or gapdh genes to demonstrate RNA equivalence in the RT reactions. One microgram of total RNA was reverse-transcribed using the ImProm-II Reverse Transcription System (Promega, Mannheim, Germany). One microliter of the RT reaction served as a template in a PCR reaction using Platinum Taq (Invitrogen, Karlsruhe, Germany). Amplicons were generated by an initial denaturation (5 min, 96°C), followed by 25 cycles of denaturation (1 min, 96°C), annealing (1 min at the annealing temperature described in Table 1), and primer extension (3 min, 72°C). The PCR reactions were terminated with a 10-min primer extension step at 72°C. Amplicons were separated on a 1% (mass/vol) agarose gel and visualized by ethidium bromide staining. Band intensities from specific samples were normalized for loading using the hspa8 band from the same sample. Expression ratios for RT-PCR data reflect values normalized for the hspa8 band: [band PD (85% O2)/hspa8 PD (85% O2)]/[band PD (21% O2)/hspa8 PD (21% O2)].

Quantitative changes in mRNA expression of genes encoding ECM and ECM-remodeling components were assessed by quantitative real-time PCR exactly as described previously (22), using a Sequence Detection System 7700 (Applied Biosystems, Foster City, CA) and the intron-sparing primer pairs indicated in Table 1. The ubiquitously expressed, pseudogene-free hydroxymethylbilane synthase (hmbg) gene was used as reference. Cycling conditions were 95°C for 6 min, followed by 45 cycles of 95°C for 20 s, 62°C for 30 s, and 73°C for 30 s. The exclusive amplification of the expected PCR product was confirmed by melting curve analysis and gel electrophoresis. Changes are expressed as: [ligand-induced fold change in cycle threshold (ΔCt) under 85% O2]/[ligand-induced fold change in ΔCt under 21% O2].
Table 1. Primers employed for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Amplicon Size, bp</th>
<th>Cycle Number</th>
<th>Annealing Temperature, °C</th>
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<tbody>
<tr>
<td>ALK-1</td>
<td>5'-AGGGCAGATGCTGAGTGGTGAG-3'</td>
<td>5'-GCGGCTAGGAGGCTGTCGTC-3'</td>
<td>735</td>
<td>29</td>
<td>58</td>
</tr>
<tr>
<td>BMPRII</td>
<td>5'-GAGCCAGAGATGCTGAGTGGTGAG-3'</td>
<td>5'-GAGAGCTGTCGTCGTCGTC-3'</td>
<td>601</td>
<td>32</td>
<td>55</td>
</tr>
<tr>
<td>CTGF</td>
<td>5'-CGGCGAGATGCTGAGTGGTGAG-3'</td>
<td>5'-AGGGCAGATGCTGAGTGGTGAG-3'</td>
<td>449</td>
<td>29</td>
<td>60</td>
</tr>
<tr>
<td>gapdh</td>
<td>5'-ACCAGAACACTGTGGATGAG-3'</td>
<td>5'-TGTGAGGAGATGTGGTGAG-3'</td>
<td>548</td>
<td>24</td>
<td>60</td>
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<tr>
<td>hspa8</td>
<td>5'-CAAGGGAAAGACCAAAAGACAT-3'</td>
<td>5'-ATACAGGGAAGAGGGTGGTGAC-3'</td>
<td>474</td>
<td>24</td>
<td>60</td>
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<tr>
<td>Id1</td>
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<td>5'-AGCCGAGATGCTGAGTGGTGAG-3'</td>
<td>409</td>
<td>25</td>
<td>55</td>
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<tr>
<td>Id2</td>
<td>5'-AGGAACTCTCGGGTGGAG-3'</td>
<td>5'-GGTGAGGAGATGTGGTGAG-3'</td>
<td>400</td>
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<td>60</td>
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<tr>
<td>Id3</td>
<td>5'-CGGCGAGATGCTGAGTGGTGAG-3'</td>
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<td>PAI-1</td>
<td>5'-TCATCAAGATGCTGAGTGGAG-3'</td>
<td>5'-GCCAGAGATGCTGAGTGGAG-3'</td>
<td>508</td>
<td>26</td>
<td>60</td>
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BMPRII, BMP type II receptor; CTGF, connective tissue growth factor; Id, inhibitor of differentiation; PAI-1, plasminogen activator inhibitor-1; MMP, matrix metalloproteinase; hspb, hydroxymethylbilane synthase gene; TIMP, tissue inhibitor of metalloproteinase; NA, not applicable.

