Regulation of Fibrillar Collagen Types I and III and Basement Membrane Type IV Collagen Gene Expression in Pressure Overloaded Rat Myocardium

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Left ventricular hypertrophy is based on cardiac myocyte growth. The hypertrophic process can be considered heterogeneous based on whether it also includes a remodeling and accumulation of fibrillar types I and III collagens that are responsible for impaired myocardial stiffness. In the heart, the messenger RNA (mRNA) for fibrillar collagen types I and III has been detected only in cardiac fibroblasts, whereas mRNA for basement membrane collagen type IV is present in both fibroblasts and myocytes. We studied the early and long-term expression of these collagenous proteins in rat myocardium after abdominal aortic banding with renal ischemia. Complementary DNA probes for rat pro-α1(I), mouse type III and mouse type IV collagens, and chicken β-actin were used. Northern and dot blot analysis on total RNA extracted from left ventricular tissue indicated a sixfold increase in steady-state levels of mRNA for collagen type I on day 3 of abdominal aortic banding, which had declined to control levels by day 7 where it remained rather constant at 4 and 8 weeks. Type III collagen showed a similar pattern of gene expression after banding. mRNA levels for type IV collagen, on the other hand, were elevated on day 1 after banding, returning to control at day 7 and remaining constant. Actin mRNA levels also increased on day 1 of banding, followed by a rapid return to control levels. Monospecific antibody to types I and III collagens and immunofluorescent light microscopy on frozen sections of the myocardium revealed that at 1 week after banding, the distribution and density of these collagens were similar to those of control animals. At 8 weeks, the density of collagen type I fibers was greater than controls, and a perivascular fibrosis involving intramyocardial coronary arteries also was present. The distribution and density of collagen types III and IV, however, remained unchanged at these intervals. Thus, fibrillar collagen gene regulation and subsequent myocardial fibrosis are integral components of renovascular hypertension. The cellular origins of the fibrillar and nonfibrillar collagen mRNAs and their temporally disparate regulation suggest that separate regulatory mechanisms, involving myocytes and fibroblasts, may be responsible for the remodeling of the myocardium. (Circulation Research 1990;67:787-794)

Pressure overload leads to myocardial hypertrophy and the remodeling of muscular and collagenous compartments of the myocardium where the accumulation of fibrillar collagen is known to impair myocardial stiffness. It has been demonstrated\(^1\)\(^-\)\(^2\) that after abdominal aortic banding, left ventricular weight rises early and reaches a plateau by day 3. Protocollagen prolyl hydroxylase, a measure of collagen forming capacity, is shown to be increased severalfold by day 2, followed by an increase in proline incorporation that reaches its peak on day 4. Hence, collagen synthesis rises promptly in response to the pressure overload. Turto\(^3\) has shown that workload hypertrophy in skel-etal muscle is similarly associated with enhanced collagen synthesis. By the use of \(^3\)H\)thymidine incor-
poration and autoradiography, Morkin and Ashford demonstrated that fibroblast proliferation also occurs with pressure overload, starting at day 2 and declining at day 7 after abdominal aortic banding. Together, these findings suggest that 1) collagen synthesis rises promptly in existing cardiac fibroblasts in response to an elevation in left ventricular pressure, and 2) fibroblast proliferation, which also may contribute to collagen accumulation in pressure overload hypertrophy, occurs somewhat thereafter. The signal and transducer that link ventricular systolic pressure to collagen synthesis are unknown but would appear to involve cardiac fibroblasts that have been shown to be responsible for types I and III collagen synthesis. Ultrastructural studies also have shown that endomyosal and perimysial collagen fibers of the myocardium become thicker and denser with experimental hypertension.

The above findings suggest that quantitative and qualitative changes occur in the biosynthesis of the myocardial extracellular matrix in hypertrophy. To gain an understanding of the underlying mechanisms of collagen biosynthesis that lead to collagen remodeling during pressure overload hypertrophy, we studied the expression of fibrillar collagen types I and III and basement membrane type IV collagen genes in pressure overloaded rat myocardium.

