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Aerosolized PPAR γ agonist Pioglitazone + Synthetic Surfactant Protein B Mimic B-YL Prevents Hyperoxia-induced Neonatal Lung Injury in Rats

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Abstract

Background—Despite improvements in perinatal care, Bronchopulmonary Dysplasia (BPD) in extremely premature infants has not decreased. Postnatal surfactant therapy provides symptomatic relief from Respiratory Distress Syndrome, but it does not translate into a reduction in BPD. Therefore, the search for effective interventions to prevent BPD continues.

Objectives—Since PPAR γ agonists have been demonstrated to promote neonatal lung maturation and injury repair, we hypothesized that a combined PPAR γ agonist [pioglitazone (PGZ)] and synthetic lung surfactant [surfactant protein B (SP-B) mimic B-YL] formulation would stimulate lung maturation and block hyperoxia-induced neonatal lung injury more effectively than either modality alone.

Methods—One-day old Sprague-Dawley rat pups were administered PGZ+B-YL via nebulization every 24h up to 72h. The pups were exposed to either 21% or 95% O₂, following which pups were sacrificed and lungs examined for markers of lung maturation [(PPAR γ , SP-C, and cholinephosphate cytidylyltransferase (CCT- α) by Western analysis), and (H³)triolein uptake] and injury repair (bronchoalveolar lavage cell count and protein content; LEF-1, Fibronectin, ALK5, and β -catenin protein levels by Western analysis].

Results—The markers of alveolar epithelial-mesenchymal maturation (SP-C, CCT- α , and triolein uptake) increased significantly in the PGZ+B-YL group, more than with either drug alone. Similarly, markers of hyperoxia-induced lung injury were blocked effectively with PGZ+B-YL treatment.

Conclusions—Nebulized PPAR γ agonist PGZ with synthetic lung surfactant accelerates lung maturation and prevents neonatal hyperoxia-induced lung injury more than with either modality alone, potentially preventing BPD more effectively.

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The authors declare no conflicts of interest.

Keywords

Bronchopulmonary Dysplasia; Chronic lung disease; Lung maturation; Prematurity; Respiratory Distress Syndrome

Introduction

Despite improvements in perinatal care, Bronchopulmonary Dysplasia (BPD) in extremely premature infants has not decreased [1,2]. The use of postnatal surfactant provides symptomatic relief from Respiratory Distress Syndrome (RDS), but it does not translate into a reduction in BPD. This is not surprising, since the administration of surfactant alone does not address the fundamental issue in RDS, i.e., lung immaturity [3,4]. In contrast, PPAR γ agonists recapitulate the natural cellular and molecular pathways that determine normal lung structure, function, and homeostasis. In multiple *in vitro* and *in vivo* experimental models of neonatal lung injury, PPAR γ agonists, administered either parenterally [5] or locally [6], enhance lung maturation and prevent neonatal lung injury [5–7]. We reasoned that combining lung surfactant with a PPAR γ agonist will complement each other's benefits and the net effect on lung injury reduction would be greater than with either intervention alone. Therefore, we hypothesized that a *combined PPAR γ agonist + surfactant formulation effectively blocks neonatal lung injury and that this effect is greater than with either modality alone.*

We used a new and innovative surfactant protein B (SP-B) peptide mimic B-YL developed for aerosolized delivery specifically in a hyperoxic environment. Using the N-terminal (residues 1-25) and C-terminal (residues 63-78) α -helical sequences of native SP-B, connected with a short β -sheet loop [8], we replaced the cysteine and methionine residues with tyrosine and leucine, respectively, to minimize inactivation by oxidative stress. Based on homology templating of the linear amino acid sequence (*NH₂-FPIPLPYYWLYRALIKRIQALIPKGGRLLPQLVYRLVLRYS-COOH*), a 3D structure of SP-B peptide mimic B-YL is shown in Fig. 1. As for the choice of PPAR γ agonist, due to the superior safety and efficacy of nebulized pioglitazone (PGZ) in blocking hyperoxia-induced neonatal lung injury [5], PGZ was used to combine with the synthetic surfactant B-YL. Using a neonatal rat model, we planned to administer a highly effective, stable, and oxidant resistant synthetic lung surfactant (B-YL) and simultaneously enhance lung maturation via PGZ to prevent hyperoxia-induced neonatal lung injury.