**Immunostaining of lung tissue sections.** Expression of TGF-β/BMP receptors and Smad molecules was assessed on 3-μm tissue sections, prepared as described above for lung morphometric analysis. After antigen retrieval (performed in a pressure cooker using 6.5 mM sodium citrate, pH 6.0) and quenching of endogenous peroxidase activity with 1% H2O2 for 24 or 48 h, after which cells were stimulated with TGF-β for determination of firefly luciferase activity exactly as recommended by the manufacturer. Tocater for the effects of ligand stimulation and hypoxia on the baseline transcriptional activity of the cells, values were normalized for the transcriptional activity of the pGL3-control vector.

**Assessment of apoptosis and cell-cycle progression by flow cytometry.** Identification of apoptotic cells was performed using allolymphocyanin-conjugated annexin V (Invitrogen), following the recommendations of the manufacturer, as described previously (45). Necrotic cells were excluded by counter-staining with 2 μg/ml propidium iodide. Cell cycle analysis was performed as described previously (45); briefly, cells were harvested by trypsinization, fixed overnight at 4°C with 75% (vol/vol) ethanol, washed, and incubated in PBS containing 10 μg/ml propidium iodide and 100 μM/ml RNase (Merck Biosciences) for 1 h at 37°C. Data were collected using a FACS Canto flow cytometer and analyzed using a FACS Diva software package (both from BD Biosciences, Heidelberg, Germany). A minimum of 10,000 cells were analyzed per sample. Gates based on forward and side scatter were set to eliminate cellular debris and cell clusters.

**Statistical analysis.** Values are expressed as means ± SD. Differences between the groups were assessed by one-way ANOVA and a Student-Newman-Keuls test for multiple comparisons, with a P value <0.05 considered significant.

**RESULTS**

Survival, growth, and respiration of neonatal mice exposed to chronic hyperoxia. For chronic hyperoxia exposure, one group of pups was exposed to 85% O2 starting at day P7 and terminating at day P28. A second group was exposed to 21% O2 over the same time period. A 100% survival was recorded for both groups of pups. This high survival contrasts with the poorer survival (60%) observed for FVB/N mice exposed to 85% O2 between days P0.5 and P28 (56), probably reflecting the well-described variability in mouse strain sensitivity to hyperoxia (20).

A marked effect of hyperoxia on growth, as assessed by trends in body mass changes, was observed (Table 2). On day 15.2 ± 0.9 g/day P14 to 7.2 ± 1.1 g/day P28, and the percentage of body mass change between day P0.5 and P28 was 10.2 ± 0.9 for both groups).
P14, pups exposed to 85% O2 exhibited a comparable body mass to age-matched pups exposed to 21% O2 over the same time period (Table 2). However, by day P28, pups exposed to 85% O2 exhibited an average body mass that was 33% lower than that of age-matched pups exposed to 21% O2 (Table 2).

A marked effect of hyperoxia on dynamic respiratory compliance (Cdyn) was also observed, since pups exposed to 85% O2 between days P1 and P28 exhibited a Cdyn 25% lower than that observed for age-matched pups exposed to 21% O2 (Table 2) at day P28. Thus, while without effect on survival, exposure to chronic hyperoxia significantly impaired growth and respiratory compliance of neonatal C57BL/6J mice.

Lung morphometry of neonatal mice after exposure to chronic hyperoxia. Alveolar development was impaired in pups exposed to 85% O2, evident by the enlarged and saccular appearance of air spaces and the reduced number of secondary crests (Fig. 1A and B), compared with pups exposed to 21% O2 (Fig. 1C and D). Quantification of these parameters supported our observations. The MLI is inversely proportional to the alveolar surface area. By day P28, pups exposed to 85% O2 exhibited a MLI approximately double that of pups exposed to 21% O2 (Table 2), indicating a dramatic reduction in alveolar surface area of pups exposed to 85% O2.

