Pentoxifylline and prevention of hyperoxia-induced lung injury in neonatal rats

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INTRODUCTION: Oxygen exposure plays an important role in the pathogenesis of bronchopulmonary dysplasia (BPD). The phosphodiesterase inhibitor pentoxifylline (PTX) has anti-inflammatory and antifibrotic effects in multiple organs. It was hypothesized that PTX would have a protective effect on hyperoxia-induced lung injury (HILI).

METHODS: Newborn Sprague-Dawley rats were exposed to >95% oxygen (O₂) and injected subcutaneously with normal saline (NS) or PTX (75 mg/kg) twice a day for 9 d. NS-injected, room air-exposed pups were controls. At days 4 and 9, lung tissue was collected to assess edema, antioxidant enzyme (AOE) activities, and vascular endothelial growth factor (VEGF) expression. At day 9, pulmonary macrophage infiltration, vascularization, and alveolarization were also examined.

RESULTS: At day 9, treatment with PTX significantly increased survival from 54% to 88% during hyperoxia. Treatment with PTX significantly decreased lung edema and macrophage infiltration. PTX treatment increased lung AOE activities including those of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). Furthermore, PTX treatment also increased the gene expression of VEGF189 and VEGF165, increased VEGF protein expression, and improved pulmonary vascularization.

DISCUSSION: These data indicate that the reduced lung edema and inflammation, increased AOE activities, and improved vascularization may be responsible for the improved survival with PTX during hyperoxia. PTX may be a potential therapy in reducing some of the features of BPD in preterm newborns.

Despite improvements in neonatal intensive care, bronchopulmonary dysplasia (BPD), the chronic lung disease of premature infants, is a major cause of long-term hospitalization, recurrent respiratory illnesses, and mortality. The etiology of BPD is complex and has been linked to oxidative stress, mechanical ventilation, infection, and inflammation, as well as genetic susceptibility (1). The pathogenesis of BPD is poorly understood and no effective therapy has yet been developed.

Oxygen toxicity in the developing lung is well known for its contribution to the pathogenesis of BPD. Evidence suggests that one important mechanism involved in lung injury during hyperoxia is direct oxidative damage through increased

production of reactive oxygen species. The pulmonary antioxidant enzyme (AOE) system, specifically superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), is a protective mechanism that confers resistance and minimizes toxicity from hyperoxia-induced reactive oxygen species (2–4). However, even though newborn animals show marked tolerance to hyperoxia as compared with adults (5,6), premature newborns have deficient endogenous AOE activities (7) and limited capacity to augment their levels during oxygen exposure (8). Induction or replacement of AOE activity may be part of the therapeutic approach to minimizing reactive oxygen species damage during hyperoxia.

Evidence has demonstrated that downregulation of vascular endothelial growth factor (VEGF) expression during hyperoxia is another important mechanism involved in lung injury and BPD (9). VEGF promotes vessel growth and remodeling, improves endothelial survival, and contributes to the maintenance of alveolar structures (10). VEGF A, in particular, is a major regulator of angiogenesis. VEGF121, VEGF165, and VEGF189 represent the predominant isoforms of VEGF A in the lung. They show different expressions and responses during lung development and injury, and their role in pulmonary vascularization is yet to be completely understood (11,12). Treatment with recombinant VEGF has improved alveolarization during hyperoxia (13), suggesting that enhancing VEGF expression may be an important strategy to prevent hyperoxia-induced lung injury (HILI) in neonates.

Pentoxifylline (PTX), a methylxanthine derivative and phosphodiesterase inhibitor, has immunomodulatory and antifibrotic properties. It is proposed to have a therapeutic role in attenuating tissue injury associated with sepsis and shock in animal models and humans, including neonates (14–16). Studies in rabbits have shown increased levels of CAT, SOD, and GPX during ischemia and reperfusion injury after PTX treatment (17). Studies have also shown that PTX is able to attenuate lung injury related to mechanical ventilation and meconium (18,19). In neonatal HILI models, PTX treatment decreased IL-6 concentration in bronchoalveolar lavage (20), reduced alveolar fibrin deposition, and prolonged survival (21). However, whether PTX treatment affects AOE and VEGF expression in neonatal HILI is unknown.

