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Modulation of IGF-Binding Protein-2 and -3 in Hyperoxic Injury in Developing Rat Lung

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ABSTRACT

Retinoids play an important role in lung development and repair. We showed that retinoic acid (RA) inhibits O_2-induced fibroblast proliferation in rat lung explants. IGF-1, which enhances the proliferation of human fetal lung fibroblasts and stimulates collagen production during lung injury, has an important role in the lung injury/repair process. Interactions of IGF-1 with its receptor are modulated by IGF-binding proteins IGFBPs. We hypothesized that RA alters IGFBP-2 and -3 in hyperoxia-exposed neonatal lung and alters collagen production. Neonatal rat lungs were cultured in room air or 95% O_2 and 5% CO_2 for 3 d with or without RA. IGFBP-2 and -3 were measured both in culture medium and in lung tissue. Type I collagen and procollagen propeptide were analyzed in the lung tissue. Hyperoxia induced an increase in type I collagen that was significantly inhibited in the presence of RA. IGFBP-2 and IGFBP-3 in the lungs were decreased in hyperoxia but significantly increased in hyperoxia plus RA. In the culture medium, IGFBP-2 and -3 were not increased with hyperoxia but significantly increased in the presence of RA plus hyperoxia. There was no increase in IGFBP-3 RNA transcript after RA treatment in either room air or O_2 exposure. In conclusion, RA modulates the secreted IGFBP-2 and -3 during O_2 exposure and inhibits the increase in collagen that occurs during lung injury. We speculate that RA protects against O_2-induced neonatal lung injury through modulation of the IGFBPs. (***Pediatr Res* 58: 222–228, 2005)**

Abbreviations
BPD, bronchopulmonary dysplasia
IGFBP, IGF-binding protein
RA, retinoic acid

Bronchopulmonary dysplasia (BPD) is the most common chronic pulmonary disease of premature infants who require prolonged ventilation and/or O_2 therapy, usually after the presence of respiratory distress syndrome (1). Airway hyperreactivity, which can persist for several years, is one of the long-term sequelae of BPD. Improved perinatal and neonatal care, surfactant therapy, and new ventilation modalities have had a modest impact on the incidence of BPD in very small neonates, indicating a need for greater understanding of the mechanisms that lead to BPD.

Histologic features of infants who die of BPD include chronic airway inflammation and squamous metaplasia of epithelial cells in airways (2). These changes are observed in animal models of hyperoxic damage to developing lung. For example, neonatal rats that are exposed to high O_2 concentrations develop alveolar epithelial thickening as well as an increased thickness of smooth muscle (3) and interstitial matrix, with abundant myofibroblasts (4). The increase in myofibroblasts in lung tissue correlates with increased type III collagen synthesis and fibrosis (5).

A recent study of biopsy and autopsy material from lungs of infants who had BPD and had received both antenatal steroids and surfactant treatment showed developmental arrest, enlarged airspaces with minimal alveolarization and variable alveolar wall cellularity, and fibrosis (6). BPD in very low birth weight infants may result from inflammatory mediators' interfering with the signaling molecules in late lung development (7). Overexpression of cytokines and growth factors interfere with normal lung development in mice (8). Ventilation and O_2 exposure of very preterm infants may amplify lung injury after antenatal cytokine exposure (9). Profibrotic cytokines have been shown to be increased in bronchoalveolar fluid of preterm infants who developed chronic lung disease (10).

Growth factors and cytokines mediate chemotactic and mitogenic responses and cell differentiation. Subepithelial fibrosis observed in the airways of patients with BPD and asthma is attributed to the ability of the epithelial cells to produce

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chemotactic cytokines and growth factors (11). IGF-1, which enhances the proliferation of human fetal lung fibroblasts and stimulates collagen production during lung injury, has an important role in the lung injury/repair process (12,13). It has been shown to be associated with changes in the expression of IGF-binding proteins (IGFBPs) and collagen expression during inflammation (14).

IGFBPs are active in the cellular environment to modulate IGF-1 functions. IGFBP-3, a 43- to 45-kDa glycoprotein, modulates IGF-1 activity and regulates cell functions both by IGF-dependent and IGF-independent mechanisms. IGFBP-3 directly inhibits IGF-1 signaling through the IGF-1 receptor by sequestering IGF-1 (15). This binding protein has also been shown to inhibit growth and induce apoptosis in a variety of cell lines that are unresponsive to mitogenic effects of IGF-1 (16,17). IGFBP-3, in addition to its action as a carrier of IGFs, modulates these actions indirectly *via* independent mechanisms involving interactions with plasma, extracellular matrix and cell surface molecules (18), and nuclear transport (19).

