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Combination of pioglitazone, a PPARγ agonist, and synthetic surfactant B-YL prevents hyperoxia-induced lung injury in adult mice lung explants

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Abstract

Introduction: Hyperoxia-induced lung injury is characterized by acute alveolar injury, disrupted epithelial-mesenchymal signaling, oxidative stress, and surfactant dysfunction, yet currently, there is no effective treatment. Although a combination of aerosolized pioglitazone (PGZ) and a synthetic lung surfactant (B-YL peptide, a surfactant protein B mimic) prevents hyperoxia-induced neonatal rat lung injury, whether it is also effective in preventing hyperoxia-induced adult lung injury is unknown.

Method: Using adult mice lung explants, we characterize the effects of 24 and 72-h (h) exposure to hyperoxia on 1) perturbations in Wingless/Int (Wnt) and Transforming Growth Factor (TGF)-β signaling pathways, which are critical mediators of lung injury, 2) aberrations of lung homeostasis and injury repair pathways, and 3) whether these hyperoxia-induced aberrations can be blocked by concomitant treatment with PGZ and B-YL combination.

Results: Our study reveals that hyperoxia exposure to adult mouse lung explants causes activation of Wnt (upregulation of key Wnt signaling intermediates β-catenin and LEF-1) and TGF-β (upregulation of key TGF-β signaling intermediates TGF-β type I receptor (ALK5) and SMAD 3) signaling pathways accompanied by an upregulation of myogenic proteins (calponin and fibronectin) and inflammatory cytokines (IL-6, IL-1β, and TNFα), and alterations in key endothelial (VEGF-A and its receptor FLT-1, and PECAM-1) markers. All of these changes were largely mitigated by the PGZ + B-YL combination.

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Author contributions

Conceptualization & Methodology: Virender K. Rehan; Investigation/Data Analysis/Visualization: Chie Kurihara, Reiko Sakurai, Tsai-Der Chuang; Interpretation of results: Chie Kurihara, Reiko Sakurai, Tsai-Der Chuang, Frans J. Walther, Virender K. Rehan; Writing-original draft preparation: Chie Kurihara, Virender K. Rehan; Writing-review and editing: Chie Kurihara, Alan J. Waring, Frans J. Walther, Virender K. Rehan. All Authors read and approved the final manuscript.

Declaration of competing interest

Authors Drs. Rehan, Walther, and Waring hold a patent on the composition and methods of administering PPARγ agonists, surfactant peptides, and phospholipids for preventing and treating neonatal lung injury (Patent #:20220047681). No other competing interest or conflict of interest to declare. All authors completed the ICMJE Form.

Conclusion: The effectiveness of the PGZ + B-YL combination in blocking hyperoxia-induced adult mice lung injury ex-vivo is promising to be an effective therapeutic approach for adult lung injury in vivo.

Keywords

Hyperoxia; Lung injury; PPARγ; Pioglitazone; Surfactant

1. Introduction

Acute lung injury (ALI) including its severe form, i.e., acute respiratory distress syndrome (ARDS), are serious conditions that are often life-threatening. However, currently, there is no effective treatment for both, which presently consists of providing only supportive respiratory care instead of treating the underlying cause [1]. Part of the reason for the failure in developing an effective treatment for ALI/ARDS has been the failure to target key molecular signaling pathways underlying ALI/ARDS pathogenesis. ALI/ARDS can result from a direct lung injury, e.g., pneumonia, exposure to supra-physiologic oxygen concentrations (hyperoxia), inhalation of cigarette smoke and other toxic substances, or from an indirect lung injury, e.g., sepsis and severe trauma with shock. We used the hyperoxia model since under deteriorating lung function in ALI/ARDS from all causes, oxygen supplementation is frequently needed, which by itself causes oxidative tissue damage. ALI/ARDS is characterized by alveolar injury, disrupted epithelial-mesenchymal signaling, oxidative stress, and surfactant dysfunction [2-5]. Molecularly, activation of TGF-β and Wnt signaling, inhibition of peroxisome proliferator-activated receptor-gamma (PPARγ) signaling, and inactivation of surfactant activity by reactive oxygen species are critical components of ALI/ARDS pathogenesis that have not been targeted concurrently.

We previously demonstrated that in the developing lung a combination of nebulized pioglitazone (PGZ), a PPARγ agonist, and a novel synthetic lung surfactant with B-YL peptide as surfactant protein B (SP–B) mimic prevents hyperoxia-induced neonatal lung injury better than either modality alone [6]. Whether this strategy is also effective in adult lung injury is not known. To address this question, we hypothesized that by blocking Wnt and TGF-β signaling pathways' activations, which are critical mediators of hyperoxia-induced lung injury and surfactant supplementation simultaneously, combined PGZ and B-YL treatment would block hyperoxia-induced adult lung injury.

2. Materials and methods

2.1. Synthetic B-YL peptide surfactant

B-YL surfactant consists of 3% of synthetic SP-B peptide mimic B-YL formulated in a mixture of synthetic surfactant lipids (dipalmitoyl-phosphatidylcholine (DPPC): palmitoyl-oleoyl-phosphatidylcholine (POPC): palmitoyl-oleoyl-phosphatidylglycerol (POPG) 5:3:2 by weight). B-YL is sulfur-free and oxidant resistant [6,9]. B-YL was created using the N-terminal and C-terminal alpha helical sequences of native surfactant protein B connected with a short beta sheet loop and replacing cysteine and methionine residues with tyrosine and leucine to minimize inactivation by oxidative stress. B-YL peptide was produced

using solid phase peptide synthesis, followed by purification with high-performance liquid chromatography. Various studies have shown that synthetic B-YL surfactant is highly surface active, stable and resistant to inactivation by oxidative stress. Surface tension lowering ability of the B-YL and PGZ in the dose used in this study, assessed using captive bubble surfactometry was equal to the clinical surfactant Infasurf [6]. The combination with PGZ does not affect the bioactivity of B-YL surfactant, which has a low viscosity, is easy to aerosolize, and can be easily produced at low cost [7,8]. These characteristics are essential in the development of an effective treatment of acute lung injury and determined our preference of B-YL surfactant over currently available clinical surfactants.

