

Responses of Pulmonary Platelet-Derived Growth Factor Peptides and Receptors to Hyperoxia and Nitric Oxide in Piglet Lungs

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ABSTRACT

The peptides platelet-derived growth factor-A (PDGF-A) and especially -B have important roles in lung development. The effect of hyperoxic exposure with and without inhaled nitric oxide (iNO) on lung expression of PDGF and its receptors is unknown. We hypothesized that hyperoxia exposure would suppress mRNA expression and protein production of these ligands and their receptors. The addition of iNO to hyperoxia may further aggravate the effects of hyperoxia. Thirteen-day-old piglets were randomized to breathe 1) room air (RA); 2) 0.96 fraction of inspired oxygen (O₂), or 3) 0.96 fraction of inspired oxygen plus 50 ppm of NO (O₂ + NO), for 5 d. Lungs were preserved for mRNA, Western immunoblot, and immunohistochemical analyses for PDGF-A and -B and their receptors PDGFR- α and - β . PDGF-B mRNA expression was greater than that of PDGF-A or PDGFR- α and - β in RA piglet lungs ($p < 0.05$). Hyperoxia with or without iNO reduced lung PDGF-B mRNA and protein expression relative to the RA group lungs ($p < 0.01$). PDGF-B

immunostain intensity was significantly increased in the alveolar macrophages, which were present in greater numbers in the hyperoxia-exposed piglet lungs, with or without NO ($p < 0.01$). PDGFR- β immunostaining was significantly increased in airway epithelial cells in O₂- and O₂ + NO-exposed piglets. PDGF-A and PDGFR- α immunostain intensity and distribution pattern were unchanged relative to the RA group. Sublethal hyperoxia decreases PDGF-B mRNA and protein expression but not PDGF-A or their receptors in piglet lungs. iNO neither aggravates nor ameliorates this effect. (*Pediatr Res* 57: 523–529, 2005)

Abbreviations

GAPDH, glyceraldehyde-3-phosphate dehydrogenase
iNO, inhaled nitric oxide
NO, nitric oxide
PDGF, platelet-derived growth factor

Platelet-derived growth factor (PDGF) is a mitogen for cells of mesenchymal origin, such as fibroblasts and smooth muscle cells. It exists as a family of homodimers—PDGF-AA, PDGF-BB, PDGF-CC, or PDGF-DD—and a heterodimer—PDGF-AB. These molecules bind to their cell surface receptors that also form either homodimers—PDGFR- $\alpha\alpha$ and PDGFR- $\beta\beta$ —or a heterodimer—PDGFR- $\alpha\beta$ (1–3). PDGF is important for both alveogenesis and angiogenesis of the normally developing lung (4). Expression of different forms of PDGF peaks in

the canalicular stage, suggesting a possible role in the development of the gas-exchanging regions of the lung (5,6). In the canalicular and saccular stages, PDGF receptor expression is seen in epithelial and mesenchymal cells (7). Stimulation of PDGF receptors by PDGF ligands induces lung development (8). Although PDGF seems to be critical for normal lung development, the isoform PDGF-B expression is also associated with abnormal cellular hyperplasia and fibrosis in lungs that are exposed to hyperoxia (9–11) or to hypoxia (12). In both situations, PDGF-B has been shown to increase its expression, at both protein and mRNA levels, whereas PDGF-A and PDGF-B receptors react with inconsistent results (9–11). In mice deficient for PDGF-A expression, there is postnatal development of emphysema with failure of alveolar septation (5).

Hyperoxic injury to rapidly growing lungs contributes to development of chronic lung disease of prematurity. Hyperoxic

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damage to the lung is accompanied by inflammatory reactions such as leukocyte infiltration and cytokine release into the lung parenchyma (13). PDGF may be involved in this process because of its chemotactic and stimulatory effects to neutrophils and monocytes (14,15). As a mitogen, PDGF may also participate in the tissue-remodeling process after inflammation by stimulating fibroblast cell growth, resulting in collagen deposition and various degrees of lung fibrosis (16,17).

Infants with a variety of pulmonary disorders are now treated with the combination of fraction of inspired oxygen of ≥ 0.95 and inhaled nitric oxide (iNO) (18). Although the addition of iNO to supplemental oxygen has been shown to reduce the need for extracorporeal membrane oxygenation treatment, a significant percentage of survivors develop bronchopulmonary dysplasia, even in full-term infants (19). In addition, there are now published disparate results of recent clinical trials in which NO and O₂ have been co-administered to preterm infants to prevent or ameliorate bronchopulmonary dysplasia. Schreiber *et al.* (20) demonstrated beneficial effects of iNO in preterm infants but only those who were less severely ill. In contrast, Van Mears *et al.* (21) and Macrae *et al.* (22) demonstrated no benefit of NO administration in preterm infants and indeed possible adverse events. It is likely that some preterm infants benefit from NO administration and some may have no benefit or demonstrate an exacerbation of pulmonary and other organ injury.