IGFBP-2, a 34-kDa protein, is produced in various cellular environments, including breast cancer cell lines (20). In type II cell growth arrest, IGFBP-2 competes with IGF-1 receptor for IGF-1 (21). It also acts through IGF-dependent and IGF-independent mechanisms. Retinoic acid (RA) has been shown to modulate IGFBP-2 at the transcriptional and the posttranscriptional levels (22). Proteolysis of IGFBP-2 is another contributory mechanism in regulating its action on cell proliferation (23).

RA and its derivatives are important regulators of structural development of the postnatal lung as shown in both experimental and clinical studies. The metabolically active forms, retinol and RA increase rapidly in the postnatal period with a concomitant decline in the retinol esters, a storage form of RA (24). In infants, supplemental vitamin A has been reported to prevent or decrease the severity of BPD (25,26). The glucocorticoid-induced inhibition of alveolarization was minimized with RA in newborn rats (27). RA-responsive genes include epidermal growth factor and the IGFs, growth factors that are involved in the alveolarization process (28). Recent reports suggest that IGFBP-2 and -3 are important in cell proliferation and collagen synthesis in lung injury (29). This together with the facts that exogenous RA decreases mesenchymal cell proliferation in hyperoxic lung injury in *in vitro* rat lung hyperoxic exposure (12) and that retinoids inhibit IGF-1 action on dermal papilla cells and surrounding matrix cells by stimulating the production of IGFBP-3 (30) prompted us to study the effect of RA on hyperoxia-induced collagen synthesis and its effect on IGFBP-2 and -3.

METHODS

Animals and reagents. The animal study protocol was approved by the Institutional Animal Research Committee. Pregnant Sprague-Dawley rats were obtained from Taconic Farm (German Town, NY) and allowed to deliver. The newborn rat pups remained with their dams in the animal facility for 3 d, at which time the pups were killed and the lungs were processed as described below. Culture dishes (35 mm) used for explant cultures were obtained from Corning Glass Works (Corning, NY). Millicell cell culture inserts were obtained from Millipore Corporation (Bedford, MA). Reagents were obtained as follows: rabbit anti-mouse IGFBP-3 polyclonal antibody was purchased from Cell Science (Norwood, MA), anti-bovine IGFBP-2 antibody and goat anti-rabbit IgG were obtained from Upstate Biotechnology (Lake Placid, NY), and anti-collagen type I antibody was obtained from Rockland Immunochemicals (Gilbertsville, PA). All-trans-retinoic acid (ATRA), penicillin G, acrylamide, DMSO, SDS, pepstatin, and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO). L-Glutamine, Hanks' balanced salt solution (HBSS), Dulbecco's modified PBS (D-PBS), and FCS were obtained from GIBCO (Grand Island, NY); and BGJb medium was obtained from Biofluids (Bethesda, MD). Trizol reagent was obtained from GIBCO-BRL (Gaithersburg, MD).

In vitro *explant culture and O_2 exposure.* Ten to 12 rat pups from one pregnant mom were used for each experiment. We conducted a total of four to five experiments for each condition. Three-day-old rat pups were killed by decapitation. The trachea and the lungs were removed *en bloc*, preserved in physiologic saline, and placed on culture dishes. The lungs were cut transversely into 1-mm slices using a sterile blade. The lung slices were cultured as described for lung explants, with modification (31). Approximately 10–15 sections were pooled and placed in six-well culture dishes on top of Millicell cell culture inserts elevated by a small platform and cultured in 2 mL of serum-free, hormone-free BGJb medium that contained added ascorbic acid (0.2 mg/mL), penicillin (50 units/100 mL), and streptomycin (50 μg/100 mL). In this design, the lung slices were situated at the membrane/culture medium interface and remained suffused with medium by capillary action through the insert. Lung slices were exposed to either 95% O_2 with 5% CO_2 (hyperoxia) or 21% O_2 with 5% CO_2 (control) in a Plexiglas chamber (Billups-Rothenberg, Del Mar, CA) for 3 d. In some experiments, cultures were treated with RA (5 or 0.5 μM). In these experiments, the control cultures received the diluent DMSO (1 μL/mL). The lung slices were turned over, and the medium was changed every day. For avoiding photoisomerization of RA, this reagent was added under yellow light, and all cultures, including controls, were maintained in the dark by covering with aluminum foil throughout the experiment.