Similarly, mice exposed to 85% O2 exhibited a significant increase in septal thickness (Table 2), which is typically attributed to the accumulation of extracellular matrix components, namely collagen and elastin fibers, in the interstitium (2). These data indicate that exposure of C57BL/6J neonates to chronic hyperoxia severely impaired alveolarization. This pathology is consistent with morphometric changes observed in FVB/N mice exposed to 85% O2 (56), as well as in preterm baboon (7) and sheep (1) models of BPD.

Effect of chronic hyperoxia on abundance of TGF-β/BMP signaling molecules in neonatal mouse lungs. Where antibodies were available, lung samples were probed by immunoblotting to investigate whether exposure to 85% O2 resulted in changes in protein expression between the 85% and 21% O2 groups. Indeed, pronounced changes were observed for some TGF-β/BMP superfamily receptors (Fig. 2A). The abundance of the type I BMP receptors ALK-3 and ALK-6 was reduced (twofold; Fig. 2, A and B) and increased (fourfold; Fig. 2, A and D), respectively, particularly at days P21 and P28. The abundance of the type I TGF-β receptor ALK-5 was reduced (fourfold) in the 85% O2 group (Fig. 2, A and C), whereas abundance of TβRII, particularly the short isoform (42), was increased (fourfold; Fig. 2, A and E).

Temporal changes in the abundance of intracellular signaling components of the TGF-β/BMP system were also observed (Fig. 3A). The abundance of Smad1, the key transducer of BMP signals, was increased in the 85% O2 group. This effect peaked at day P14 (Fig. 3, A and B) and remained evident at day P21. In contrast, the abundance of Smad3, a transducer of TGF-β signals, was reduced between days P7 and P28 (Fig. 3, A and C). Protein levels of the co-Smad, Smad4, which transduces both TGF-β and BMP signals, were dramatically (sixfold) increased at days P21 and P28 (Fig. 3, A and D). Thus chronic hyperoxia markedly changed the abundance of TGF-β/BMP superfamily proteins in the lungs of neonatal mice.

Protein expression of ALK-1 and BMPRII could not be evaluated by immunoblot, since there are no commercial antibodies available against ALK-1 and a commercial antibody against BMPRII did not work in our hands (data not shown). Therefore, gene expression in the lungs of neonatal mice exposed to 85% or 21% O2 between days P1 and P28 was assessed by semiquantitative RT-PCR (Fig. 2F). Levels of mRNA encoding ALK-1 were upregulated in the 85% O2 group, evident at days P7, P21, and P28 (Fig. 2, F and G). In contrast, the exception of day P7, levels of BMPRII mRNA were unchanged under hyperoxic conditions (Fig. 2, F and H). Thus, in addition to the changes in TGF-β/BMP superfamily expression observed by immunoblot (Figs. 2A and 3), chronic hyperoxia can also alter ALK-1 gene expression in the lungs of neonatal mice. It is important to note, however, that in the hyperoxia-injured lung, mRNA levels do not necessarily correlate with protein abundance. Indeed, for some proteins, such as surfactant protein D, mRNA levels and protein abundance are inversely correlated (57). Therefore, these RT-PCR data should be interpreted with caution.

Effect of chronic hyperoxia on localization of TGF-β/BMP superfamily proteins in neonatal mice. In pups exposed to both 85% O2 and 21% O2, ALK-3 staining was evident in both the airway epithelium and septae (Fig. 4A). The ALK-6 staining, which was evident in the septae as well as in the airway epithelium of pups exposed to 21% O2, was more intense in lungs of pups exposed to 85% O2 (Fig. 4A). Similarly, whereas little or no staining for TβRII was evident in lungs exposed to 21% O2, staining (particularly in the airway subepithelial layer) was evident after exposure to 85% O2 (Fig. 4A). These trends are consistent with a fourfold elevation in ALK-6 (Fig. 2, A and D) and TβRII (Fig. 2, A and E) protein abundance in hypoxia-exposed lungs assessed by immunoblot.

