Hypoxia suppresses elastin repair by rat lung fibroblasts

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Berk, John L., Christine A. Hatch, Shirley M. Morris, Phillip J. Stone, and Ronald H. Goldstein. Hypoxia suppresses elastin repair by rat lung fibroblasts. Am J Physiol Lung Cell Mol Physiol 289: L931–L936, 2005.—Macrophage and neutrophil proteinases damage lung elastin, disrupting alveolar epithelium and filling alveoli with inflammatory exudate. Alveolar collapse and regional hypoxia occur. Whether low oxygen tension alters fibroblast-mediated lung repair is unknown. To determine the effect of chronic hypoxia on repair of enzyme-induced elastin disruption, primary rat lung fibroblasts produced elastin matrix for 5 wk before treatment with porcine pancreatic elastase (PPE). After exposure to PPE or saline, cultures recovered for 2 wk in normoxia (21% O2) or hypoxia (3% O2). Hypoxia suppressed regeneration of hot alkali-resistant elastin, achieving only 49% of the repair achieved in normoxic cultures. Vascular smooth muscle cells and lung fibroblasts repair elastin by two pathways: de novo synthesis and salvage repair. Although both pathways were affected, hypoxia predominantly inhibited de novo synthesis, decreasing formation of new elastin matrix by 63% while inhibiting salvage repair by only 36%. Prolonged hypoxia alone downregulated steady-state levels of elastin mRNA by 45%, whereas PPE had no significant effect on elastin gene expression. Electron microscopy documented preservation of intracellular organelles and intact nuclei. Together, these data suggest that regional hypoxia limits lung elastin repair following protease injury at least in part by inhibiting elastin gene expression.

tropelein; elastase; lung injury; elastic fiber; extracellular matrix

ELASTIN IS ESSENTIAL to lung physiology. In animal models of emphysema, exposure to macrophage- or neutrophil-derived proteinases disrupts elastin scaffolding, altering alveolar architecture and impairing gas exchange (9, 12). Ultrastructural studies after elastase treatment demonstrate rapid loss of elastin in alveolar walls and septal tips (11, 17), interruption of alveolar epithelial cell tight junctions, and flooding of terminal air spaces (14). Ultimately, alveolar collapse produces regions of tissue hypoxia.

Several studies document fibroblast repair of elastin matrix in the lung. One day following elastase injury, in situ hybridization studies demonstrated increased expression of elastin mRNA at the free margins of damaged alveoli septa in hamster lung (11). Four days postinjury, transmission electron microscopy revealed formation of new fibrillar elastin adjacent to synthetically active interstitial fibroblasts, the principal producer of new elastin molecules in extravascular lung (11, 20). One month after injury, alveolar elastin regained structural continuity and pretreatment levels of total lung elastin (20).

Despite evidence for repair following elastase treatment, morphometric analyses of lung tissue indicate progressive decreases in alveolar number and increased mean linear intercept (24), persistently abnormal gas diffusion and lung volumes (12), and misshapen elastin (17). Aberrant lung remodeling and alveolar collapse suggest that regional tissue hypoxia may alter elastin repair by interstitial lung fibroblasts.

The effects of hypoxia on matrix production are organ specific. In the lung, hypoxia suppresses elastin mRNA levels in interstitial lung fibroblasts by predominantly posttranscriptional mechanisms (3). Additionally, low oxygen tension inhibits amino acid uptake through the L-transport system in lung fibroblasts (2).

In vitro repair of proteolytically damaged elastin by lung fibroblasts occurs by two pathways (21). De novo synthesis, the principal mechanism of repair, requires production of nascent tropoelastin molecules, while salvage repair reestablishes hot alkali resistance by reforming intermolecular desmosine and isodesmosine bonds (20, 21). The need for new elastin formation in de novo repair, the sensitivity of elastin gene expression to low oxygen tension, and the presence of alveolar collapse in elastase-treated lung tissue suggest that hypoxia may disrupt repair in animal models of emphysema.

MATERIALS AND METHODS

Isolation of lung interstitial cells. Lipid interstitial cells (LIC) were isolated from the lungs of 8-day-old Sprague-Dawley rats (Charles River Breeding Laboratory, Wilmington, MA) as previously described (1). Briefly, after sodium pentobarbital anesthesia, the lungs were exposed, perfused with a balanced buffer solution, and surgically excised. Minced lung tissue was enzymatically dissociated. The cell suspension was filtered, washed, and resuspended.

