Collagen and Elastin Metabolism in Hypertensive Pulmonary Arteries of Rats

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We evaluated the processes controlling the accumulation of collagen and elastin in main pulmonary arteries of rats during an episode of hypoxic pulmonary hypertension. Explant cultures of main pulmonary arteries were incubated with [3H]proline to measure collagen and protein synthesis and percent collagen synthesis. Elastin synthesis was measured by [14C]valine incorporation into insoluble elastin. Relative collagen synthesis increased twofold (from 1.1±0.2×10^3 to 2.0±1.0×10^3 disintegrations per minute [14C]hydroxyproline/vessel/hr/mg protein), relative collagen synthesis doubled (from 2% to 4–5% of total protein synthesis), and elastin synthesis increased ninefold (from 0.4±0.2×10^4 to 3.6±0.6×10^4 dpm [14C]valine/vessel/hr/mg protein) in early hypertension. The level of proc(1) collagen RNA paralleled the relative collagen synthetic rate during the study period. Within 7 days of recovery from hypoxia, collagen and elastin contents were normal. We conclude that collagen and elastin in main pulmonary arteries are synthesized rapidly during an episode of hypoxic pulmonary hypertension and that collagen and elastin are rapidly removed from the hypertensive vessel during normoxic recovery. (Circulation Research 1990;66:968–978)

Hypoxia causes a rise in pulmonary blood pressure by hypoxic vasoconstriction, and continued hypoxia causes further hypertension due to structural remodeling of the blood vessel walls and increased blood viscosity due to secondary polycythemia. The structural changes consisting of increased medial smooth muscle cells and accumulation of extracellular connective tissue contribute to the maintenance of hypertension. Pulmonary blood pressure gradually lowers after return to ambient air due to the slow reversal of the anatomic changes and polycythemia after relief of hypoxia.

Connective tissue is thought to play an important role in remodeling of hypoxic hypertensive pulmonary arteries. Mecham and associates and Stenmark and colleagues showed substantial increases in collagen and elastin synthesis in intralobar pulmonary arteries from newborn calves exposed to hypoxia; this increase is associated with marked thickening of the adventitia by elastic fibers. Collagen and elastin synthesis is increased in pulmonary arteries from rats with pulmonary hypertension induced by monocrotaline, a pyrrolizidine alkaloid that is metabolized to an active metabolite that causes damage to endothelial cells, which ultimately leads to the structural changes associated with pulmonary hypertension. Treatment of rats exposed to chronic hypoxia with antifibrotic agents partly prevents collagen accumulation and ameliorates hypertension. These studies suggest that substantial changes in collagen and elastin metabolism are present in hypertensive pulmonary blood vessels and that excess accumulation of connective tissue contributes to elevated pulmonary blood pressure.

The relation between the synthesis of connective tissue in hypertensive pulmonary arteries and blood pressure is incompletely described. The aim of this study was to measure the collagen and elastin synthetic rates in main pulmonary arteries during an episode of hypoxic pulmonary hypertension in the rat and relate these changes to pulmonary blood pressure. The molecular events regulating vascular collagen synthesis were assessed by measuring the steady-state messenger RNA (mRNA) levels for proc(1) collagen. Our results suggest that collagen and elastin synthesis are markedly increased in early hypertension and that the relative rates of...
collagen and elastin synthesis decline as blood pressure increases with continued hypoxia. There were parallel changes in rate of collagen synthesis and steady-state mRNA level for proα1(I) collagen, consistent with regulation at a pretranslational level. Recovery from hypertension was associated with reduction of blood pressure and a rapid decrease of collagen and elastin contents.