Materials and Methods

Synthetic surfactant

B-YL peptide was synthesized on a Symphony Multiple Peptide Synthesizer (Protein Technologies, Tucson, AZ) using a FastMoc™ protocol on a H-Ser(OtBu)-HMPB NovaPEG resin, as described previously [8,9]. Purified B-YL peptide was freeze-dried and its mass confirmed by MALDI-TOF mass spectrometry. B-YL surfactant was formulated to contain 35 mg/ml of 5:3:2 DPPC:POPC:POPG combined with 3.0% (by wt) of B-YL. Synthetic surfactant components were combined in organic solvent (e.g., chloroform for lipids and

TFE for peptide), dried under nitrogen, exposed to house vacuum to remove residual solvent, resuspended by hand- vortexing in 0.15 M NaCl adjusted to pH 7.0, heated to 65°C intermittently for 30 min, and refrigerated for >12 h prior to use. PGZ was mixed into B-YL surfactant as an ethanol suspension (4 mg/ml pure ethanol), followed by removal of the ethanol by evaporation. The surface tension lowering ability of surfactant preparations was assessed under dynamic compression at 37°C by captive bubble surfactometry [8]. We routinely analyze surfactant samples of 1 µL (35 mg phospholipids/mL) in ~1.5 ml of Goerke's buffer with 10% sucrose (~ 25 µg surfactant/mL) in the captive bubble surfactometer and perform all measurements in quadruplicate. Lipids only are used as negative control and the clinical surfactant Infasurf® as positive control.

Animal Protocol and Study Design

All studies were approved by the Institutional Review Board and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Briefly, following our previously described protocol, one-day-old *ad libitum* breastfeeding rat pups were exposed to either normoxia (21% O₂) or hyperoxia (95% O₂) for 72 h [6,7]. Hyperoxia exposed animals were administered diluent, B-YL surfactant [100 mg/kg body weight, (BW)], PGZ (1 mg/kg), or PGZ (1 mg/kg) + B-YL surfactant (100 mg/kg) in a 1 ml volume via nebulization over 30 min every 24 h up to 72 h. Aerosolization of B-YL surfactant and PGZ was achieved with a vibrating mesh nebulizer (Aeroneb Pro® nebulizer, Aerogen Inc., Mountain View, CA)[9–10], which aerosolizes liquid formulations without affecting compound's composition and concentration. This small volume (10 ml maximum), median diameter nebulizer delivers a low velocity aerosol with a particle size <3.0 µm and a flow rate >0.1 ml/min, is FDA-approved for human use, and does not need a gas source [11]. Control (normoxia group) pups were administered the diluent similarly via nebulization.

All pups were killed at 72 h, and lungs were either snap-frozen for Western analysis of markers of lung homeostasis [SP-C and cholinephosphate cytidyltransferase (CCT)-α] and injury repair (LEF-1 and Fibronectin) or cultured as explants to determine the rate of [³H] triolein uptake, a functional marker of surfactant phospholipid synthesis. A separate set of animals was used for bronchoalveolar lavage (BAL) collection. Retrieved BAL solutions were centrifuged at 400g for 10 min. Sediments were analyzed for total cell counts and supernatants were snap-frozen in liquid nitrogen for subsequent analysis of inflammatory markers, such as IL-6 and interferon (IF)-γ (ELISA kits from R&D Systems).

Western analysis

Following previously described methods [6,7,12], Western analysis was performed on lung tissue to determine protein levels of alveolar epithelial (SP-C and CCTα) and alveolar mesenchymal (PPAR-γ) differentiation and injury repair (LEF-1 and Fibronectin) markers. Primary antibodies (all from Santa Cruz Biotechnology, Dallas, TX, USA) used included: PPARγ (1:500), SP-C (1:250), CCT-α (1:200), LEF1 (1:200), Fibronectin (1:250), BAX (1:500), Bcl-2 (1:200), and GAPDH (1:10,000), which was from Millipore, Billerica, MA, USA.

Immunofluorescence

Tissue immunofluorescence staining for ALK5 (1:100) and β -catenin (1:100) was performed following previously described methods [7].

Real time qRT-PCR for lung cytokine assay

Tissue RNA extraction and q-RT-PCR were performed according to previously described methods [5]. RT-PCR primers used included IL6: F5'-CTTCCTACCCCAACTTCCAA-3' and R5'-ACCACAGTGAGGAATGTCCA-3' (191 bp); Interferon- γ : F5'-CCAAGTTCGAGGTGAACAAC-3'; R5'-ACTCCTTTTCCGCTTCT-3' (110 bp). The normalization control was 18S ribosomal RNA. The relative fold-change for each gene was calculated using the $2^{-\Delta\Delta CT}$ method.

Choline incorporation in saturated phosphatidylcholine assay

[3 H]choline (NEN Dupont, Boston, MA) incorporation into saturated phosphatidylcholine, which is the major surface-active lipid substrate of surfactant was determined in cultured lung explants following our previously described protocol [12].

Triglyceride uptake assay

[3 H]triolein (PerkinElmer, Boston, MA, USA) uptake, a key marker of alveolar lipofibroblast (LIF) function, was used to quantitate triglyceride uptake by fetal rat lung explants using our previously described protocol [12].

Statistical analysis

Student t-test and ANOVA, in combination with the Tukey's *post-hoc* test, as appropriate, were used to detect group differences. The number of samples for each analysis was 4–6, from three separate sets of experiments, i.e., N=3. Results are expressed as means \pm SEM.