Preparation and concentration of culture medium. Culture media from individual dishes were collected from explant cultures that were exposed to 95% O_2 and to room air with 5% CO_2, with and without RA treatment. Culture medium was concentrated 25- to 100-fold by ultrafiltration using Centricon microconcentrators (10,000 kDa molecular weight cut-off; Amicon, Beverly, MA). Proteins were separated by PAGE and probed with anti–IGFBP-3 antibody as described below.

ELISA for IGFBP-3. For determination of IGFBP-3 concentration, DSL-10-6600 Active IGFBP-3 ELISA kit (DSL, Webster, TX), an enzymatic "two-step sandwich" immunoassay, was used. A polyclonal antibody to IGFBP-3 was prebound to the surface of the microtiter plate. After incubation with the culture medium, a second polyclonal anti–IGFBP-3 antibody linked to horseradish peroxidase was added. The microtiter plates then were incubated with tetramethylbenzidine. Addition of sulfuric acid stopped the color reaction, and the absorbance was measured at 450-nm wavelength. The specificity of detection and capture antibodies for IGFBP-3 in this assay was demonstrated by the lack of response to either IGF-1 (500 ng/mL) or insulin (200 ng/mL).

Western blot analysis. Western blotting was performed to analyze IGFBP-2 and -3 proteins in lung explants and in the culture media. Tissue was harvested into an ice-cold solution of PBS (pH 7.4) that contained protease inhibitors (leupeptin 2 μg/mL, aprotinin 1 μg/mL, 1 mM phenylmethylsulfonyl fluoride, and antipain 2 μg/mL). After the sonicated sample was centrifuged, an aliquot was removed for protein determination, and the samples were stored at −80°C until use. Lung tissue protein and the protein from the concentrated culture media were measured using a protein microassay kit (Pierce, Rockford, IL). A 50-μg protein sample was separated by PAGE on a 10% acrylamide-SDS gel then transferred to a nitrocellulose membrane. The membrane was washed in TBST buffer [20 mM Tris (pH 7.6), 136 mM NaCl, and 0.25% Tween-20] and then blocked for 18 h at 4°C using 5% nonfat dry milk in TBST. Western blots were probed using a monoclonal anti-mouse IGFBP-3 antibody (1:1000; Cell Sciences, Norwood, MA) overnight at 4°C to demonstrate the presence of IGFBP-3 or with IGFBP-2 antibody (1:2000) to demonstrate IGFBP-2 (Upstate Biotechnology). IGFBP-3 antibody has a $<$0.2% cross-reactivity for IGFBP-2, and IGFBP-2 antibody has $<$0.5% cross-reactivity with IGFBP-3 according to the manufacturer's specifications. After a sequence of washes in TBST, the membrane was incubated with goat anti-rabbit IgG (1:5000 in TBST) for 1 h at room temperature. Antigens were identified using chemiluminescence (Amersham Biosciences, Piscataway, NJ) and exposure to x-ray film. Comparisons of relative changes were made by densitometry scanning. Relative molecular weights were estimated using prestained molecular weight standards. To control for loading differences in the Western blots, actin was used as an internal standard for lung tissue samples and Ponceau stain was used for samples from condition media.

Type I collagen and procollagen propeptide analysis. Affinity-purified anti-collagen type I antibody (rabbit) with minimum cross-reactivity to types II to VI collagens (Rockland Immunochemicals) was used was used to determine

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levels of collagen type I. Rabbit anti-human procollagen antibody (LF 39; provided by Dr. L. Fisher, National Institutes of Health/National Institute of Dental and Craniofacial Research, Bethesda, MD) (32) was used in 1:1000 concentration to determine the level of procollagen propeptide. After antigens were incubated with the secondary antibody as described above, they were identified by chemiluminescence.

RNA isolation and RNase protection assay. Three-day-old neonatal rat lung explant cultures that were exposed to 95% O_2 and 5% CO_2 or room air with 5% CO_2 for 72 h were snap-frozen in liquid nitrogen, then kept in 1.5 mL of Trizol (GIBCO-BRL, Gaithersburg, MD) at −60°C until further analysis. RNA was isolated from the lungs of four different experiments using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Using a MAXI-Script Kit (Ambion, Austin, TX), a 301-nucleotide ^{32}P-labeled antisense riboprobe was transcribed using a rat IGFBP-3 cDNA fragment (a gift from Dr. Wayne Price, University of North Carolina Chapel Hill, Chapel Hill, NC). As an internal standard, a 115-nucleotide ^{32}P-labeled antisense riboprobe was also transcribed using a 28S cDNA fragment (Ambion). In all blots, probe alone and probe mix (BP-3 and 28S) were run. The probe mix allowed us to probe the same RNA sample for two genes of interest, BP-3 and 28S. Ten micrograms of RNA was incubated with the probes overnight at 56°C and then digested with RNase A/T1 and proteinase K. Protected fragments were precipitated and separated using a 4.75% acrylamide Tris-borate EDTA–urea gel, dried, and then visualized by autoradiography.