2.2. Lung explant hyperoxia exposure model

All animal procedures were performed following the National Institutes of Health guidelines for the care and use of laboratory animals and approved by The Lundquist Institute Animal Care and Use Committee (IACUC approval number 31355–01) and comply with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Adult male (3 to 6-month-old) C57BL/6 mice were purchased from Charles River Laboratories and housed in humidity- and temperature-controlled rooms on a 12 h (h) light and 12 h dark cycle and allowed food and water ad libitum. These mice were sacrificed using euthasol (sodium pentobarbital 390 + 50 mg/ml phenytoin; Virbac Animal Health) to harvest lungs and placed in 15% fetal bovine serum (FBS)/Waymouth medium on ice. Three mice per experiment were used, which was replicated at least 3 times. Lungs were cut into ~1-mm cubes aseptically and were pooled, randomized, and cultured in six-well plates with 15% FBS/Waymouth medium while rocking on an oscillating platform (3 cycles/min). Lungs were incubated at 37 °C simultaneously to minimize potential confounders under one of the following four conditions: 1) untreated control, 2) B-YL (100 mg/kg), 3) PGZ (1 mg/kg), and 4) B-YL (100 mg/kg) + PGZ (1 mg/kg); both B-YL and PGZ dosages were based on previously used doses [6]. Lung explants were exposed to either 21%O2 (normoxia) or 95%O2 (hyperoxia) for 24 or 72 h using sealed chambers (Billups-Rothenberg Inc., Del Mar, CA).

At 24 or 72 h, lungs were harvested and snap-frozen in liquid nitrogen and processed for western blotting and real-time quantitative reverse transcription PCR (q-RT-PCR) for lung homeostasis, injury repair, and inflammatory markers.

2.3. Protein extraction and western blotting

Liquid nitrogen flash-frozen lungs were homogenized with tissue homogenizer (Fisher Scientific, Pittsburg, PA) in Radio-immunoprecipitation assay lysis buffer containing 1 mM Ethyl-enediaminetetraacetic acid and egtazic acid (Boston Bioproducts, Ashland, MA), 1 mM phenylmethylsulfonyl fluoride, and complete proteinase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Fifty micrograms of total protein for each sample were denatured using sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer and electrophoresed in 10% SDS polyacrylamide gel. Next, samples were transferred onto 0.45 μm nitrocellulose membrane and blocked with 5% milk in tris-buffered saline-Tween and then probed with primary antibodies [surfactant

protein-C (SP–C; 1:200, cat#SC-13979), CTP:phosphocholine cytidylyltransferase alpha (CCTα; 1:200, cat#SC-376107), CCAAT/enhancer-binding protein alpha (C/EBPα; 1:150, cat#SC-365318), BCL-2 associated X protein (BAX; 1:150, cat#SC-493), β-catenin (1:2500, cat#SC-7963) (all from Santa Cruz Biotechnology, Santa Cruz, CA), lymphoid enhancer binding factor 1 (LEF-1; 1:800, cat#14972), B-cell lymphoma 2 (BCL-2; 1:500, cat#26593), SMAD3 (1:800, cat#25494-1-AP), SMAD7 (1:800, cat#25840-1-AP), Fibronectin (1:3000, cat#15613), PPARγ (1:1000, cat#16643) (all from Proteintech, Rosemont, IL), calponin (1:2500, cat#2687, MilliporeSigma, Burlington, MA), activin receptor-like kinase 5 (ALK5; 1:500, cat#AB31013, Abcam, Cambridge, MA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:3000, cat#MAB374, MilliporeSigma, Burlington, MA)] overnight at 4 °C followed by appropriate secondary antibody and Pierce Super-Signal Chemiluminescent substrate (Thermo Fischer Scientific, Rancho Palos Verdes, CA). ImageJ software (National Institutes of Health, Bethesda, MD) was used to quantitate protein bands. Bands were measured by densitometry and expressed relative to accompanying GAPDH bands as relative units.

2.4. RNA isolation and qRT-PCR

Tissue RNA extraction and qRT PCR were performed according to the previously described method [10]. Primer sequences were: TGF- β1 (sense5′-CGAAGCGGACTACTATGCTAAA-3’; anti-sense5′-TCCCGAATGTCTGACGTATTG-3′), TGF- β3 (sense5′-CGCTACATAGGTGGCAAGAA-3’; anti-sense5′-CAAGTTGGACTCTCTCCTCAAC-3′), tumor necrosis factor alpha (TNFα; sense5′-TTGTCTACTCCCAGGTTCTCT-3’; anti--sense5′-GAGGTTGACTTTCTCCTGGTATG-3′), IL-6 (sense5′-GAGTGGCTAAGGACCAAGACC-3’; anti-sense5′-AACGCACTAGGTTTGCCGA-3′), IL-1β (sense5′-AGCTTCAGGCAGGCAGTATC-3’; anti-sense5′-AAGGTCCACGGGAAAGACAC-3); platelet endothelial cell adhesion molecule 1 (PECAM-1; sense5′-CACCCATCACTTACCACCTTATG-3’; anti-sense5′-TGTCTCTGGTGGGCTTATCT-3′), vascular endothelial growth factor receptor-1 (FLT-1; sense5′-CAACGTCACAGTCACCCTAAA-3’; anti--sense5′- CTCTCCTACTGTCCCATGTTATTC-3′), vascular endothelial growth factor (VEGF-A; sense5′-GCAGACCAAAGAAAGACAGAAC-3’; anti-sense5′-CAGTGAACGCTCCAGGATTTA-3′). Normalization control was Tuba1a: 5′-CTCTCTGTGGATTACGGAAAGAAG-3’ (forward), 5′-GGTGGTGAGGATGGAATTGTAG-3’ (reverse) [11]. All reactions were run in triplicate; relative expression was determined using the comparative cycle threshold method (2–ΔΔCT), recommended by the supplier (Applied Biosystems). Abundance values were expressed as fold changes compared with the corresponding control group.