Results

We first determined whether combining PGZ with B-YL affects B-YL's bioactivity by measuring its surface activity with captive bubble surfactometry (CBS) [8,10,13] in the presence and absence of PGZ [B-YL surfactant (100 mg/kg) and PGZ (1 mg/kg)] and compared with a clinical surfactant (Infasurf®) as positive and lipids alone (DPPC:POPC:POPG 5:3:2 wt:wt:wt) as negative controls, respectively. Clinical surfactant contains both SP-B and SP-C in contrast with B-YL surfactant that only contains a highly active SP-B peptide mimic. Minimum surface tension values of B-YL surfactant \pm PGZ were similar to those of Infasurf (Fig. 2).

Based on the ampholytic properties, the addition of B-YL to a PGA is not expected to interfere with PGZ's PPAR γ agonist activity [14]. Therefore, we next confirmed that the addition of B-YL to PGZ did not interfere with its PPAR γ agonist activity, and, hence its lung maturation effect. Embryonic day 19 fetal rat lung explants were cultured for 24 h with or without B-YL [100 mg/kg fetal body weight (BW)] alone or with PGZ (0.5, 1 or 2 mg/kg BW). Following which PPAR γ , SP-C, and CCT- α protein levels, along with choline incorporation into disaturated phosphatidylcholine (DSPC), all markers of lung maturation,

were determined. As shown in Fig. 3, explants treated with PGZ+B-YL demonstrated the expected increases in PPAR γ , SP-C, and CCT- α protein levels along with an increase in choline incorporation into DSPC, indicating that combining B-YL with PGZ does not affect PGZ's lung maturation activity.

Having confirmed that combining B-YL and PGZ does not interfere with the surface tension reducing and lung maturational activity, respectively, of B-YL and PGZ, under *in vitro* conditions, we next determined if this combinatorial approach prevents hyperoxia-induced neonatal lung injury under *in vivo* conditions. Postnatal day (PND) 1 rat pups were exposed to either normoxia or hyperoxia up to 72 h. Hyperoxia-induced decrease in PPAR γ (A), SP-C (B), CCT- α (C) and increase in LEF-1 (D) and Fibronectin (E) protein levels were blocked by the concomitant administration of either PGZ or PGZ+B-YL. This was true also for hyperoxia-induced decrease in triolein uptake (F). As evidenced by the effects on the protein levels of SP-C and CCT- α , and triolein uptake, PGZ+B-YL administration had a more robust effect; In contrast, B-YL administration alone had no effect on any of these parameters.

Blockage of hyperoxia-induced neonatal lung injury with combined PGZ+B-YL nebulization is also evidenced by the normalization of hyperoxia induced alterations in BAL cell count and protein content (Fig. 5A), as well as whole lung apoptosis (Bcl2/Bax) (Fig. 5B) and lung inflammatory markers IL-6 and Interferon (IF)- γ (Fig. 5C). Interestingly, even though B-YL administration alone did not have any effect on most molecular markers of lung maturation and injury repair; it did suppress the hyperoxia-induced increase in IL-6 and IF- γ expression. Lastly, since hyperoxia-induced neonatal lung injury has been previously shown to be mediated via TGF- β and Wnt activations, lung sections from control, hyperoxia, and hyperoxia + B-YL+PGZ groups were stained for ALK5 and β -catenin, two key intermediates of TGF- β and Wnt pathway activations, respectively. In line with the data on other markers of lung injury, hyperoxia-induced increase in ALK5 and β -catenin staining was blocked in the B-YL+PGZ group (Fig. 6).

Discussion

Using both *in vitro* and *ex vivo* studies we found that combining PGZ with the SP-B mimic B-YL doesn't interfere with PGZ's PPAR γ 's agonist activity and B-YL's surface tension lowering activity. Furthermore, in a neonatal rat model of hyperoxia-induced lung injury, nebulized combined PGZ+B-YL formulation complemented each other's benefits as reflected by a greater net reduction in lung injury compared with either intervention alone. For example, on hyperoxia exposure, there was relatively greater preservation of alveolar homeostatic markers PPAR γ , SP-C, CCT- α , and triolein uptake, suggesting potentially greater protection against hyperoxia-induced neonatal lung injury with the PGZ+B-YL formulation than with either component alone.

Prematurity is the underlying cause of lung immaturity leading to RDS. Various approaches including antenatal steroids, administration of exogenous surfactant, and non-invasive and gentler modes of ventilation have shown only limited benefits in preventing RDS and BPD. Only antenatal steroid therapy has been consistently shown to enhance lung maturation and

decrease the incidence and severity of RDS. However, it is differentially more effective in females [15,16], is associated with significant side effects, and does not result in a reduction in BPD. In contrast, in experimental models, administration of PPAR γ agonists has been shown reduce lung injury and enhance fetal/neonatal lung maturation safely in both males and females [17,18].