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In this study, we hypothesized that PTX administration would improve survival in hyperoxia, which would be associated with enhancement of AOE activities and upregulation of pulmonary VEGF expression in a newborn rat model of HILL. Our findings suggest a therapeutic potential of PTX in reducing some of the features of BPD in preterm newborns.

RESULTS

PTX Improves Survival

Pups exposed to hyperoxia for 9 d had a significantly increased mortality as compared with those exposed to room air (RA). However, treatment with PTX dramatically improved survival from 54% to 88% during hyperoxia ($P < 0.001$; **Figure 1**). Survival between O_2 + normal saline (NS) and O_2 + PTX groups was similar until day 6. After day 7, mortality in the O_2 + PTX group remained stable until the experiment ended on day 9, whereas the mortality in the O_2 + NS group continued to increase.

PTX Reduces Lung Edema

By day 4, there was no difference in lung wet-to-dry-weight ratio between animals raised in RA and animals exposed to O_2 . At day 9, lung wet-to-dry-weight ratio was significantly increased in O_2 -exposed groups. However, animals treated with PTX showed a significantly lower lung wet-to-dry-weight ratio as compared with the O_2 + NS group (**Figure 2**), suggesting less pulmonary edema in the PTX-treated group.

PTX Decreases Macrophage Infiltration

At day 9, macrophages were barely detected in RA + NS lungs. In contrast, in O_2 + NS lungs, there were many macrophages in alveolar airspaces, whereas fewer macrophages were detected in O_2 + PTX lungs (**Figure 3**). Quantification of macrophages demonstrated an eightfold increase in O_2 + NS lungs as compared with RA + NS lungs. Treatment with PTX produced significantly less macrophage infiltration as

compared with O_2 + NS lungs. These results suggest that PTX is protective against hyperoxia-induced macrophage infiltration.

PTX Enhances AOE Activity

To assess the effect of hyperoxia and PTX treatment on the pulmonary antioxidant defense system against free radicals, we studied CAT, SOD, and GPX activities. No differences were observed in these enzyme activities among the three study groups at day 4 (**Figure 4a**). At day 9, as compared with the RA + NS group, pups in the O_2 + PTX group showed significant increases in CAT (236.2 ± 59.7 vs. 416.2 ± 91.1 , $P < 0.001$), total SOD ($11,631.4 \pm 4,282.6$ vs. $55,758 \pm 9,579.1$, $P < 0.001$), and GPX (0.26 ± 0.1 vs. 1.03 ± 0.4 , $P < 0.001$) all expressed as activity units/mg DNA (**Figure 4b**). CAT activity was also significantly elevated in the O_2 + PTX group as compared with the O_2 + NS group (416.2 ± 91.1 vs. 292.5 ± 52.4 , $P < 0.001$; **Figure 4b**). These changes were not a result of differences in lung protein or DNA content between the O_2 + PTX and O_2 + NS groups (data not shown).

PTX Increases VEGF Expression and Improves Vascular Development

We investigated lung VEGF189, VEGF165, and VEGF121 expression using semiquantitative reverse-transcriptase PCR. There was no difference in the expression of the three isoforms at day 4 among the three study groups (data not shown). In contrast, at day 9, the O_2 + NS group had significantly decreased expression of VEGF189 and VEGF165 as compared with the RA + NS group (VEGF189: 0.31 ± 0.07 vs. 0.4 ± 0.02 , $P < 0.05$; VEGF165: 0.29 ± 0.06 vs. 0.37 ± 0.01 , $P = 0.001$). However, PTX treatment significantly increased the expression of these two isoforms as compared with O_2 + NS pups (VEGF189: 0.61 ± 0.005 vs. 0.31 ± 0.07 , $P < 0.001$; VEGF165: 0.37 ± 0.02 vs. 0.29 ± 0.06 , $P < 0.05$) (**Figure 5a**). There was no change in the expression of VEGF121 after hyperoxia or exposure to PTX. To evaluate the correlation between VEGF isoform expression and protein expression, we performed

Figure 1. PTX improves survival. Kaplan-Meier survival curve of newborn rats exposed to room air and normal saline (RA + NS; open diamonds), hyperoxia and NS (O_2 + NS; open triangles), or hyperoxia and PTX (O_2 + PTX; crosses). $n = 57$ /group. Data are expressed as percentage of pups surviving at the observed time point. * $P < 0.001$, RA group vs. hyperoxia groups. † $P < 0.001$, O_2 + PTX vs. O_2 + NS. NS, normal saline; O_2 , oxygen; PTX, pentoxifylline; RA, room air.