Protein abundance of Smad1 in the lungs of pups at day P28 was similar in the 21% and 85% O2 groups (although it was significantly elevated at days P14 and P21; Fig. 3, A and B). Consistent with these data, Smad1 staining, evident in the endothelial layer, as well as in the airway epithelium, was of a similar intensity in lung sections from pups exposed to 21% and 85% O2 (Fig. 4B). In contrast, strong Smad3 staining was
evident in the airway epithelium and the septae in the lungs of pups exposed to 21% O2 (Fig. 4B), although this staining was less intense in the lungs of 85% O2-exposed pups (Fig. 4B), reflecting the twofold decrease in Smad3 protein abundance evident in immunoblots (Fig. 3, A and C). A similar correlation was observed in the case of Smad4, which exhibits a dramatic sixfold increase in protein abundance in the lungs of 85% O2-exposed pups (Fig. 3, A and D). Smad4 staining was localized primarily to the endothelium in the lungs of pups exposed to 21% O2 (Fig. 4B). After exposure to 85% O2, staining was more intense and was now also evident in the airway epithelium and the alveolar septae (Fig. 4B). Together, these data indicate that chronic exposure changes both the protein abundance and localization of components of the TGF-β/BMP superfamily.

**Effect of chronic hyperoxia on TGF-β/BMP signaling in neonatal mouse lungs.** To address whether the dramatic alterations in the expression of TGF-β and BMP signaling molecules we observe actually translate to altered TGF-β and BMP signaling in the neonatal mouse lung in response to high oxygen concentrations, we assessed the expression of two TGF-β responsive genes, PAI-1 and CTGF, as well as three BMP-responsive genes, encoding Id1, Id2, and Id3. The PAI-1 mRNA expression was consistently upregulated in lungs of pups exposed to 85% O2, in which CTGF mRNA levels were also prominently upregulated, particularly at days P14 and P21 (Fig. 5A). Whereas no quantitative statement could be made regarding Id1 mRNA levels, the mRNA levels of Id2 and Id3 were decreased, notably at days P14 and P21, in lungs of pups exposed to 85% O2 (Fig. 5A). These data suggested that TGF-β signaling was enhanced, whereas BMP signaling was dampened in lungs of pups exposed to 85% O2. In further support of this idea, we assessed the degree of phosphorylation of Smad1 and Smad2, which are specific transducers of BMP and TGF-β signals, respectively. Indeed, at day P14, exposure to 85% O2 promoted both a significant decrease in Smad1 phosphorylation and a significant increase in Smad2 phosphorylation (Fig. 5, B and C). By day P28, Smad1 phosphorylation was restored.
to levels observed; however, Smad2 phosphorylation remained elevated in the 85% O2 group (Fig. 5, B and C). Together, these data indicate that TGF-β signaling is most likely enhanced, whereas BMP signaling is decreased, in lungs from pups exposed to 85% O2.

Effect of hyperoxia on TGF-β/BMP signaling in epithelial and fibroblast cell-lines. Since the impaired TGF-β/BMP signaling we observed in the lungs of pups may have been attributable to the undernourishment (clearly evident by reduced body mass) of pups exposed to 85% O2, we sought to validate our observations in vitro in cell culture. Therefore, TGF-β and BMP signaling was assessed in NIH/3T3 (fibroblast) and MLE-12 (epithelial) cells, using a luciferase-based transcriptional reporter assay, employing the TGF-β-responsive reporter p(CAGA)12 or the BMP-responsive reporter pId120. Baseline transcriptional activity of the cells was also measured by a loading control.
tered by hyperoxia (~11% reduced) and TGF-β1 (reduced by as much as 17%), although not by BMP-2, as assessed by changes in the expression of the constitutively active pGL3-control construct (data not shown). Therefore, induction of transcription was normalized for pGL3-control transcriptional activity. Exposure of both MLE-12 and NIH/3T3 cells to 85% O2 for 24 or 48 h dramatically (3- to 4-fold) increased their sensitivity to TGF-β1 stimulation (Fig. 6, A and B). In contrast, MLE-12 cells were poorly responsive to BMP-2 stimulation, and MLE-12 responsiveness to BMP-2 was not affected by elevated oxygen concentrations (Fig. 6C). NIH/3T3 cells were responsive to BMP-2 stimulation, and after 48 h of exposure to 85% O2, the responsiveness of NIH/3T3 cells to BMP-2 was significantly decreased (Fig. 6D). These data nicely support our contention that hyperoxia has opposing effects on TGF-β and BMP signaling, where TGF-β signaling is enhanced and BMP signaling is dampened.