2.5. Statistical analysis

Student t-test and analysis of variance (ANOVA), as appropriate, were used to detect group differences and a p-value of <0.05 was considered statistically significant (N = 3 mice/group, experiments repeated three times). The results are expressed as means± SEM; data are shown using GraphPad Prism software.

3. Results

3.1. Effect of PGZ and B-YL on lung homeostasis markers

We determined the effect of PGZ and B-YL under normoxic conditions on key epithelial (SP–C and CCTα) and mesenchymal (PPARγ) markers of lung homeostasis. With PGZ + B-YL treatment, SP-C and CCTα protein levels increased at both 24 and 72 h time points (Fig. 1a and b).

PGZ alone treatment also increased CCTα at both time points, while SP-C protein levels were increased only at 72 h. For both CCTα and SP-C, there was a trend towards a more pronounced increase with PGZ + B-YL versus PGZ alone. In contrast, although PPARγ protein levels were not affected by any treatment at 24 h, these increased equally with PGZ alone and PGZ + B-YL treatments at 72 h. Taken together, the effects of PGZ and B-YL treatment on lung homeostasis markers in adult lungs are like those seen in neonatal lungs [6].

3.2. Effect of PGZ and B-YL surfactant on hyperoxia-induced activation of TGF-β and Wnt signaling pathways

Since TGF-β and Wnt signaling activations are critical mediators of injury repair response in almost all organs, including the lung, we next evaluated activations of these pathways in hyperoxia-induced adult lung injury and how these are affected by various treatments. At 72 h time point, the levels of the key canonical Wnt pathway proteins β-catenin and LEF-1 increased with hyperoxia, and this increase was attenuated with PGZ alone and PGZ + B-YL combined treatments (Fig. 2a). Similarly, hyperoxia exposure activated TGF-β signaling as evidenced by increased protein levels of ALK 5 and SMAD 3 (Fig. 2b). Concomitant PGZ + B-YL treatment blocked increases in both proteins, while PGZ alone blocked the increase in ALK5 only. Although there was a trend towards an increase in SMAD 7 protein levels with hyperoxia exposure with or without any treatment, it did not reach statistical significance in any experimental group. The activation of TGF-β on exposure to hyperoxia and its blockage by PGZ + B-YL was also supported by the mRNA levels of TGF-β1 at 24 and 72 h and TGF-β3 at 24 h (Fig. 2c).

We next examined protein levels of lung myogenic proteins calponin and fibronectin, both of which are targets of TGF-β and Wnt signaling. Hyperoxia-induced increase in protein levels of both calponin and fibronectin was blocked by PGZ alone and PGZ + B-YL treatments (Fig. 3).

Since PPARγ signaling antagonizes Wnt and TGF-β activations under both homeostatic and injury repair conditions [12], we next determined the protein levels of key PPARγ signaling intermediates PPARγ and C/EBPα (Fig. 4).

On exposure of adult mouse lung explants to hyperoxia, C/EBPα protein level decreased significantly; however, in contrast to studies using several other models of lung injury, PPARγ protein levels did not decrease. Importantly, nevertheless, compared to controls, PGZ + B-YL treatment increased PPARγ protein levels. Notably, both PGZ and PGZ + B-YL treatments blocked hyperoxia-induced decreases in C/EBPα levels.

3.3. Effect of PGZ and B-YL on hyperoxia-induced alterations of inflammatory cytokines and cellular apoptosis

Hyperoxia exposure resulted in significant alterations in inflammatory cytokines (IL-6, IL-1β, and TNFα), which are known mediators of lung injury (Fig. 5a). Following 72 h exposure to hyperoxia, expression of all inflammatory cytokines examined was significantly increased, and this increase was either blocked (IL-6, IL-1β in PGZ only and PGZ + B-YL treated groups) or showed an improving trend (TNFα in the PGZ + B-YL treated group). Additionally, as reflected by the protein levels of BCL-2 and BAX (Fig. 5b), exposure to hyperoxia increased apoptosis. Per other data on the protective effects of PGZ and PGZ + B-YL, the hyperoxia-induced increase in apoptosis was also blocked by PGZ only and PGZ + B-YL treated groups.

Lastly, as shown in Fig. 5c, mRNA expression of endothelial markers showed an increase in VEGF-A, VEGF-receptor FLT-1, and PECAM-1 in 24 h hyperoxia exposure group. This increase was blocked in all treatment groups with the most pronounced effect in the PGZ + B-YL treated group. However, at 72 h, the effect of hyperoxia on endothelial markers was variable, i.e., an increase in VEGF-A, a decrease in FLT-1, and no change in PECAM expression, with an inconsistent effect of interventions.

4. Discussion

Acute lung injury and ARDS are serious conditions, which can be life-threatening, yet there is no effective treatment. This ex-vivo study confirmed activations in TGF-β and Wnt signaling pathways in adult lung injury. Importantly, it showed that hyperoxia-induced activations in these pathways in adult lung injury were effectively blocked by combined treatment with PGZ and B-YL. The PGZ + B-YL combination also attenuated the hyperoxia-induced increases in inflammatory cytokines, cellular apoptosis, myogenic protein levels, and alterations in endothelial injury markers. Additionally, PGZ + B-YL treatment increased protein levels of PPARγ, SP-C, and CCTα in normoxia, indicating its pro-homeostatic effect. Although PGZ alone also had significant lung protectant and pro-homeostatic effects, overall, these effects were more pronounced with PGZ + B-YL combination versus PGZ alone. These data suggest the possible effectiveness of the PGZ + B-YL combination in blocking hyperoxia-induced adult lung injury, likely mediated by blocking TGF-β and Wnt signaling activations, both of which are critical mediators of adult lung injury.