Data Analysis. Densitometry was used to get a quantitative analysis for Western blots. Data are represented as mean ± SEM. Statistical analysis was performed using one-way ANOVA to determine the effect of O_2 and RA with Bonferroni correction for multiple comparisons. Differences between hyperoxia and normoxia with and without RA were considered statistically significant at $p < 0.05$.

RESULTS

Modulation of RA on type I collagen and collagen synthesis in hyperoxia-exposed neonatal rat lung. We showed previously that exposure of neonatal rat lung in explant culture to 95% O_2 for 72 h leads to an increase in mesenchymal cell proliferation (12). We estimated the effect of hyperoxia on collagen content using affinity-purified type 1 collagen antibody and collagen synthesis by analyzing procollagen propeptide antibody (32). Because type 1 collagen is a major structural protein in the lung interstitium and is produced more during lung fibrosis in chronic lung diseases, we evaluated type I collagen in lung homogenate using Western blot analysis followed by densitometry. Hyperoxia induced an increase in type 1 collagen that was inhibited by RA (5 and 0.5 μM; Fig. 1*A*). Computerized densitometry of the Western blot membranes confirmed these results ($n = 3$; $p < 0.05$; Fig. 1*B*). This is in accordance with Krupsky *et al.* (33), who observed a similar decrease in α1 collagen in RA-treated human lung fibroblast cultures.

We also examined collagen production by analyzing procollagen propeptide (32). The carboxy-terminal propeptide of procollagen type I, an index of collagen type I synthesis, has been used as a marker for collagen synthesis in relation to fibrosis in other systems (34). Hyperoxia increased procollagen propeptide in neonatal rat lung. Under these experimental conditions, RA did not have any effect on the synthesis of collagen (Fig. 2*A*). Computerized densitometry confirmed the significant increase in procollagen after hyperoxia ($p < 0.05$; $n = 3$). This increase was not changed in the presence of RA (5 and 0.5 μM; Fig. 2*B*).

Effect of hyperoxia and RA on lung tissue IGFBP-2 and IGFBP-3 content. On the basis of our previous studies showing that hyperoxia increases cell proliferation and increased IGF-1 expression in epithelial cells, we asked whether the effects of O_2 on lung explant culture is associated with changes in IGFBP-2 and -3. Because RA has been shown to inhibit cell proliferation by interfering with the IGF system (20,21), we were interested in finding out whether RA has any effect on hyperoxia-induced changes on these binding proteins. Western blots were performed to determine the amount of IGFBP-2 and -3 in lung tissue that was exposed to room air and hyperoxia for 72 h with and without RA. IGFBP-2 was decreased in the lung tissue by O_2 exposure. RA at 5- and 0.5-μM concentration increased IGFBP-2 in the lung tissue that was exposed to hyperoxia, and the increase was more with RA (5 μM; Fig. 3*A*). This was confirmed by densitometry (Fig. 3*B*) and in agreement with findings described in a human lung epithelial cell line (35).

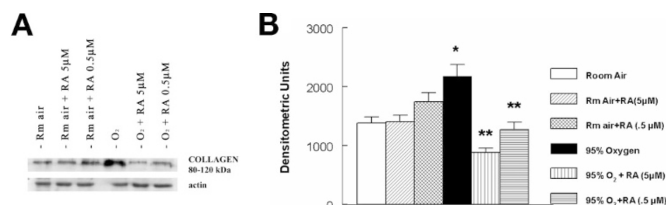

Figure 1. Effect of hyperoxia on type I collagen in neonatal rat lung. (*A*) Representative Western blot for type I collagen in rat lung tissue that was exposed to room air or 95% O_2 for 72 h with and without RA. (*B*) Densitometric analyses of type I collagen measured in lung homogenate. The histogram shows quantitative representations of type 1 collagen levels. The increase was blocked in the presence of RA (5 and 0.5 μM). Bars represent the mean ± SEM of $n = 3$ experiments; *$p < 0.05$ *vs* room air; **$p < 0.05$ *vs* O_2 treatment alone.