Figure 2. PTX decreases lung edema. At 9 days, hyperoxia increased wet-to-dry-weight ratio in the presence of NS (black bars) or PTX (gray bars) as compared with the RA group (white bars). However, PTX treatment decreased wet-to-dry-weight ratio as compared with NS during hyperoxia. $n = 13$ – 14 /group, * $P < 0.001$ and ** $P < 0.01$ as compared with RA; † $P < 0.01$ as compared with O_2 + NS. NS, normal saline; O_2 , oxygen; PTX, pentoxifylline; RA, room air.

Figure 3. PTX decreases macrophage infiltration. Macrophage infiltration was assessed on Mac3-stained lung tissue sections. Hyperoxia increased macrophage infiltration in airspaces as compared with the RA group, but treatment with PTX attenuated hyperoxia-induced macrophage infiltration. $n = 6/\text{group}$. * $P < 0.001$ as compared with RA + NS; † $P < 0.001$ as compared with O₂ + NS. Original magnification: $\times 40$. Bar = 50 μm . NS, normal saline; O₂, oxygen; PTX, pentoxifylline; RA, room air.

enzyme-linked immunosorbent assay at day 9. Although there was no significant decrease in VEGF concentration when comparing the O₂ + NS and the RA + NS groups, treatment with PTX significantly increased VEGF expression in comparison with NS-exposed animals during hyperoxia (106.8 ± 39.8 vs. 49.8 ± 41.4 , $P < 0.01$; **Figure 5b**).

To determine whether increased VEGF expression by PTX leads to improved vascular development, we assessed vascular density on von Willebrand factor-stained lung tissue sections. The lungs from the O₂ + NS group had 65% decreased vascular density as compared with the normoxia group. In contrast, treatment with PTX significantly increased vascular density during hyperoxia as compared with the O₂ + NS group (4.7 ± 0.5 vs. 3.4 ± 0.8 , $P < 0.01$; **Figure 5c**).

Effects of PTX on Alveolar Structure and Fibrosis

To evaluate the effect of PTX treatment on alveolar development, we performed lung histology and morphometry on day 9. On histological examination, lungs in the normoxia group displayed normal alveolarization. In contrast, lungs from hyperoxia groups, both NS and PTX, demonstrated distal airspace enlargement, decreased septation, and a reduction in complexity (data not shown). Morphometric analysis demonstrated a significant decrease in radial alveolar count and a significant increase in mean linear intercept in the O₂ groups (as compared with the RA group) and there was no significant difference between the O₂ + NS and O₂ + PTX groups (data not shown). We assessed connective tissue growth factor (CTGF) and α -smooth muscle actin (α -SMA) expression as markers for fibrosis by western blot analysis on days 4 and 9. On day 4, there was no difference in CTGF and α -SMA expression among the groups. On day 9, hyperoxia exposure significantly increased

CTGF and α -SMA expression as compared with normoxia exposure, but there was no significant difference between O₂ + NS and O₂ + PTX groups (data not shown).

DISCUSSION

Consistent with our hypothesis, we have found that therapy with PTX (a phosphodiesterase inhibitor) during 9 d of hyperoxia exposure increased AOE activity, increased VEGF expression, improved vascular formation, decreased pulmonary edema and macrophage infiltration, and improved survival in newborn rats. These data highlight some of the potential mechanisms by which PTX protects against neonatal HILI.