One of the biggest impediments towards finding an effective preventive/therapeutic intervention against BPD has been a failure to target the fundamental cellular and molecular pathways centrally involved in lung development and injury repair [3]. Combining surfactant with a PPAR γ agonist aims to not only reduce the manifestations of RDS acutely, but also to recapitulate the natural cellular and molecular pathways that **i)** determine the normal lung structure, function, and homeostasis; **ii)** are disrupted by oxotrauma, inflammation, and volutrauma; and **iii)** have been shown to be normalized by PPAR γ agonists in multiple *in vitro* and *in vivo* models [4–7,18]. This approach is based on the paradigm that the developing lung mesenchyme, dominated by Wnt signaling, the default pathway for muscle development, inhibits C/EBP α and PPAR γ , thereby actively inhibiting the adipogenic programming of the developing alveolar mesenchyme [19]. During lung morphogenesis, the mesenchymal default Wnt pathway is inhibited and the adipogenic pathway is dis-inhibited, resulting in the formation of lipid-laden alveolar interstitial adipothelial fibroblasts~LIFs [19]. Lipofibroblasts are critical for lung homeostasis and injury repair, since they actively provide triglyceride substrate to ATII cells for surfactant synthesis, support ATII cell growth and differentiation, and act as an important defense against oxidant lung injury [19,20]. On exposure to volutrauma, oxotrauma, and infection, *pulmonary LIFs transdifferentiate to a myogenic phenotype, i.e., myofibroblasts (MYFs)* [18,21]. Myofibroblasts do not support ATII cell growth or differentiation, whereas LIFs do [20], hence the critical importance of maintaining the proper balance of LIFs and MYFs to direct lung development. Previous studies have demonstrated that all of the above mentioned BPD inducers cause downregulation of alveolar LIF PPAR γ expression [18], and thus inhibit normal lung development, but PPAR γ agonists mitigate and in some cases even reverse the lung damage [18,20–22]. However, it is important to point out that although we have focused on PPAR γ 's role in LIF biology, it also exerts a prominent injury repair effect via a variety of other mechanisms [23].

Other advantages of our approach include the potential delivery of the nebulized PGZ+B-YL formulation directly to the lung, which should result in maximum therapeutic effect, limit systemic side effects, and circumvent the issues pertaining to bioavailability and first-pass metabolism if administered orally. Furthermore, aerosolized delivery of PGZ+B-YL eliminates intubation and the related complications. However, detailed pharmacokinetics studies are needed to determine the bio-distribution of PGZ and B-YL in the lung, blood, and other organs and to determine if there are any systemic effects before the translation of the proposed approach to bedside.

As alluded to above, B-YL is an innovative surfactant developed specifically to resist hyperoxia-induced inactivation [24]. It is both stable and highly active in its native state and after addition of PGZ during *in vitro* testing (Fig. 2). Moreover, it has a low viscosity, aerosolizes easily, and can be produced inexpensively, all highly relevant attributes for its

translation to clinical practice. Since B-YL+ PGZ formulation effectively blocked lung injury, even following an extremely high O₂ exposure, i.e., 95% O₂ exposure for 72h, a scenario which is rarely used in contemporary neonatal practice, we are confident that it will also be effective against the usual lower levels of hyperoxia exposure frequently encountered in neonatal intensive care units. While PGZ was used as a prototype PPAR γ agonist in this study, other PPAR γ agonists might prove to be safer and even more effective than PGZ. It is also worth noting that the lung maturational effect of PPAR γ agonists is not restricted only to PGZ, but is also seen with other PPAR γ agonists such as curcumin, troglitazone, RGZ, and PGJ₂ [4–7,18,21,25].

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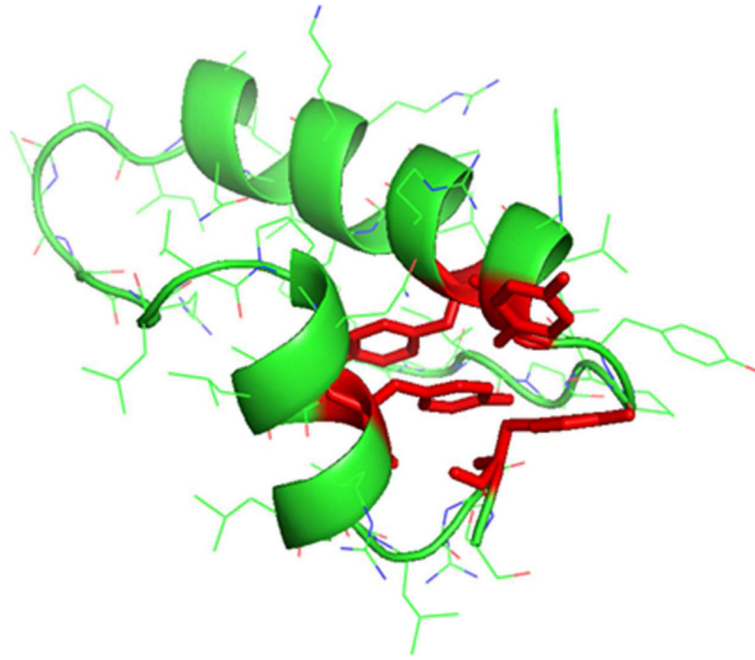