Materials and Methods

Materials

Six-week-old Sprague-Dawley (CrI-CD[SD]BR) male rats weighing 180–250 g were obtained from Charles River Breeding Laboratories, Wilmington, Massachusetts. Bottled custom grade 10% O2-90% N2 was supplied by Jersey Welding Supply, Piscataway, New Jersey. Solutions were Krebs-Ringer bicarbonate, which contained (mM) NaCl 128, KCl 6, NaHPO4 18, KH2PO4 16, MgCl 2.5, and NaHCO3 5, pH 7.4, and 1× saline sodium citrate (SSC), which contained 150 mM NaCl and 15 mM trisodium citrate. Denhardt’s solution and 3-(N-morpholino)propanesulfonic acid (MOPS) solution were prepared using standard methods.11 L-Proline, L-ascorbic acid, spermidine, herring sperm DNA, and Tris-HCl were purchased from Sigma Chemical, St. Louis, Missouri. Sarkosyl was obtained from ICN Biomedicals, Plainview, New York, and Sephadex G-50 was obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey. Formamide, sodium dodecyl sulfate, cesium chloride, guanidinium thiocyanate, agarose, Tris-HCl, and EcoRI, BamHI, and HindIII restriction endonuclease were supplied by International Biotechnologies, New Haven, Connecticut. 32P]-Uridine 5′-triphosphate (dUTP) (3,200 mCi/mmol) was purchased from ICN Radiochemicals, Irvine, California, and 35Cl]-l-proline (260 mCi/mmol) and 3HCl]-valine (264 mCi/mmol) were purchased from New England Nuclear, Boston, Massachusetts. ATP, GTP, CTP, ribonuclease inhibitor, SP6 RNA polymerase, reticulocyte lysate RNA, and DNase were obtained from Promega Biotec, Madison, Wisconsin. Calf thymus DNA was supplied by Calbiochem-Behring, San Diego, California. Nitrocellulose (BA85, 0.45 μm) was purchased from Schleicher & Schuell, Keene, New Hampshire. Clostridial collagenase (type III) was obtained from Advance Biofactures, Lynbrook, New York. Liquid scintillation fluid (Liquiscint) was purchased from National Diagnostics, Somerville, New Jersey. Kodak X-Omat AR X-ray film was purchased from E.I. duPont de Nemours & Co, Wilmington, Delaware. All other chemicals were of analytical grade.

Animal Handling

Animals were held in an animal care facility 5–7 days before use and were fed standard rat chow and water ad libitum. Animals exposed to hypoxia were allowed free access to food and water. Control ani-
mals breathed air and were kept in cages in the same room and were pair-fed to hypoxic animals by weighing the food consumed by hypoxic animals and feeding the same amount of food to controls to ensure that final body weights were similar. Body weights were carefully matched since food deprivation can affect tissue mRNA levels.12,13 Animals were kept on a 12-hour light-dark cycle.

Experimental Protocols

Cohorts of 12–14 animals were randomly selected for control or hypoxic studies. Animals were exposed to hypoxia for 1, 2, 3, 4, 5, 7, or 10 days or to hypoxia for 10 days followed by room air for 1, 3, 7, or 14 days (study days 11, 13, 17, and 24, respectively). Age- and weight-matched control animals were studied on the same days. Separate groups of animals were used for studies of protein and mRNA.

Exposure Conditions

Animals were exposed to 10% O2-90% N2 or room air at ambient pressure in chambers as previously described.6 Gas tensions under hypoxia for PO2 ranged from 74 to 80 mm Hg and for PCO2 from 3–5 mm Hg.

Right Ventricular Pressure, Hematocrit, and Ventricular Weights

Mean right ventricular pressure was measured via a catheter placed in the right ventricle in rats anesthetized with 30 mg/kg i.p. pentobarbital; in some animals, aortic blood pressure was also obtained as previously described.6 Readings were taken after the animal had breathed room air for 5 minutes, a time sufficient to eliminate hypoxic vasoconstriction in chronically hypoxic rats.14 The animals were killed by cutting the abdominal aorta, and hematocrit and the ratio of ventricular weights were obtained.5

Preparation of Tissues

A segment of pulmonary artery including the trunk, the left extrapulmonary branch, and 2–3 mm of the right extrapulmonary branch was excised intact, and loose connective tissue was removed. The vessel was opened longitudinally, washed in incubation medium, blotted dry, and weighed. The thoracic aorta from the aortic valve to the diaphragm was removed, trimmed of all branches and loose connective tissue (adventitia was retained), and opened longitudinally. A midthoracic segment was cut into two pieces 10 mm in length, and each piece was randomly allocated for protein or mRNA studies.

Collagen and Protein Assays

Each pulmonary artery or aortic segment (20–50 mg wet weight) was placed in 5 ml Krebs-Ringer bicarbonate containing 5.6 mM dextrose, 0.5 mM ascorbic acid, 0.9 mM L-proline, and 10 μCi/ml [3H]proline. The amount of exogenous proline was 10 times the physiological level of free proline in rat plasma.15 The tissue was incubated under ambient air for 4 hours at 37°C, 25 oscillations/min, in a water
shaker bath. After incubation, the tissue was heated to 100°C for 10 minutes. The tissue was homogenized in ice-cold incubation medium for 3 minutes at 10,000 rpm (Polytron, Brinkmann Instruments, Westbury, New York). The tissue homogenate was dialyzed against 4 l deionized distilled water at 4°C for 3 days with two changes daily, lyophilized, and resuspended in 2.2 ml of a solution containing (mM) Tris-HCl 10, CaCl2 5, and N-ethylmaleimide 2.5, pH 7.5.