The lung's ability to respond to oxidative stress depends largely on its capacity to upregulate protective antioxidants. Newborn experimental animals are more tolerant than adults to hyperoxia. However, preterm experimental animals and presumably newborn infants as well have deficient endogenous AOE activity and limited capacity to augment their levels of protective AOE during oxygen exposure to overcome oxidative stress (7,8). Studies have established positive correlations between relative resistance to hyperoxia and increase of some or all of the pulmonary AOE in newborn animals and humans (22,23). Bucher *et al.* demonstrated a significant increase in GPX and SOD but not in CAT activity in newborn rats exposed to O₂ >95% for 6 d and in all three AOE when exposed for 12 d (2). Ilizarov *et al.* demonstrated that overexpression of manganese-SOD improves survival of pulmonary epithelial cells during hyperoxia and CAT offers additional protection when coexpressed with manganese-SOD (24). Treatment with PTX has been shown to increase AOE levels during ischemia and reperfusion injury in different tissues and organs (17); however, no studies have been reported on its effect on AOE levels during prolonged hyperoxia. Although the absolute AOE activities at day 4 were found to actually be higher than on day 9, we found a significant increase in GPX activity after 9 d of hyperoxia in control rats, whereas treatment with PTX increased the AOE activities of CAT, SOD, and GPX, indicating that PTX may increase tolerance to hyperoxia by improving cellular antioxidant defense mechanisms. To the best of our knowledge, this is the first study showing a PTX effect on lung AOE activity levels during prolonged hyperoxia.

VEGF plays a central role in normal lung development. Inhibition of angiogenesis reduces alveolarization (25) and VEGF expression is decreased in infants dying of BPD (26). As previously reported (27), we found suppression of VEGF expression in newborn rat lungs during hyperoxia. VEGF is a potent endothelial cell mitogen that stimulates angiogenesis, promotes vessel remodeling, enhances endothelial survival, and maintains alveolar structures. Different isoforms have different affinities for heparin and receptors, and distinct tempo-spatial expression of these isoforms suggests different function. Mice expressing only VEGF121 had impaired lung vascular and airspace formation, indicating an essential role for the heparin sulfate-binding VEGF165 and VEGF189 isoforms in lung development (28). In our study, we demonstrated that PTX protects against hyperoxia-induced

Figure 4. PTX enhances antioxidant enzyme activity. **(a)** Comparison of CAT, total SOD, and GPX activities at day 4 did not show differences between the three study groups ($n = 7/\text{group}$). **(b)** At day 9, as compared with normoxia, hyperoxia alone increased GPX activity and PTX treatment with hyperoxia increased CAT, SOD, and GPX activities. CAT activity was elevated in the O₂ + PTX group as compared with the O₂ + NS group. $n = 12\text{--}14/\text{group}$. * $P < 0.001$ as compared with the RA + NS group; † $P < 0.001$ as compared with the O₂ + NS group. CAT, catalase; GPX, glutathione peroxidase; NS, normal saline; O₂, oxygen; PTX, pentoxifylline; RA, room air; SOD, superoxide dismutase.

downregulation of VEGF189 and VEGF165 isoform expression as well as downregulation of VEGF protein expression. Kunig *et al.* reported enhanced vascularization in animals treated with VEGF during recovery after hyperoxia (13). Our study demonstrated that PTX treatment during hyperoxia is able to markedly increase pulmonary vascular density, which may be induced by increased VEGF expression. We found that VEGF121 expression was constant in all three study groups, indicating that VEGF121 may not be influenced by oxygen stress as seen in a rabbit model (28). Previous studies have shown that VEGF121 is a predominant form before embryonic day 14, indicating that this isoform has a unique role early in lung development (29), with perhaps a less important role toward birth, leading to a decreased expression and a lack of response during oxygen exposure.

As expected, lung edema was decreased in rat pups treated with PTX during hyperoxia. This decreased lung edema may be associated with increased activity in the AOE system as a result of PTX, which could lead to decreases in reactive oxygen species tissue damage and inflammation, thus

resulting in decreased microvascular permeability. In addition, upregulation of VEGF expression by PTX may stabilize the endothelium, preventing further development of pulmonary leakage.

Previous studies have shown that hyperoxia induces fibrotic gene expression such as that of CTGF (30). Furthermore, anti-CTGF therapy attenuates hyperoxia-induced alveolar damage and vascular remodeling (31). Multiple studies in kidney and liver have demonstrated that PTX downregulates the expression of CTGF (32,33); therefore, we expected that PTX would decrease CTGF expression and lung fibrosis during hyperoxia. Consistent with previous studies, we found that hyperoxia increases CTGF and α -SMA expression. However, we failed to observe downregulation of CTGF and α -SMA expression by PTX. We also did not observe a beneficial effect of PTX on alveolarization during hyperoxia.