**Effect of hyperoxia on TGF-β- and BMP-induced apoptosis of ATII and PASMC.** The hyperoxia-induced apoptosis of ATII cells is believed to be an important factor in oxygen toxicity.

**Fig. 5. Induction of TGF-β/BMP signaling in the lungs of neonatal mice chronically exposed to hyperoxia.** A: expression of mRNA of the TGF-β-inducible genes encoding plasminogen activator inhibitor-1 (PAI-1) and connective tissue growth factor (CTGF), and the BMP-inducible genes encoding inhibitor of differentiation 1 (Id1), Id2, and Id3, in lungs extracted at days P7, P14, P21, and P28 from neonatal mice exposed to 21% O2 or 85% O2 from day P1. The mRNA expression was assessed by semiquantitative RT-PCR. The gapdh gene was used to demonstrate RNA equivalence in the RT reactions. Baseline phosphorylation of Smad proteins was also assessed in lungs extracted at days P14 and P28 from neonatal mice exposed to 21% O2 or 85% O2 from day P1 (B). Data were quantified by densitometry, by comparing the ratio of phospho-Smad (pSmad) to total Smad in hyperoxic vs. normoxic lungs (C). The fold change in Smad phosphorylation was calculated by \[ \frac{\text{pSmad}}{\text{total Smad (85% O2)}}/\frac{\text{pSmad}}{\text{total Smad (21% O2)}}; n = 3–4 \text{ for each bar.} *P < 0.05. \]

**Fig. 6. Effect of hyperoxia on TGF-β and BMP signaling assessed by a luciferase reporter assay.** Mouse lung epithelial-12 (MLE-12) (A and C) or NIH/3T3 (B and D) cells were transfected with the TGF-β-responsive reporter p(CAGA)12 (A and B) or the BMP-responsive reporter p(lD120) (C and D) and maintained in 21% O2 (black bars) or 85% O2 (gray bars) for 24 or 48 h prior to stimulation with TGF-β (2 ng/ml) or BMP-2 (20 ng/ml) for 12 h. Ligand-inducible luciferase activity was normalized for luciferase activity of the constitutively active pGL3-control vector to account for effects of hyperoxia or TGF-β/BMP ligands on baseline transcriptional activity. Determinations were made for 3 separate experiments, each in quadruplicate. *P < 0.05; **P < 0.01.
and the development of BPD (10). Since TGF-β (27) and BMP ligands (31) have both been ascribed with proapoptotic activity, we sought to determine whether exposure of ATII cells and PASMC altered their sensitivity to TGF-β- or BMP-induced apoptosis. Primary ATII cells and primary PASMC were exposed to 21% O₂ or 85% O₂ for 24 h prior to stimulation with TGF-β1 or BMP-2, or addition of vehicle alone in the case of control experiments, after which cells were assessed for apoptosis after an additional 24 or 48 h. No significant differences in the percentage of apoptotic cells were observed between control and BMP-2-stimulated groups. However, both 24 and 48 h after TGF-β1 stimulation, the percentage of apoptotic cells in the 85% O₂ group was significantly increased compared with the 21% O₂ group (Fig. 7, A and C). In contrast, neither TGF-β1 nor BMP-2 induced apoptosis in PASMC, irrespective of the oxygen concentration. These data indicated that exposure of ATII cells to 85% O₂ for as little as 24 h could abnormally increase the sensitivity of ATII cells to TGF-β1-induced apoptosis.

![Graph showing the effect of hyperoxia on TGF-β- and BMP-induced apoptosis of primary alveolar type II (ATII) cells and primary pulmonary artery smooth muscle cells (PASMC).](http://www.ajplung.org)