A 1.2-ml aliquot of the tissue homogenate was hydrolyzed in 6N HCl at 116°C for 48 hours, a 0.1-ml aliquot of the hydrolysate was diluted 1:10 with water, and a 0.2-ml aliquot was reacted in triplicate with ninhydrin for determination of total protein using leucine as standard. Total hydroxyproline was determined in triplicate on a 0.1-ml aliquot of the hydrolysate using the colorimetric method of Kivirikko and associates. Results were expressed as protein or hydroxyproline content per vessel; results were also expressed as hydroxyproline per protein. Hydroxyproline was used as an index of collagen since approximately 83% of hydroxyproline in normal rat pulmonary artery tissue is derived from collagen.

The relative rate of [14C]proline incorporated into protein was determined by adding 20 ml scintillation fluid to 0.1 ml hydrolysate and determining counts per minute with 80% efficiency using a liquid scintillation counter (model SL-30, Intertechnique, Dover, New Jersey). [14C]Hydroxyproline was determined on a 1.0-ml aliquot of the hydrolysate by the method of Juva and Prockop. From a total of 25 ml eluted from the silicic acid column, a 0.1-ml aliquot was used for the colorimetric assay, and 1.0 ml was mixed with 20 ml scintillation fluid; counts per minute was determined. The incorporation of [14C]proline into [14C]hydroxyproline was found to be linear over time as determined by the level of [14C]hydroxyproline in tissue samples at 1, 2, 3, and 4 hours. Relative collagen synthesis was estimated as disintegrations per minute of [14C]hydroxyproline per vessel per hour and relative protein synthesis as disintegrations per minute of [14C]proline incorporated into protein per vessel per hour. Results were also expressed per protein content.

The percent of total protein synthesis devoted to collagen synthesis was determined by the method of Peterkofsky and Diegelmann. Tissue protein labeled with [14C]proline was digested with bacterial collagenase followed by dialysis. This assay distinguishes newly synthesized collagen from all other proteins because collagen is uniquely susceptible to bacterial collagenase. The proportion of protein labeled with [14C]proline isolated by dialysis after collagenase digestion is compared with total radioactivity and is used as an index of percent protein synthesis devoted to collagen synthesis. A 1.0-ml aliquot of the tissue homogenate dissolved in collagenase buffer was sonicated (Microultrasound Cell Dispersor, Kontes, Vineland, New Jersey); a 0.5-ml aliquot of the dissolved material was placed in a small dialysis bag, and 90 units purified bacterial collagene was added. Dialysis of the sample and of a blank not containing collagenase was performed as described by Berg.

A 1.0-ml aliquot of the solution outside the dialysis bag and the entire contents of the bag were added separately to 15 ml scintillation fluid and counted. The percentage of collagen synthesized was calculated according to a formula devised by Breul and associates.

### Elastin Synthesis

In separate animals, the pulmonary artery or aorta was isolated as described above. The relative rate of synthesis of insoluble elastin was estimated by a modification of the method described by Keeley and Johnson. The vessels were preincubated in a water shaker bath at 37°C in 1.0 ml Krebs-Ringer bicarbonate supplemented with (mM) dextrose 5.6, ascorbic acid 0.5, and 1-valine 2.4, pH 7.4, under 95% O2-5% CO2. All incubation medium was pregassed with a mixture of 95% O2-5% CO2 for 10 minutes. After a 30-minute preincubation period, the tissue was washed twice in incubation medium lacking valine, transferred to 1.0 ml fresh incubation medium containing 10 μCi/ml [14C]-valine, and incubated for 4 hours. After incubation, the tissue was rinsed twice with incubation medium containing 4.0 mM valine and was added to 1.0 ml of 0.01 M phosphate buffer containing 1.0% (wt/vol) sodium dodecyl sulfate, pH 7.0, and heated to 100°C for 15 minutes. The sample was centrifuged at 10,000g at 25°C for 10 minutes, and the supernatant was removed; extraction was continued overnight at 20°C in the same buffer and centrifuged again, and the tissue was saved. The tissue was extracted overnight in a solution of 70% formic acid and 1% (wt/vol) cyanogen bromide at 20°C and centrifuged at 10,000g for 10 minutes at 20°C. The residue was hydrolyzed in 6N HCl at 100°C for 48 hours, evaporated, lyophilized, and reconstituted in 0.5 ml water. A 0.1-ml aliquot of this hydrolysate was added to 5.0 ml scintillation fluid and counted. The residue after cyanogen bromide–formic acid extraction was considered insoluble elastin, as confirmed by amino acid analysis. Radioactivity of the residue was taken as a measure of the relative rate of insoluble elastin synthesis during the incubation period, and results were expressed as disintegrations per minute of [14C]valine incorporated per vessel per hour. Results were also expressed per protein.

