Fibrillar Collagen and Myocardial Stiffness in the Intact Hypertrophied Rat Left Ventricle


This study tested the hypothesis that with hypertrophy, the proportion, distribution, and structural alignment of fibrillar collagen are important determinants of myocardial stiffness. Toward this end, the collagen volume fraction (morphometry), the transmural or subendocardial distribution of collagen, and the structural arrangement of fibrillar collagens (picrosirius red) were examined in the hypertrophied ventricle secondary to pressure overload (abdominal aorta banding or perinephritis), isoproterenol, and pressure overload plus isoproterenol. In the same hearts, the slopes of the systolic and diastolic stress-strain relations of the left ventricle, representing its active and passive stiffness, respectively, were obtained. In comparison with controls, we found 1) for a moderate rise in transmural collagen, active and passive stiffness increased with pressure-overload hypertrophy; 2) following isoproterenol alone there was a marked increase in subendocardial collagen, and active and passive stiffness increased; 3) in pressure-overload hypertrophy plus isoproterenol, active stiffness declined. Passive stiffness was increased except when fibrosis and thinning of the interventricular septum occurred, in which case it decreased; and 4) fibrillar collagens involved in remodeling included the formation of collagen strands and fibers in a greater number of previously collagen-free intermuscular spaces in pressure-overload hypertrophy, or a dense crisscrossing lattice of fibers that encircled muscle fibers after isoproterenol. Thus, an increase in fibrillar collagen in pressure-overload hypertrophy is partially adaptive in that it enhances the tensile strength and three-dimensional delivery of force by the myocardium, but at the expense of reducing distensibility. The appearance of a dense collagen meshwork within the subendocardium after isoproterenol can be considered pathological in that it entraps muscle fibers causing active stiffness to fall while impairing distensibility. Finally, fibrosis may paradoxically reduce passive stiffness if it leads to a thinning of the interventricular septum. (Circulation Research 1989;64:1041-1050)

The myocardium is a composite material that includes muscular and interstitial compartments whose relative proportions, stiffness, and physical arrangement will determine myocardial viscoelasticity. Fibrillar type I and III collagens are major structural components of the interstitium. An increased content or transformed structure of these fibrillar collagens with respect to cardiac muscle fibers could adversely influence diastolic and systolic myocardial stiffness. In this connection, a substantial increase in collagen volume fraction has been observed in man with aortic stenosis or hypertension and in the nonhuman primate and smaller mammal with experimental or genetic hypertension. This accumulation of collagen was noted to be either diffusely distributed throughout the myocardium or confined to the subendocardium, or both, and has been held responsible for the abnormal passive stiffness of excised cardiac muscle and the impaired diastolic stiffness and pumping capacity of the intact ventricle. In the hypertensive postmortem human heart, Caulfield has noted two distinct patterns of collagen ultrastructure remodeling. The first involves an increased diameter to various components of the matrix (e.g., fibers interconnecting myocytes) while the second includes the appearance of a dense meshwork to replace the finer collagen weave that surrounds myocytes. The functional consequences to these patterns of collagen remodeling, particularly their influence on the diastolic and systolic stiffness of the intact, hypertrophied myocardium, remain unknown. This study was therefore undertaken to...
test the hypothesis that in the hypertrophied rat left ventricle, collagen volume fraction, distribution, and structural nature are each important determinants of myocardial stiffness. Accordingly, we examined the systolic and diastolic stress-strain relations, or active and passive stiffness, respectively, of the hypertrophied left ventricle following pressure overload (abdominal aorta banding or perinephritis), isoproterenol, and pressure overload plus isoproterenol.

These models were chosen so that we might examine a broad range of collagen volume fraction similar to that reported in humans and where subendocardial fibrosis was additive to the collagen remodeling seen with pressure overload alone and a structural remodeling of collagen could be expected. Collagen content was assessed by morphometric technique of trichrome-stained tissue while the fibrillar nature and structural features of collagen were examined by taking advantage of its enhanced birefringence provided by the picrosirius red technique and polarization microscopy.