Whether iNO improves or aggravates oxygen toxicity has not been resolved. NO toxicity is usually attributed to its oxidative capabilities, especially that of its metabolites nitrogen dioxide (NO₂) and peroxynitrite (23–25). In the presence of oxygen, NO reacts readily in a concentration-dependent manner to form peroxynitrite, a potent oxidant that is capable of oxidizing lipids and proteins. Peroxynitrite inactivates both α -1-antitrypsin (26) and tissue inhibitors of metalloproteinases and may activate matrix metalloproteinases (27,28).

Garat *et al.* (29) demonstrated that survival of adult rats that were exposed to hyperoxia was not improved by iNO. However, inhalation of 10 ppm of NO, co-administered with O₂, was associated with reduction of a marker of oxidative injury but had no effect on alveolar barrier permeability to protein. Inhalation of 100 ppm of NO increased vascular permeability to protein. Thus, iNO can either reduce or increase the early consequences of hyperoxic lung injury.

Nelin *et al.* (30) and Gutierrez *et al.* (31) also studied survival of adult rats with NO and O₂ administration. Both demonstrated improvement in duration of survival with 100 ppm of NO; however, overall survival was very brief, with a median of only 51 h. Pulmonary alveolar cell culture studies (31) showed reduced injury with co-administration of NO and O₂. Our group has shown that, at least compared with neonatal piglets (32), rat pups demonstrate substantial endogenous pulmonary NO production. Thus, the relevance of adult rat models to the questions raised in preterm infants is unclear.

Additional studies that have investigated the interaction of O₂ and NO have evaluated the effects on surfactant biology. Issa *et al.* (33) demonstrated that iNO decreased hyperoxia-induced surfactant abnormality in rabbit pups that were delivered on day 19 of gestation and then were exposed to NO at 14

ppm for 20 h, beginning several hours after delivery. Compared with hyperoxia-exposed animals only, iNO prevented the effects of oxygen-induced reduction in the large surfactant aggregates and surface activity. In addition, surfactant protein B concentration in alveolar lavage fluid was relatively preserved in the NO studies. However, Robbins *et al.* (34) demonstrated that 48 h of exposure to 100% oxygen and to NO, in neonatal piglets, resulted in an exacerbation in the surfactant dysfunction as measured by minimum surface tension. From both these clinical and animal science-based results, it is clear that investigation in relevant animal models of clinically relevant doses of NO for clinically relevant periods of time continues to be important (35).

With this as background, we sought to analyze the effect of iNO on the expression of PDGF-A and -B mRNA and peptides and their receptors PDGFR- α and - β in lungs of animals that were exposed to sublethal oxygen toxicity, with or without concomitant iNO administration. We used a 13-d-old piglet model because, at this age, piglets have relatively mature lungs that demonstrate secondary septation and manifest rapid lung growth, achieving an average 25% increase in lung wet or dry weight in 1 wk (unpublished). This pattern—although not this rate—of growth is similar to that in term newborn human lungs (36). Because PDGF and its receptors have critical roles in lung morphogenesis and because hyperoxia contributes to the alveolar and microvascular hypoplasia found in bronchopulmonary dysplasia, we hypothesized that hyperoxic exposure would suppress expression of PDGF and of its receptors in rapidly growing lungs. We further sought to determine whether concurrent iNO administration would aggravate or ameliorate the hyperoxic injury.

METHODS

Animal exposure. The animal exposure method has been reported previously (13,37). Briefly, we randomly separated 10- to 17-d-old piglets into three groups and placed them for 5 d in chambers that contained 1) room air (RA), 2) $>95\%$ oxygen (O₂), or 3) O₂ + 50 ppm of NO (O₂ + NO). All animals weighed between 2.7 and 3.7 kg at the start of the exposure, and mean age (13.5 d) and mean weight (3.1 kg) were not different among the three groups.

Humidified medical grade O₂ and NO (Puritan Bennett, Overland Park, KS) were introduced at flow rates sufficient to maintain chamber concentration at $\geq 96\%$ and 50 ppm, respectively, throughout the experiment. Activated charcoal was used to scavenge CO₂ and NO₂. Gas concentration at chamber outlet port was monitored for O₂, CO₂, NO, and NO₂. At the end of exposure, piglets were anesthetized with 2 mg/kg xylazine, 20 mg/kg ketamine, and 40 mg/kg sodium pentobarbital and killed with a high dose of pentobarbital. Lungs were harvested promptly. The lower and middle lobes of the right lung were lavaged with six aliquots of 25 mL of PBS. Part of the left lung was cut into small pieces, quickly frozen in liquid nitrogen, and stored at -80°C for protein and RNA studies. The upper lobe of the right lung was fixed in 4% formalin and used for immunohistochemical analysis.