### Desmosine

Desmosine in the residue after formic acid–cyanogen bromide extraction was used as an index of insoluble elastin. A 0.2-ml aliquot was diluted to a final volume of 10 ml with isotonic veronal-buffered saline (0.85% NaCl, 0.01 M sodium diethylbarbiturate) after adjusting the pH to 7.2 with 1N NaOH. A 0.1-ml aliquot of the diluted solution was assayed for desmosine using a monospecific radioimmunoassay, according to the method of Yu and associates, and the results were expressed as desmosine per vessel and as desmosine per protein.
RNA Extraction

In separate animals, the pulmonary artery and aortic segments were isolated and immediately frozen at −70°C after weighing. Vascular tissue was pooled from four to six animals and minced with a sterile scalpel. Total RNA was extracted using a guanidinium isothiocyanate–CsCl extraction procedure modified from that described by Gilsin and colleagues.24 The minced tissue was added to 5 vol ice-cold extraction buffer containing (M) guanidinium isothiocyanate 4, sodium citrate 5, β-mercapto-ethanol 0.1, and 0.5% (wt/vol) sarkosyl, pH 7.0, and homogenized at 10,000 rpm. CsCl (1 g) was added per 2.5 ml homogenate, and the mixture was layered on top of a CsCl cushion (5.7 M CsCl and 0.1 M EDTA, pH 7.5) and centrifuged at 35,000 rpm for 16 hours at 20°C (model L8-70M Ultracentrifuge, Beckman Instruments, Fullerton, California). RNA pellets were dissolved in 10 mM Tris-HCl, 5 mM EDTA, and 1% (wt/vol) sodium dodecyl sulfate, pH 7.4, extracted with an equal volume of chloroform:butanol (4:1), precipitated with ethanol, and dissolved in water. The amount of RNA was determined spectrophotometrically at 260 nm. Stock concentrations were adjusted to 40 µg/ml and stored at −70°C.

Radiolabeled RNA Transcripts

Linearized SP6 recombinants containing complementary DNA (cDNA) fragments for human proα1 (I) collagen and γ-actin were used to synthesize 32P-labeled single-stranded RNA transcripts using a commercially available kit (Riboprobe, Promega-Biotec).25 Transcripts were generated by incubating 1 µg SP6 recombinant DNA in a 50-µl reaction mixture containing 40 mM Tris-HCl, pH 7.5, 6 mM MgCl2, 2 mM spermidine, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 200 µCi [32P]UTP, 0.01 M dithiothreitol, 50 units ribonuclease inhibitor, and 10 units SP6. The reaction mixture was incubated for 1 hour at 37°C; 100 µg RNA carrier, 50 units ribonuclease inhibitor, and 0.5 µg DNAse were added, and the reaction mixture was incubated at 37°C for 15 minutes. The reaction mixture was extracted with a phenol:chloroform:isoamyl alcohol solution (50:50:1), and the radiolabeled RNA was separated by chromatography on a Sephadex G-50 column and ethanol precipitated. The specific activity of the 32P-labeled RNA was determined, and about 8×106 dpm was used in the hybridization procedure.

Dot Blot Hybridization Assay of Total RNA

Dot (or “slot”) blot hybridization was carried out according to the method of Pierce and associates.26 For each experimental and control group, filters were prepared on the same day and were hybridized using the same solutions and probes. However, hybridization studies were done at different times for each paired group (except all day 0 controls were done on the same day) so that absolute levels of mRNA cannot be compared on different days. Total RNA was serially diluted and applied to presoaked nitrocellulose (100–500 ng total RNA/well) with 10×SSC and 1% formaldehyde using an apparatus for dot hybridization (Minifold II, Schleicher & Schuell). Samples containing 200 ng yeast tRNA were applied to each filter to determine nonspecific binding of the probe. Each slot was washed three times with 200 µl of 10×SSC; the filter was air dried, baked at 80°C under vacuum for 2 hours, and sealed in a clear plastic bag. The prehybridization and hybridization solution consisted of 5×SSC and 1% (wt/vol) sarkosyl. Hybridization was performed by adding the radiolabeled probe and hybridization buffer to the filter and incubating for 12–18 hours at 65°C with gentle shaking. After washing twice at 65°C with a posthybridization buffer containing 2×SSC and 0.5% (wt/vol) sarkosyl and twice with 2×SSC for 10 minutes each at 65°C, the nitrocellulose was exposed to x-ray film in a cassette backed by intensifying screens (Lightning Plus, E.I. duPont de Nemours & Co) at −70°C for 10 minutes to 3 hours.

