Curcumin Protects the Developing Lung Against Long-Term Hyperoxic Injury

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Running Title: Curcumin protects against neonatal lung injury

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Curcumin, a potent anti-inflammatory and antioxidant agent, modulates PPARγ signaling, a key molecule in the etiology of Bronchopulmonary Dysplasia (BPD). We have previously shown curcumin’s acute protection against neonatal hyperoxia-induced lung injury. However, its longer-term protection against BPD is not known. Hypothesizing that concurrent treatment with curcumin protects the developing lung against hyperoxia-induced lung injury long-term, we determined if curcumin protects against hyperoxic neonatal rat lung injury for the first 5 days of life, as determined at postnatal day (PND) 21. One day old rat pups were exposed to either 21% or 95% O₂ for 5 days with or without curcumin treatment (5 mg/kg) administered intraperitoneally once daily, following which the pups grew up to PND21 in room air. At PND21 lung development was determined, including gross and cellular structural and functional effects, and molecular mediators of inflammatory injury. To gain mechanistic insights, embryonic day 19 fetal rat lung fibroblasts were examined for markers of apoptosis and MAPK activation following in vitro exposure to hyperoxia for 24h in the presence or absence of curcumin (5μM). Curcumin effectively blocked hyperoxia-induced lung injury based on systematic analysis of markers for lung injury [apoptosis, Bcl-2/Bax, collagen III, fibronectin, vimentin, calponin, and elastin-related genes] and lung morphology (radial alveolar count and alveolar septal thickness). Mechanistically, curcumin prevented the hyperoxia-induced increases in cleaved caspase-3 and the phosphorylation of Erk 1/2. Molecular effects of curcumin, both structural and cytoprotective, suggest that its actions against hyperoxia-induced lung injury are mediated via Erk1/2 activation, and that it is a potential intervention against BPD.
Key Words- curcumin, hyperoxia, Brochopulmonary Dysplasia, apoptosis, inflammation
Introduction

With the improvements in neonatal care in general, and respiratory care in particular, premature infants exposed to insults such as hyperoxia, volutrauma, and infection develop the “new” Bronchopulmonary Dysplasia (BPD), typified by abnormal lung cytoarchitecture due to deranged alveolarization, with minimal large and small airway changes, and comparatively mild inflammation and fibrosis (32). Infants who develop BPD are at significantly higher risk for morbidity and mortality (2; 14); they are unable to oxygenate efficiently, and often require oxygen supplementation during childhood (8). Although the pathogenesis of BPD is not well understood, hyperoxia, inflammation, and/or ventilatory exposure of the premature lung are the principal causative factors, and there are currently no effective interventions to prevent or palliate BPD (3).

Mesenchymal interstitial fibroblasts play a key role in lung development and injury/repair (11; 15; 24; 28; 30). Briefly, during the process of lung development, alveolar interstitial fibroblasts (AIFs) differentiate into lipid-laden lipofibroblasts, which actively promote alveolar epithelial cell proliferation and differentiation (15; 30). However, AIFs are not terminally differentiated, so they can lose their lipogenic phenotype, transdifferentiating into a myogenic phenotype, i.e., myofibroblasts, which are not conducive to lung epithelial cell growth and differentiation (24; 30). In fact, such abnormally located myofibroblasts are the “hallmark” of all chronic lung diseases, including BPD (31).
The ‘molecular switch’ that determines the lipo- and myofibroblast phenotypes is peroxisome proliferator-activated receptor gamma (PPARγ), a member of the retinoid X-receptor heterodimer family of the retinoid/steroid/thyroid hormone superfamily of ligand-activated nuclear receptors (7). We have previously shown that maintaining a lipofibroblast phenotype prevents hyperoxia- and inflammation-induced neonatal lung injury (5; 18; 28). Given curcumin’s potent antioxidant, anti-inflammatory, and PPARγ-stimulatory properties, in a previous study, we had shown that curcumin treatment could prevent the pathologic effects of hyperoxic exposure on lung development in newborn rats up to 7 days of life (22). In the current study, we have extended these observations by showing the longer-term preventive effects of the 5 day regimen of newborn hyperoxia with or without curcumin treatment at 21 days of life, consistent with its potential for the treatment and prevention of BPD. We hypothesize that curcumin, by upregulating homeostatic AIF PPARγ signaling, enhances neonatal lung maturation and prevents hyperoxia-induced neonatal lung injury, and that this protective effect is mediated by blocking hyperoxia-induced activation of TGF-β signaling.