Western blots of IGFBP-3 in the lung tissue after hyperoxia with and without RA are shown in Fig. 4*A*. Densitometry of the blots in Fig. 4*B* confirms a significant decrease in lung tissue IGFBP-3 after hyperoxia, similar to effects on lung tissue IGFBP-2 ($p < 0.05$; $n = 3$). There was a significant increase in IGFBP-3 in hyperoxia conditions in the presence of RA (5 and 0.5 μM), again similar to results for IGFBP-2 ($p < 0.05$; $n = 3$).

RA effect on IGFBP-2 and -3 content in culture medium of hyperoxia-exposed lung. IGFBP-2 and -3 proteins in concentrated culture medium were analyzed by Western blot to determine the potential role of the secreted binding proteins in IGF-1 and IGF-1 receptor interaction in hyperoxic injury. Incubation of the Western blots with anti-bovine IGFBP-2 antibody revealed a 34-kDa double band, consistent with the known M_r of IGFBP-2 (20). A representative Western blot of the secreted IGFBP-2 is shown in Fig. 5*A*. IGFBP-2 was recognized both in room-air conditions and after O_2 exposure. Computerized densitometry showed that there was no significant change in IGFBP-2 released into the culture medium after hyperoxia ($p > 0.05$; $n = 3$). However, it was significantly increased in the presence of RA in 95% O_2 compared with O_2 alone ($p < 0.05$; $n = 3$; Fig. 5*B*).

Incubation of the Western blots with anti-mouse IGFBP-3 antibody revealed a 43- to 45-kDa double band, representing glycosylated forms consistent with the known M_r of IGFBP-3 (35). Representative Western blot of the released IGFBP-3 is shown in Fig. 6*A*. IGFBP-3 was recognized as a double band both in room air and after O_2 exposure. IGFBP-3 in the culture

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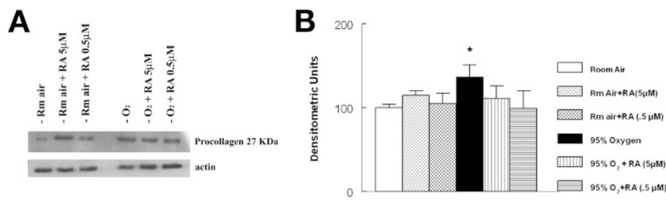

Figure 2. Effect of hyperoxia on procollagen propeptide in neonatal rat lung. (*A*) Representative Western blot for procollagen propeptide in rat lung tissue that was exposed to room air or 95% O_2 for 72 h with and without RA. (*B*) Densitometric analyses of procollagen measured in lung homogenate. The histogram shows quantitative representations of procollagen propeptide levels. Bars represent the mean ± SEM of $n = 3$ experiments; $*p < 0.05$ *vs* room air.

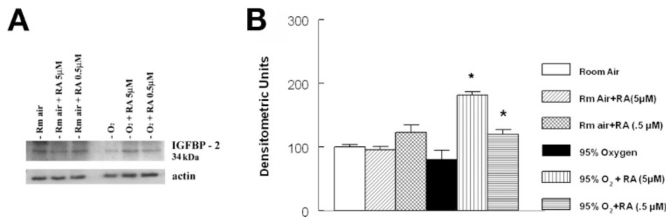

Figure 3. Effect of hyperoxia on tissue IGFBP-2 in neonatal rat lung. (*A*) Representative Western blots for IGFBP-2 protein in rat lung tissue that was exposed to room air or 95% O_2 without and with RA (5 and 0.5 μM) for 72 h. (*B*) Densitometric analysis of lung tissue IGFBP-2. The histogram shows quantitative representations of IGFBP-2 levels. Bars represent mean and SEM of $n = 3$ experiment; $*p < 0.05$ *vs* O_2 treatment alone.

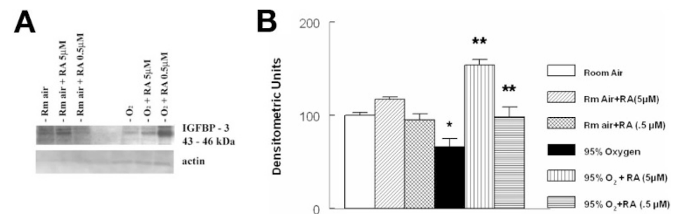

Figure 4. Effect of hyperoxia on cellular IGFBP-3 in neonatal rat lung. (*A*) Representative Western blot for IGFBP-3 protein in rat lung tissue that was exposed to room air or 95% O_2 for 72 h with and without RA. (*B*) The histogram shows quantitative representations of lung tissue IGFBP-3 levels. Bars represent mean ± SEM of $n = 3$ experiments; $*p < 0.05$ *vs* room air; $**p < 0.05$ *vs* O_2 treatment alone.