Quantitation of Radioactivity

Quantitation of filter-bound radioactivity was carried out by scanning the nitrocellulose filter using a computer-assisted beta scanner (Automated Microbiology System, San Diego, California). This scanner counts with an efficiency of 18% compared with counts obtained by a scintillation counter (model LS 1801, Beckman Instruments). The results were corrected for the efficiency of the beta scanner and quantitated by subtracting the nonspecific binding (tRNA sample). Results were plotted as disintegrations per minute per slot against total RNA applied per slot. Linearity of the plot was determined by linear regression analysis,27 and plots were accepted at r>0.95.

Northern Blot Analysis

Total RNA was denatured in a solution containing 2 M formaldehyde, 1×MOPS, and 50% formaldehyde by heating to 65°C for 10 minutes, followed by quick chilling on ice. RNA samples (3 µg) were applied to a 1.0% (wt/vol) agarose gel containing formaldehyde as a denaturant and electrophoresed in 1×MOPS buffer using a horizontal gel electrophoresis unit (model MPH, International Biotechnologies). RNA was transferred to a nitrocellulose filter using a plastic transfer system (Ann Arbor Plastics, Ann Arbor, Michigan) overnight at 4°C in the presence of 10×SSC as described by Thomas.28 After transfer, the nitrocellulose was briefly rinsed in 2×SSC and baked at 80°C under vacuum for 2 hours. The filter was soaked in 3×SSC for 5 minutes and prehybridized at 65°C for 1 hour in a solution of 3×SSC, 10×Denhardt’s solution, 0.1% (wt/vol) sodium dodecyl sulfate, and 10 µg/ml denatured herring sperm DNA. Hybridization was performed with approximately 200 ng of 32P-labeled cDNA probes (1×106 dpm/µg) in 8–12 ml hybridization solution mentioned above at 65°C overnight. RNA markers were 28S and 18S human ribosomal RNA. After hybrid-
ization, the filters were washed at 65°C four times for 15 minutes each in 250 ml buffer consisting of 3xSSC, 10×Denhardt’s solution, and 0.1% (wt/vol) sodium dodecyl sulfate, sealed in plastic bags, and exposed to x-ray film in a cassette with image intensifiers at −70°C for 3–24 hours.

Preparation of Recombinant Plasmids

A human proc1(I) collagen cDNA, Hf677,20 generously donated by Dr. D.J. Prockop, Jefferson Medical College, Philadelphia, Pennsylvania, was digested with EcoR1. A 1,500 base pair (bp) restriction fragment containing a DNA sequence coding for most of the carboxyl propeptide and part of the helical region of human proc1(I) collagen was isolated and subcloned into an SP6 vector. Recombinants containing correctly oriented DNA inserts were linearized by digestion with AvaI. A human γ-actin cDNA, pHF-γ-A-3'ut,30 generously donated by Dr. P. Gunning, Stanford University School of Medicine, Palo Alto, California, was digested with BamHI-HindIII, and a 700 bp restriction fragment was inserted into the SP6 Riboprobe (Promega-Biotec) vector and linearized with HindIII. This restriction fragment contains sequences coding for part of the 3' nontranslated region of human γ-actin mRNA.

Statistical Analysis

Values are given as mean±SEM. Two-way analysis of variance was performed31 followed by a Duncan's post hoc test.32 Correlations of data were made by linear regression analysis using the least-squares method and analyzed by the standard error of the estimate (r).27 Nonparametric data (animal survival) were analyzed using a χ² analysis with Yates' correction.33 A value of p<0.05 was considered significant.

Results

Animals

Survival was 169 of 172 (98%) in controls and 146 of 178 (82%) in hypoxic animals (χ²=13.9, p<0.01). Hypoxic animals lost approximately 3% of initial body weight in the first 3 days but thereafter gained 27% of initial body weight by day 17. There were no significant differences in mean body weight between control and hypoxic groups at any time.

Hemodynamics

Mean right ventricular pressure was increased above age-matched controls after 1 day of hypoxic exposure and increased progressively until day 10 (Figure 1A). A partial reduction in blood pressure occurred 3 days after withdrawal of hypoxia, and blood pressure was at control level 14 days after recovery from hypoxia (9.6±0.5 mm Hg [mean±SEM] in control [n=7] and 10.3±0.4 mm Hg in hypoxic [n=6]; p=NS). Right ventricular hypertrophy, indicated by the ratio of the right ventricle to the left ventricle plus the septum, paralleled the increase in right ventricular pressure and was normal on day 14 of recovery (0.23±0.01 in control [n=7] and 0.24±0.01 in hypoxic [n=6]; p=NS) (Figure 1B). Hematocrit increased during hypoxia (Figure 1C) and returned to normal 14 days after recovery (44±2% in control [n=7] and 45±1% in hypoxic [n=7]; p=NS). There was no difference in mean aortic blood pressure in control and hypoxic animals on day 7 (128±5 mm Hg in control [n=6] and 136±3 mm Hg in hypoxic [n=5]; p=NS).