Immunohistochemistry. Formalin-fixed, paraffin-embedded lung tissues were cut into 4- μm sections and mounted on positively charged glass slides. After deparaffinization and rehydration, sections were incubated with rabbit polyclonal antibodies against PDGF peptides and PDGFR- α or a mouse MAb against PDGFR- β (Santa Cruz Biotechnology, Santa Cruz, CA), followed by rabbit or mouse biotinylated secondary antibodies and then a preformed avidin-biotinylated horseradish peroxidase macromolecular complex (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA). Antigens were visualized with 3, 3'-diaminobenzidine substrate (Sigma Chemical Co., St. Louis, MO). The immunostains were semiquantified with light microscopy by two observers who were blinded to treatment group, using a 0–3 scoring system, as follows: 0 = no staining; 1 = light staining; 2 = moderate staining; and 3 = heavy staining. Observations of the two observers were averaged.

Western blot analysis. Piglet lung tissue was homogenized in RIPA buffer that contained $1 \times$ PBS; 0.1% SDS; 1% Igepal CA-630 (Sigma Chemical Co.); 0.5% sodium deoxycholate; and protease inhibitors, including 10 $\mu\text{g/g}$ tissue PMSF, 30 $\mu\text{L/mL}$ Aprotinin (Sigma Chemical Co.; cat. no. A6279), and 10 $\mu\text{L/mL}$ 100 mM of sodium orthovanadate. The homogenate was centrifuged for 10 min at $10,000 \times g$ (4°C). The supernatant was applied to a 10% Bis-Tris acrylamide gel with 40 μg protein/lane. After electrophoresis, proteins were transferred to a nitrocellulose membrane and the membrane was carried on for Western blot. Reagents used in the procedure were a nonspecific blocking buffer Western Breeze (Invitrogen kit, cat. no. 46-7003, 46-7004); rabbit antihuman polyclonal PDGF-A, PDGFR- α , and PDGF-B and mouse antihuman MAb to PDGFR- β were obtained from (Santa Cruz Biotechnology; cat. no. SC-128, SC-338, SC-127, and SC-6252, respectively). β -Actin antibody was purchased from (Santa Cruz Biotechnology) as was secondary antibody conjugated to horseradish peroxidase. Detection was by an enhanced chemiluminescent technique (Amersham Biosciences, Piscataway, NJ; cat. no. RPN2106). Western blot results were analyzed quantitatively using a densitometer and the Image-Quant software (Amersham Biosciences). Values were expressed as a ratio of signal of interest to internal standard, β -actin.

Cloning of pig mRNA sequences. Degenerate oligonucleotide primers (20 bp) for PDGF-A and -B, PDGF receptors (PDGFR- α and - β), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized on the basis of the published sequences (PubMed) for human, rat, and mouse. Piglet (*Sus scrofa*) lung tissue total RNA was isolated using Tri Reagent (Sigma Chemical Co.) and subjected to reverse transcription (RT) using RETROscript reverse transcription kit (Ambion Diagnostics, Austin, TX). PCR was carried out using the degenerate primers and the reverse transcribed cDNA. PCR products of the appropriate size were cloned into pCR II-TOPO vector (Invitrogen Corp., Carlsbad, CA) and sequenced. Sequence results were analyzed with BLAST on PubMed. BLAST results and sequence comparisons of PDGF molecules are compared (Fig. 1), which indicates high sequence homology (ranging from 76% to 92%) between cloned pig sequences and sequences of other species.

Quantitative analysis of lung tissue mRNA using real-time PCR technique. On the basis of cloned sequences from piglet, PCR primers were synthesized for PDGF-A, PDGF-B, PDGFR- α , PDGFR- β , and pig GAPDH with designated PCR products for 200 bp. Isolated pig lung tissue total RNA was treated with DNA Free Kit (Ambion Diagnostics) to remove any residual DNA contamination and was reverse-transcribed using the RETROscript kit. The RT product was diluted 5-, 50-, 500-, and 5000-fold, and each was subjected to quantitative PCR analysis using the AmpliTag Gold DNA polymerase and SYBR Green I (Applied Biosystems, formerly PE Biosystems, Foster City, CA) on iCycler (Bio-Rad Laboratories, Hercules, CA). Primers that yielded a linear relationship between RT dilutions and PCR quantification were selected for real-time quantification experiment. Appropriate template dilutions (1:10–1:100 for most experiments) were decided at the same time. The GAPDH was used as a housekeeping gene for the analysis of PDGFs or PDGF receptors, the genes of interest. GAPDH was used on the basis of observations of others that its expression is not significantly affected by exposure to oxidizing agents (38) and because of our own findings of no significant difference among groups in GAPDH mRNA threshold cycle of exponential increase. Each experimental group for real-time quantitative analysis on iCycler included both the housekeeping gene and the genes of interest. The relative quantity of mRNA for each experimental group was calculated with the following equation: Relative mRNA = 2^{-} (threshold cycle of genes of interest – threshold cycle of housekeeping gene).

Statistics. Statistical analysis was performed using either MS-Excel or INSTAT (GraphPad Software, San Diego, CA). Parametric data were analyzed with one-way ANOVA, and nonparametric data were analyzed with Kruskal-Wallis test. A $p < 0.05$ was considered significant. Results are expressed as mean \pm SD unless noted otherwise.