**Fig. 7.** Effect of hyperoxia on TGF-β- and BMP-induced apoptosis of primary alveolar type II (ATII) cells and primary pulmonary artery smooth muscle cells (PASMC). Primary ATII cells (A) and primary PASMC (B) were maintained under 21% O₂ or 85% O₂ for 24 h prior to stimulation with vehicle [0.8% (mass/vol) NaCl] alone (●), TGF-β (2 ng/ml; ○), or BMP-2 (100 ng/ml; □). Cells were examined for induction of apoptosis by flow cytometry 24 and 48 h after ligand stimulation. Data represent the mean fold change in the percentage of apoptotic cells in the 85% O₂ group vs. the percentage of apoptotic cells in the 21% O₂ group at the time points indicated ± SD (n = 3). *P < 0.05. Staurosporine (Stauro; 100 nM, 24 h) was employed as a positive control for induction of apoptosis in both cells types in 21% O₂ (insets). C: original flow cytometry scattergrams from a 72-h time point of an arbitrarily selected representative experiment with ATII cells.
Effect of hyperoxia on TGF-β- and BMP-induced proliferation of ATII and PASMC. Since both TGF-β and BMP ligands can have pro- and antiproliferation activity in variety of cell types (27, 31), we assessed the effects of TGF-β1 and BMP-2 on the proliferation of ATII cells and PASMC after exposure to 85% O₂. Primary ATII cells and primary PASMC were exposed to 21% O₂ or 85% O₂ for 24 h prior to stimulation with TGF-β1 or BMP-2, or addition of vehicle alone in the case of control experiments, after which cells were assessed for apoptosis after an additional 48 h (Fig. 8). No significant changes in cell proliferation were observed, for ATII cells or for PASMC, for either ligand.

Effect of hyperoxia on TGF-β- and BMP-induced extracellular matrix proteins by lung fibroblasts. The production of ECM and ECM-remodeling enzymes (and their inhibitors) by interstitial fibroblasts is a key step in the alveolarization process, and expression of many of these proteins may be stimulated by TGF-β. Exposure of primary lung fibroblasts to 85% O₂ increased the basal expression of both Smad4 and TβRII and increased the basal phosphorylation of Smad2, as assessed by immunoblot (Fig. 9A). These data are consistent with our observations made in whole lung extracts from pups chronically exposed to 85% O₂.

Exposure of fibroblasts to 85% O₂ also significantly altered the basal mRNA levels of several important ECM or ECM-remodeling proteins: mRNA levels for the α₁ chains of collagens I and III as well as tropoelastin and tenascin-C were reduced, whereas tissue inhibitor of metalloproteinase-1 (TIMP-1) mRNA levels were increased, and matrix metalloproteinase (MMP)-1, MMP-2, and TIMP-3 mRNA levels were unchanged (Fig. 9B).

We then assessed whether exposure to 85% O₂ could alter the induction of these genes by TGF-β1 (2 ng/ml) or BMP-2, BMP-4, or BMP-7 (all at 100 ng/ml). The α₁ chains of collagens I and III, and MMP-1, MMP-2, and TIMP-3 were not TGF-β-inducible in 21% O₂, which is consistent with other reports (21) indicating that none of these genes are normally TGF-β-inducible in fibroblasts, in contrast, for example, to collagens IV, VIII, and XV. However, levels of mRNA encoding TIMP-1 (Fig. 9G), tropoelastin (Fig. 9f), and tenasin-C (Fig. 9J) were significantly increased in the presence of TGF-β in 21% O₂. Of particular interest to our study, the ability of TGF-β to increase mRNA levels of TGF-β1 and tropoelastin was significantly enhanced after 24 h of exposure to 85% O₂ (Fig. 9, G and J). Of further interest, collagen I mRNA levels were increased by TGF-β in 85% O₂ but not 21% O₂ (Fig. 9C). Levels of tenasin-C mRNA were also increased by TGF-β in 85% O₂ (Fig. 9J); however, this increase was not significantly different with respect to that observed in 21% O₂. With the exception of a small (but significant) decrease in collagen I mRNA levels in response to BMP-7 (Fig. 9C), none of the genes we investigated were regulated by BMP (Fig. 9, C–J). In sum, our data indicate that exposure of primary lung fibroblasts to 85% O₂ significantly increases the capacity of TGF-β to upregulate levels of mRNA encoding extracellular matrix components.