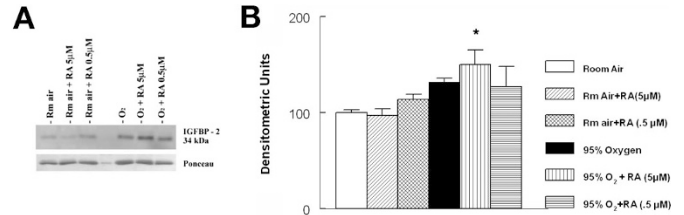

Figure 5. Effect of hyperoxia on IGFBP-2 in culture media from explant cultures that were treated with or without RA (5 and 0.5 μM). (*A*) Representative Western blots for IGFBP-2 from lung explant culture media after room air and O_2 exposure. (*B*) The histogram shows quantitative representations of protein levels of IGFBP-2 in culture media. Bars represent the mean ± SEM of $n = 3$ experiments; $*p < 0.05$ *vs* O_2 treatment alone.

medium was significantly increased with RA alone in room air ($p < 0.05$). IGFBP-3 seemed to be increased after 72 h of 95% O_2 compared with room air, but it was not statistically significant ($p > 0.05$). With O_2 exposure plus RA (5 and 0.5 μM), IGFBP-3 was increased significantly in the culture medium ($n = 3$; $p < 0.05$; Fig. 6*B*).

To determine the concentration of IGFBP-3 in the culture medium, we used an enzymatic "two-step sandwich" immunoassay. IGFBP-3 was significantly increased in the culture medium by RA and by hyperoxia. The increase with hyperoxia was increased further in the presence of RA (5 μM; Fig. 6*C*).

Effect of RA on IGFBP-3 mRNA expression in hyperoxia-exposed lung. RNA protection assay was used to determine whether the effects of hyperoxia and of RA on the expression of IGFBP-3 in the neonatal rat lung explant system were related to changes in mRNA levels. There was no significant change in IGFBP-3 mRNA expression with RA both in room air and hyperoxia ($p = 0.07$). There was no significant change in IGFBP-3 expression after hyperoxic exposure itself (Fig. 7).

DISCUSSION

We chose to use this *in vitro* lung culture system because it maintains parenchymal architecture and cell–cell contacts. Many workers have used similar systems in which cultures are maintained at the air–liquid interface to overcome the limitations of diffusion to study adult rat lung under hyperoxic exposure (36) and under normoxia (37). For example, we and others have used thin slices of neonatal and fetal lung explants cultured on Gelfoam saturated with serum-free medium (31,37,38). In these studies, a normal pulmonary parenchyma, with the exception of endothelial cells, was maintained for up to 60 d as assessed by light and electron microscopy.

We previously showed that O_2 exposure caused a proliferative response in this model similar to that seen *in vivo* with hyperoxic rat lung models (12,39,40) and in an adult rat lung organ culture model (36). Kelleher *et al.* (41) showed that lung fibroblasts that were isolated from rat lung after *in vivo* hyperoxic exposure had a higher proliferative potential compared with those from air-exposed rats.

IGF-1 is one of the factors that play an important role in development and cell proliferation and participate in the cellular response to injury (42–44). IGF-1 biosynthesis is enhanced during wound healing (45,46) and takes part in tissue repair because of its ability to enhance cell proliferation as well as collagen production (45).

Vitamin A seems to protect neonatal lung that is exposed to hyperoxia. Deficiency of vitamin A has been associated with the development of BPD, and improved pulmonary outcome is seen in infants with vitamin A supplementation (47). We showed in our earlier studies that RA, an active metabolite of vitamin A, caused an inhibition of hyperoxia-induced proliferative response in the *in vitro* lung when cultured in the presence of all-trans RA (5 μM) (12).

Retinoids have been shown to reduce immunoreactive IGF-1 in the culture medium in association with induction of apoptosis in breast cancer cell lines (45). In the present study, we used a model that incorporates simultaneous hyperoxic lung injury and RA exposure to study changes in collagen synthesis and in cellular and secreted IGFBP-2 and -3. The effect of RA is seen in the binding proteins both in lung tissue and in the culture medium of O_2-exposed lungs. The effect of RA on culture