Protein

Protein content per vessel doubled within 3 days of exposure to hypoxia (Figure 2A). This was followed by a slower rate of increase after day 3. Protein content decreased to normal within 7 days of recovery. The relative rate of protein synthesis per vessel was increased on day 1, continued to increase over the hypoxic period, and decreased to control after 7 days of recovery (Figure 2B). The relative rate of protein synthesis expressed per milligram protein was increased only on day 1; after day 1, the relative rates of protein synthesis per milligram protein were not significantly different compared with control (Figure 2C.)
Protein was and brackets with vessel.34 Compared per vessel.35 Hydroxyproline content increased 700% during the 10-day exposure period (Figure 3A). Within 3 days of return to air, the hydroxyproline content of the hypertensive vessel was not different from the age-matched control vessel (Figure 3A). In the hypoxic group, the relative rate of collagen synthesis per vessel increased markedly and was eightfold greater than age-matched control group on day 3 (Figure 3B). Relative collagen synthesis rate per vessel at day 10 was less than day 3 and was at control levels 1 week after recovery from hypoxia (Figure 3B). The percent of protein synthesis devoted to collagen was 2% in control and 4–5% in the hypoxic group (Figure 3C). In control groups, the ratio of hydroxyproline to protein increased from 15±5 μg/mg on day 0 to 30±3 μg/mg on day 17 (p<0.05), reflecting the greater increase in hydroxyproline compared with protein content during normal growth (Figure 4A). The ratio of hydroxyproline to protein in hypoxic vessels was the same as control at each time point, reflecting proportional increases in collagen and protein contents in the hypertensive pulmonary arteries (Figure 4A). The relative rate of collagen synthesis per milligram protein was increased on days 3 and 7 compared with controls (Figure 4B). After day 7, the values were not significantly different compared with control. There was no difference in the relative rate of collagen synthesis in aortic tissue at 7 days (19±1×10³ dpm/vessel/hr in control [n=5] and 18±3×10³ dpm/vessel/hr [n=6] in hypoxic).

**Elastin**

Desmosine content per vessel in the control group did not increase over the 17-day period (Figure 5A). In hypoxic animals, desmosine content increased 22% by day 3 and 43% by day 10 compared with age-matched controls. Desmosine content decreased within 1 week of return to air (Figure 5A). Radiolabeled valine incorporation into insoluble elastin expressed per vessel increased on day 1 of hypoxia, increased to a peak value on day 3, and decreased between days 3 and 10 but remained above control levels (Figure 5B). Relative elastin synthesis per vessel was at control levels 7 days after return to room air. The ratio of desmosine to protein was reduced in hypertensive vessels on days 3–10, but the value on day 17 was similar to control (Figure 6A). These data indicate that in the hypertensive vessels...
there was a greater increase in protein compared with elastin than in the control vessels, but during recovery from hypoxia, the ratio of elastin to protein was similar to control values. Relative elastin synthetic rate corrected for tissue protein in the hypoxic groups was increased on days 3, 7, and 10 but was not different from controls on days 1 or 17 (Figure 6B).

We observed no difference in elastin synthesis in aortas on day 7 of hypoxia since the radiolabel incorporated into insoluble elastin was $2.3 \pm 0.4 \times 10^9$ dpm/vessel/hr for controls ($n=5$) and $2.1 \pm 0.4 \times 10^9$ dpm/vessel/hr for the hypoxic group ($n=4$).

**Procollagen and Actin mRNA**

Hybridization with the proα1(I) collagen probe to total RNA extracted from pulmonary artery tissue produced a single major band at the 28S region on Northern blot analysis (Figure 7A). The position of the bands was similar in hypertensive and control pulmonary arteries. Quantitation of dot blot hybridization using a probe for proα1(I) collagen for aorta and pulmonary artery is shown in Figure 7B. The hybridization kinetics were linear for the serial dilutions of RNA on the nitrocellulose filter, indicating an excess of probe during the hybridization reaction. The dot blot analysis for RNA extracted from aortic tissues on day 7 of hypoxic exposure showed no difference in the slopes of the plots relating radioactivity per slot versus total RNA applied. In contrast, radioactivity per slot was greater in RNA from pulmonary arteries of rats exposed to hypoxia compared with RNA from controls for the same amount of total RNA applied. This was reflected by a steeper slope when counts per slot were related to total RNA per slot in the RNA from hypertensive arteries. The relative changes in mRNA for proα1(I) collagen and γ-actin over the study period are shown in Table 1. We considered a change in proα1(I) mRNA to be significant if it was less than 92% or greater than 108% of control since measurements of mRNA levels from five separate groups of tissue from control
animals differed by 8% or less (Table 1). These results could be compared in absolute terms since all hybridization studies were done on the same day. The steady-state mRNA for proα1(1) collagen was increased most on day 3 (285%), and less marked increases were present on days 7 and 10 (Table 1). The slope relating counts of the γ-actin probe to total RNA per slot was increased 212% on day 3, but at the other times was within 88–112% of control (Table 1).