DISCUSSION

Alveolarization begins with branching of distal airway sacs

cules into immature alveoli, in the canicular phase of lung
development, beginning on embryonic day E16 in mice, and con-

continues through the saccular phase and into the alveolar phase, occurring between days P5 and P21 (9). During this time, ATII cells proliferate and differentiate into type I cells. Alveolar septae divide the terminal respiratory sacs, increasing the number of alveoli (9). The septae are supported by
the ECM, composed of a collagen scaffold, to which glycoproteins (like tenasin) and fibrinous proteins (like elastin) are interwoven (41). The ECM is deposited by fibroblasts and is continuously remodeled by matrix remodeling proteases (41). Epithelial growth occurs concomitantly with that of lung capillaries (46). This alveolarization process is mediated by paracrine, autocrine, and juxtacrine communication between the epithelium and endothelium, and their associated fibroblasts, undertaken by signaling molecules including TGF-β/BMP (17).

Injury to the developing lung, like oxygen toxicity, may disrupt critical signaling pathways that regulate lung development, leading to developmental arrest. In the case of premature infants, who usually present during the canalicular stage, a pronounced arrest of alveolarization is observed culminating in a pathology typical of BPD (2). In this study, we investigated whether exposure of neonatal mice to hyperoxia during the critical alveolarization period had any effect on the expression and function of the TGF-β/BMP system, a key signaling pathway in lung development. Elevated levels of TGF-β ligands have been detected in neonates with BPD undergoing oxygen therapy (24) and in animal models of BPD (5). Furthermore, exposure of ATII cells to 95% O2 promotes association of Smad2/3/4 complexes with DNA (34), indicating that hyperoxia upregulated TGF-β signaling. However, no study to date has addressed the effect of hyperoxia on the expression of nonligand components of the TGF-β/BMP signaling pathways.

The results that we present in the current study indicate that temporal changes in the expression and localization of key signaling components of the TGF-β/BMP system do occur under hyperoxic conditions. Our data clearly indicate that hyperoxia can "tweak" TGF-β signaling, and that TGF-β-mediated effects are generally potentiated by hyperoxia, whereas BMP signaling is generally dampened.

How could dysregulated TGF-β/BMP signaling arrest alveolarization? TGF-β has potent antiproliferative properties on epithelial cells (43, 58) and some types of smooth muscle cells...
(45). The proliferation and differentiation of ATII cells are key steps in the alveolarization process. It is known that TGF-β can arrest proliferation of ATII cells (43) and prevent keratinocyte growth factor-stimulated ATII cell proliferation (58). We demonstrated in our study that after exposure to 85% O₂ for as little as 24 h, primary mouse ATII cells are significantly more sensitive to the proapoptotic effects of TGF-β, although primary smooth muscle cells derived from the pulmonary artery remained unaffected. Considering these data, it may well be that enhanced TGF-β signaling in the alveolar epithelium after exposure to hyperoxia promotes apoptosis (and thus prevents proliferation and differentiation) of ATII cells, thereby contributing to the hypoplasia associated with BPD (2).

We report in our study that neither TGF-β nor BMP-2 promoted the proliferation of ATII cells or PASMC. Although BMPs are accredited with pro-proliferative properties (31), it is noteworthy that we observe a downregulation of ALK-3 and an upregulation of ALK-6 at the protein levels in pups exposed to hyperoxia, since these two receptors have opposing functions: ALK-3 promotes proliferation, whereas ALK-6 is antiproliferative, promoting mitotic arrest and apoptosis (38). These data would suggest that hyperoxia promotes a shift away from ALK-3-mediated proliferation, in favor of ALK-6-mediated growth arrest. Indeed, we did observe increased ATII cell apoptosis in the presence of BMP-2, although the extent was not statistically significant with respect to vehicle controls.

In the context of cell proliferation, the abnormally enhanced TGF-β signaling during exposure to hyperoxia together with the elevated ALK-1 levels we have observed are noteworthy, since ALK-1 is primarily expressed in the endothelium, and ALK-1 activity inhibits proliferation of endothelial cells by inducing G₀/G₁ arrest (23). These two phenomena could, in part, underlie the capillary hypoplasia observed in animal models of BPD (8). However, since we have not focused on endothelial cells in this study, this idea remains entirely speculative.