RESULTS

mRNA analysis. Quantitative mRNA analysis by real-time PCR technique showed that in RA breathing piglets, lung PDGF-B mRNA expression is 5- to 10-fold higher than that of PDGF-A and of the receptors PDGFR- α and - β ($p < 0.01$; Fig. 2). Breathing hyperoxia with or without NO resulted in a significant decline in lung PDGF-B mRNA expression compared with RA control ($p < 0.001$). There was no significant difference in the suppressive effect of hyperoxia alone *versus* that of hyperoxia with concurrent iNO on lung PDGF mRNA expression. Hyperoxia with or without NO breathing did not

significantly alter mRNA expression of PDGF-A or of the receptors PDGFR- α and - β . There was no significant difference in initiation of exponential doubling cycle for GAPDH mRNA among the three groups when the same initial amount of total RNA was present.

PDGF-A, PDGF-B, and Western blot analysis. Results of Western blot analyses of lung PDGF-A and PDGF-B proteins paralleled the changes seen in mRNA expression (Figs. 3 and 4). PDGF-B protein expression declined significantly in the lungs of both the piglets with hyperoxia and the piglets with hyperoxia and NO breathing compared with RA controls ($p < 0.05$). PDGF-A protein expression was unchanged.

Immunohistochemical analysis. Intense immunostaining reactivity of airway epithelial cells, from the bronchi to the alveolar ducts, as well as of septal wall cells, vascular endothelium, and alveolar macrophages, was seen for both PDGF peptides and their receptor proteins PDGFR- α and - β in all RA-exposed piglet lungs (Fig. 5). For PDGF-A and the α receptor, in the presence of RA, O_2 , and $\text{O}_2 + \text{NO}$, the immunostaining intensity score was high, and the distribution did not change in the epithelium, endothelium, macrophage, or alveolar septa. These findings indicate that the PDGF ligand and receptors are constitutively near maximally up-regulated at this stage of lung growth and O_2 or $\text{O}_2 + \text{NO}$ did not influence this result. Typical staining at the bronchiolar level is shown in Fig. 6.

PDGF-B immunoreactivity expression did not significantly change with exposure to hyperoxia or with hyperoxia + NO, except in macrophages. Oxygen and $\text{O}_2 + \text{NO}$ significantly increased both the number of macrophages and the intensity of the expression ($p < 0.01$ and < 0.05 , respectively). It is interesting that the expression of PDGFR- β significantly increased in the epithelium at all levels of the airways in the O_2 - and $\text{O}_2 + \text{NO}$ -exposed animals ($p < 0.05$; Fig. 5). It also increased significantly in the alveolar septum ($p < 0.05$). There were also increases in the vascular endothelium and macrophage staining intensity, but they did not reach significance.

DISCUSSION

Various studies have focused on adult animal models to correlate O_2 -mediated pulmonary cellular hyperplasia to changes in expression of various growth factor genes. Such models are simplistic in that the adult lung being a nonproliferating organ is unlikely to exhibit dramatic alterations in growth factor expression after the onset of a proliferative lung insult. In contrast, the neonatal lung is an actively proliferating organ, even in the absence of injury. However, very few studies have focused on the injury-mediated changes in the neonatal lung.

In this study, we investigated the effect of hyperoxia with or without concurrent iNO on expression of lung PDGF peptides and their receptors in the rapidly growing neonatal lung. We found that hyperoxia with concurrent iNO, like hyperoxic breathing alone for 5 d, had a suppressive effect on lung PDGF-B mRNA transcripts and protein expression but had no effect on PDGF-A or on both PDGF receptors expression, as assessed in total lung homogenates. However, we also found

Amino acid sequence alignment of pig PDGF cDNA clones

PDGF-A

Pig : AEEAEIPREVIERLAHSQIHSIRDQLRLEIDSVGAEDPLETSLRAHGHHGAKHASEKRPVPPVRRKRSIEEAI PAVCKTRTVIYEIPRS
 Human: AEEAEIPREVIERLARSQIHSIRDQLRLEIDSVGSSEDSLDTSLRAHGVHATKHVPEKRPLPIRRKRSIEEAVPAVCKTRTVIYEIPRS
 Mouse: AEEAEIPRELIERLARSQIHSIRDQLRLEIDSVGAEDALETSLRAHGSHAINHVPEKRVPPIRRKRSIEEAVPAVCKTRTVIYEIPRS
AEEAEIPRE+IERLA SQIHSIRDQLRLEIDSVGAED LETSLRAHG H H EKRP+P+RRKRSIEEA+PAVCKTRTVIYEIPRS