Methods and Materials

In vivo hyperoxia exposure system and animal protocol. First-time pregnant Sprague-Dawley rat dams were housed in humidity- and temperature-controlled rooms on a 12:12-h light-dark cycle, and allowed food and water ad libitum. On day 22 of pregnancy, the dams delivered naturally. The pups were pooled, randomized, and returned to the nursing dams.
One set of pups was maintained in 95% O2, while the other set was maintained in room air (21% O2). Nursing dams were rotated between hyperoxia- and room air-exposed litters every 24 h to prevent O2 toxicity in the nursing dams. Continuous 95% O2 exposure was achieved in a Plexiglas chamber (77 x 64 x 37 cm) flow-through system. The O2 level inside the Plexiglas chamber was monitored continuously with an O2 analyzer (MAXO2, Ceramatec, Maxtec Inc., Salt Lake City, Utah). Experimentally, pups were grouped as controls (21% O2 of newborn rats or 5 days + placebo intraperitoneal saline administration), 21% O2 + curcumin, hyperoxia only (95% O2 of newborn rats for 5 days + placebo), or hyperoxia with curcumin (95% O2 for 5 days + curcumin). Curcumin (Sigma-Aldrich, St. Louis, MO) was first dissolved in dimethylsulfoxide (16 μg/μl), and administered based on the body weight of each animal (5 mg/kg), further diluted with sterile saline to a 50 μl volume, and administered intraperitoneally with a microsyringe once a day. After the 5-day experimental period, both hyperoxia and normoxia-exposed animals were maintained in 21% O2 until day 21 of life, when the pups were killed using 0.1 ml of Euthasol (390 mg/ml pentobarbital sodium + 50 mg/ml phenytoin; Virbac Animal Health, Inc., Fort Worth, Texas) per pup. At sacrifice, the lungs were collected and processed for Western analysis, immunofluorescence staining, triglyceride uptake, or fibroblast isolation (see below). All animal procedures were performed following National Institutes of Health guidelines for the care and use of laboratory animals, and were approved by the Los Angeles Biomedical Research Institute Animal Care and Use Committee.

Lung Morphometry. Lung morphometry was assessed by determination of radial alveolar count by an investigator unaware of the treatment groups, as described by us previously (5).
**Western analysis.** The isolated lungs were flash-frozen in liquid nitrogen, and then homogenized and sonicated in ice-cold RIPA lysis buffer (500 μl/25 mg of lung tissue) containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS (catalog no. BP-115, Boston Bioproducts, Ashland, MA) with protease inhibitor cocktail tablet (Complete Mini, EDTA-free, Roche Diagnostics GmbH Mannheim, Germany). For checking phospho proteins, phosphatase inhibitor Cocktail 3 (p0044-1ML, Sigma-Aldrich, St. Louis, MO) was added along with fresh 1mM phenylmethanesulfonyl fluoride. After centrifugation at 13,200 g for 5 min at 4°C, the supernatant was used for Western blot analysis for fibronectin, vimentin, calponin, phosphorylated Smad3, Smad7, ALK-5, Bcl-2, Bax. The protein concentration of the supernatant was measured using the Pierce BCA Protein Assay Kit (Pod#23225, Thermo SCIENTIFIC, Rockford, IL), with bovine serum albumin as the protein standard. Aliquots of the supernatant, each containing 50μg of protein, were separated by SDS-PAGE and electrically transferred to nitrocellulose membranes. Nonspecific binding sites were blocked with Tris-buffered saline (0.1% TBS) containing 5% nonfat dry powdered milk (wt/vol) for 1 h at room temperature. After a brief rinse with TBS containing 0.1% Tween 20 (TBST), the protein blots were incubated in primary antibodies at the following dilutions, [fibronectin (1:250 dilution; catalog no. sc-9068), Smad6/7 (1:250 dilution; catalog no. sc-7004), ALK-5 (TGFβ R1 1:200 dilution; catalog no. sc-398), Bcl-2 (1:350 dilution; catalog no. sc-492), and Bax (1:600 dilution; catalog no. sc-493), all from Santa Cruz Biotechnology, Santa Cruz, CA; phosphorylated Smad3 (1:250 dilution; catalog no. 9514, Cell Signaling); calponin (1:3000; catalog no. C2687), vimentin (1:500 catalog no. V6630), were purchased from Sigma-Aldrich, St. Louis, MO; and GAPDH (1:10,000 dilution; catalog no. 5160, Cell Signaling).
The blots were incubated at 4°C overnight, and then with the appropriate secondary antibody for 1h at room temperature. After three washes in TBST, the blots were exposed to X-ray film using SuperSignal West Pico chemiluminescent substrate (Thermo SCIENTIFIC, Rockford, IL) and developed. The relative densities of the protein bands were determined with UNSCAN-IT software (Silk Scientific, Orem, UT), and normalized to GAPDH as an internal control.