As supported by multiple human and animal studies, TGF-β activation plays a pivotal role in injury repair in almost all organs, including the lung [13-15]. In addition, activation of Wnt signaling in adult lung injury is supported by numerous ALI models and ARDS patients [16-18]. Importantly, TGF-β activation and its interaction with canonical Wnt pathway intermediates disrupts the homeostatic epithelial-mesenchymal interactions [6,19-23]. Although a complex network of signaling molecules is involved in these interactions, the primary TGF-β signaling intermediates include ALK 5, and SMAD 3 and 7. The major Wnt signaling pathway intermediates include Wnt receptors, Frizzled and lipoprotein receptor-related proteins 5 and 6, and other intracellular mediators such as Disheveled, Axin, APC, and glycogen synthase kinase 3β. Interactions of these intermediates lead to β-catenin

stabilization, resulting in its nuclear translocation where it binds to LEF-1 to induce target gene transcription. Therefore, in this study, we examined key signaling molecules of TGF-β (ALK 5, SMAD 3 and 7) and Wnt (β-catenin and LEF-1) pathways and their downstream targets, including cellular apoptosis (BCL-2 and BAX), inflammatory markers (IL-6, IL-1β, and TNFα), myogenic markers (fibronectin and calponin) and endothelial markers (VEGF-A, FLT-1, and PECAM-1). In addition, the expression of key Wnt pathway antagonists PPARγ and C/EBPα was determined.

In line with our prior study that examined neonatal rat lung injury [6], in this study, using adult mouse lung explants, we observed that hyperoxia-induced TGF-β activation (upregulation of ALK 5 and SMAD 3 protein levels) was effectively blocked by PGZ + B-YL combination. However, in contrast to our previous study, the increase in SMAD 7 protein level, which is an autoinhibitory response to TGF-β activation, was not observed; this may be due to a relatively shorter duration of hyperoxia exposure (72 h) in this study versus the longer (7-day) exposure in the previous study and/or a relatively sample size (N = 3/group).

Furthermore, interestingly, in contrast to hyperoxia-induced PPARγ downregulation in the neonatal lung rat model, in adult mouse lung explants, PPARγ protein levels were unaffected at both time points examined, suggesting that alterations in TGF-β and Wnt signaling pathways play a more dominant role in the adult lung injury than in neonatal lung injury. Nevertheless, the evidence from numerous adult organ injury models, including the adult lung, points to antagonistic effects of PPARγ and Wnt signaling pathways [12,24,25]. Therefore, it is not surprising that upregulation of PPARγ signaling (increases in PPARγ and C/EBPα protein levels) with both PGZ alone and PGZ + B-YL combined treatments was associated with blockage of hyperoxia-induced activation in Wnt signaling (increased β-catenin and LEF-1 protein levels) and its downstream myogenic targets such as fibronectin and calponin. Hyperoxia-induced increases in inflammatory cytokines and apoptosis were also effectively blocked by the PGZ + B-YL combination at 72 h but not at 24 h, which may be a function of treatment duration. Regarding the effects of hyperoxia on pulmonary endothelial markers, unlike our findings, hyperoxia has been shown to decrease VEGF and FLT-1 levels in other lung injury models. This difference also might be due to a relatively shorter duration of hyperoxia exposure and the use of lung explants in the current study versus previous in-vivo studies [26,27]. However, similar to our observation, upregulation of PECAM-1 has been reported in hyperoxia-induced lung injury in an adult mouse model [28]. It may be essential to note that since the lung injury and its repair response is quite complex that involves multiple pathways and numerous intermediates, the differential beneficial effects of PGZ + B-YL observed at 24 h and 72 h time points for some of the markers examined are not entirely surprising. However, it is reassuring that the beneficial trends were observed at both time points.

We chose to study the hyperoxia model since oxygen supplementation is commonly used in most patients hospitalized with ALI [12] which by itself causes oxidative tissue damage. Although both surfactant and PPARγ agonist treatments have been tried individually in various ALI/ARDS models, the benefits of either drug alone are limited. For example, exogenous surfactant treatment in patients with ALI/ARDS significantly lowered inspired

oxygen need; however, the mortality did not decrease [29]. The lack of mortality benefit may be explained, at least partially, due to only a limited delivery of surfactant to the distal lung in these studies [30]. In addition, although PPARγ agonist administration has been shown to ameliorate pulmonary inflammation, e.g., a decrease in neutrophil influx and tissue injury in a lipopolysaccharide-induced lung injury model [31], the sole treatment with PGZ did not benefit endotoxin-induced lung inflammation in humans [31,32].

To promote injury repair and block lung surfactant dysfunction and oxidant damage simultaneously, we combined PGZ with a novel synthetic surfactant B-YL. This combined PGZ + B-YL approach targets key signaling pathways disrupted in ALI/ARDS and provides an oxidant-resistant surfactant B-YL. B-YL is an innovative surfactant developed to resist hyperoxia-induced inactivation; it is relatively stable and highly active in its native form. We previously demonstrated that combining B-YL with PGZ does not affect B-YL's surfactant bioactivity, and B-YL does not affect PGZ's PPARγ agonist activity. Moreover, when delivered via nebulization, combining B-YL with PGZ improved PGZ's delivery by about 30% versus PGZ delivered alone [6]. Although the ex vivo design and a relatively small sample size/group are important limitations of this study, our findings provide proof of the possible efficacy of combined PGZ + B-YL treatment to mitigate lung injury when administered in vivo.

5. Conclusions

In summary, using an adult mouse lung model, this study demonstrates that combined PGZ and B-YL treatment mitigates hyperoxia-induced activation of TGF-β and Wnt signaling and the accompanying increases in inflammatory cytokines, myogenic proteins and cellular apoptosis. Although PGZ alone also exhibited significant protective effects, overall, the effects were more pronounced with the PGZ + B-YL combination versus PGZ alone. The effectiveness of the PGZ + B-YL combination in blocking hyperoxia-induced adult lung injury ex-vivo begs us to test this promising therapeutic approach in-vivo.