LIC were isolated by discontinuous Percoll gradient centrifugation and resuspended in minimal essential medium containing 5% fetal bovine serum, 0.37 g sodium pyruvate/100 ml, 100 units penicillin/ml, and 100 μg streptomycin/ml and seeded into 75-cm2 flasks (Falcon Plastics, Los Angeles, CA). Cultures were maintained in a humidified 5% CO2/95% air incubator at 37°C. After 5 days, the cells were passed following treatment with trypsin and resedded in 25-cm2 flasks at 0.5 x 106 cells per cm2. When confluent, cells were made quiescent for the duration of the experiment by reduction of the serum content of the media to 0.4%. Fresh media replaced spent media every 3 days.

General protocol. On postpassage day 10, confluent LIC cultures were pulse labeled with 1.7 μCi [14C]U-lysine for 24 h. Tracer uptake by LIC was determined by the difference in counts in media before and after 24 h of incubation. After 5 wk, the cell layers were washed twice with physiological buffer solution (PBS) to remove elastase inhibitors present in serum. Porcine pancreatic elastase (PPE; Elastin Products, Owensville, MO) was used to disrupt elastin matrix. PBS or PBS with PPE (25 μg per flask) was added to 25-cm2 flasks and incubated for 45 min at 37°C (19).

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To assess the extent of injury, we harvested some control and enzyme-treated flasks immediately after incubation with PBS (CON-initital) or PBS with PPE (PPE-initial). Fresh medium containing 5% serum was added to the remaining flasks for 1 day to inactivate residual elastase. The following day, and every 3 days thereafter, all cultures were refed media with 0.4% serum. Twenty-four hours after PPE treatment, cultures were incubated at 37°C in 5% CO₂/95% air (normoxia) or in a humidified sealed chamber (Billups-Rothenburg, Del Mar, CA) gassed with 3% O₂ containing 5% CO₂/balance N₂ mixtures (hypoxia) (Medical-Technical Gases, Medford, MA) (3). Chambers were regassed after medium changes every 3 days.

Biochemical analyses. Aliquots of enzyme incubation media were lyophilized, hydrolyzed in 6 N HCl, and subjected to amino acid analysis (Beckman model 6300, System Gold software; Palo Alto, CA). The radioactivity (dpm) of a separate aliquot of acid hydrolysate was assessed by liquid scintillation spectrometry with external quench correction (Packard Model 1900 TR; Packard Instruments, Meriden, CT). To quantify enzyme-solubilized elastin in the incubation media, elastin-specific cross-link amino acids, desmosine (DES) and isodesmosine (IDES), were quantified. The amount of solubilized rat elastin (μg) was calculated by multiplying DES + IDES (nM) by 43, based on the content of DES + IDES in elastin (2 residues/1,000) and the average residue weight (85) in elastin (19).

Lactate dehydrogenase (LDH) activity was assessed in aliquots of the enzyme incubation media, as a measure of cell lysis (20), and in the cell layer, which was scraped from flasks in cold water. The suspension was homogenized with a glass Dounce. Aliquots of the homogenate were either treated with hot alkali to isolate intact insoluble elastin or assayed for LDH activity. Cell layer protein resistant to hot alkali treatment (0.1 N NaOH at 98°C for 45 min) represented intact elastin, as confirmed by amino acid analysis (10, 18). Proteolytically damaged elastin was extracted by hot alkali treatment. The purity of alkali treated elastin was confirmed by amino acid analysis, displaying a high percentage of nonpolar amino acids (18).

Electron microscopy. Cultures were fixed for 2 h at 4°C in 1% glutaraldehyde buffered with 0.1 M sodium phosphate, pH 7.1. After being rinsed in phosphate buffer, samples were postfixed in 1% osmium tetroxide, dehydrated with ethyl alcohols, and embedded in Polybed (Polysciences, Warrington, PA). Thin sections were cut with a diamond knife on an ultramicrotome, mounted on collodion-covered nickel grids, and stained with 0.1% palladium chloride, 1% aqueous uranyl acetate, and lead citrate (13).

Calculation of salvage repair and de novo synthesis. The percent salvage repair (Eq. 1) was defined as the percent restoration of elastin-associated radioactivity (EAR) in cultures after recovery from elastase treatment (PPEfinal) vs. the EAR in control cultures (CONfinal) (21). This value was corrected for the percent hot alkali-resistant radioactivity in cell layers immediately after elastase treatment (initial).

\[
\%\text{salvage repair} = \left( \frac{\text{EAR}_{\text{PPEfinal}}/\text{EAR}_{\text{CONfinal}} - \text{EAR}_{\text{PPEinitial}}/\text{EAR}_{\text{CONinitial}}} \right) \times 100
\]

The amount of elastin protein repaired by the salvage repair mechanism (Eq. 2) equaled the percent salvage repair times the total amount of elastin protein in 25-cm² flasks immediately before elastase treatment.

\[
\text{salvage repaired elastin protein} = \left( \frac{\%\text{salvage repair} \times \text{elastin protein (mg/flask)}}{100} \right)
\]

The amount of elastin arising from de novo synthesis following elastase injury was defined as that portion of recovered elastin protein not attributable to salvage repair. Therefore, de novo synthesis of elastin protein (Eq. 3) equaled the final quantity of elastin protein (per flask) minus the amount present immediately after elastase treatment, minus elastin protein repaired by the salvage mechanism.