Fig. 1. Predicted molecular model of B-YL derived from the primary amino acid sequence using the homology templating program i-Tasser [26]

The secondary structure of the SP-B peptide mimic B-YL is that of a helix hairpin with the helical elements shown as green ribbons connected by a bend domain shown as a green tube. The helix hairpin structure is stabilized by the interaction of the tyrosine side chains in red highlight at the N and C terminal domains that emulate the disulfide interactions and the Saposin protein fold of the native SP-B sequence.

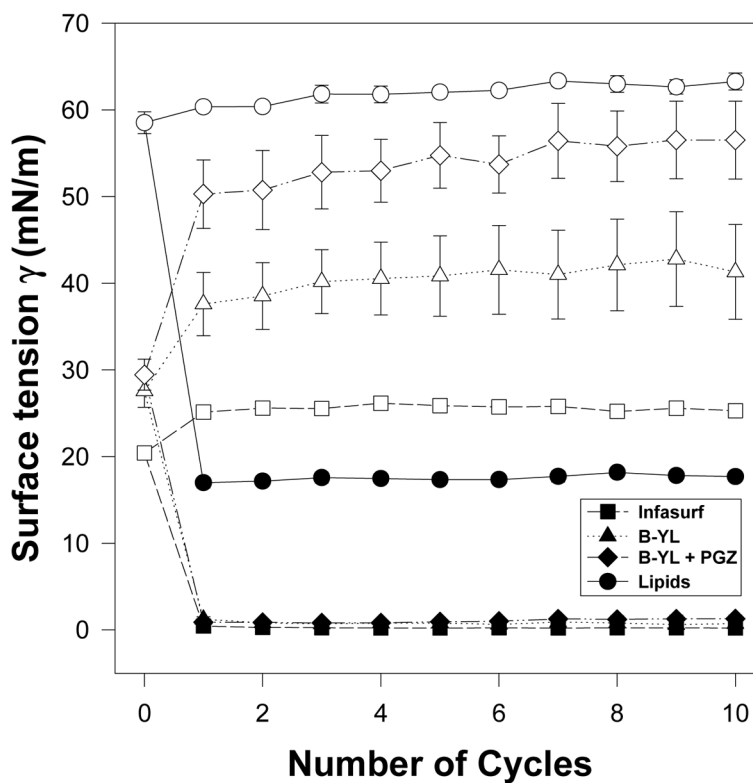


Fig. 2. Comparison of B-YL, PGZ+B-YL, lipids alone, and Infasurf® on surface tension reduction activity

Surface activity of the B-YL (100 mg/kg) was measured with captive bubble surfactometry in the presence and absence of PGZ (1 mg/kg) and compared with a clinical surfactant (Infasurf®) as positive control and lipids alone (DPPC:POPC:POPG 5:3:2 wt:wt:wt) as negative control. Minimum surface tension values are indicated with black symbols and maximum values with similar white symbols. Minimum surface tension values of B-YL surfactant \pm PGZ were similar to those of Infasurf®. Values are mean \pm SEM of N=4–5.