**Discussion**

Our results show substantial and rapid increases in relative rates of connective tissue protein synthesis in explants of main pulmonary arteries of rats after onset of hypoxic pulmonary hypertension. Within the first few days after blood pressure elevation, relative protein synthesis expressed per vessel was increased modestly (1.7-fold), and marked increases were found in the relative synthetic rates per vessel of collagen (eightfold) and insoluble elastin (ninefold). To evaluate these changes relative to the size of blood vessels, we expressed the results as synthetic rate per milligram tissue protein. Protein synthesis per milligram protein was increased only during the first day of hypertension; thereafter, the increase in protein synthetic rate was proportional to increased vessel size. In contrast, collagen and elastin synthetic rates per milligram protein were increased throughout most of the hypertensive period. A greater percent of total protein synthesis was devoted to colla-

**TABLE 1. Slot Blot Analysis**

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Data are results from RNA extracted from four to six pulmonary arteries for each group. Numbers are slopes of slot blot analysis in disintegrations per minute times $10^9$ divided by 100 ng total RNA per slot. Change is calculated as follows: [(slope of hypoxic−slope of control)/slope of control]×100. Control refers to animals exposed to room air; hypoxic refers to animals exposed to 10% O₂ for up to 10 days or exposed to hypoxia for 10 days and studied 7 days after recovery (day 17). Day 0 refers to two separate control groups studied to assess the range of repeated measurements.
Collagen synthesis in the hypertensive pulmonary arteries (4–5%) than in the control arteries (2%). This finding may reflect either production of more collagen per cell or an alteration in cell population with a greater proportion of collagen-producing cells.

In the hypertensive vessels, the contents of collagen and insoluble elastin were not increased when normalized to protein. This finding is consistent with other observations that show that the proportion of collagen and elastin is unchanged or decreased in hypertensive vessels. The substantial increases in relative synthetic rates of collagen and elastin without enrichment in connective tissue content may be due to concomitant increases in degradation of collagen and elastin. Increased activities of enzymes capable of degrading collagen and elastin have been measured in aortic tissue and cells of rats and are increased in hypertensive aortas. It is possible that enhanced collagenolytic and elastolytic activities occur in the hypoxic model and explain why connective tissue content is not enriched.

The increases in steady-state mRNA levels for γ-actin (212%) and procollagen(I) collagen (285%) were of similar magnitude on day 3. However, after day 3, the level of γ-actin mRNA was at control levels whereas that of procollagen(I) collagen remained increased throughout the hypertensive period. The temporal changes of the mRNA levels for γ-actin, a cellular protein, and for procollagen(I) collagen parallel the changes in protein and collagen synthetic rates: a transient early increase in protein synthesis per protein and a prolonged increase in collagen synthesis per protein during hypoxic exposure. The first few days after exposure to hypoxia is a period of intense cellular proliferation in the main pulmonary artery of the rat. Meyrick and Reid found an early (2–5-day) burst of DNA synthesis in adventitial fibroblasts in hilar pulmonary arteries of rats exposed to hypoxia. The increase in mRNA level for γ-actin on day 3 is consistent with a marked increase in cellular protein synthesis during this period of cellular proliferation. We postulate that the early transient increase in protein synthesis probably reflects increased synthetic rates of both cellular and extracellular proteins.

The level of regulation of collagen synthesis was assessed by comparing the relative changes in collagen synthetic rate and steady-state mRNA level for procollagen(I) collagen. We found that the relative quantity of mRNA was parallel to the relative collagen production rate throughout the study period. Our results are consistent with changes reported in neonatal porcine aorta and in lobar pulmonary arteries of calves maintained in a hypoxic environment, which found proportional increases in mRNA level for procollagen(I) collagen and collagen synthetic rate. Our findings suggest a predominately pretranslational level of control of collagen synthesis in adult vascular tissue.