Pig : QVDPTSANFLIWPP
 Human: QVDPTSANFLIWPP
 Mouse: QVDPTSANFLIWPP
QVDPTSANFLIWPP

PDGF-B

Pig : KMLS DHSIRS FDDLQRL LHRDSVDE DGA DLNLTXXXXXXXXXXXXXXXXXXXXX SPTVAEPAVTAECKTRTEVFEISRRLIDRTNANFL
 Human: EMLS DHSIRS FDDLQRL LHGDPGEEDGAELDLNMTRSHSGGELESLARGRRSLGSLTIAEPAMIAECKTRTEVFEISRRLIDRTNANFL
 Mouse: EMLS DHSIRS FDDLQRL LHRDSVDE DGA ELNLTXXXXXXXXXXXXXXXXXXXXX SPTVAEPAVTAECKTRTEVFEISRRLIDRTNANFL
+MLS DHSIRS FDDLQRL LHRDSVDE DGA +LDLN+T S AEPA+ AECKTRTEVFEISRRLIDRTNANFL

Pig : VWPPC VEVQRC SGCCNRRNVQCRPTQVQLRPVQVRKIEIVRKKPTFKKATVTL EDHLACKCETV-VARPVTRSPGSSQEQRAKTPQXXX
 Human: VWPPC VEVQRC SGCCNRRNVQCRPTQVQLRPVQVRKIEIVRKKPIFKKATVTL EDHLACKCETVAAAARPVTRSPGSSQEQRAKTPQTRV
 Mouse: VWPPC VEVQRC SGCCNRRNVQCRASQVQMRPVQVRKIEIVRKKPIFKKATVTL EDHLACKCETIVTPRPVTRSPGTSREQRAKTPQARV
VWPPC VEVQRC SGCCNRRNVQCR QVQ R PVQVRKIEIVRKKP FKKATVTL EDHLACKCET RPVTRSPG SQEQRAKTPQ

Pig : XXXXXXXX RPPK GKHRKFKH TH
 Human: TIRTVRVRPPK GKHRKFKH TH
 Mouse: TIRTVRIRPPK GKHRKFKH TH
RPPK GKHRKFKH TH

PDGF-R-Alpha

Pig : IPCRTTDPETPVTLSSDGVVHASYSRQGFNGFTFTVGPYICEATVRGKKFQTI PFNVYALKATSELDLEMEALKTVYKAGESIVVTCVAV
 Human: IPCRTTDPETPVTLHNSGVPVASYDSRQGFNGFTFTVGPYICEATVRGKKFQTI PFNVYALKATSELDLEMEALKTVYKGETIVVTCVAV
 Mouse: IPCRTTDPETQVTLHNNGR LVPASYDSRQGFNGFTFSVGPYICEATVKGRTFKTSEFNVYALKATSELDLEMEALQTVYKAGETIVVTCVAV
IPCRTTDPET VTL +++G+V ASYDSRQGFNGFT+VGPYICEATV+GKKF+T FNVYALKATSELDLEMEAL TVYK+GE+IVVTCVAV

Pig : FNNEVV DLQW TYPGQVKGK GITM LEE TKVPSIKLVYTL MVEATVKD S DGEYCAARQATREVKEMKKVTISVHERGFIEIKPNFSPLEAV
 Human: FNNEVV DLQW TYPGEVKGK GITM LEE IKVPSIKLVYTL TVEATVKD S DGEYCAARQATREVKEMKKVTISVHEKGFIEIKPTFSQLEAV
 Mouse: FNNEVV DLQW TYPGEVNRK GITM LEE IKLPSIKLVYTL TPKATVKD S DGEYCAARQATREVKEMKKVTISVHEKGFVEIEPTFGQLEAV
FNNEVV DLQW TYPG+VKGK GITM LEE KVPSIKLVYTL VPEATVKD S DGEYCAARQATREVKEMKKVTISVHE+GFIEI P FS LEAV

PDGF-R-Beta

Pig : NVPVRVLELS ESH PASGEQTIRCRGRGMPQP HLTWSTCS DLKRCRPRELPPTPLGNSSEESQLETNVTYWPQEQEFVSTLRLRRVDQ
 Human: NVPVRVLELS ESH PDSGEQTVRCRGRGMPQPNI IWSACRDLKRCRPRELPPTLLGNSSEESQLETNVTYWEEQEFVSTLRLQHVDQ
 Mouse: NVPVRVLELS ESH PANGEQTIRCRGRGMPQPNTWSTCRDLKRCRPRKLSPTPLGNSSEESQLETNVTFWEEDQEFVSTLRLRHVDQ
NVPVRVLELS ESH P SGEQT+RCRGRGMPQP++ WS C DLKRCRPRELPPT LGNSSEESQLETNVTW++QE+EVVSTLRLR VDQ

Pig : PLSVRCTLHNL LGHGAQEVTVVPHSLPFQXXXXXXXXXXXXXXXXXXXXXXXXX MLWQKKPRY EIRWKVIESVSSD GHEYIYVDHMQLPYDS
 Human: PLSVRCTLHNL AVGQD TQEVIVVPHSLPFKVVVISAILALVLT IISLILIMLWQKKPRY EIRWKVIESVSSD GHEYIYVDPMQLPYDS
 Mouse: PLSVRCTLHNL QNSMGGDSQEVTVVPHSLPFKVVVISAILALVLT V IISLILIMLWQKKPRY EIRWKVIESVSSD GHEYIYVDVQLPYDS
PLSVRC L N +G +QEVTVVPHSLPF+ MLWQKKPRY EIRWKVIESVSSD GHEYIYVD MQLPYDS

