Hyperoxia inhibits fetal rat lung fibroblast proliferation and expression of procollagens

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Hussain, Naveed, Fengying Wu, Constance Christian, and Mitchell J. Kresch. Hyperoxia inhibits fetal rat lung fibroblast proliferation and expression of procollagens. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L726–L732, 1997.—The direct effects of hyperoxia on collagen production by fetal lung fibroblasts are unknown and would be important to the understanding of the molecular mechanisms involved in bronchopulmonary dysplasia in premature infants. We studied the effect of hyperoxia on 1) proliferation, 2) mRNA levels for type I and III procollagens, and 3) net collagen production in primary cultures of fetal rat lung fibroblasts. Fibroblasts from 19-day-old rat fetuses (term is 22 days) were obtained. Test plates were incubated in hyperoxia and controls in room air for varying time periods. Cell viability in both conditions was >97% as determined by trypsin blue exclusion. Fibroblast proliferation in nonconfluent cultures was found to be significantly reduced with exposure to hyperoxia (P < 0.001). Steady-state levels of type I and III procollagen mRNAs, analyzed on Northern blots hybridized to 32P cDNA probes, were significantly decreased in hyperoxia (P < 0.01). This effect was noted as early as 4 h of exposure to hyperoxia and persisted for 5 days. There was a significant inverse correlation between duration of exposure to O2 and steady-state levels of mRNA for α1(I)-procollagen (r = –0.904) and α1(III)-procollagen (r = –0.971). There were no significant changes in steady-state levels of β-actin mRNA. We also found a significant decrease in collagen synthesis in hyperoxia-exposed fibroblasts (P < 0.05). We conclude that hyperoxia directly affects a reduction in fetal lung fibroblast proliferation and net collagen production at a pretranslational level.

EXPOSURE OF THE LUNGS of human premature infants to high concentrations of O2 is a major factor in the development of bronchopulmonary dysplasia, which is characterized by inflammation and fibrosis (5). The major contributors to fibrosis in this situation are collagen types I and III produced by lung fibroblasts (12). Similar changes are observed in the O2-exposed lungs of numerous species, including the rat, as reviewed by Balentine (3). The mechanism by which hyperoxia ultimately leads to lung fibrosis is unclear. Understanding whether this is a direct effect of hyperoxia on fibroblasts or secondary to the influence of O2 on other cell types is important. The little that is known suggests that hyperoxia inhibits proliferation of cultured fibroblasts derived from adult animals (29) or cell lines (4), but it is not known if fetal lung fibroblasts have the same response. Also, the direct effect of hyperoxia on lung fibroblast collagen expression is unknown. We formulated the hypothesis that O2 directly affects expression of types I and III collagen by fibroblasts derived from fetal rat lungs. In this report, we describe the direct effects of hyperoxia on 1) fetal lung fibroblast proliferation, 2) steady-state levels of mRNA for α1-chains of type I and type III procollagens, and 3) net collagen production and noncollagen protein synthesis by fetal rat lung fibroblasts.

METHODS AND MATERIALS

Isolation and culture of fetal lung fibroblasts. Primary cultures of fibroblasts from fetal rat lungs were obtained as previously described by Smith (27) and Kresch et al. (22). Briefly, the lungs of 19-day gestation fetal rats (term is 22 days) were removed, dissected free from connective tissue and nonpulmonary pulmonary tissue, and cultured as explants for 48 h in serum-free Waymouth MB 752/1 medium with penicillin and streptomycin in 95% O2-5% CO2 at 37°C. During this time, endothelial and blood cells do not survive. The explant tissue was then harvested, and the cells were dissociated using a solution of collagenase, trypsin, and deoxyribonuclease. Cells were suspended in minimal essential medium (MEM) with 100 U/ml penicillin and 100 µg/ml kanamycin containing 10% fetal bovine serum (FBS). The mixed cell suspension was subjected to differential adhesion on plastic tissue culture dishes (Costar, Cambridge, MA), and fibroblasts were separated by their property of adhering to the plastic. Nonadherent cells were washed off the plate. Alveolar macrophages are not present in fetal lungs and were not a source of contamination of fibroblast cultures. Fibroblasts were cultured at 37°C in MEM containing 100 U/ml penicillin, 100 mg/ml kanamycin, and 2.5 mg/ml amphotericin B (PKA) and 10% FBS. All cultures contained 94 ± 2% (mean ± SE) fibroblasts as determined by morphological appearance under phase contrast microscopy. Exposure to hyperoxia. Control samples were kept in room air-5%CO2 environment at 37°C. The O2 tension in the media was 70–80 mmHg. Test samples were kept in a Plexiglas chamber on a rocking platform (Bellco Glass, Vineland, NJ), maintaining a constant environment of 95% O2-5% CO2 at 37°C for various time points studied. This maintained an O2 tension of 480–520 mmHg in the media. The pH was not significantly affected (7.30–7.34 in controls vs. 7.28–7.31 in hyperoxia). Cell viability at each time point was 97 ± 2% (mean ± SE) in both conditions measured by trypan blue exclusion as described by Freshney (16).