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Data availability

Data will be made available on request.

Abbreviations:

PGZ	Pioglitazone
Wnt	Wingless/Int
TGF	Transforming Growth Factor
ALI	Acute lung injury

ARDS	acute respiratory distress syndrome
PPARγ	peroxisome proliferator-activated receptor-gamma
SP-B	surfactant protein B
DPPC	dipalmitoyl-phosphatidylcholine
POPC	palmitoyl-oleoyl-phosphatidylcholine
POPG	palmitoyl-oleoyl-phosphatidylglycerol
ARRIVE	Animal Research: Reporting of In Vivo Experiments
h	hour
FBS	fetal bovine serum
qRT-PCR	real-time quantitative reverse transcription PCR
SP-C	surfactant protein C
CCTα	CTP:phosphocholine cytidylyltransferase alpha
C/EBPα	CCAAT/enhancer-binding protein alpha
BCL-2	B-cell lymphoma 2
BAX	BCL-2 associated X protein
LEF-1	lymphoid enhancer binding factor 1
ALK5	activin receptor-like kinase 5
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
TNFα	tumor necrosis factor alpha
PECAM-1	platelet endothelial cell adhesion molecule 1
FLT-1	vascular endothelial growth factor receptor-1
VEGF	vascular endothelial growth factor
ANOVA	analysis of variance

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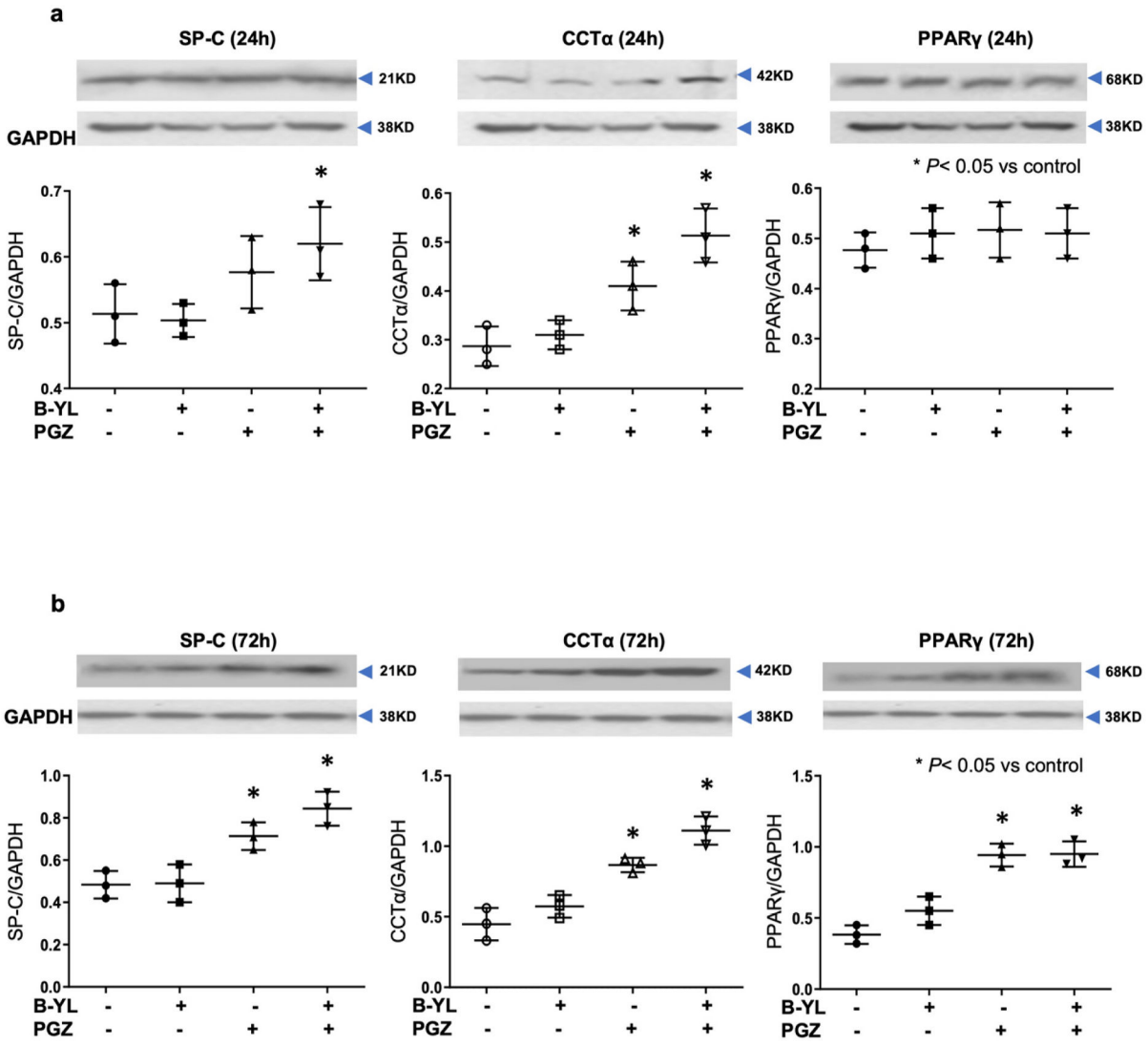

Fig. 1. Effect of PGZ, B-YL, and PGZ + B-YL on lung homeostasis.