**Materials and Methods**

**Animal Model**

Male Sprague-Dawley rats (180–200 g) were used for all experiments. Before surgery, the animals were anesthetized with 0.1–0.3 mg intraperitoneal methohexitall. A midline abdominal incision was used to expose the suprarenal abdominal aorta and to isolate a 0.5-cm segment of this vessel. Constriction of the aorta and right renal artery was produced by a silk ligature as previously reported. The abdomen was closed, and the animals were allowed food and water ad libitum. After 1, 3, and 7 days and 4 and 8 weeks, animals were anesthetized with ketamine hydrochloride (70 mg/kg i.p.). The trachea was cannulated and connected to a constant volume respirator (model 683, Harvard Apparatus, South Natick, Mass.). The left carotid artery was cannulated for the measurement of arterial pressure (Statham P23Gb, Gould Instruments, Inc., Cleveland). A median sternotomy then was performed, the heart was removed, and left ventricular tissue was processed as indicated below. Age- and sex-matched control animals were studied at similar intervals.

**RNA Extraction**

Total RNA was extracted by the procedure of Chirgwin et al with minor modifications as previously described. Briefly, hearts were rapidly dissected, the atria and the right ventricle were removed, and the left ventricular myocardium was homogenized in 4 M guanidine thiocyanate solution on ice. The homogenate was cleared of cell debris by centrifugation at 9,500 rpm for 10 minutes. RNA was pelleted on a cesium chloride cushion, then redissolved in Tris EDTA pH 7.4 and purified by three ethanol precipitations. Total RNA was quantified by absorbance at 260 nm, assuming 40 mg/ml for each unit of absorbance.

**RNA and DNA Hybridization**

Steady-state levels of messenger RNA (mRNA) were determined by Northern and dot blot hybridization analyses. For Northern blot studies, 10 μg of total RNA was denatured in 50% formamide, 17.5% formaldehyde, and 1× MOPS buffer (20 mM morpholinopropanesulfonic acid at pH 7.0, 5 mM sodium
Acetate, and 1 mM Na$_2$-EDTA, pH 8.0); electrophoresed in a 1% agarose gel; transferred to a GeneScreen Filter (New England Nuclear, Boston); and baked 2 hours at 80°C in a vacuum oven. Blots were prehybridized in 5× standard saline citrate (SSC), 0.1% sodium dodecyl sulfate, 5× Denhardt’s solution, 50% deionized formamide, 0.1 M phosphate buffer pH 7.0, and 100 mg/ml calf thymus DNA for 24 hours at 42°C as described. Hybrids were performed at 42°C using the same buffer containing the appropriate radioactive probes to obtain $3 \times 10^{8}$ counts/min/ml hybridization medium. The complementary DNA (cDNA) was radioactively labeled by random primer extension as described by Feinberg and Vogelstein using Amersham Multiprime DNA labeling systems according to the manufacturer’s manual (Amersham, England). [32P]dCTP (specific activity 3,000 Ci/mM, Amersham) was included in the reaction mixture to obtain a specific activity of 2 to $6 \times 10^{8}$ counts/min/mg DNA. Recombinant plasmids used as probes were as follows: rat $\alpha_1$ (I) collagen; recombinant plasmid complementary to rat pro-$\alpha_1$ (I) containing 1,500 base pairs $\alpha_1$ (I) sequences specific for the entire 3’ noncoding and carboxy-terminal propeptide regions that hybridize to 4.2 and 4.5 kilobases mRNA; mouse type III collagen DNA fragment that codes for the amino-terminal propeptide of mouse $\alpha_1$ (III) collagen and hybridizes to mRNA slightly larger than $\alpha_2$ (I) mRNA; mouse type IV collagen plasmid clone homologous to collagen type IV (a2) mRNA; and a full-length cDNA for chicken $\beta$-actin. After hybridization, the membranes were washed and exposed to Kodak XAR-5 film at −70°C. For dot blot studies, the total RNA was incubated for 5 minutes at 65°C in 3% formaldehyde and then was serially diluted (6, 3, and 1.5 μg). It then was spotted (6, 3, and 1.5 μg) on the GeneScreen membrane and baked before prehybridization as described above. For quantification and comparison of relative amounts of mRNA, dots were scanned by densitometry. The areas under the curve were digitized and compared in relative units. Quantities of 3 μg were arbitrarily chosen for comparison and presentation in all cases.