Cell proliferation in hyperoxia. Nonconfluent fibroblasts obtained within 12 h after adhesion on 60-mm tissue culture plates were washed with warm phosphate-buffered saline (PBS), and the medium was changed to either MEM + PKA + 10% FBS (regular medium) or MEM + PKA + 10% carbon-stripped FBS (CSFBS; carbon-stripped medium). This was done to control for any effects of carbon-stripped serum on cell proliferation. [Methyl-3H]thymidine (2 µCi/ml; specific activity 6.7 Ci/mmol) was added to each plate. Replicate samples were kept in separate incubators at 37°C and were exposed to normoxia (room air-5% CO2) or hyperoxia (95% O2-5% CO2) for various periods of time (2–96 h) before full confluence. Then the plates were washed with PBS, and the cell mono-
layer was released from the plate with 1 ml of 0.05% trypsin treatment for 15 min. A fraction of this was taken for counting in the hemocytometer, and the rest was used for the assay of [3H]thymidine incorporation as described by Smith et al. (28).

Briefly, 5 ml of absolute methanol were added to precipitate DNA. After refrigeration overnight, the material was centrifuged at 2,000 g for 20 min, and the pellet was washed with 5 ml of methanol and was recentrifuged. The supernatant was decanted, and the pellet was solubilized in 1 ml of a solution of 2% Triton X-100 and 2% sodium dodecyl sulfate (SDS). This was then mixed with 10 ml of Opti-Fluor liquid scintillation cocktail and was counted in a Beckman Scintillation Spectrometer. The results were expressed as counts per minute (cpm) per 106 cells.

In three separate experiments, we studied the effect of 48 h of exposure to O2 or room air on confluent cultures of fibroblasts. Thymidine incorporation was studied as described above.

RNA extraction and Northern analysis. Confluent fibroblasts obtained 36–48 h after adhesion on 60-mm tissue culture plates were washed with warm sterile PBS, and the medium was changed to MEM + PKA + 10% CSFS BS. Replicate samples were kept in separate incubators and were exposed to room air-5% CO2 or hyperoxia (95% O2–5% CO2) for various time points. At the end of each time point, 10 µl of cell media from each condition was taken and concentrated using a Centricon 100 concentrator (Amicon, Beverly, MA) spun at 1,000 revolutions/min (rpm) for 6 h. The 100 µl concentrate was acidified to a final concentration of 0.5 M acetic acid by 10.220.32.246 on June 20, 2017 http://ajplung.physiology.org/ Downloaded from

...ing by 32P by random priming and were used for Northern blot analysis. After prehybridization with 60% formamide, 6× SSC, 5× Denhardt’s reagent, and 0.2% SDS with 100 µg/ml denatured salmon sperm DNA, hybridization was performed at 42°C in a solution containing 50% formamide, 5× SSC, Denhardt’s reagent, 0.2% SDS, 10% dextran sulfate, and 1× 106 cpm/ml labeled cDNA probe. The blots were washed at room temperature one time in 1× SSC and 0.1% SDS followed by three washes at 68°C in 0.2× SSC and 0.1% SDS. Autoradiography or β-scanning was carried out to obtain an optimal signal for comparison.