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\text{de novo elastin protein} = \text{elastin protein(PPEfinal} - \text{PPEinitial}) - \text{salvage repaired elastin}
\]

In control cultures, de novo synthesis of elastin was calculated as elastin protein increase from the time of elastase treatment in parallel cultures to final harvest.

RNA isolation and northern analysis. Total cellular RNA was isolated using guanidium thiocyanate (American Bioanalytical, Natick, MA) following the methods of Chirgwin et al. (5). RNA was quantified by absorbance at 260 nm. Purity was assessed by absorbance at 280 and 320 nm. RNA (10 μg) was fractionated by electrophoresis on a 1% agarose-6% formaldehyde gel, transferred to a nylon filter (Hybond), and immobilized to the filter by UV cross-linking (Stratalinker, Stratagene, CA). The loading of RNA samples was monitored by ethidium bromide staining of ribosomal bands fractionated on agarose-formaldehyde gels and by radiolabeling of the 18S ribosome. Hybridization was performed using 0.5–1.0 × 10⁶ cpm/lane labeled probe (specific activity 4–10 × 10⁶ cpm/μg). The filter was washed according to methods described by Thomas (22) and exposed to X-ray film for autoradiography at several different times to ensure that the bands could be quantified by densitometry within the linear range. A rat elastin cDNA probe, γRE2 (16) (kindly provided by Dr. J. A. Foster, Boston University School of Medicine), was labeled with [32P]dCTP using a multiprime labeling kit (Amersham). A 20-bp oligonucleotide complementary to the 18S ribosome was end-labeled with [γ32P]ATP using T4 polynucleotide kinase (New England Biolabs).

Statistics. Mean values and SE were compared among three or more treatment groups by the Scheffe’s test (Statview 4.01; Abacus Concepts, Berkeley, CA); Student’s t-test was used to compare statistical differences between two groups. Probability values < 0.05 were considered significant.

RESULTS

Extent of enzyme-induced elastin damage. Elastin-producing neonatal rat lung fibroblasts, LIC, were pulse labeled with [3H]lysine and maintained in culture. Five weeks later, matrix-rich LIC cultures were treated with pancreatic porcine elastase (PPE) or PBS (control).

Elastin content of culture matrix was determined by hot alkali extraction, a treatment to which only intact, cross-linked elastin is resistant. PPE rendered elastin susceptible to hot alkali treatment. At the time of PPE treatment, control cultures contained 79.25 ± 5.07 μg/flask (mean ± SE, n = 4) of elastin. PPE treatment reduced the elastin content to 11.50 ± 0.96 μg/flask (15% of control values) and EAR to 3.03 ± 0.63 dpm/flask (mean ± SE, n = 4; 13% of controls).

LDH activity in the media and cell layer was used to assess cell injury. Immediately after treatment, cell layer LDH levels did not differ between PPE-treated and PBS-treated cultures (1.23 ± 0.08 vs. 1.40 ± 0.06 IU, mean ± SE, n = 4).

Elastin production under varying oxygen conditions. To examine the effect of chronic low oxygen tension on elastin matrix production and cell viability in the absence of proteolytic injury, we maintained untreated LIC cultures in hypoxic or normoxic conditions for 14 days. Electron micrographs of cultures kept hypoxic for 14 days revealed intact mitochondria, rough endoplasmic reticulum, and normal cell membrane (Fig. 1).
In normoxic conditions, elastin levels increased 46% over baseline values (79.25 ± 5.07 to 115.6 ± 3.96 g, mean ± SE, P = 0.0001). When cultures were maintained in hypoxic conditions, elastin levels did not increase over the same 14-day period (79.25 ± 5.07 to 78.4 ± 5.41 g, n = 5) (Fig. 2A). EAR (dpm/µg) fell 28% in normoxic cultures vs. a 5% decrease in hypoxic cultures (P < 0.0001) (Fig. 2B). The decrease in EAR among normoxic cultures reflects synthesis and cross-linking of new elastin. The lack of change in total elastin levels or EAR among hypoxic cultures reflects minor incorporation of new elastin into the extracellular matrix.