Pig : TWELPRDQLV LGR TLGSGAFGQVVEATAHGLSHS QATMKVAVKMLKSTARSSEKQALMSELKIMSHLGP HLNVV
 Human: TWELPRDQLV LGR TLGSGAFGQVVEATAHGLSHS QATMKVAVKMLKSTARSSEKQALMSELKIMSHLGP HLNVV
 Mouse: TWELPRDQLV LGR TLGSGAFGQVVEATAHGLSHS QATMKVAVKMLKSTARSSEKQALMSELKIMSHLGP HLNVV
TWELPRDQLV LGR TLGSGAFGQVVEATAHGLSHS QATMKVAVKMLKSTARSSEKQALMSELK+MSHLGP HLNVV

Sequence identity between pig and other species

	PDGF-A	PDGF-B	PDGF-R-Alpha	PDGF-R-Beta
Human	88%	77%	92%	82%
Mouse	89%	76%	80%	81%

Figure 1. This sequence comparison is a direct result from BLAST search. Only a partial sequence of each molecule was obtained and BLASTed. The bottom line sequence of each paragraph indicates identities or similarities between each species. PDGF-R-Alpha and PDGF-R-Beta represent PDGF receptors.

increased immunostaining intensity of PDGF-B in macrophages and of the receptor PDGFR- β in alveolar septa and airway epithelial cells. These latter results suggest that there was up-regulated expression in response to hyperoxia with or without iNO.

PDGF peptides and their receptors have been studied in the lungs of adult and immature animals that were treated with hyperoxia but not in the lungs of young growing animals (10).

Full-term human infants who experience pulmonary hypertension are treated with hyperoxia and iNO for several days' duration (18,19). However, the effect of administering fraction of inspired oxygen of 0.95 with concurrent iNO on expression of lung PDGF peptides and their receptors has not been examined in animals of any age. The results from the current study provide the first insight into the potential implications of such use on PDGF-regulated events in the growing lung.

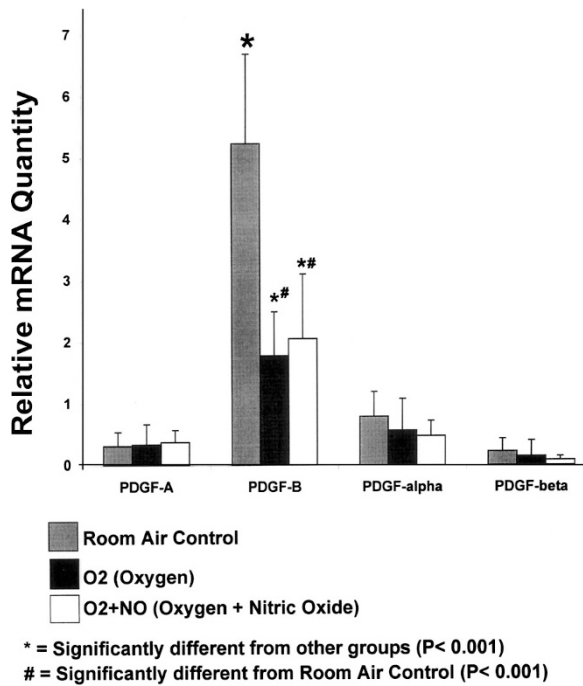


Figure 2. Quantitative mRNA analysis using real-time PCR. There is a significant difference between PDGF-B and PDGF-A and the α and β receptors. The mRNA level of PDGF-B is 5- to 10-fold higher than PDGF-A and PDGFR- α and - β proteins in RA. The presence of O₂, with or without NO, significantly reduced PDGF-B mRNA expression compared with RA controls ($p < 0.001$). NO did not seem to modify the effect of hyperoxia. $n = 5, 4,$ and $4,$ respectively, for the three groups.

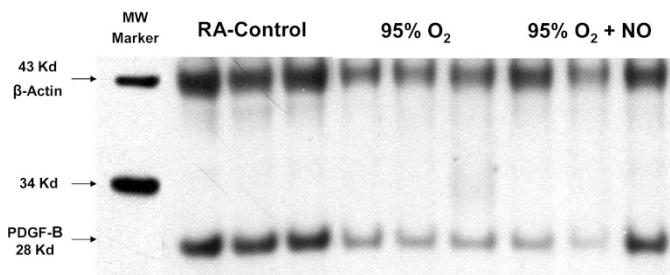


Figure 3. Example of Western blot for the PDGF-B in the lungs that were treated with 95% O₂ and 95% O₂ + NO under nonreducing conditions. Each lane was loaded with 40 μ g of protein.