Northern analysis of mRNA from nonconfluent and confluent fibroblast cultures. In some experiments, fibroblasts isolated within 12 h after adhesion on plastic tissue culture plates were washed with PBS, and the cells were incubated in MEM + PKA + 10% CSFS BS. This time was designated 0 h for these experiments. Replicate samples were kept in separate incubators at 37°C and were exposed to room air-5% CO2 or hyperoxia (95% O2–5% CO2) for various times (2–120 h). These cultures were initially nonconfluent and attained full confluence between 24 and 48 h. No changes were made in the media during the course of these experiments. At each specified time, appropriate control and test cultures were harvested for RNA isolation and Northern analysis as described above. These experiments were different in that the time of initial plating of fibroblasts was taken as 0 h to study the effects of O2 on nonconfluent cultures, whereas in other experiments involving RNA or protein analyses, to study the effects of O2 on confluent cultures, 0 h was the time at which cultures had reached complete confluence.

Collagen protein synthesis. Experiments were done in parallel to those mentioned above for mRNA assessments with confluent fibroblast cultures. In the culture plates that were used for collagen protein studies, 10 µCi/ml of [3H]proline (specific activity 25 Ci/mmol) were added to the culture media 2 h before completion of the time period. At the end of the study period, medium was removed from the cell layer and was placed in a glass tube. The cells in each dish were scraped into 1 ml of homogenization buffer (1 M NaCl, 50 mM tris(hydroxymethyl)aminomethane-HCl (pH 7.4), 2.5 mM EDTA, 1 mM N-ethylmaleimide, and 0.2 mM phenylmethylsulfonyl fluoride) and were added back to their respective medium. Each sample was adjusted to 15% trichloroacetic acid (TCA) and was sonicated two times for 10 s on ice.

Pretreated proteins were collected by centrifugation at 4,000 g for 20 min, washed one time with 10% TCA and one time with 10 mM potassium acetate in absolute ethanol, and dissolved in 0.5 M NaOH. The incorporation of [3H]proline into collagenase-digestible and noncollagen protein was determined using purified bacterial collagenase by the method of Peterkofsky and Diegelmman (24). The amount of radioactivity solubilized by collagenase is a measurement of the collagen synthesized.

The remaining radioactivity in the precipitate represents the noncollagen protein synthesized. The percentage of collagen present in a portion of analyzed protein substrate was determined on the basis of the following calculations from Diegelmman and Peterkofsky (13): percentage of collagen synthesis = [cpm in collagen digest/(cpm in residue × 5.4)] + (cpm in collagen digest) × 100. DNA content was determined using diamidinophenylindole to correct for cell numbers in each dish (8). Results are shown as the percentage of collagen synthesis per microgram of DNA.

To further delineate the effects of hyperoxia on α1(I)-, α2(I)-, and α3(III)-net collagen production, we used SDS-polyacrylamide gel electrophoresis (PAGE) of labeled proteins extracted from fibroblasts. Experiments were done similar to those mentioned above for RNA measurements except that tritiated proline was added to the culture media for 24 h of labeling. After the end of hyperoxic exposure at 24 and 48 h, the media were collected separately. One milliliter of medium from each condition was taken and concentrated using a Centricon 100 concentrator (Amicon, Beverly, MA) spun at 1,000 revolutions/min (rpm) for 6 h. The 100 µl concentrate was acidified to a final concentration of 0.5 M acetic acid solution. This was digested with pepsin (2 mg/ml final concentration) for 6 h at 16°C. The acetic acid was neutralized with equimolar solution of NaOH, and a repeat Centricon spin at 1,000 rpm was done for 1 h to get a final volume of ~75 µl. This was mixed with protein sample buffer containing 5 M urea and was boiled for 5 min. This was loaded on a 5% polyacrylamide gel, and electrophoresis was performed using the Sykes et al. modification (30) of the Laemmli method (23).