Elastin recovery under low oxygen conditions. To determine whether low oxygen tension impairs lung fibroblast repair of elastin, PPE-treated cultures recovered in normoxic or hypoxic conditions for 14 days. Cell viability was assessed by measuring LDH activity in the cell layers. LDH activity did not fall in the cell layer of normoxic or hypoxic cultures following protease treatment (Fig. 3). In normoxic conditions, alkali-resistant elastin levels rose more than sixfold (11.5 ± 1.0 to 78.8 ± 6.1 µg, n = 5) in cultures recovered in hypoxic conditions (Fig. 4A). The inhibitory effect of low oxygen levels on elastin repair was detectable after 9 days (elastin 42.8 µg/flask in hypoxia vs. 62.2 µg/flask in normoxia, P = 0.02) and persisted at 14 days (elastin 44.8 µg/flask in hypoxia vs. 78.8 µg/flask in normoxia, P = 0.0001). EAR decreased 46% in PPE-treated cultures kept normoxic vs. a 29% decline in cultures recovered under hypoxic conditions (Fig. 4B). The larger fall in specific activity and greater rise in alkali-resistant elastin in normoxic conditions suggest that hypoxia inhibits elastin repair dependent on new elastin production.

**Effect of chronic hypoxia on elastin gene expression.** To determine the effect of prolonged hypoxia on elastin gene expression in interstitial lung fibroblasts, we exposed LIC to normoxic or hypoxic conditions for 14 days following treatment with PPE or PBS. Recovery in normoxia following PPE induced minimal change in steady-state levels of elastin mRNA. In contrast, recovery in hypoxic conditions downregulated elastin mRNA by 44% following treatment with PPE or PBS (Fig. 5).

**Mechanisms of repair under varying oxygen concentrations.** We examined the mechanism of elastin repair under standard and low oxygen conditions by comparing the amounts of elastin and EAR present in untreated and PPE-treated cells after 14 days recovery in normoxic or hypoxic conditions. In hypoxia, EAR fell 28% in normoxic cultures vs. a 5% decrease in hypoxic cultures (P < 0.0001) (Fig. 2B). The decrease in EAR among normoxic cultures reflects synthesis and cross-linking of new elastin. The lack of change in total elastin levels or EAR among hypoxic cultures reflects minor incorporation of new elastin into the extracellular matrix.
PPE-treated cultures kept normoxic, elastin protein and EAR recovered to 78.8 g/flask (68% of control) and 11,323 dpm/flask (47% of control), respectively. Salvage repair is defined biochemically as restoration of alkali-resistance in protease-damaged elastin (21). Therefore, increases in alkali-resistant elastin-associated radioactivity reflect salvage repair. After PPE treatment, EAR was 13% of untreated controls, rising to 47% after 14 days recovery in normoxia. These data indicate that 34% (47–13%) of the radioactively labeled elastin, or 27 g/flask (0.34 79.3 g), underwent salvage repair in normoxic culture conditions. De novo synthesis represents elastin reconstitution not attributable to salvage repair. Under normoxic conditions, de novo synthesis produced 40.3 g [78.8 g (PPEfinal) – 27 g (salvage repair) – 11.5 g (PPEinitial)] of alkali-resistant elastin protein.

Hypoxia suppressed elastin repair at all time points of recovery following protease exposure. After 14 days of hypoxia, elastin protein levels rose modestly to 44.8 µg/flask (57% of untreated controls maintained in hypoxia) with a specific activity of 8,476 dpm/flask (47% of controls), respectively. Salvage repair is defined biochemically as restoration of alkali-resistance in protease-damaged elastin (21). Therefore, increases in alkali-resistant elastin-associated radioactivity reflect salvage repair. After PPE treatment, EAR was 13% of untreated controls, rising to 47% after 14 days recovery in normoxia. These data indicate that 34% (47–13%) of the radioactively labeled elastin, or 27 µg/flask (0.34 × 79.3 µg), underwent salvage repair in normoxic culture conditions. De novo synthesis represents elastin reconstitution not attributable to salvage repair. Under normoxic conditions, de novo synthesis produced 40.3 µg [78.8 µg (PPEfinal) – 27 µg (salvage repair) – 11.5 µg (PPEinitial)] of alkali-resistant elastin protein.