Beneficial effects of PTX treatment on survival have been reported in sepsis and in HILI models (21,34). Our study demonstrated survival improvement from 54% to 88% with PTX. Our results, showing a positive effect of PTX on

Figure 5. PTX increases VEGF expression and improves vascular development. **(a)** At day 9, semiquantitative reverse-transcriptase PCR analysis demonstrated a significant decrease of lung VEGF189 and VEGF165 expression in the O₂ + NS group as compared with the RA + NS group. PTX treatment significantly increased expression of VEGF189 and VEGF165 as compared with the O₂ + NS group. RA + NS group, white bars; O₂ + NS group, black bars; O₂ + PTX group, gray bars. *n* = 6/group. **P* < 0.001 as compared with RA + NS; †*P* < 0.001 as compared with O₂ + NS. **(b)** Enzyme-linked immunosorbent assay demonstrated a significant increase of VEGF expression in the O₂ + PTX group as compared with the O₂ + NS group. *n* = 7/group. †*P* < 0.05. **(c)** Immunofluorescence staining with an anti-vWF antibody. As compared with RA + NS lungs, the number of vWF-positive vessels (arrows) was significantly decreased in the O₂ + NS group. PTX treatment increased pulmonary vascular density by 40% as compared with the O₂ + NS group. *n* = 5/group. **P* < 0.001 as compared with the RA + NS group; †*P* < 0.01 as compared with the O₂ + NS group. Original magnification: ×20. Bar = 50 μm. NS, normal saline; O₂, oxygen; PTX, pentoxifylline; RA, room air; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.

increasing AOE activity, upregulating VEGF, and decreasing pulmonary edema during oxygen exposure, show that PTX may be sufficient to improve survival but inadequate to blunt the effect of oxygen on alveolarization and fibrosis. However,

other unexplored factors might have also accounted for the improved survival of newborn rats treated with PTX, including reduction in activation of nuclear factor-κB and production of tumor necrosis factor-α, leading to attenuated lung injury (35), and the release of endothelium-derived nitric oxide by PTX in the pulmonary vascular bed, resulting in improved oxygenation (36).

There are several potential limitations of this study. First, different routes of administration, dosage, and length of treatment from hours to weeks for PTX have been used in numerous studies. The twice-a-day dosing used in this experiment might have not been optimal to exert all the potential benefits of PTX in the lungs of the newborn. However, this dosing was already found to be safe in preterm rats and beneficial in HILI as reported by ter Horst *et al.* (21). Second, the animals most susceptible to oxygen-induced injury were not analyzed due to their death. Whether PTX treatment during hyperoxia would have had similar effects on lungs of the pups that died remains unknown.

In summary, we found that PTX increases AOE activities, upregulates VEGF expression, improves lung vascularization, and decreases pulmonary edema and macrophage infiltration in newborn rats with hyperoxic lung injury. These enzymatic and molecular changes may ultimately have led to the improved survival during 9 d of hyperoxia. Further studies are needed to determine other mechanisms of PTX in lung protection as well as the most appropriate dose to obtain the maximal response. Nonetheless, these findings suggest that PTX therapy may play a role in the reduction of some of the features of BPD in premature infants.

METHODS

Animal Model and Experimental Protocol

Timed pregnant Sprague-Dawley rats were obtained from Charles River (Portage, MI). Pups were delivered naturally at term gestation, pooled, and randomly assigned to three groups to receive RA plus placebo, NS (RA + NS); hyperoxia (>95% O₂) plus NS (O₂ + NS); or hyperoxia plus PTX (O₂ + PTX; Aventis Pharma, Mexico City, Mexico). Hyperoxia was achieved in a sealed Plexiglas chamber as previously described (31). PTX (75 mg/kg equivalent to 3.75 ml/kg) based on the efficacy and safety data previously reported by ter Horst *et al.* (21) or NS (same volume) was given via subcutaneous injection before O₂ exposure and then twice a day during continuous exposure to RA or O₂ for 4–9 d. Dams were rotated daily between RA and O₂ to avoid oxygen toxicity. Animals were killed on day 4 and 9 with intraperitoneal injections of Euthazol (0.15 ml/kg; Virbac AH, Fort Worth, TX) for subsequent studies. The research protocol and procedures were reviewed and approved by the Animal Care and Use Committee at the University of Miami.