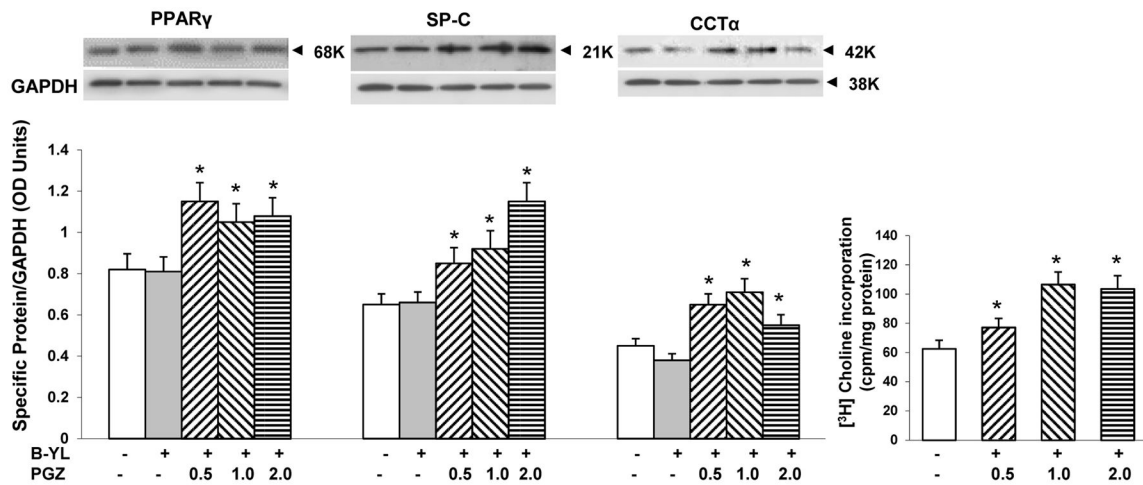


Fig. 3. Effect of B-YL on PGZ's PPAR γ agonist activity

Embryonic day 19 fetal rat lung explants, cultured for 24 h, under either control condition (without B-YL or PGZ supplementation) or treated with B-YL (100 mg/kg BW) alone or with PGZ (0.5, 1 or 2 mg/kg BW) + B-YL (100 mg/kg BW), demonstrated significant increases in PPAR γ , SP-C, and CCT- α protein levels, as determined by Western analysis, along with a significant increase in [3 H]choline incorporation into disaturated phosphatidylcholine ($p < 0.05$ vs. control; $N=3$).

Fig. 4 A

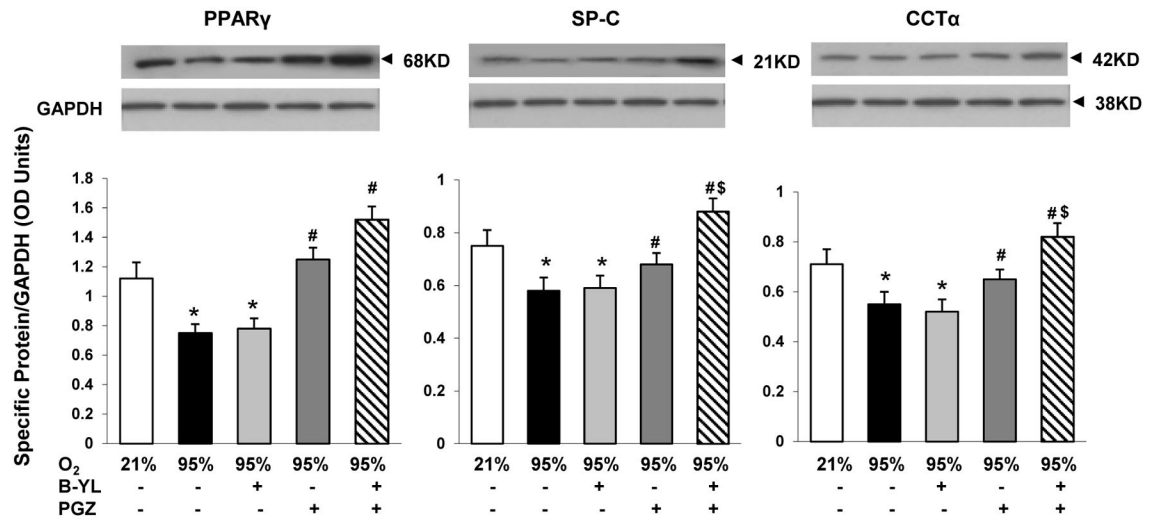


Fig. 4 B

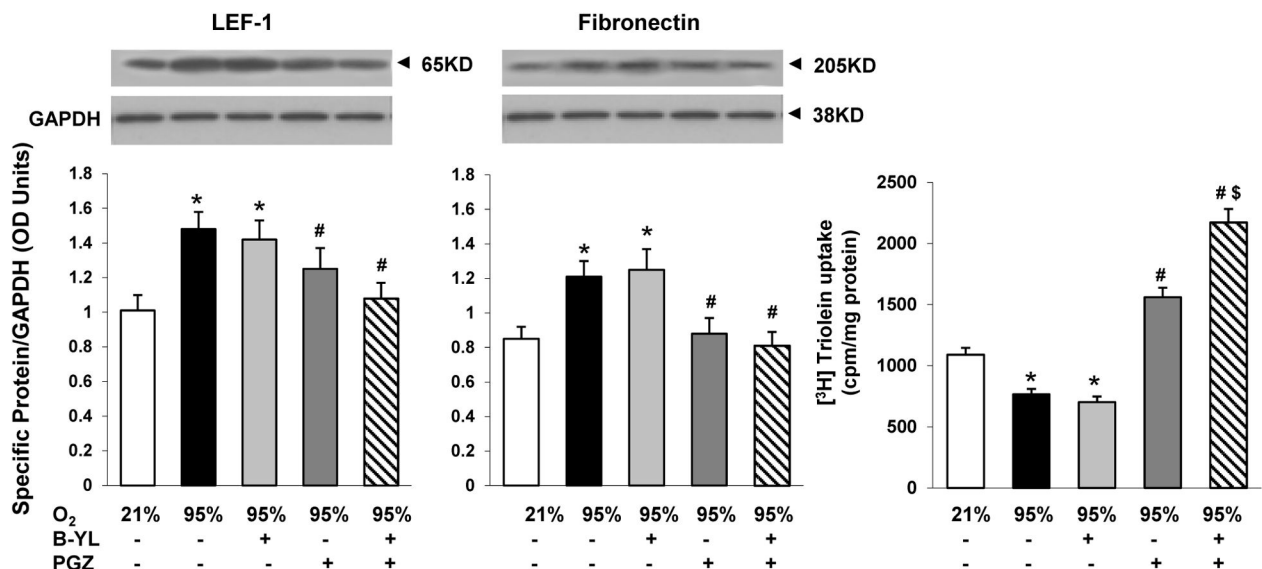


Fig. 4. Nebulized PGZ+B-YL blocks hyperoxia-induced alterations in selective markers of neonatal lung maturation more effectively than either modality alone