**Immunofluorescent Staining**

Tissue preparation and staining were performed as previously described. Briefly, banded and control rats were decapitated, and hearts were dissected, washed with phosphate buffered saline, and frozen in isopentane at −55°C. The frozen sections (15–20 μm) were cut at −20°C in a cryostat, mounted, and air-dried at room temperature. The primary antisera to rat tail tendon collagen type I and rat skin type III and mouse type IV collagen were raised in rabbits. An IgG-enriched fraction was precipitated with ammonium sulfate. The monospecificity of these antibodies was tested by enzyme-linked immunosorbent assay as described previously. Heart sections were overlaid with 50–100 μl of 1:50 dilution of antibodies and incubated at room temperature. Sections were washed in 1× phosphate buffered saline and then incubated with 50–100 μl of 1:50 dilution of fluorescein isothiocyanate–conjugated goat anti-rabbit IgG at room temperature. As negative control, parallel sections were incubated with IgG prepared from rabbit preimmune serum. Visualization of stained tissue was performed using an Olympus model BH2 epifluorescent microscope. Photographs were taken using Trix-400 film.

**In Situ Hybridization**

Animals were anesthetized, and hearts were rapidly excised and washed in 1× phosphate buffered saline.
to clear the ventricle of blood. The hearts then were frozen at −55°C in isopentane. Sections were cut 16 μm thick in a cryostat at −20°C. A modification of the procedure described by Shivers et al17 was used as previously reported.

Prehybridization buffer contained 50% formamide, 0.6 M NaCl, 10 mM Tris (pH 7.5), 0.02% Ficoll (type 400), 0.02% polyvinylpyrrolidone (PVP-40, Sigma Chemical Co., St. Louis), 0.02% bovine serum albumin (fraction V), 1 mM EDTA, 0.05% yeast total RNA (type III), 0.005% transfer RNA (type IX), 0.05% herring sperm DNA, 0.05% inorganic sodium pyrophosphate, 10 mM L-methionine, and 20 mM 2-mercaptoethanol. The mixture was heat-denatured in boiling water for 10 minutes before application. Ten to 20 μl (depending on the area to be covered) of prehybridization medium was applied to each section. Sections were incubated at 37°C overnight.

Hybridization buffer contained the same components as prehybridization buffer with the following additions: 10% dextran sulfate (MW 8,000), 0.05% polyadenylic acid, and 5 mM unlabeled ribonucleotides and deoxynucleotides. 35S-labeled cDNA probes were added to the hybridization medium to obtain a specific activity of 1×109 counts/min/10 ml hybridization buffer. The hybridization buffer was placed in boiling water for 10 minutes before application.

Prehybridization buffer was removed from the slides by brief washing in 2× SSC buffer. Hybridization was conducted at 37°C overnight. The cDNA probes were radioactively labeled as described above with the exception of using a different radioisotope, [35S]dCTP (specific activity, 1,000 Ci/mmol, Amer sham). This was included in the reaction mixture to obtain a specific activity of 107–108 counts/min/mg DNA. Recombinant plasmids used as probes were as described above. Plasmid PBR 322 was used as a negative control.

Detection of Bound Complementary DNA Probes

Slides were dipped in Kodak NTB-3 nuclear track emulsion diluted 1:1 with distilled water, dried at room temperature for 10 minutes, and exposed in desiccant-containing, light-tight boxes at −20°C for 1–3 hours. Slides were developed in Unicolor developer B&W diluted 1:3 for 4 minutes and fixed for 2 minutes. After fixation, slides were rinsed in distilled water and stained with Giemsa (4% in phosphate buffered saline) for 10 minutes. After two rinses in distilled water, slides were dehydrated in graded ethanol (70%, 90%, and absolute) and mounted with cytoseal mounting medium (Stephens Scientific Division, Cornwell Corporation, Oak Ridge, N.J.). Reduced silver grains reflecting bound probes were
visualized by dark field imaging. Phase-contrast light microscopy also was performed on each section.

**Results**

**Experimental Hypertension**

One and 8 weeks after abdominal aortic banding, systolic arterial pressure exceeded 180 mm Hg (versus 130 mm Hg in control) in most animals. This level of experimental hypertension is in keeping with our previous observations in this model and was associated with a rise in left ventricular mass and body weight of 10–30%.

**Steady-State Levels of Messenger RNA**

The abundance of mRNA for collagen types I, III, and IV and actin in left ventricular tissue from rats with abdominal aortic banding and normal age-matched control rats was quantified by Northern and dot blot analyses and densitometric scanning of hybridization signals. Left ventricular tissue ranged from 500 to 820 mg per heart for control rats and 550 to 850 mg per heart in banded rats. No increase in left ventricular weight was observed at days 1, 3, and 7 after banding. However, at 4 and 8 weeks after abdominal aortic banding, there was a 12–15% increase in left ventricular weight compared with that of control rat hearts.