**Immunofluorescence staining.** For tissue immunofluorescence staining for the relevant proteins, rat lungs were inflated *in situ* with 4% paraformaldehyde in phosphate buffer at a standard inflation pressure of 20 cm H$_2$O for 4h at 4°C. The lungs were subsequently transferred to PBS containing 30% sucrose (wt/vol) until equilibrated in the cold (4°C). After fixation, 5-μm paraffin sections were treated three times with Histo-Clear$^\text{TM}$ (National Diagnostics, Atlanta, GA) for 5 min, and then rehydrated by a sequential ethanol wash. Sections were then washed twice for 10 min with PBS, and blocked for 1h in PBS-5% normal goat serum-0.2% Triton X-100. Sections were incubated with primary antibodies for 1h at room temperature, and then with the appropriate secondary antibody for 1 h, also at room temperature. Antibodies included collagen type III [primary antibody, 1:100 dilution; catalog no. C 7805, Sigma-Aldrich, St. Louis, MO; secondary antibody, 1:100 dilution; Alexa Fluor, goat anti-mouse 568 (red)]; calponin [primary antibody, 1:250 dilution, catalog no. C 2687, Sigma-Aldrich, St. Louis, MO; secondary antibody, 1:100 dilution, Alexa Fluor, anti-rabbit 488 (green)]; vimentin [primary antibody 1:100 dilution, catalog no. V 6630, Sigma; secondary antibody 1:100 dilution Alexa Fluor, anti-mouse 568 (red)]; elastin [primary antibody, 1:100 dilution, catalog no. AB2039, Millipore, Billerica, MA; secondary antibody, 1:100 dilution; Alexa Fluor, goat anti-mouse 568 (red)].
In vitro studies. Fetal rat lung fibroblasts were isolated from e19 Sprague-Dawley rat pups, and cultured at 37°C using slight modifications of our previously described methods (29). For MAP Kinase activation studies, cells were exposed to hyperoxia for either 10 minutes or 24h with or without pretreatment for 1h with either curcumin (10μM) or UO126 (an Erk1/2 pathway inhibitor at 10μM). Control cells were kept in 21% O2. Cells were also cultured on microscopy slides for immunofluorescent analysis for phospho-p42, p44 MAP Kinase (primary antibody 1:1000 dilution catalog no. 9101, Cell Signaling, Danvers, MA), phospho-SAPK/JNK (primary antibody 1:1000 dilution catalog no. 9255, Cell Signaling, Danvers, MA) and phospho-p38 MAP Kinase (primary antibody 1:1000 dilution catalog no. 2269211, Cell Signaling, Danvers, MA). MAP Kinase activation was also confirmed by Western analysis for p-Erk1/2, p-SAPK/JNK, and p-p38. After stripping, the same membranes were reprobed for total Erk 1/2, SAPK/JNK, and p38 proteins. Protein lysates of cells from 24h exposure groups were probed for fibronectin, Bcl-2 and Bax expression by Western analysis.
Cell proliferation: Cell proliferation was determined based on thymidine incorporation as described previously (23).