Previous investigators have shown that the adult rat has low constitutive lung levels of PDGF-B mRNA and that, in the presence of hyperoxia, PDGF-B mRNA is elevated 2.5-fold by day 3 and remains high for 7 d. This early rise in mRNA precedes the increase in protein synthesis and other responses that reflect remodeling with hyperoxia, suggesting that PDGF proteins modulate lung injury and repair (9). Powell *et al.* (10), studying hyperoxia in adult rats, showed that within hours of exposure, mRNA levels of the α and β receptors increase, return to normal by 1 d, and decline thereafter in the presence of hyperoxia. The PDGF-A mRNA also rose within hours and then returned to normal levels, even though the hyperoxic exposure continued. In their study, the PDGF-B chain mRNA also responded to hyperoxia by increasing 10-fold on day 3 of exposure. Again, this increase preceded the day 4 proliferative response of microvascular adventitial fibroblasts, precursor

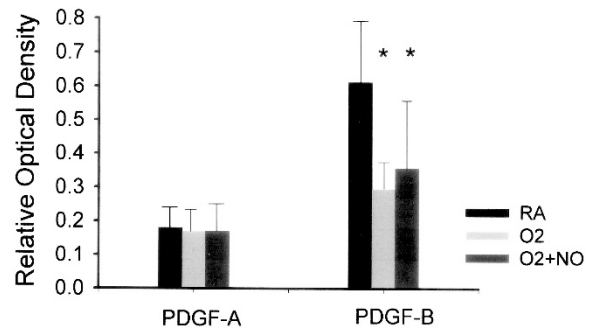


Figure 4. Western blot analysis. PDGF-A in RA-treated animals did not change with O₂ or O₂ + NO exposure, whereas PDGFR- β showed a significant decrease in both O₂- and O₂ + NO-treated animals ($p < 0.05$); $n = 5, 4,$ and 4 for RA, O₂, and O₂ + NO, respectively.

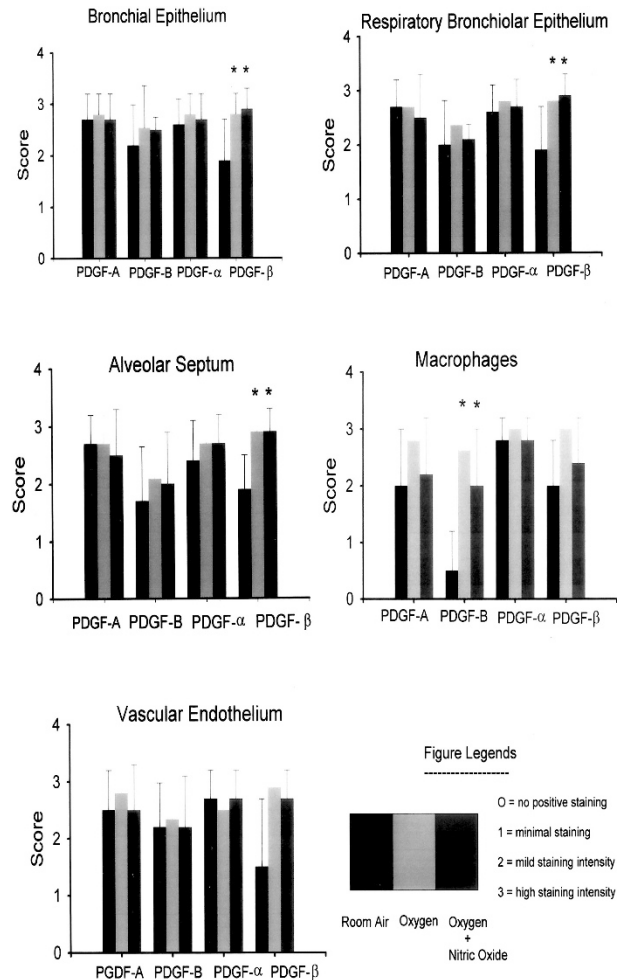


Figure 5. PDGF-A and PDGF-B and PDGFR- α did not significantly change in airway epithelium or vascular endothelium in O₂- or O₂ + NO- compared with RA-treated animals. However, PDGFR- β reactivity was significantly increased ($p < 0.05$) in the macrophages that were exposed to O₂ and O₂ + NO. The expression of PDGFR- β was significantly increased in the epithelium at all levels of the airway and in the alveolar septa in the O₂- and O₂ + NO-exposed animals; $n = 5, 4,$ and 4 for RA, O₂, and O₂ + NO, respectively.

smooth muscle cells, and epithelial cells. In adult rats with acute lung injury from bleomycin, the mitogenic potency of PDGF-B was more than 5 times that of PDGF-A (26). Fur-