For biochemical assays and molecular analyses, lungs were perfused with ice-cold NS via the right ventricle until white. The perfused lungs were then excised, trimmed of extraparenchymal tracheal-bronchial and vascular tissue, weighed, frozen in liquid nitrogen, and stored in a –80°C freezer. For histological and morphometric analyses, lungs were infused with 4% paraformaldehyde in phosphate-buffered saline via a tracheal catheter under 20 cm H₂O pressure for 5 min and then fixed in 4% paraformaldehyde overnight at 4°C. Dehydrated lung tissues were paraffin-embedded and 5-μm tissue sections were prepared.

Unperfused lungs were excised *en bloc* and dissected away from the heart and thymus. The right upper lobe was immediately removed, blotted dry, and weighed immediately after removal. The lungs were then dried for 4 d in an oven at 60°C and reweighed. The wet-to-dry-weight ratio was then calculated.

Lung tissue sections were immunostained for Mac3, a macrophage-specific marker (BD Biosciences, San Jose, CA). The number of Mac3-positive cells in the alveolar airspaces was counted from 10 random images taken with the $\times 40$ objective on each slide.

Frozen lung tissue was pooled in groups from two rat pups and homogenized in ice-cold phosphate-buffered saline. The lung homogenates were assayed by standard spectrophotometric techniques for activities of total SOD with the xanthine/xanthine oxidase method (37), of CAT using the rate of reduction of hydrogen peroxide (38), and of GPX using the rate of oxidation of nicotinamide adenine dinucleotide phosphate using cumene hydroxylperoxide as substrate (39). Lung homogenates were also assayed for total DNA content according to the Schmit-Thannhauser-Schneider method (40). Purified reference standards for all these assays were obtained commercially (Sigma Chemical, St Louis, MO).

Alveolarization was assessed on hematoxylin-eosin stained tissue sections by radial alveolar count and by mean linear intercept as previously described (41,42).

To assess vascular development, immunofluorescence staining for von Willebrand factor (Dako, Carpinteria, CA), an endothelial-specific marker, was performed. Ten random images were taken with the $\times 20$ objective on each von Willebrand factor-stained slide. The vascular density was expressed as the average number of von Willebrand factor-positive vessels (15–50 μm) counted per high-power field as previously described (42).

RNA Isolation and Semiquantitative Reverse-Transcriptase PCR of VEGF Splice Variants

Total RNA isolation and cDNA reverse transcription were performed as previously described (42). Reverse-transcriptase PCR was performed using a pair of rat VEGF primers: sense, 5'-CCAGCACATAGGAGAGATGAGCTTC-3' and antisense, 5'-GGTGTGGTGGTGACATGGTTAATC-3', which resulted in three bands (262, 394, and 466 bp) corresponding to the three principal VEGF isoforms VEGF121, VEGF165, and VEGF189, respectively, expressed in rats (43). As a control, reverse-transcriptase PCR was also performed with β -actin-specific primers. The amplified cDNA fragments were then separated on 2% agarose gels and visualized by ethidium bromide staining. The intensity of the cDNA products was determined with the Quantity One Imaging Analysis Program (Bio-Rad, Hercules, CA). The relative mRNA levels of each VEGF splicing variant were determined after normalization to β -actin.

Measurement of VEGF Concentration and Western Blot Analysis

Total VEGF protein concentration in lung homogenates was analyzed using a commercial enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Total protein isolation and western blot analysis were performed as previously described (42).

Statistical Analysis

Data are expressed as mean $\pm$ SD. Comparison among the groups was performed by ANOVA followed by the Holm-Sidak method as a *post hoc* analysis. For comparison of survival curves, Kaplan-Meier analysis followed by a log-rank test was used. A $P < 0.05$ was considered significant.

STATEMENT OF FINANCIAL DISCLOSURE

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