Triglyceride uptake: Triglyceride uptake rate was determined using \[^{3}H\]triolein uptake, performed according to our previously described method (21).

Real Time RT-PCR. RNA extraction from postnatal day (PND)21 lung tissue and q-RT-PCR was performed according to previously described methods (19). RT-PCR primers used included: Elastin: F5′-ACCTGGGTTTGGACCTTTCTCCTA-3′ and R5′-GGGTCCCCAGAAGATCACTTTC-3′ (171 bp); Tropoelastin: F5′-AGAAGCCTCGACATTAGATTTGGT-3′ and R5′-GGAGCTATTCCCAGTGAGAAGT-3′ (139 bp); FGFr4: F5′-CCTGACCTTTGGACCATCATT-3′ and R5′-AGCAGTAGCCTCGAGTCAGA-3′ (249 bp); FGFr3: F5′-GTTCACCCATGACCTGCTAC-3′ and R5′-AACACGCAGGAAGTCTTGTC-3′ (194 bp); Fibrillin 5: F5′-ACCTGGTCCATAGCCTTTCTCA-3′ and R5′-ACGTGTTCCCATAGCCTTTCTC-3′ (113 bp); Fibrillin 2: F5′-ACCTGGGACCTGCTACAACACTCT-3′ and R5′-CCGTTATAGCTTCTGTAGCAAAAGC-3′ (126 bp); Lox: F5′-AAACGGAAAAACAACAAAGAAGGT-3′ and R5′-TGCTGATTTAAACACTCAAAATCCA-3′ (83 bp). The normalization control was 18S ribosomal RNA. Data were analyzed using a threshold level of fluorescence that was in the linear range of the PCR reaction. The CT value for 18S ribosomal RNA was subtracted from the CT value of the gene to obtain a delta CT (ΔCT) value. The relative fold-change for each
gene was calculated using the ΔΔCT method. Results were expressed as the mean ± SE, and
considered statistically significant at P < 0.05.

Statistical analysis- Experiments were reproduced at least three times. Statistical differences
between the groups were evaluated by one-way ANOVA followed by Newman-Keuls post hoc test and unpaired Student’s t-test as appropriate. P<0.05 was considered to be statistically significant. Values are means ± SE.

Results

Effect of Curcumin on Hyperoxia-Induced Gross Structural Changes

21 day old rat lungs exposed to 95% O₂ for the first 5 days of life resulted in a significant decrease in alveolar count (Fig. 1A), an effect that was blocked by concomitant treatment with curcumin (5 mg/kg, administered intraperitoneally once daily). Curcumin treatment also blocked the decrease in lung alveolar septal thickness (Fig. 1B) caused by the 5 day exposure to 95% O₂.

Effect of Curcumin on Alterations in Apoptotic and Mesenchymal Markers of Airway Reactivity

As for the hyperoxic effect on apoptosis, Western blot analysis of the whole lung lysates showed a decreased Bcl-2/Bax ratio (Fig. 2A), confirmed by immunofluorescence staining as a decrease in Bcl-2 and increase in Bax (Fig. 2B, C) protein levels, which was blocked by curcumin treatment. Mesenchymal markers of airway reactivity- fibronectin, vimentin, and calponin (Fig 3A, B, C)- remained increased even at PND21 after 5 days exposure to
276 Hyperoxia from PND 1 to 5, an effect that was also confirmed by immunofluorescence staining (Fig 3D, E); this was also blocked by curcumin treatment. As for the effect of hyperoxia on interstitial connective tissue, there was a significant increase in staining for collagen III (Fig. 3F), which was also blocked by curcumin treatment. To confirm the cell-specificity of hyperoxia-induced mesenchymal changes and how curcumin modulates these changes, we next performed double staining for a fibroblast marker, \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA), and an alveolar type II cell marker, p180 lamellar body protein. As is clear from Fig. 3G, the hyperoxia exposed group showed increased \( \alpha \)-SMA staining, restricted specifically to the mesenchymal compartment as evidenced by the lack of co-staining with p180, and this increase was blocked in the curcumin treated group. Turning to the expression of genes relevant to the matrix, we observed significant up-regulation of elastin, tropoelastin, FGFr4, FGFr3, Fibrillin5, Fibrillin2 and Lox (Fig. 4A) by RT-PCR; these effects were blocked by curcumin treatment, with the exception of FGFr4 and r3. The hyperoxia-induced upregulation of elastin and its blockade by curcumin were also confirmed by immunofluorescence (Fig. 4B).