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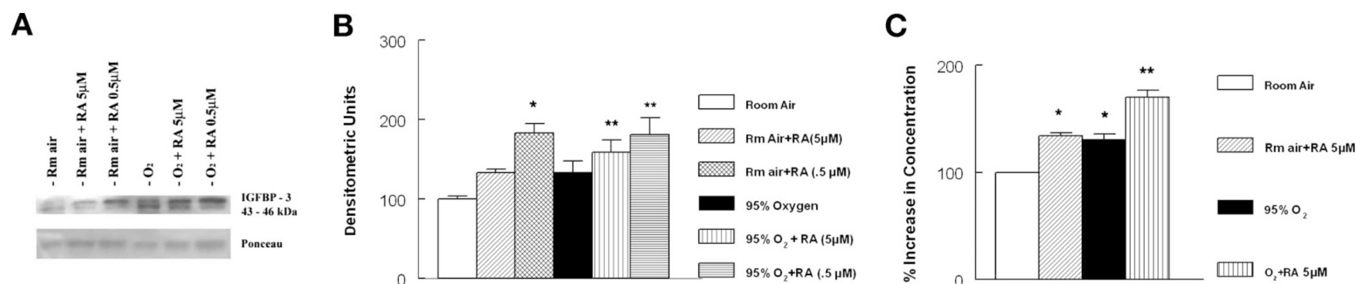

Figure 6. Effect of hyperoxia on IGFBP-3 in culture media from explant cultures that were treated with or without RA (5 and 0.5 mM). (*A*) Representative Western blot for IGFBP-3 from lung explant culture media after room air and O_2 exposure. (*B*) The histogram shows quantitative representations of protein levels of IGFBP-3 in culture media. Bars represent the mean ± SEM of $n = 3$ experiments; *$p < 0.05$ *vs* room air condition and O_2 treatment alone. (*C*) Total IGFBP-3 in culture media as measured by ELISA. Bars represent mean and SEM of $n = 3$ experiments; *$p < 0.05$ *vs* room air conditions; **$p < 0.05$ *vs* O_2 treatment alone.

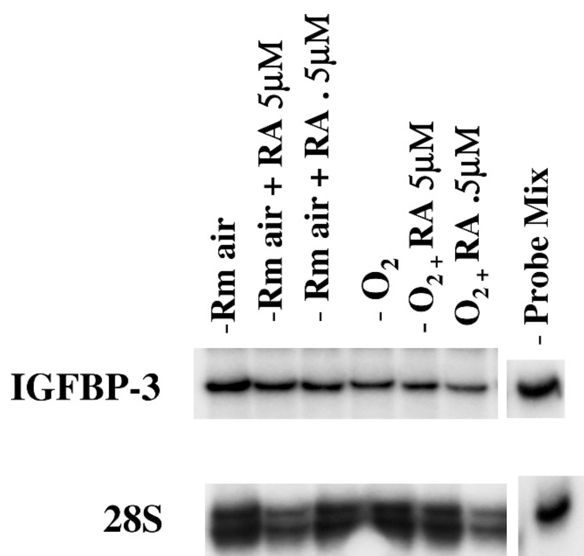

Figure 7. Effect of hyperoxia on IGFBP-3 mRNA in neonatal rat lung. Autoradiograph of RNAse protection assay of IGFBP-3 mRNA of lung explant culture that was exposed to room air or 95% O_2 for 72 h with and without RA. 28S was used as an internal control. (*A*) RNAse protection assay showing the IGFBP-3 protected bands in a representative sample of neonatal rat lung with two different concentrations of RA in room air and hyperoxia. Probe mix: IGFBP-3 and 28S. (*B*) Histogram represents densitometric analysis of IGFBP-3 bands corrected for 28S. Values are means ± SEM from $n = 4$ experiments.

medium content of IGFBP-2 seen in our study was also seen in type II epithelial cell line cultures (21). It is possible that IGFBP-2 acts through a mechanism involving a competition for IGF-1 and IGF-1 receptor, although the affinity of IGFBP-2 for binding IGF-1 is less than that of IGFBP-3. IGFBP-2 and -3 are mainly inhibitory for IGF-1 action by binding to it, rendering it inactive and leading to inhibition in cell proliferation. In addition, IGFBP-2 and especially IGFBP-3 may act independent of IGF-1 to influence cell proliferation (15). The mechanism by which RA can modulate cell proliferation is under investigation because it involves several different pathways. Glucocorticosteroid-induced growth arrest of rat lung alveolar type II epithelial cells has been associated with increased production of IGFBP-2 (48).

IGFBP-3 binds to IGF-1 with high affinity and sequesters IGF-1 from binding to its receptor. Our present results indicate that IGFBP-3 is increased in the presence of RA at 5 and 0.5 μM in lung explants and in culture medium after hyperoxia. This agrees with studies using human dermal papilla cells in which retinoids inhibit IGF-1 action by stimulating IGFBP-3 production by these cells (30). In that system and in the current study, it is possible that IGFBP-3 forms a complex with IGF-1 to reduce the concentration of available IGF-1. A similar potent inhibitory effect of RA (100 nM) was seen in MAC-T cell proliferation (29). Retinoids have been shown to have growth inhibitory and proapoptotic factors for many cell lines (49). However, it is unclear why IGFBP-3 is increased more at a lower concentration of RA (0.5 μM) both in the tissue and in culture medium.