In addition to a direct effect on cell proliferation and apoptosis, dysregulation of TGF-β signaling would also impact ECM deposition and remodeling, another key step in the alveolarization process (41). TGF-β regulates the secretion of some matrix-metabolizing enzymes: the MMPs and their cognate inhibitors, TIMPs. Throughout the canalicular, saccular, and alveolar phases of normal lung development, MMP-1, MMP-2, MMP-9, and TIMP-2 are strongly expressed in humans (29) and mice (44), and during normal development, MMP-9 expression peaks during alveolarization (5), indicating the importance of matrix remodeling in this process. In our study, we have illustrated that TGF-β stimulation dramatically elevates levels of mRNA encoding TIMP-1 in fibroblasts cultured in 85% O₂, compared with fibroblasts cultured in 21% O₂, whereas mRNA levels of MMP-1 and MMP-2 were either unaffected or decreased. These data are consistent with reports that TIMP-1 protein expression is increased in rat (16) and mouse (5) models of hyperoxia-induced arrest of alveolarization, although no changes in MMP-2 levels were observed. These data are also consistent with reports of elevated TIMP-1 levels in BPD (12).

TGF-β regulates secretion of some components of the ECM, including collagens, elastin, and tenascin-C (41), and their deposition plays a key role in alveolarization. TGF-β can stimulate collagen secretion by primary lung fibroblasts (13) and upregulates expression of collagen (27), elastin (30), and tenascin and fibronectin (59) in lung tissue. In our study, we have illustrated that when fibroblasts are cultured in 85% O₂, levels of mRNA encoding collagen Iα₁, tropoelastin, and tenascin-C in the primary lung fibroblasts are abnormally increased in response to TGF-β stimulation. Taken together, our data collectively suggest that abnormal upregulation of the TGF-β system in the lung upon exposure to hyperoxia would swing the balance in favor of interstitial ECM deposition and would prevent turnover or breakdown of ECM components. This is consistent with the increased thickening of alveolar septa observed in this and other studies (1, 7), as well as the detection of increased amounts of ECM components in the interstitium in BPD (35, 48) and in several animal models of BPD (1, 7, 39).

In sum, we illustrate in our study that hyperoxia dysregulates both the expression of components of the TGF-β/BMP signaling machinery and TGF-β and BMP signaling per se. We have further demonstrated that this dysregulated signaling has at least two functional consequences relevant to hyperoxia-induced arrest of alveolarization and BPD: 1) hyperoxia increases the sensitivity of ATII cells to TGF-β-induced apoptosis, and 2) hyperoxia modulates the expression of ECM and ECM-remodeling components induced by TGF-β in fibroblasts.

The ability of all-trans retinoic acid (RA) to rescue failed septation (28) and attenuate oxygen-induced inhibition of lung septation (50) lends credence to our hypothesis. RA is a TGF-β antagonist, since RA prevented TGF-β-stimulated ECM production by lung fibroblasts (40), downregulates TGF-β receptor expression (32), and reverses hyperoxia-induced cell-cycle arrest (34). It may well be that RA exerts its protective effects by downregulating TGF-β signaling, which we illustrate in our study is abnormally upregulated by hyperoxia.

BPD is a significant complication of premature birth, affecting up to 10,000 newborns annually, and has long-term respiratory consequences that reach beyond childhood (47). Intensive research is currently encouraged to delineate signaling pathways that underlie the alveolar and capillary hypoplasia that are the hallmarks of “new BPD” (18). Such research has recently paid off, with the discovery that lung-specific vascular endothelial growth factor gene transfer restored alveolar development in a hyperoxia model of BPD (47). The data we present in the current study implicate a second growth factor signaling pathway, that of the TGF-β/BMP superfamily, in the pathogenesis of BPD. It should be emphasized that our data do not unequivocally demonstrate a causal effect between dysregulated TGF-β signaling and BPD. However, the elevated TGF-β signaling observed in the lungs of neonates exposed to hyperoxia, together with the increased sensitivity of lung fibroblasts and epithelial cells to TGF-β after hyperoxic exposure, are consistent with the increased epithelial cell apoptosis and elevated ECM deposition observed in BPD. Given that the TGF-β/BMP pathways are also amenable to pharmacological and genetic manipulation in the lung, they may provide alternative avenues for the management of this debilitating disorder.

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HYPEROXIA MODULATES TGF-β/BMP SIGNALING IN NEONATES

REFERENCES