Effect of Curcumin on Hyperoxia-Induced Alterations in Triglyceride Uptake ex vivo

Sustained deleterious effects of hyperoxia were reflected by a significant decrease in the rate of triolein uptake, a marker for alveolar interstitial fibroblast function, by cultured lung explants of PND21 lungs, an effect reversed by curcumin treatment (Fig. 5).

Effect of Curcumin on Hyperoxia-Induced Alterations in Alveolar Fibroblast Proliferation and Apoptosis in vitro
Cell proliferation, as determined by the thymidine incorporation assay, was significantly decreased by 95% O₂ exposure of the cultured e19 fibroblasts for 24h (Fig. 6A), an effect that was reversed in a dose-dependent manner by the curcumin treatment. Consistent with the effects of hyperoxia on apoptosis, treatment of e19 lung fibroblasts with 95% O₂ in culture resulted in an increase in cleaved caspase 3, an effect that was significantly reduced by the curcumin treatment (Fig. 6B). This effect was also confirmed by immunostaining; following 6h synchronization in 0.1% fetal bovine serum (FBS), cultured e19 fibroblasts were exposed to 95% O₂ for 16h, resulting in an increase in staining for cleaved-caspase-3 (Fig 6C), an effect that was blocked by 1h pre-treatment with curcumin.

**Effect of Curcumin on Hyperoxia-Induced Activation of MAP Kinase Signaling**

To probe the mechanism of curcumin action, we focused on MAP Kinases, using cultured lung fibroblasts to discern which of the various MAP Kinases were affected by curcumin. Following a 6h synchronization in 0.1% FBS, e19 fibroblasts were exposed to 95% O₂ for 10 min, which resulted in significant increases in Erk1/2 activation, expressed as the ratio of phosphorylated/total Erk1/2 protein levels; curcumin pretreatment blocked Erk activation (Fig. 7A). In accord with these observed effects of curcumin on MAP Kinase signaling, under the same experimental conditions there was an increase in nuclear staining for Erk 1/2, which was blocked by curcumin (Fig. 7D). Conversely, although hyperoxia increased the amount of phospho-JNK protein, expressed as a ratio to total-JNK, the effect was not affected by curcumin (Fig. 7B); likewise, p38, expressed as a ratio to total p38, was unaffected by hyperoxia (Fig. 7C), indicating the specificity of curcumin’s effect on MAP Kinase activation. E19 fibroblasts, pretreated with either UO126, a specific inhibitor of
322ErK1/2, or curcumin (5μM) were exposed to 95% O₂ for 10 min. Both UO126 and curcumin 323blocked Erk1/2 activation (Fig. 8A). 24h exposure of E19 fibroblasts to 95% O₂ with or 324without UO126 or curcumin blocked the hyperoxia-induced increases in fibronectin, and the 325increased Bax and decreased Bel-2 proteins (Fig. 8B).

Effect of Curcumin on Hyperoxia-Induced Activation of TGF-β Signaling

Previously, we have shown that exposure to hyperoxia activates TGF-β signaling in the 329neonatal lung, an effect blocked by curcumin (5). It is interesting to note the spontaneous 330resolution of increased p-Smad3 and Smad 7 proteins at PND21 following the initial 5 day 331exposure to hyperoxia (Fig. 9A and 9B). However, Alk-5 (a TGF-β receptor) protein was 332still significantly increased at PND21 in the hyperoxia exposure group, with at least partial 333attenuation in the curcumin-treated group (Fig. 9C). Of note, Smad-7, an inhibitor of TGF-β 334activation, was markedly increased, even at PND21, in the hyperoxia-exposed, curcumin- 335treated group (Fig. 9B). Immunostaining for Alk-5 (Fig. 9D) and Smad 7 (Fig. 9E) on tissue 336section from flash-frozen PND21 lung tissue from different experimental conditions 337corroborated the in vitro Alk-5 and Smad7 data, further validating the in vivo blockade of 338TGF-β activation by curcumin.