Briefly, this involved changing to reducing conditions with β-mercaptoethanol after an initial run to delineate the separation of the subunits of type I and III collagens. The gel was then fixed with 10% (vol/vol) Glacial acetic acid and 30% (vol/vol) methanol and was kept in Enhance for 1 h. After immersing in cold water for 0.5 h and drying the gel, the radioactive emission by tritiated proline incorporated in the
collagen was captured on a Kodak X-OMAT AR photographic film after an 8-day exposure at −80°C (20, 21). Statistics. All data are means ± SE of three to five experiments. Statistical significance was determined using the Student’s t-test or analysis of variance with post hoc Bonferroni/Dunn test as appropriate. Correlations were determined using Spearman correlation. Differences were considered significant at P < 0.05.

Materials. Timed pregnant Sprague-Dawley rats were purchased from the Charles River Breeding Laboratories (Wilmington, MA). All culture media were purchased from Gibco-BRL (Grand Island, NY). Culture supplies and plasticware were obtained from Costar (Cambridge, MA). Collagenase and trypsin for cell dissociation were obtained from Worthington (Freehold, NJ). Radios isotopes were purchased from New England Nuclear (Boston, MA). Opti-Fluor was bought from Packard (Meriden, CT). FBS was purchased from Hydcone (Logan, UT). All other chemicals and reagents, including purified bacterial collagenase for the assay, were purchased from Sigma Chemical (St. Louis, MO). FBS (Hydcone lot no. 11112207) was carbon stripped by the method of Yoshiizato et al. (34) as modified by Tanswell et al. (31).

RESULTS

Cell viability. When cells and tissues are exposed to high concentrations of O2, they exhibit toxic effects leading to cell death (3, 17). However, fibroblasts from fetal rat lungs in these experiments were resistant to the cytotoxic effects of hyperoxia, as their cell viability was 97 ± 2% at all time points studied up to 5 days. The mRNA levels for the housekeeping gene β-actin also remained fairly constant (see below), indicating that these cells were maintaining their vital function.

Cell proliferation. Data for the effect of hyperoxia on 
[3H]thymidine incorporation by fetal rat fibroblasts cultured in media containing either regular or CSFSB are shown in Fig. 1. In nonconfluent fibroblasts cultured in regular medium (MEM + PKA + 10% FBS) and exposed to room air conditions, [3H]thymidine incorporation per 106 cells increased 67-fold, from

![Figure 1](http://ajplung.physiology.org/)

**Fig. 1.** Hyperoxia inhibits proliferation of fetal rat lung fibroblasts. Cells were cultured in either media containing charcoal-stripped fetal bovine serum or regular fetal bovine serum. [3H]thymidine incorporation was expressed as counts per minute (cpm) per 106 cells. Data are means ± SE of 7 samples. Proliferation of cells exposed to 95% O2 (O2) was significantly inhibited compared with room air-exposed cultures (RA) at 24, 72, and 96 h of exposure (*P < 0.05, §P < 0.01, and ¶P < 0.001, respectively). Cell proliferation in carbon-stripped medium was lower than regular medium but the percentage of inhibition of proliferation in hyperoxia was similar in both media at all time points studied.

<table>
<thead>
<tr>
<th>Time, h</th>
<th>a1(Ⅰ)-procollagen mRNA</th>
<th>a1(Ⅲ)-procollagen mRNA</th>
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<tr>
<td>0</td>
<td>100</td>
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<tr>
<td>48</td>
<td>65 ± 12*</td>
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<td>120</td>
<td>36 ± 10*</td>
<td>23 ± 12*</td>
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*Data are means ± SE given as percent change from sample of confluent fibroblasts at 0 h; n = 3–5 experiments. Time-dependent change in a1(Ⅰ) and a1(Ⅲ)-procollagen mRNA levels in cultures of fetal rat lung fibroblasts exposed to hyperoxia. Densitometric units were compared after normalizing with the message of β-actin. The steady-state levels of both a1(Ⅰ) and a1(Ⅲ)-procollagen mRNAs were significantly decreased in hyperoxia at 48 and 120 h of exposure. *P < 0.01 vs. control at 0 h.

Cell proliferation was significantly decreased in the hyperoxia-exposed fibroblast cultures incubated in both regular and carbon-stripped media at 24 (P < 0.05), 72 (P < 0.01) and 96 (P < 0.001) h. There was no effect of 48 h of exposure to hyperoxia on cell proliferation in confluent fibroblast cell cultures (regular medium: room air 6,399 ± 600 cpm/106 cells vs. 02 5,912 ± 37 cpm/106 cells, P = not significant; carbon-stripped medium: room air 5,620 ± 935 cpm/106 cells vs. O2 3,623 ± 78 cpm/106 cells, P = not significant).