Adult mice lung explants were cultured in normoxia for **a)** 24 h or **b)** 72 h in either control medium or medium treated with B-YL (100 mg/kg), PGZ (1 mg/kg), or B-YL (1 mg/kg) + PGZ (100 mg/kg) to determine effects on epithelial (SP–C and CCTα) and mesenchymal (PPARγ) markers of lung homeostasis. There was an increase in SP-C and CCTα, as determined by Western analysis when treated with PGZ + B-YL for 24 or 72 h. With PGZ-only treatment, the SP-C protein level was not affected at 24 h but increased at 72 h; CCTα protein levels increased at both 24 and 72 h time points. Although PPARγ protein was not affected at 24 h, at 72 h, it increased with either PGZ-only or PGZ + B-YL treatment. Values are means ± SE (*$p < 0.05$ vs control: N = 3).

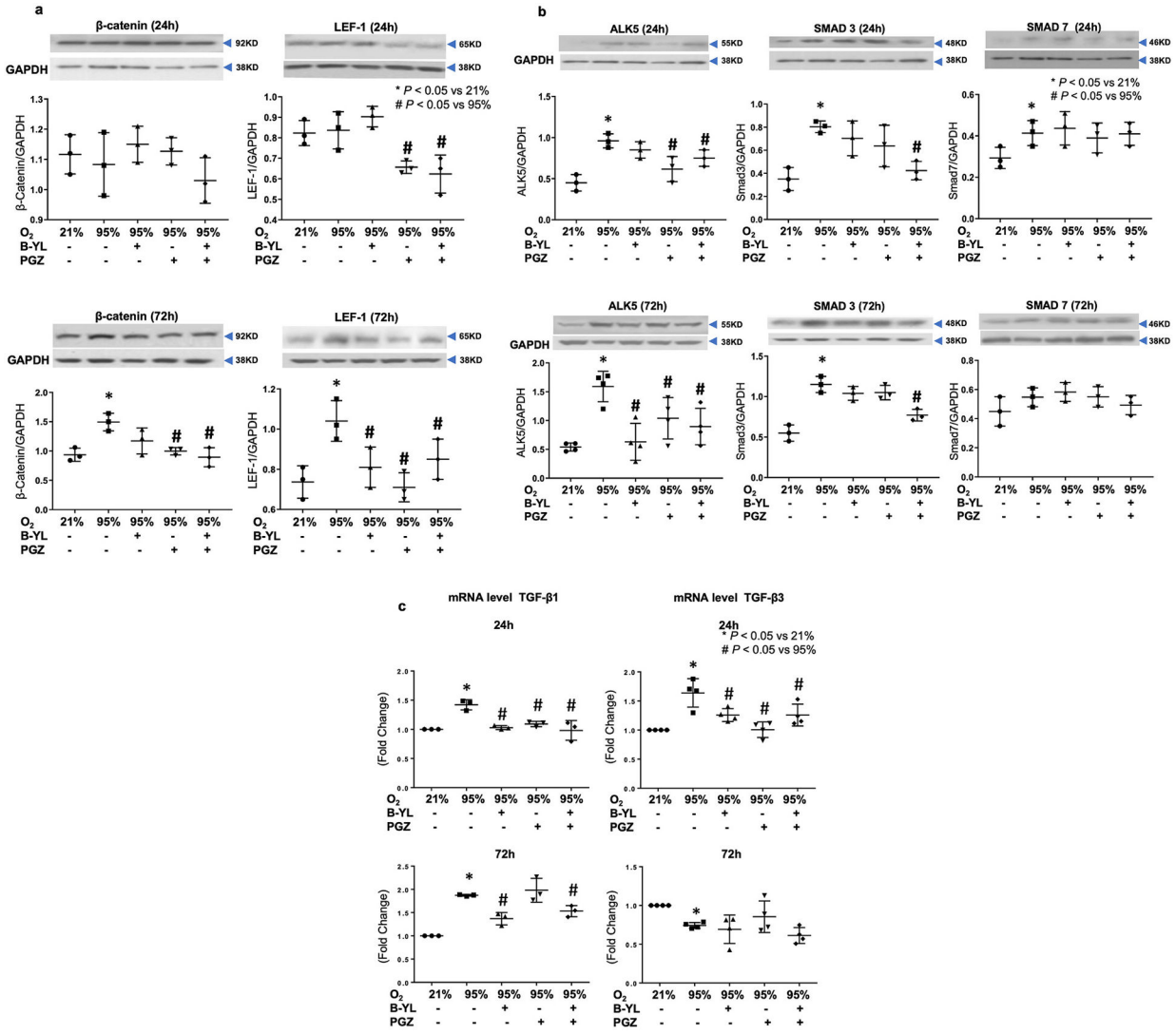

Fig. 2. Effect of PGZ, B-YL, and PGZ + B-YL on hyperoxia-induced activation of Wnt (β-catenin and LEF-1) and TGF-β (ALK5 and SMAD 3) signaling pathways.

Adult mice lung explants were cultured in normoxia or hyperoxia ± B-YL, PGZ, or PGZ + B-YL for 24 or 72 h. **a)** Western analysis was used to measure β-catenin levels, which were unchanged in all groups at 24 h but increased with hyperoxia and this increase ameliorated with PGZ-only and PGZ + B-YL treatments at 72 h. LEF-1 protein level was not affected following 24 h exposure to hyperoxia, but was decreased in PGZ-only and PGZ + B-YL treated groups. At 72 h, the LEF-1 protein level increased in the hyperoxia exposure group, and this increase was blocked in all treatment groups. **b)** ALK5 and SMAD 3 protein levels increased in the hyperoxia exposure groups, and this effect was ameliorated by concurrent treatment with PGZ + B-YL for 24 and 72 h. However, at 72 h, with PGZ-only treatment, ALK5 protein level decreased, while no effect was seen on SMAD 3 protein levels. There was an increase in SMAD 7 protein level with hyperoxia exposure at 24 h, an effect that was not impacted with any treatment. **c)** Using qRT-PCR, TGF-β1 and β3 mRNA expression was determined. TGF-β1 expression increased at both 24 h and 72 h time-points following exposure to hyperoxia; however, TGF-β3 expression was increased only at the 24 h time-

point, which was decreased in treatment groups. However, there was a decrease in TGF-β3 with hyperoxia at 72 h time-point with no changes in the treated groups. Representative protein bands and densitometric values relative to the GAPDH of each group are shown. Values are means ± SE (*$p < 0.05$ vs 21% O2, and #$p < 0.05$ vs 95% O2 control: N = 3).