Figure 1 (panels A–D) shows the time course of changes in steady-state levels of mRNA for collagen types I, III, and IV and actin after abdominal aorta banding as quantified by dot blot analysis. Collagen type I mRNA reached a peak at day 3 of banding, corresponding to a fivefold (p<0.01) increase in mRNA levels compared with nonbanded control rats. At day 7, mRNA levels had declined and reached a plateau at 4 weeks, where it remained constant 8 weeks after banding. The mRNA levels, however, were slightly higher than those of control hearts even at 8 weeks (Figure 1A). The same pattern of increased levels of mRNA for collagen type I also was evident in Northern blot analysis, as shown in Figure 2.

For type III collagen, the same temporal pattern of increased mRNA levels was observed after aortic banding (Figure 1B). At day 3, mRNA levels reached a peak corresponding to a 1.7-fold (p=0.05) increase in mRNA levels compared with control rats. This increase was followed by a decline at day 7.

The steady-state level of mRNA for type IV collagen, however, was elevated in the left ventricle of the banded rats at day 1 (1.3-fold, p=0.06), remained elevated at day 3, and then returned to control levels by day 7, where it remained constant at 4 and 8 weeks (Figure 1C). Densitometric scanning of dot blot analysis indicated a prompt increase (2.5-fold, p<0.01) in steady-state level of actin mRNA in the
banded animals at day 1, followed by a rapid return to control level by day 3, where it remained constant at 1, 4 and 8 weeks (Figure 1D).

**Immunofluorescence Light Microscopy**

The results of antibody staining on frozen sections of the left ventricle showed that the distribution of fibrillar collagen types I and III in the banded animals was similar to that seen in controls. In comparison, however, type I fibers were thicker and denser in the interstitial space and surrounding intramyocardial coronary arteries after 8 weeks of experimental hypertension (Figures 3 and 4). There were no detectable differences in the accumulation or distribution of fibrillar type III collagen in the ventricle of banded animals versus controls (Figure 5). Similarly, immunofluorescent staining for type IV collagen, which is located mainly in the basement membrane, did not reveal any apparent differences in the accumulation of type IV collagen between hypertrophied and control hearts (data not shown).

**In Situ Hybridization**

To verify potential regional redistribution of mRNA for collagen types I and III in the myocardium caused by experimental hypertension, in situ hybridization was performed on cryostat sections of the heart at day 3 after aortic banding. mRNA for types I and III collagens was found in the interstitium, around the collagen struts. These hybridization signals for both collagen types were significantly increased in the ventricle of the rat with hypertension compared with controls (Figure 6); however, no focal areas of mRNA accumulation were observed.

**Discussion**

Previous studies indicate that collagen volume fraction of the hypertrophied left ventricle was significantly increased at 4 and 8 weeks of experimental hypertension. This finding could be explained by the fact that for mature collagen fibers to be deposited, extracellular posttranslational modifications of the secreted collagen molecules are necessary. These modifications consist of intermolecular and intramolecular covalent cross-linking that lead to the formation of mature collagen fibers. Moreover, the twofold accumulation of fibrillar collagen that was observed in those studies, and which presented as a generalized interstitial fibrosis and a perivascular fibrosis of intramyocardial coronary arteries, was associated with abnormalities in diastolic and systolic myocardial stiffness.5,6,8 These findings suggest that quantitative and qualitative changes had occurred in the biosynthesis of myocardial collagen in our model of renovascular hypertension. Accordingly, this study was undertaken to gain insight into underlying mechanisms of collagen biosynthesis that lead to myocardial fibrosis in this model of experimental hypertension.
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Previously, we have demonstrated by Northern blot analysis and in situ hybridization on cardiac tissue, isolated cardiac cells, and cultured cardiac cells that mRNA for collagen types I and III can be detected only in cardiac fibroblasts, whereas mRNA for collagen type IV is present both in cardiac myocytes as well as in fibroblasts. Therefore, considering the cellular origin of those collagens, it could be envisaged that cardiac fibroblasts and myocytes are differentially affected in renovascular hypertension by signals (e.g., hemodynamic and hormonal) that come into play at different intervals after abdominal aortic banding with right renal ischemia. Interestingly, when chicken β-actin cDNA probe was used as a control, dot blot analysis of mRNA levels showed a somewhat similar pattern to that of type IV collagen. The hybridization signals with cDNA for β-actin represent both myocyte-specific and nonmyocyte actin isotypes. This is due to the high degree of sequence conservation between different actin isotypes.