The current study shows that IGFBP-3 is increased at the protein level after RA treatment. It is possible that the induced IGFBP-3 may act as a proapoptotic factor inhibiting cell proliferation, as seen in proliferating cell lines (50). The increase in IGFBP-3 was higher in the culture medium compared with the lung tissue in the presence of RA after hyperoxic exposure. This is consistent with studies done in a human lung adenocarcinoma cell line by Besnard *et al.* (35). In their study, growth arrest of human alveolar epithelial cells to hyperoxia was associated with an increased expression of IGFBP-2 and IGFBP-3; however, IGFBP-2 was localized mainly in the intracellular compartment and secreted less in culture media. These findings reflect distinct expression patterns for these two binding proteins in intracellular compartment. The reason for the small increase in IGFBP-2 and -3 in culture medium that we see after hyperoxic exposure is unclear. Cazals *et al.* (51) showed that IGFBPs accumulate to higher levels in culture medium of SV40T-T2 cells whose proliferation has been arrested by hyperoxia. We are reporting a similar increase in these binding proteins in the culture medium of rat lung explants. In contrast, proliferating astrocytes showed increased cytochemical expression of IGFBP-2 with decrease in culture medium as a result of the presence of proteases (52).

In this study, we found a small but nonsignificant decrease in the mRNA expression of IGFBP-3 in neonatal rat lung after hyperoxic exposure. Han *et al.* (53) observed a decrease in IGFBP-3 mRNA after a 6-d and a 14-d exposure to 85% O_2 in adult rat lung. It is not clear from our results whether the reduced mRNA with increased protein in the media represents increased mRNA translation, increased

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protein secretion, increased protein stability, or a combination of these factors. Similar increase in IGFBP-3 in culture medium was seen in breast cancer cell lines in response to RA, suggesting that RA acts principally at a translational or posttranslational level (20).

Increased cell proliferation with injury is often associated with an increase in collagen synthesis. Therefore, we studied collagen production in neonatal lung by analyzing the content of procollagen propeptide and type I collagen. As part of the repair process after lung inflammation, fibroblasts proliferate and produce large amounts of collagen. It has been shown that IGF-1 increases collagen synthesis in several systems, including human lung fibroblasts (54,55). In our study, after hyperoxia exposure in neonatal lung explants, we observed an increase in collagen production as evidenced by an increase in type I collagen and procollagen propeptide. This increase in type I collagen was significantly inhibited in the presence of RA. This agrees with the study of Kropsky *et al.* (33), who found that RA-treated human lung fibroblasts showed a decrease in steady-state collagen mRNA level. In contrast, RA treatment *in vivo* led to increased collagen staining in air-space walls in hyperoxia exposure of neonatal rats (56). The mechanism by which RA alters lung collagen remains unknown. We have shown in our previous studies that the increase in cell proliferation of neonatal rat lung explants after exposure to 95% O_2 for 72 h was inhibited in the presence of RA and in the presence of neutralizing antibody to IGF-1 (12,38). We expect to perform more experiments using the neutralizing antibodies to establish the mechanisms. The difference between these two studies could be due to several factors, including the type of hyperoxia exposure (*in vivo versus in vitro*), duration of hyperoxia, the dose of RA used, and stability of the collagen molecule.

In conclusion, we have shown that in hyperoxia-exposed neonatal rat lung explants, IGFBP-2 and -3 levels increase in response to RA. The growth-inhibitory effect of RA may be due to increased IGFBP-2 and -3. Our results have some limitations in interpreting cellular IGFBPs because they are synthesized on the endoplasmic reticulum and are exported from the cell in a constitutive or a continuous manner. Constitutive secretion can be increased in response to certain growth factors (57) and cytokine stimulation (58). Because the interaction between IGF-1 and IGF-1 receptor is modulated by IGFBPs and these binding proteins affect cell proliferation and collagen synthesis, the changes in IGFBP-2 and -3 in association with an inhibition of collagen synthesis in our current study suggest that these binding proteins may influence the biologic action of IGF-1 in our hyperoxia model. Whether IGFBP-2 and/or IGFBP-3 acts by IGF-1–dependent or IGF-1–independent pathways in our system requires further studies.

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