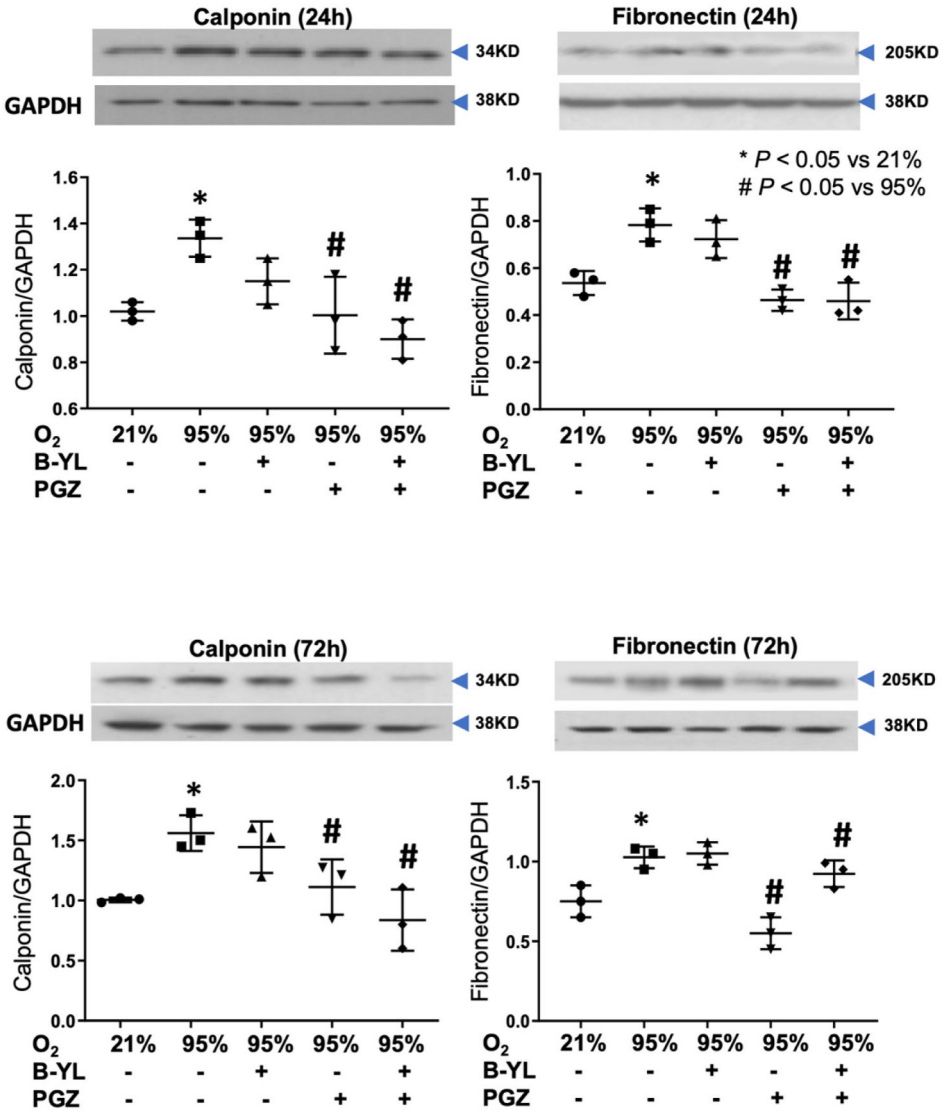

Fig. 3. Effect of PGZ, B-YL, and PGZ + B-YL on hyperoxia-induced changes in lung myogenic proteins.

Western analysis was used to measure calponin and fibronectin protein levels. Calponin was increased on exposure to hyperoxia, and this increase was blocked with PGZ-only and PGZ + B-YL treatment groups at both 24 h and 72 h time points. Fibronectin was increased on exposure to hyperoxia at 24 h and 72 h time points. The hyperoxia-induced increase in fibronectin protein level was ameliorated with PGZ-only and PGZ + B-YL treated groups at both time points. Representative protein bands and densitometric values relative to the GAPDH of each group are shown. Values are means ± SE (*p < 0.05 vs 21% O_2, and #p < 0.05 vs 95% O_2 control: N = 3).

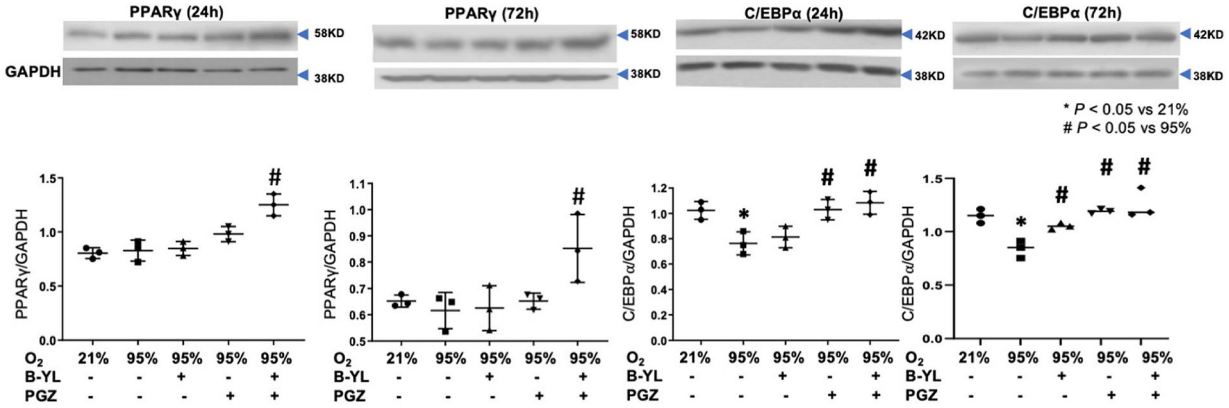

Fig. 4. Effect of PGZ, B-YL, and PGZ + B-YL on hyperoxia-induced changes in PPARγ and C/EBPα protein levels.