Materials and Methods

Animal Model

Forty-four male, age-matched Wistar rats weighing 300–350 g were studied in six different experimental groups. Group I (n=11) served as sham-operated controls. Animals were anesthetized (ketamine, 100 mg/kg i.p.), and the abdomen was opened by midline incision. No further surgical procedure was performed. Group II (n=7) animals received a single daily dose (500 μg/kg s.c.) of isoproterenol hydrochloride in 0.1% phosphate buffer for 10 days to induce myocardial fibrosis. No surgery was performed on these animals. In group III (n=10), abdominal aorta constriction was performed. After midline abdominal incision a 0.5 cm segment of aorta was dissected free. A 0.9-mm diameter wire was then placed on its anterior surface, and surgical silk was used to bind the wire and aorta. The wire was withdrawn from the ligature so that the remaining orifice was 0.9 mm. The abdomen was then closed. Animals were allowed food and water ad libitum and were killed after 8 weeks. In group IV (n=5), subcutaneous isoproterenol was administered for 10 days after 8 weeks of aortic banding. Thereafter, the animals were killed. Group V animals (n=7) underwent abdominal surgery to wrap the left kidney in cellophane; the right kidney was not manipulated or removed. These animals were followed for 10 weeks and then killed. Group VI animals (n=4) had perinephritis hypertension for 10 weeks after which time they received the same dose of isoproterenol for 10 days before they were killed 3 weeks later.

Physiological Studies

At time of death, animals were again anesthetized, intubated, and ventilated, and carotid arterial pressure was recorded. The chest was entered by median sternotomy. Hearts were then quickly removed, the thoracic aorta was cannulated, and the hearts were perfused in retrograde fashion using a constant perfusion pressure reservoir and warmed, modified Krebs-Henseleit bicarbonate buffer (37°C, pH 7.40–7.45) bubbled with 95% O2-5% CO2. A compliant balloon, attached to a stiff tube and connected to a three-way stopcock with pressure transducer and 100-μl syringe, was inserted into the left ventricle via the mitral valve orifice. The pressure and volume of the balloon were recorded during controlled variations of balloon volume in the isovolumetrically beating ventricle. The right ventricle was vented.

Coronary perfusion pressure was selected as previously reported and averaged 109 mm Hg for all hearts. Steady-state left ventricular pressure was recorded from each heart during either increments or decrements (0.01 ml) in balloon volume over the left ventricular end diastolic pressure range 0–25 mm Hg. For each heart, two sets of pressure-volume data were recorded. Reproducible (±10%) results were combined for analysis of the systolic and diastolic stress-strain relations. The elastic property and volume of each balloon was determined as previously reported.

To assess myocardial stiffness for hearts of different left ventricular weight and size, stress (σ; g/cm²) and strain (ε) for the midwall of the left ventricle were calculated assuming a spherical geometry as previously reported:

\[
\sigma = \frac{1.36 \times LVP \times V^{2/3}}{(V + 0.943 \times LVW)^{2/3} - V^{2/3}}
\]

\[
e = \frac{V^{1/3} + (V + 0.943 \times LVW)^{1/3}}{[(V + 0.943 \times LVW)^{2/3} + (V^{0.93} + (V_s + 0.943 \times LVW)^{1/3})^{1/3}] - 1}
\]

where V is chamber volume (ml), V_s is volume at end diastolic of 0 mm Hg, LVV is left ventricular weight (g), and LVP is end diastolic or systolic pressure (mm Hg). Developed stress was derived by subtracting peak isovolumetric and end diastolic stress. The slope of the developed systolic and the diastolic stress-strain relations were obtained by fitting regression lines to the stress-strain relations. Active stiffness was taken as the slope of the developed stress-strain relation and passive stiffness as the slope of the diastolic stress-strain relation.