Discussion

BPD was first described by Northway et al. (16), and redefined three decades later by Jobe 343(9) as the ‘new BPD’ in an era of less invasive mechanical ventilation. It is essentially a 344disease of prematurity caused by a wide variety of proinflammatory conditions, including
barotrauma, oxotrauma, and infection (3). All these agonal insults have been found to funnel through a common pathway, causing delayed alveolarization and myofibroblast proliferation (28). Conversely, by stimulating the lipofibroblast phenotype (30), as determined by increased PPARγ expression (7; 20), we have been able to effectively prevent all these injuries(3; 5; 17; 18; 28). Because of the proximate relationship between lipofibroblast maturation and these diverse effectors of BPD, we have devised a developmental model for the experimental prevention and treatment of lung injury leading to a “BPD-like” picture (28), which integrates all of these factors mechanistically. In the present series of studies, we have extended our original findings of the effect of curcumin on lung fibroblast differentiation at 7 days of age out to 21 days, focusing on the fibroblasts since they provide all the growth factors necessary for normal lung development.

Curcumin was previously found to stimulate the key genes necessary for lipofibroblast differentiation, which protects the lung against oxidant injury (22). Furthermore, oxidant injury, which downregulates these genes, was inhibited by curcumin treatment both in vitro and in vivo, thereby preserving the molecular and structural integrity of the alveoli. At the cell-molecular level, curcumin sustained the lipofibroblast phenotype, maintaining its ability to take up triolein, which is cytoprotective against oxidant injury (29), and provides substrate for surfactant phospholipid synthesis by the neighboring alveolar type II cell (27). Mechanistically, it inhibited oxidative stress and hyperoxic activation of TGF-β signaling, which is thought to be an important causal pathway in the pathogenesis of BPD (1; 5).
Curcumin, an active ingredient of the Indian spice turmeric, is known to have potent antioxidant, anti-inflammatory, and antimicrobial properties, making it an interesting dietary therapy for BPD (6). The dose of curcumin used by us was based on its optimal anti-TGF-β effects at 5 mg/kg body weight in our previous experiments (22). This dose is markedly lower than those used in adults by others, either by the oral or parenteral route (6; 10; 12; 25). Our previous investigation into the mechanism of curcumin’s cytoprotective effect on neonatal lung injury suggested blockade of hyperoxia-induced TGF-β activation, which modulates fibroblast differentiation via both canonical (Smad) and non-canonical (MAPK) pathways (13; 23; 33; 35). In line with previous observations in a variety of models, we now for the first time document blockade of hyperoxia-induced activation of TGF-β and its down-stream non-canonical mediator Erk1/2 by curcumin (4; 23; 26). Since curcumin seems to block both the canonical and non-canonical TGF-β activations, it likely explains the near-complete abrogation of hyperoxia-induced neonatal lung injury in our model. Furthermore, extension of the protective effect of curcumin against hyperoxia-induced neonatal lung injury up to PND21 in this current study indicates long-term efficacy of curcumin for the treatment and prevention of BPD. We have examined the effects of curcumin at multiple structural and functional levels, in combination with cellular and molecular perturbations. Using this hierarchical approach, we have found that curcumin acts comprehensively to prevent the deleterious effects of hyperoxic lung injury. Similarly, curcumin has been shown to inhibit bleomycin-induced pulmonary fibrosis by modulating TGF-β signaling (25). Curcumin has also been shown to counteract TGF-β-mediated downregulation of PPARγ in hepatic stellate cells, which are the liver homolog of AIFs (34). However, our study is the first to show that
Parenterally administered curcumin prevents hyperoxia-induced neonatal lung injury long-term, suggesting its potential usefulness in preventing BPD.