Time-dependent decrease in procollagen mRNA levels in hyperoxia. In confluent fibroblast cultures, we found a decrease in the steady-state mRNA levels of both a1(Ⅰ)- and a1(Ⅲ)-procollagens with hyperoxia exposure at all time points studied, starting as early as 4 h (data not shown) and continuing up to 48 and 120 h (Table 1). Intact and comparable RNA loading per lane was confirmed by ethidium bromide staining and also by β-actin mRNA levels that did not change as a result of hyperoxia. Densitometric analysis of the autoradiographs was done, and the steady-state mRNA levels of a1(Ⅰ) and a1(Ⅲ)-procollagens in room air controls were normalized to 100% for comparison. The mRNA levels of a1(Ⅰ)-procollagen in fibroblasts exposed to hyperoxia were 65 and 36% of controls kept in room air at 48 and 120 h, respectively, as shown in Table 1 (P < 0.01). Similarly, the steady-state mRNA levels of a1(Ⅲ)-procollagen in fibroblasts cultured in hyperoxia were 56 and 23% of controls kept in room air at 48 and 120 h, respectively (P < 0.01). There was a significant inverse...
correlation between steady-state mRNA levels and time of exposure to hyperoxia for \( \alpha_1(1) \)-procollagen \((r = -0.904, P < 0.001) \) and \( \alpha_1(III) \)-procollagen \((r = -0.971, P \leq 0.001) \). This was a selective effect on mRNA collagen expression because the steady-state levels of \( \beta \)-actin mRNA did not change.

Time-dependent changes in procollagen mRNA levels in room air. Interestingly, there was an increase in the steady-state levels of \( \alpha_1(1) \)-procollagen mRNA in fibroblasts cultured in room air in the first 48 h (Fig. 2A, lanes 1, 3, and 5). A similar increase was seen in levels of \( \alpha_1(III) \)-procollagen mRNA (data not shown). This was followed by a decrease in mRNA levels for these procollagenes at 120 h in culture (Fig. 2B, lane 3, and Fig. 3, lane 3). The decrease in expression of procollagen mRNA at 120 h may be a reflection of the feedback inhibition of gene transcription by collagen peptides that has been previously shown by others (10, 33).

Changes in procollagen mRNAs in nonconfluent and confluent fibroblast cultures. When nonconfluent fibroblasts soon after adhesion were exposed to room air or hyperoxia, the procollagen mRNA levels in hyperoxia-exposed cultures were lower than the room air-exposed cultures at each time point (Fig. 2A). In the first 24 h of culture, before the fibroblasts became confluent, the procollagen mRNA levels in hyperoxia increased to a lesser degree over time than the corresponding cultures exposed to room air. However, on continued culture of fibroblasts from 48 to 120 h (Fig. 2B), after full confluence was attained, the procollagen mRNA levels in hyperoxia were markedly reduced compared with the corresponding room air cultures (Fig. 2, A, lanes 5 and 6, and B).

Percentage of collagen protein synthesis. To demonstrate that the decreases in mRNA levels were reflected by a concomitant decrease in net collagen protein production in the fibroblasts, we studied the incorporation of \( ^{3}H \)proline in collagen protein. The percentage of collagen synthesis at each time was calculated according to Peterkofsky and Diegelmann (24). Figure 4 shows the percentage of collagen synthesis at 24 h of hyperoxia exposure. We observed a 30% decrease in the percentage of net collagen protein synthesis in hyperoxia compared with room air \((P < 0.05)\).