Hypoxia suppressed elastin repair at all time points of recovery following protease exposure. After 14 days of hypoxia, elastin protein levels rose modestly to 44.8 µg/flask (57% of untreated controls maintained in hypoxia) with a specific activity of 8,476 dpm/flask (47% of controls), respectively. Given an immediate post-PPE elastin-associated radioactivity of 13%, these data indicate that 27% (40–13%) of the radioactively labeled elastin, or 27 µg/flask (0.34 × 79.3 µg), underwent salvage repair. De novo synthesis in hypoxia generated only 11.9 µg [44.8 µg (PPEfinal) – 21.4 µg (salvage repair) – 11.5 µg (PPEinitial)] of alkali-resistant elastin (Fig. 6). Compared with recovery in normoxia, low oxygen tension suppressed de novo synthesis of elastin by 70% (P < 0.0001) while inhibiting salvage repair by only 21% (P = 0.15).

**DISCUSSION**

Chronic low oxygen tension suppressed repair of protease-damaged elastin by interstitial lung fibroblasts to less than half the amount achieved under standard culture conditions. Two pathways of elastin repair occur in lung fibroblasts: a salvage mechanism that reestablishes intermolecular bonds between damaged elastin and de novo synthesis that replaces disrupted matrix with new tropoelastin molecules. Hypoxia predominantly affected de novo synthesis, decreasing formation of new elastin matrix by 70%, while inhibiting salvage repair by only 21%. Morphologically, cells were unaffected by low oxygen tension. Cell layer-derived LDH levels rose under all recovery conditions, suggesting that cell loss did not limit elastin matrix repair by lung fibroblasts.
Hypoxia inhibited de novo synthesis to greater degree than it did salvage repair. In prior studies, we showed that short-term hypoxia (≤24 h) downregulated elastin gene expression in neonatal rat lung fibroblasts by a complex interplay of transcriptional and posttranscriptional events (3). We now show that extended exposure to hypoxia suppressed steady-state levels of elastin mRNA by 45% relative to expression in normoxic cultures. At the same time, hypoxia decreased elastin repair through de novo synthesis by 70%. The fall in elastin mRNA and de novo synthesis suggests that hypoxia suppressed repair of elastin in part by downregulating elastin gene expression through pretranslational mechanisms.

Hypoxia could also affect translation of elastin message. Durmowicz et al. (6) reported that 120 h of 3% O₂ preferentially suppressed translation of elastin mRNA in isolated rat pulmonary vascular smooth muscle cells, inducing lesser decreases in cell layer total protein levels. Similarly, 0% O₂ for <18 h suppresses total protein synthesis by 30–60% in bovine pulmonary artery endothelial cells (25). Regulation of mRNA translation typically occurs at the initiation step. In rat hepatocytes, hypoxia inhibits protein synthesis by inducing translation initiation factor eIF-4E to complex with its inhibitory binding protein, 4E-BP1. By tying eIF-4E subunit, hypoxia impairs binding of the 5' cap structure of mRNA to the 40S ribosome subunit, blocking translation (23). Alternatively, hypoxia could limit amino acid uptake, impairing protein formation (2).

Chronic low oxygen tension also blunted salvage repair of elastase damage by 21%. Although not well delineated, salvage mechanisms render enzyme-nicked fibers hot alkali resistant by reforming intermolecular bonds between deaminated lysyl residues on parallel elastin (20, 21). This repair pathway requires lysyl oxidase (LOX), a cross-linking enzyme whose activity is upregulated by brief exposure to mild hypoxia (12–13% O₂) and downregulated by moderate hypoxia (5% O₂) (4, 7). Long-term exposure to 3% O₂ may have different effects on LOX, however. In our experimental system, decreased enzyme activity could occur through downregulation of LOX mRNA, impaired translation of LOX message, or decreased enzyme activity from altered redox conditions. Further work is needed to determine the effect of chronic hypoxia on LOX activity.

The importance of salvage repair in vivo is illustrated by blocking cross-link formation following various lung injuries. In the presence of lathyrins such as β-aminoproprionitrile and penicillamine, endotracheal elastase induces significantly greater histological emphysema as measured by alveolar septal intercept and alveolar surface area (8). In one animal model of pulmonary fibrosis, inhibiting intermolecular bond generation prevented the profibrotic response to cadmium chloride, ex-
posing the destructive character of the inflammatory reaction (15). By inhibiting de novo synthesis, hypoxia should amplify the contribution of salvage mechanisms on tissue repair.

In summary, chronic low oxygen tension inhibited lung fibroblast repair of elastin at least in part by suppressing elastin mRNA levels and de novo synthesis of tropoelastin. The effects of hypoxia on tropoelastin formation exceeded decreases in elastin mRNA, implicating further downregulation at the translational level. Together, our data suggest that low oxygen tension suppresses repair of proteolytic injury in the lung.

GRANTS

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