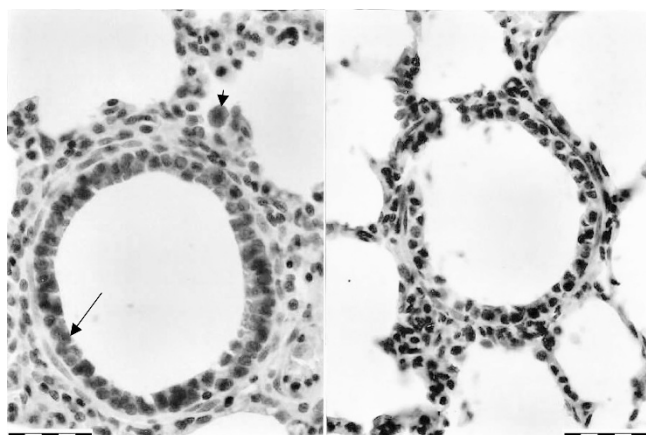


Figure 6. Immunohistochemical staining of piglet lung. (A) O₂-treated sample. Bronchiole epithelium (indicated by arrow) is stained intensely (score 3) with anti-PDGF-B antibody. An alveolar macrophage (arrowhead) is also intensely stained. (B) RA group sample showing bronchiole epithelium. No anti-PDGF-B staining is observed (score 0). Scale bar = 6 μ m.

Furthermore, the role of PDGF-BB as a critical growth factor in early postnatal rat lung growth has been demonstrated by Buch *et al.* (39) who, using both neutralizing antibodies to PDGF-BB and a truncated soluble PDGFR- β , observed a significant reduction of DNA synthesis in the first week of life. They showed that newborn rats that breathed RA had a significant rise in lung PDGF-B, PDGF-A, and PDGFR- β mRNA at 6 d of life but a decrease in PDGFR- α mRNA, compared with levels at birth (day 0). Negligible PDGF-B immunoreactivity was seen at birth, increasing by day 4, declining at day 6, and increasing again at day 10 of life. Similarly, PDGF-A and the α receptor immunoreactivity varied from 0 to 14 d, with the intensity and distribution changing as various tissue elements, *e.g.* epithelium and microvasculature matured and underwent growth and differentiation (39). Exposure of these newborn rats to 60% O₂ significantly increased the mRNA for PDGF-B and that of the α and β receptor but not PDGF-A. PDGF-A and -B and the α receptor immunoreactivity were reduced and delayed in hyperoxia, consistent with overall inhibition of lung growth (39). Evidence of a role for PDGF-B in lung growth was shown by a reduction in lung DNA synthesis after treatment with PDGF-BB neutralizing antibodies.

The expression of PDGF and its receptors in the immature lung in the presence of hyperoxia is much more complicated than in the mature adult lung because of the rapid growth and tissue remodeling and differentiation that occurs with immaturity. Therefore, it can be anticipated that experiments on immature rats, lambs, piglets, and baboons may give various PDGF responses to hyperoxia, depending on the stage of development and the species.

In our study, after 5 d of hyperoxia, with or without NO, the piglet showed a significant decrease in mRNA expression of PDGF-B mRNA but not PDGF-A mRNA, or PDGFR- α , or PDGFR- β . This was paralleled by a significant fall in the PDGF-B protein in whole-lung homogenate. The PDGF-A protein did not change. These findings suggest that this relatively short but potent hyperoxic stimulus depressed the mitogenic effect of PDGF-B. However, within 48 h of stopping the

hyperoxia, the PDGF-B protein, as determined by Western blot analysis, had recovered to control levels (data not shown). NO did not seem to have an independent significant influence on the levels of PDGF messenger or protein.

Our piglet model is in a more rapid lung growth phase than the adult animal but is unlike the neonatal rat model of Buch *et al.* (39). The piglet is analogous to the human full-term infant, which is also in a rapid lung growth phase, but the piglet lung is more mature.

The increased immunostaining intensity that we observed for PDGF-B and the β receptor in certain lung tissue and cell types after exposure to hyperoxia with or without NO at first would seem to conflict with our findings from hybridization studies of the lung homogenates. However, this seeming discrepancy between results from hybridization studies of the total lung homogenate and immunohistochemical analysis should not be surprising. Changes in lung tissue and cell-specific level of expression of a protein can occur in response to a stimulus and may not be consistent with results determined from studying the homogenate (40). This could arise if there are changes in cell population or in their numbers. The lung is made of many cell types (41). The capillary endothelial cells constitute two thirds of the parenchymal cell population, and the alveolar epithelial cells represent less than one third. Hyperoxia is known to alter cell types and numbers in the lungs and especially has a propensity to damage vascular endothelial and airway epithelial cells. Indeed, we found a significant rise in the number of alveolar macrophages and their immunostaining intensity for PDGF-B, in the hyperoxia-exposed piglet lungs, with or without NO, compared with the RA group.

Limitations of the current study include the limited number of time points evaluated and dose of NO studied. However, these notwithstanding, the study identifies perturbations that occur in lung expression of PDGF-B and its receptors in response to combined NO-hyperoxia exposure and therefore offers insight into potential complications of such use on lung growth and development.

In summary, hyperoxia with or without iNO suppresses total lung PDGF mRNA and protein expression in young growing piglet lungs. Concurrent iNO at 50 ppm does not aggravate or ameliorate these effects of hyperoxia on PDGF peptides or on expression of their receptors in this sublethal oxygen exposure model. Alterations in levels of lung PDGF could have permanent consequences for lung growth and development after the exposure period is completed. Confirming this possibility will require longer term follow-up studies.

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