This finding provides additional evidence that cardiac myocytes and cardiac fibroblasts both are affected by the experimental hypertension in our model, but at different times. Our histological data involving in situ hybridization for type I collagen also may be an indication of fibroblast proliferation as well as increased mRNA levels. Fibroblast proliferation has been shown to occur with pressure overload after abdominal aortic banding. Therefore, although increased mRNA for different collagen types could be viewed as an independent response, it is also accompanied by fibroblast proliferation. Indeed, it has been shown that gene expression in cardiac hypertrophy is a complex process. Within 2 days of pressure overload induced by abdominal aortic banding, fetal isoforms of α-actin and sarcomeric isomyosin are reexpressed in adult myocardium. Furthermore, the c-fos and c-myc proto-oncogenes and a major heat shock protein (hsp 70) are induced in the ventricular myocardium within 1 hour after imposition of pressure overload. Although these findings are attributed mainly to cardiac myocytes, the possible involvement of connective tissue cells in these events has not been excluded. Starksen et al showed that in cellular hypertrophy induced by exposure to α-adrenergic agonists, the level of mRNA for c-myc proto-oncogenes increased 10-fold. These findings suggest that cardiac hypertrophy could lead to quantitative as well as qualitative changes in gene expression for different proteins. Transient increase of mRNA levels for proto-oncogenes c-fos and c-jun, as well as a transcriptional factor EGR-1 (early growth response), has been demonstrated after adrenergic stimulation of the neonatal rat myocardial cell. On the other hand, the c-myc proto-oncogene

The results of our present studies have three distinct features. 1) Like other proteins, myocardial collagen gene expression is altered in experimental hypertension. Steady-state levels of mRNA for fibrillar collagen types I and III and basement membrane type IV were increased after abdominal aortic banding. 2) Fibrillar collagen types I and III and basement membrane collagen type IV gene expression are regulated at different times after aortic banding. Steady-state levels of mRNA for collagen types I and III reached a peak at day 3 of pressure overload. The mRNA levels then declined for both collagen types at day 7 and reached a plateau at 4 and 8 weeks of banding. Collagen type IV mRNA levels, however, increased at day 1 after abdominal aortic banding. 3) Although changes in mRNA levels for type I collagen occur at early stages of pressure overload, collagen accumulation and fibrosis as detected by immunofluorescence light microscopy can be observed only at later stages of experimental hypertension. This finding could be explained by the fact that for mature collagen fibers to be deposited,
has been shown to modulate cardiac hypertrophy in transgenic mice. Two distinct features of macromolecular biosynthesis in the extracellular matrix need to be considered: 1) selection of the genes that will be expressed during hypertrophy, and 2) control of regulatory mechanisms that will amplify or attenuate the expression of these selected genes. The nature of regulatory mechanisms responsible for the observed mRNA levels for collagen types I, III, and IV in pressure overload hypertrophy is not clear. Hemodynamic changes induced by aortic banding may lead to the release of neurotransmitters and growth factors, such as norepinephrine and transforming growth factor-β (TGF-β), and the influx of trace elements, such as Ca²⁺, into myocytes and fibroblasts. Any of these substances, acting alone or in combination with potential transducers, ultimately may initiate a cascade of events that may lead to the regulation of collagen gene expression in myocytes and fibroblasts. Potential regulatory factors may act at the transcriptional level.

Increased levels of collagen mRNA also may be due to an increased stability of the message. In this case, regulatory factors that may alter the cytoplasmic half-life of the mRNA for collagen types I, III, and IV must be considered. It has been shown that in cultured liver cells, the steady-state levels of liver-specific mRNAs will change in response to regulatory factors, such as hormones and growth factors, and that these changes are not correlated with similar changes in transcription rates for those mRNAs. These studies suggest posttranscriptional mechanisms responsible for regulating the abundance of mRNAs in liver cell culture. If an analogy could be made between the cellular responses to environmental alterations in culture medium and to those generated in vivo in hypertrophy because of pressure overload, potential alterations in the concentration of growth factors, hormones, and trace elements that may occur with experimental hypertension would potentially modulate the steady-state levels of collagen mRNA in the myocardium.

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References


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