To determine if this was a specific inhibition in collagen synthesis versus a nonspecific inhibition of total protein synthesis, we compared the collagenase-digestible protein fraction with the noncollagenase-digestible fraction, which is representative of general protein synthesis. Figure 5 shows the percent change in each fraction with increasing duration of exposure to hyperoxia, considering 0 h levels to be 100%. The percent decrease in collagen protein was highly correlated to time of hyperoxia exposure \((r = -0.944, P < 0.001) \). The absolute decrease at 12 and 24 h of exposure was also statistically significant \((P < 0.05) \). Conversely, noncollagen protein levels showed a positive correlation with time of hyperoxia exposure \((r = +0.916, P < 0.001) \). The absolute increase in noncollagen protein levels at 24 h of hyperoxia was also statistically significant \((P < 0.05) \). This confirmed our observation that the time-dependent effect of hyperoxia was specific for collagen and not a nonspecific or toxic decrease in total cellular protein synthesis.

As shown in Fig. 6, we also measured net collagen production using SDS-PAGE of labeled cellular proteins to confirm these effects and to further examine the effects on net production of \( \alpha_1(1) \)- and \( \alpha_2(1) \)-collagens by fetal lung fibroblasts exposed to hyperoxia for 24–48 h. We found a significant decrease in net production of both collagens by fibroblasts exposed to hyperoxia at both 24 and 48 h. We were unable to detect \( \alpha_1(III) \)-collagen proteins in either control or experimental cultures using this method.

**DISCUSSION**

The pulmonary response to hyperoxic exposure is characterized by extensive injury to airway, parenchy-
mal, and vascular structures leading to inflammation and tissue remodeling that may ultimately result in complete repair or fibrosis (12). This situation is further influenced by immaturity of specific lung cells in the premature infant that develops bronchopulmonary dysplasia (6). Few studies have addressed the direct effects of O2 on specific lung cells. Those studies dealing with fibroblasts have utilized established cell lines (4) or primary cultures of cells obtained from adult animals (29). No study before this has reported on the direct effects of hyperoxia on cellular proliferation or expression of collagen synthesis in primary cultures of fibroblasts derived from fetal rat lungs. This model may be more relevant to an understanding of the molecular events involved in hyperoxic injury to premature lungs.

Hyperoxia inhibits fibroblast proliferation. We have shown that hyperoxia decreases proliferation of cultured fetal rat lung fibroblasts after 24–96 h exposure. Cell proliferation in hyperoxia was ~10–30% of that in room air, given optimal temperature, pH, and CO2 environment. This inhibitory effect was seen with both carbon-stripped and regular media. Experiments done in parallel with regular and carbon-stripped media show that carbon stripping (which removes hormones...
such as glucocorticoids that influence cellular proliferation and collagen expression) decreases proliferation, but the effects of hyperoxia are still significant in both media conditions studied. We conclude that hyperoxia does inhibit fibroblast proliferation.

The decrease in fibroblast proliferation with hyperoxia concurs with other reports of similar inhibition noted in fibroblast cell lines and primary cultures from mature adult lungs (1, 17). However, in vivo exposure of lungs to elevated levels of O2 leads to increased fibroblast proliferation (12). Shapiro et al. (26) reported that exposure of lung slices in organ culture to hyperoxia for 3 days also caused increased proliferation of parenchymal cells that returned to baseline after 7 days of exposure. The specific cells affected, however, were not described in that study. Kelleher et al. (19) recently showed that lung fibroblasts isolated from rats that were exposed to hyperoxia proliferated more rapidly than fibroblasts obtained from rats exposed to room air. The fibroblasts isolated from hyperoxia-exposed animals were more sensitive to the proliferative effects of insulin-like growth factor I, and these cells had a higher expression of c-Ha-ras, which is an essential protein for progression through the cell cycle (19). Unfortunately, these experiments were performed on cells isolated after the tissue was exposed, which makes it difficult to exclude primary or secondary influences of other cell types that were present. Moreover, these cells were cultured in serum that may contain hormones and growth factors that might confound the interpretation of these results. Nevertheless, these studies indicate that cell-cell interactions with neighboring cells are important in the in vivo responses.