The metabolic turnover of collagen and elastin in adult animals is believed to be low. However, collagen and elastin contents of the hypertensive pulmonary arteries were found to be normal within 3 and 7 days, respectively, after removal of the hypoxic stimulus. Rapid resorption of extracellular connective tissue occurs in special situations, such as rapid growth and development and wound healing, and during involution of the uterus after parturition. Normally, collagen half-life is several weeks to months, but in the involuting uterus, the half-life approaches 30 hours. The rapid breakdown of collagen and other proteins in the uterus is attributed to an increase in activity of tissue collagenase and other proteases during the first 3 days of the postpartum period. We speculate that the rapid reduction in collagen content in recovering blood vessels might be due to proteolytic degradation of connective tissue.

It is likely that increased blood pressure and not hypoxia was the stimulus for matrix protein synthesis since increased collagen and elastin synthesis was found in pulmonary arteries but not aortas of rats exposed to hypoxia. Several studies suggest that mechanical factors influence vascular connective tissue composition and synthesis: collagen synthesis is increased in arteries but not in veins in systemic hypertension; vessels with high tangential tensions contain abundant collagen and elastin fibers; vessels with the greatest pulsatile distension have the highest rates of collagen synthesis; and tension applied to rings of pulmonary and systemic arteries stimulates collagen synthesis. We postulate that in our model elevated blood pressure produced by hypoxic vasoconstriction of arterioles passively distends main pulmonary arteries and stimulates synthesis of collagen and elastin.

There was no direct relation between blood pressure and the rates of collagen or elastin synthesis in the main pulmonary artery. For example, mean right ventricular pressures during the early hypoxic and early recovery periods were similar, but the corresponding rates of collagen and elastin synthesis were markedly different. Clearly, stimulation of vascular matrix proteins by hypertension is different in normal and remodeled pulmonary artery. We speculate that the normal pulmonary artery responds to low increases in blood pressure by increased connective tissue synthesis and that within a few days increased connective tissue provides tensile strength to the wall that counteracts the increased wall tension. In the remodeled vessel, wall tension is reduced even though blood pressure remains elevated. It is possible that wall tension is the key physiological stimulus controlling rates of collagen and elastin synthesis in hypertensive blood vessels.

It is worthwhile to compare changes in connective tissue content in the hypertensive pulmonary artery to the changes in the hyptrophied heart caused by pressure overload. Ventricular hypertrophy in response to hemodynamic stress is assumed to result in fibrous replacement of muscle cells, which is thought not to regress after the stimulus is removed. Animal
studies have shown increased collagen concentration and lack of regression when ventricular hypertrophy is induced abruptly by marked increases in afterload.\textsuperscript{53–55} However, if hypertrophy develops slowly, collagen increases proportionately to muscle mass and can regress completely as hypertrophy recedes.\textsuperscript{56,57} An abrupt severe load is thought to produce myocardial necrosis leading to irreversible connective tissue replacement, but under less severe loading conditions, collagen maintains a normal relation to muscle mass.\textsuperscript{56}

In the hypertensive pulmonary artery, our results showing proportional changes in collagen and protein are consistent with the idea that increased connective tissue in remodeled pulmonary arteries is an adaptive response that maintains the normal relation of extra-cellular matrix proteins to muscle mass.

In conclusion, exposure of rats to an episode of hypoxia produces remarkably fast changes in collagen and elastin metabolism in the main pulmonary artery resulting in no change in the proportion of collagen and elastin relative to the increased mass of the hypertensive vessel. We speculate that mechanical factors involving wall tension influence collagen and elastin metabolism in the main pulmonary artery in hypoxic pulmonary hypertension. Whether hypoxia per se contributes to vascular wall connective tissue synthesis in hypoxic hypertensive pulmonary arteries cannot be determined from our results.

Acknowledgments

We thank Dr. Charles D. Boyd for helpful suggestions, Carol L. Ruppert, Sändra Hayes, Angellia Finkinger, and James Sumka for technical assistance, and Marcella Spioch for preparation of the manuscript.

References


sion and arterial adventitial changes in newborn calves at 4,300 M. \textit{J Appl Physiol} 1987;62:821–830


32. Duncan DB: T tests and intervals for comparisons suggested by the data. \textit{Biometrics} 1975;31:339–359


Other


KEY WORDS  • pulmonary hypertension  • collagen  • elastin  • hypertension  • hypoxia  • pulmonary circulation  • pulmonary artery
Collagen and elastin metabolism in hypertensive pulmonary arteries of rats.
G J Poiani, C A Tozzi, S E Yohn, R A Pierce, S A Belsky, R A Berg, S Y Yu, S B Deak and D J Riley

Circ Res. 1990;66:968-978
doi: 10.1161/01.RES.66.4.968

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/66/4/968

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