Exposure to hyperoxia did not alter PPARγ protein levels at either 24 or 72 h timepoint, but these levels increased with PGZ + B-YL treatment at both time points. C/EBPα protein level decreased on exposure to hyperoxia, and this decrease was blocked by PGZ and PGZ + B-YL treatments at 24 and 72 h. Representative protein bands and densitometric values relative to the GAPDH of each group are shown. Values are means±SE; n = 3. (*p < 0.05 vs 21% O2, and #p < 0.05 vs 95% O2 control; N = 3).

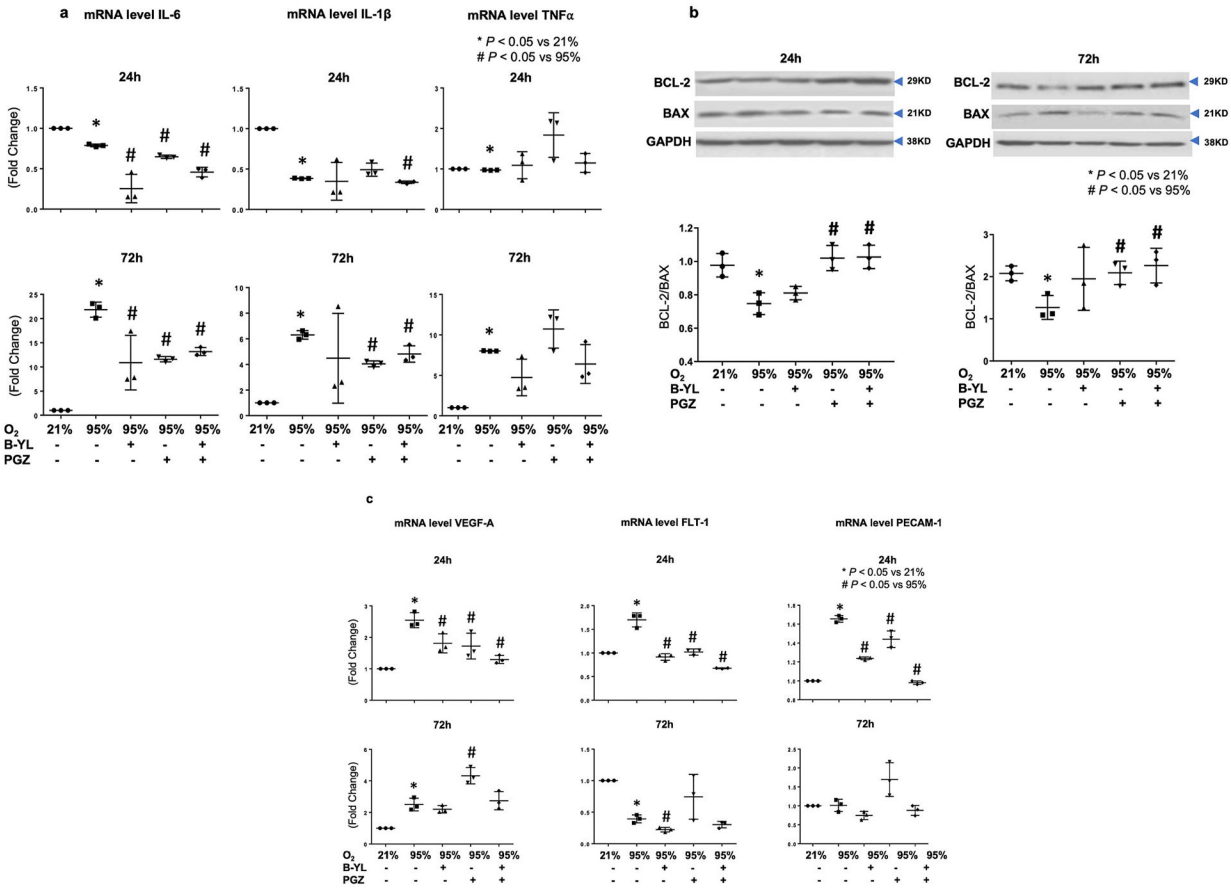

Fig. 5. Effect of PGZ, B-YL, and PGZ + B-YL on hyperoxia-induced changes in inflammatory and apoptosis markers.

Adult mice lung explants were exposed to hyperoxia ± B-YL, PGZ, or PGZ + B-YL for 24 h or 72 h. qRT-PCR was used to measure inflammatory cytokines and Western analysis for apoptosis. **a)** Following 72 h exposure to hyperoxia, expression of all inflammatory cytokines examined was significantly increased, and this increase was either blocked (IL-6, IL-1β in PGZ only and PGZ + B-YL treated groups) or showed an improving trend (TNFα in the PGZ + B-YL treated group). **b)** Hyperoxia-induced decrease in BCL-2/BAX ratio at 24 h and 72 h was blocked by PGZ only and PGZ + B-YL treatments. **c)** mRNA expression of VEGF-A, VEGF-receptor FLT-1, and PECAM-1 increased following 24 h exposure to hyperoxia; this increase was blocked in all treatment groups with the most pronounced effect observed in the PGZ + B-YL treated group. However, at 72 h, the effect of hyperoxia on endothelial markers was Variable, i.e., an increase in VEGF-A, a decrease in FLT-1, and no change in PECAM mRNA expression, with an inconsistent effect of interventions. Values are means ± SE (*p < 0.05 vs 21% O_2, and #p < 0.05 vs 95% O_2 control: N = 3).