Postnatal day one rat pups were exposed to hyperoxia \pm B-YL, PGZ, or PGZ+B-YL for 72 h. Hyperoxia-induced decrease in PPAR γ (A), SP-C (B), CCT- α (C) and increase in LEF-1 (D) and Fibronectin (E) protein levels were blocked by concomitant administration of either PGZ or PGZ+B-YL. This was also true for hyperoxia-induced decrease in triolein uptake (F). PGZ+B-YL administration had a more robust effect, as evidenced by the effects on the protein levels of SP-C and CCT- α and the triolein uptake; however, B-YL administration alone had no effect on any of these parameters. *, $p < 0.05$ vs. control; #, $p < 0.05$ vs. 95% O₂; control; \$, $p < 0.05$ vs. PGZ; N=3.

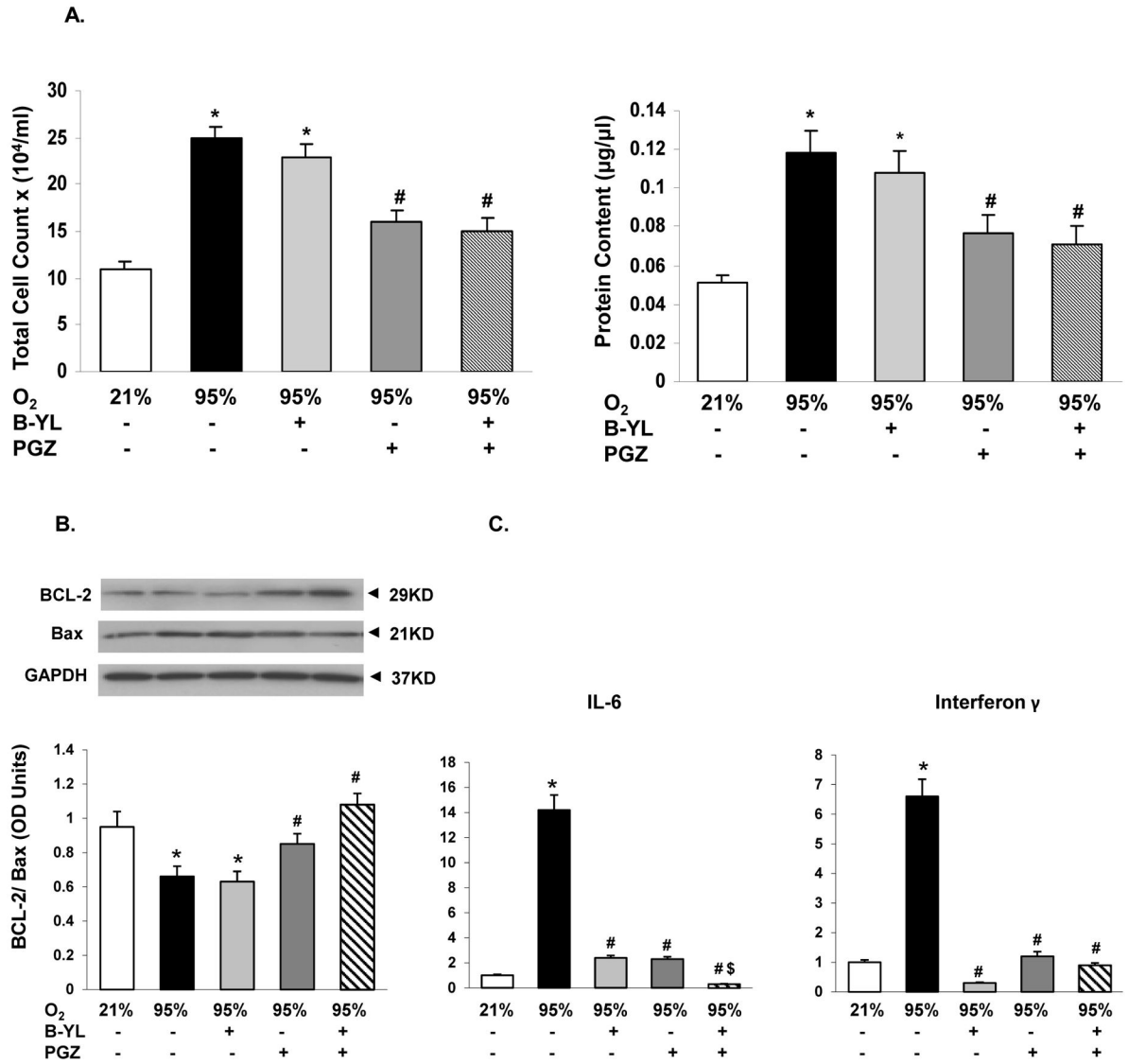


Fig. 5. Nebulized PGZ+B-YL blocks hyperoxia-induced alterations in selective markers of neonatal lung injury more effectively than either modality alone