Curcumin provides in vivo protection against hyperoxia-induced damage. 21 day old rats pups exposed to 95% O₂ for the first 5 postnatal days, and then followed subsequently in room air demonstrated a significant decrease in alveolar count (Fig 1A) and significantly increased septal thickness (Fig 1B), both of which were blocked by concurrent treatment with curcumin (5 mg/kg, administered intraperitoneally once daily). Values are means ± SE; n = 9 animals; n = 20 sections.

Curcumin provides in vivo protection against hyperoxia-induced lung cell apoptosis. In vivo exposure to 95% O₂ for 5 days resulted in a decreased Bcl-2/Bax ratio (Fig 2A), an effect that was blocked by curcumin treatment (5 mg/kg, administered intraperitoneally once daily) commencing at birth. Values are means ± SE; n = 3. This observation was corroborated by immunofluorescence staining of Bcl-2 (Fig 2B) and Bax (Fig 2C).

Protective effects of curcumin against hyperoxia-induced increase in markers of pulmonary damage. In vivo exposure to 95% O₂ for 5 days resulted in significant increases in Fibronectin (Fig. 3A), Vimentin (Fig. 3B), and Calponin (Fig. 3C) protein levels, which were all blocked by once-daily curcumin treatment (5 mg/kg, administered intraperitoneally once daily). Values are means ± SE; n = 3. Immunofluorescence staining of
Paraformaldehyde-fixed, paraffin-embedded PND21 lung sections for Vimentin (Fig. 3D), Calponin (Fig. 3E), and collagen III (Fig. 3F) corroborated these data. Double staining of PND21 lung tissue for the mesenchymal compartment marker, α-smooth muscle actin (α-SMA), and epithelial alveolar type II cell marker, lamellar body (Fig. 3G), showed increased α-SMA staining (arrow pointing to green stain), restricted specifically to the mesenchymal compartment [lack of any evidence of co-staining with p180(arrowhead pointing to red stain)].

Effect of curcumin on hyperoxia-induced alterations in elastin-related genes. In vivo exposure to 95% O₂ for 5 days resulted in significant increases in elastin, tropoelastin, FGFr4, FGFr3, fibrillin5, fibrillin2 and lox (Fig. 4A), all of which were blocked by concomitant curcumin treatment (5 mg/kg, administered intraperitoneally once daily) (Real Time PCR data are shown). Values are means ± SE; n = 3. The upregulation of elastin by hyperoxia, and its blockade by curcumin treatment were confirmed by immunofluorescence (Fig. 4B).

Hyperoxia-induced decrease in triolein uptake, a functional marker for lipofibroblasts, is blocked by curcumin administration. Hyperoxia exposure of PND21 lung explants significantly decreased triolein uptake, an effect that was blocked by curcumin administration (5 mg/kg, administered intraperitoneally once daily). Values are means ± SE; n = 6.
Curcumin inhibits hyperoxia-induced decrease in cell proliferation and apoptosis in embryonic day 19 (e19) fetal rat lung lipofibroblasts (LIF). 24h hyperoxia-induced decrease in thymidine uptake was significantly blocked by concomitant treatment with curcumin (Fig. 6A); n = 6. E19 LIF exposure to 95% O2 resulted in an increase in cleaved caspase 3, and was also significantly blocked by pre-treatment with curcumin (5 µM); n = 3 (Fig. 6B). Curcumin also inhibited the increase in immunofluorescence staining for cleaved-caspase-3 (Fig. 6C). Values are means ± SE.

Curcumin inhibits hyperoxia-induced p44/p42 activation. Following a 6h synchronization, e19 LIFs were exposed to 95% O2 for 10 min, resulting in a significant increase in both phospho-p44 and phospho-p42, which were both blocked by 1h pretreatment with curcumin (Fig. 7A). Similar data were found by immunofluorescence staining (Fig. 7D). In contrast, hyperoxia-induced activation of SAPK/JNK was not blocked by curcumin pre-treatment (Fig. 7B); hyperoxia did not induce activation of p-38 MAP Kinase (Fig. 7C).