Hyperoxia decreases levels of procollagen mRNA in confluent cultures of fibroblasts. Our experiments demonstrate that hyperoxia results in a time-dependent decrease in steady-state mRNA levels for both types α1(I)- and α1(III)-procollagens in confluent fetal lung fibroblast cultures. This is a novel finding because previous studies by Houssset et al. (17) with mixed cell cultures have shown that exposure to elevated O2 levels leads to an increase in α1(I)-procollagen mRNA. Similarly, Armstrong et al. (2) demonstrated that oxidant exposure of rats increased the lung content of type I collagen mRNA. This discrepancy between the responses of primary cell cultures and mixed lung cell cultures can be reconciled if it may be suggested that other cells, especially alveolar macrophages, and proinflammatory cytokines [e.g., tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, and interleukins] are involved in mediating the effects of hyperoxia in vivo. Recent evidence has shown that alveolar macrophage-depleted rats have a significantly higher tolerance to hyperoxia than controls (5). Also, TNF-α has been shown to enhance susceptibility of lung cells to O2 toxicity, and administration of anti-TNF antibodies protects against this toxicity (18).

Biphasic time-dependent effects in steady-state collagen mRNA in fibroblasts cultured in room air. We found an increase in the levels of α1(I)-procollagen mRNA in lung fibroblasts cultured in room air over the first 48 h after plating, which was followed by a decrease in steady-state levels at ~120 h after plating (Fig. 2B). The same was true for α1(III)-procollagen mRNA (Fig. 3). Whereas it is possible that this later decrease in levels of mRNA is due to the time-dependent effects on cell proliferation, this later decrease in mRNA occurs well after the fibroblasts have reached confluence, making this possibility less likely. A more likely explanation for these findings is that there is an initial time-related increase in the mRNA levels of procollagens when fibroblasts are freshly cultured; once cells have reached confluence and enough collagen protein has been translated and secreted, then feedback inhibition of collagen expression would lead to reduced steady-state levels of procollagen mRNA. The role of collagen peptides on pretranslational regulation of collagen synthesis has been well documented (10, 33). Comparison of results from different studies must take these effects of time and confluence of cultures into account.

Effect of hyperoxia on collagen expression is pretranslational. In our study, the decrease in mRNA levels was detectable by 4–6 h exposure of fibroblasts to high O2 levels and became progressively more pronounced with increasing times of exposure up to 5 days without affecting the cell viability. The steady-state levels of β-actin mRNAs were not affected, suggesting that the effect on procollagen mRNAs was selective and was not a global shut down of cell machinery due to the toxic effects of O2. The demonstration that there was a specific decrease in net collagen production, with respect to noncollagenous proteins (Fig. 6), supports our conclusion that the changes shown are specific and not general. In fact, the increase in noncollagen protein synthesis at 24 h of exposure indicates that the cellular mechanisms that support protein synthesis remain intact. We therefore conclude that hyperoxia directly affects a decrease in collagen gene expression at the pretranslational level in fetal rat lung fibroblasts.

The mechanism by which hyperoxia directly affects fibroblast collagen expression is unknown. It has recently been suggested by Duncan et al. (14) that the transcription factor nuclear factor-1, which is influenced by the cell redox state, may regulate the promoter for both collagen types I and III. Falanga et al. (15) have shown that low O2 tensions increase mRNA levels of α1(I)-procollagen in fibroblast cell cultures. Our finding that high O2 tensions decrease the levels of both α1(I)- and α1(III)-procollagen mRNAs in fibroblast cultures may be another clue that changes in the cell redox state may regulate collagen expression.

In conclusion, we have shown that the direct effects of hyperoxia on fetal rat lung fibroblast cultures are 1) a decrease in proliferation and 2) a pretranslational decrease in types I and III procollagen expression. Because the opposite effects, i.e., increased proliferation and collagen expression, are seen in vivo and in tissue or organ cultures, we speculate that other cell types (alveolar macrophages) or cytokines (e.g., TNF-α, TGF-β, and interleukins) may mediate the profibrotic effects of hyperoxia.
We thank Drs. Roger Thrall and Alex Lichtler for helpful review of the manuscript. We are also grateful to Drs. Barbara Kream and Joel Pachtor for the cDNA probes and Eileen Roark and Penny Kelly for technical support and assistance.

This work as supported in part by grants from the Charles H. Hood Foundation (to M. J. Kresch) and Patterson trust (to M. J. Kresch). Address for reprint requests: N. Hussain, Dept. of Pediatrics, Univ. of Connecticut School of Medicine, Mail Code #203, Farmington, CT 06030.

Received 13 March 1996; accepted in final form 17 June 1997.

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