Postnatal day one rat pups were exposed to hyperoxia \pm B-YL, PGZ, or PGZ+B-YL for 72 h. Hyperoxia-induced alterations in bronchoalveolar lavage total cell count(A) and protein content (B), apoptosis marker Bcl2/Bax ratio (C) and inflammatory markers IL-6 and interferon- γ (D) were blocked by concomitant administration of either PGZ or PGZ+B-YL.

*, $p < 0.05$ vs. cont; #, $p < 0.05$ vs. 95% O₂; cont; \$, $p < 0.05$ vs. PGZ N=3.

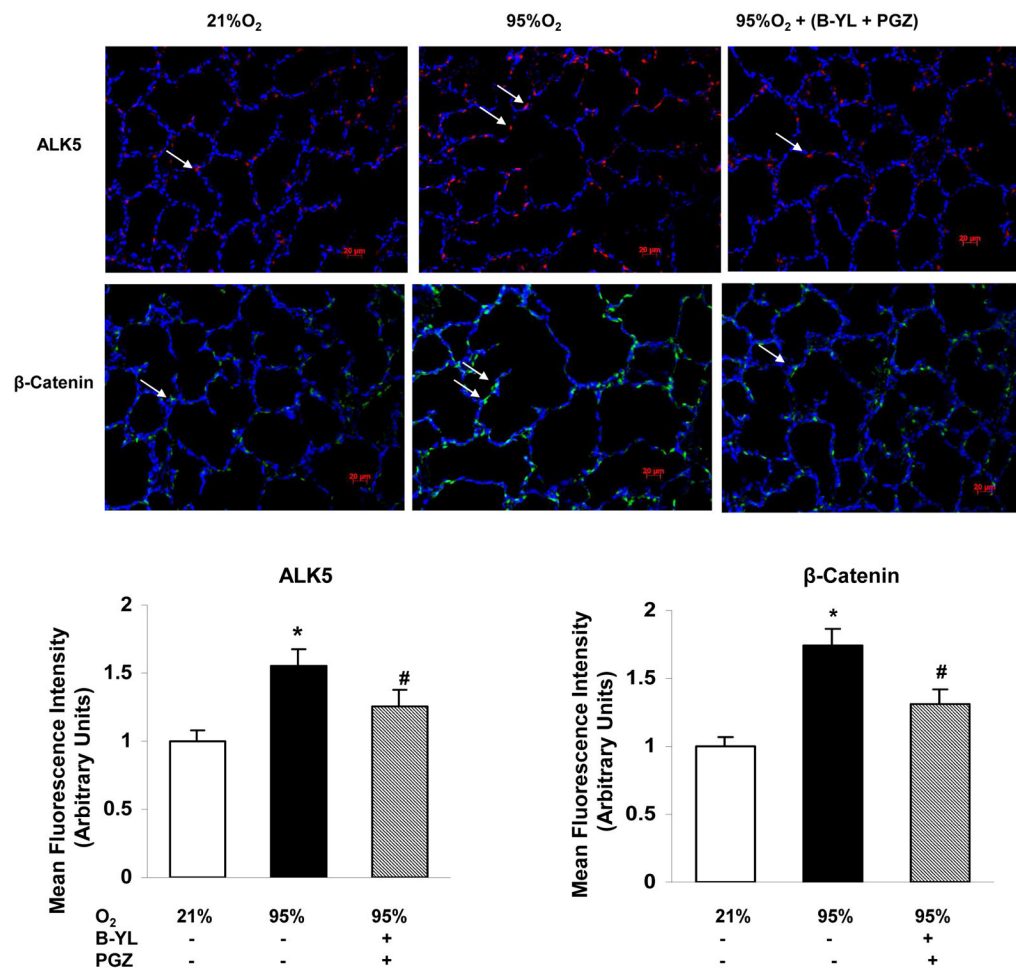


Fig. 6. Nebulized PGZ+B-YL blocks hyperoxia-induced activations of TGF- β and Wnt signaling pathways

Postnatal day one rat pups were exposed to hyperoxia \pm PGZ+B-YL for 72h. Hyperoxia-induced activation in TGF- β [determined by ALK5 protein levels, red staining] and Wnt (determined by β -catenin protein levels, green staining) signaling was blocked by concomitant PGZ+B-YL administration. Representative immunostaining pictures and relative mean fluorescence values are shown; N=3.