Effect of curcumin on hyperoxia-induced activation of MAP kinase (Erk1/2) signaling pathway, and the hyperoxia-induced increase in fibronectin and apoptosis. E19 LIFs were exposed to 95% O2 for 10 min, resulting in significant increases in both phospho-p44 and phospho-p42. 1h pretreatment with UO126 (Erk1/2 inhibitor) or curcumin completely blocked hyperoxia-induced phosphorylation of Erk1/2. (Fig. 8A). E19 LIF exposure to 95%
O2 for 24h significantly increased fibronectin (Fig. 8B), and significantly decreased the BCL-2/Bax ratio (Fig. 8C). 1h pretreatment with UO126 or curcumin partially blocked this effect.

Curcumin protects against hyperoxia-induced activation of the TGF-β pathway. Western blot analysis shows spontaneous resolution of increased p-Smad3 and Smad 7 proteins at PND21 following the initial 5 day exposure to hyperoxia (Fig. 9A and 9B). However, Alk-5 (a TGF-β receptor) protein was still significantly increased at PND21 in the hyperoxia exposure group, with at least partial attenuation in the curcumin-treated group (Fig. 9C). Smad-7 was markedly increased, even at PND21, in the hyperoxia-exposed, curcumin treated group (Fig. 9B). Immunostaining for Alk-5 (Fig. 9D) and Smad 7 (Fig. 9E) on PND21 lung tissue corroborated the in vitro Alk-5 and Smad7 data. Values are means ± SE; n = 3.
21%O₂ Curcumin 21%O₂ 95%O₂ Curcumin 95%O₂

*<0.01 vs. 21% O₂
#<0.01 vs. 95% O₂

N=20

Fig 1A Radial Alveolar Count

N=100

Fig 1B Alveolar Septal Thickness (µM)
Fig 2A

- BCL-2
- Bax
- GAPDH

*<0.05 vs. 21% O₂
#<0.05 vs. 95% O₂
Fig 3A

Fig 3B

Fig 3C

*<0.05 vs. 21% O₂
#<0.05 vs. 95% O₂
Cur 95% O₂

95% O₂

21% O₂

Fig 3D

Fig 3E
Fig 4A

Relative Elastin-Related Gene mRNA levels (fold change)

- Elastin
- Tropoelastin
- FGFr4
- FGFr3
- Fibrillin5
- Fibrillin2
- Lox

*<0.05 vs. 21% O₂
#<0.05 vs. 95% O₂

- 21%
- 95%
- Cur95%
Fig 4B

Cur 95%O₂

95%O₂

21%O₂
Fig 5

[3H] Triolein Uptake (cpm/mg protein)

- <0.05 vs. 21% O₂
- #<0.05 vs. 95% O₂

N=6
Fig 6A

Fig 6B

Cleaved Caspase-3
GAPDH

[3H] Thymidine uptake (cpm)

Cleaved Caspase3/GAPDH

O₂: 21% 95% 95% 95% 95%
Cur: 0 0 1 5 10 (µM)

Curcumin: 0 0 5 (µM)
Fig 7A

$O_2$ Cur 21% 95% 95% 10 (µM)

Phospho-Erk1/2

Total-Erk1/2

Fig 7B

P-JNK

T-JNK

Fig 7C

P-p38

T-p38

*<0.01 vs. 21% $O_2$

#<0.01 vs. 95% $O_2$
**Fig 8A**

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**P-Erk1/2**

*<0.01 vs. 21% O₂
#<0.01 vs. 95% O₂

**T-Erk1/2**

**Phospho-p44/Total-p44**

**Phospho-p42/Total-p42**
Fig 8B

- Fibronectin
- GAPDH

Fig 8C

- BCL2
- Bax
- GAPDH

*<0.05 vs. 21% O₂
#<0.05 vs. 95% O₂
Fig 9A

P-Smad3

GAPDH

Fig 9B

Smad7

GAPDH

Fig 9C

ALK5

GAPDH

*<0.05 vs. 21% O2

#<0.05 vs. 95% O2

N=3

Fig 9A

Fig 9B

Fig 9C