Collagen Morphometry, Morphology, and Concentration

After the physiological studies, hearts were removed from the perfusion apparatus, and their atria, great vessels, and valvular structures were trimmed away. Right and left ventricles (plus septum) were separated and weighed. Coronal (1 mm) sections of the left ventricle were obtained for light and scanning electron microscopy.

Collagen volume fraction was assessed using a computer-assisted procedure as previously reported. Five-micron thick paraffin sections, fixed
in 10% buffered formaldehyde and stained with Gomori’s trichrome, were placed in a projection microscope. The section was divided into four quadrants with the center of the section as the origin. From each quadrant, four fields were randomly selected from the subendocardial (two fields) and subepicardial (two fields) regions. Each field was then transferred to a digitizing pad connected to a cursor-computer assembly. Segments representing connective tissue and muscle were identified and traced. A computer program was used to calculate the traced areas as the volume fraction of collagen and muscle. Collagen volume fraction was calculated as the sum of all connective tissue areas divided by the sum of all connective and muscle areas in all fields. Areas of connective tissue surrounding intramyocardial coronary arteries were excluded given our assumption that they would not likely influence mechanics. The presence of muscle necrosis was examined based on the following criteria: the loss of nuclei and cross striations, the presence of inflammatory cells, and the confluence of fibrotic tissue replacing muscle.

Our sections were stained with the picrosirius red technique as modified by Dolber and Spach. Junqueira et al. reported that because of collagen’s birefringence, differences in collagen fiber diameter can be distinguished by the acidic dye Sirius red and polarized light, where thick fibers appear yellow, or yellow-red, while thin fibers are green.

Statistical Analysis

All group data are expressed as mean±SD and were compared by analysis of variance; when a significant F value was found, the groups were compared using Bonferroni bounds or Student-Newman-Keuls multiple-range test. For function studies, we analyzed all diastolic data for the six groups combined and for each group separately versus all developed data from the six groups combined and for each group separately using least-squares regression analysis. However, before analysis, the developed stress at V₀ was subtracted from all other developed stress data in each experiment so that each developed stress-strain relation intersected the x and y axes at the origin. For each group, the developed stress at V₀ was grouped, averaged, and compared as described above. An intergroup comparison of the diastolic and developed stress-strain relations was performed using the analysis of covariance. If a significant difference was found, dummy variables were used to test for equality of the control slope with the slope of each of the experimental groups.

Results

Hemodynamics and Left Ventricular Hypertrophy

Systolic arterial pressure and the ratio of left to right ventricular weights are summarized in Table 1 for each experimental group. Both abdominal aorta banding and perinephritis produced a significant increase in arterial pressure, and each model alone, or with isoproterenol, was associated with left ventricular hypertrophy. Isoproterenol alone also led to left ventricular hypertrophy. There was the suggestion that the addition of isoproterenol to the pressure overload groups caused additional hypertrophy, but this was not statistically significant for groups IV and VI with respect to groups III and V.

Collagen Volume Fraction and Distribution

The morphometrically determined collagen volume fractions for all groups are given in Table 1. In the pressure-overload groups, there was a significant elevation in collagen concentration throughout the left ventricular myocardium in comparison with age- and sex-matched controls. The addition of isoproterenol to the pressure-overload models provided a significant additional increment in collagen volume fraction that was distributed primarily within the subendocardium of the left ventricle and interventricular septum. In group VI, where the fibrotic process had 3 weeks to develop, the septum had become thinner than that seen in groups II or IV.

Fibrillar Collagen Structure and Collagen Types

In the normal myocardium (see Figure 1), there were thick and thin collagen fibers, often colocalized, within intermuscular spaces. Strands of straight
FIGURE 1. Polarizing light microscopy and Sirius red stain of control rat myocardium (group I). Top panel: Thick and thin collagen fibrils have a yellow and light green appearance, respectively. Magnification, ×100. Collagen strands have a perpendicular orientation to muscle (arrow). They occasionally appear branched. Colocalized thick and thin collagen fibers (arrowhead) course through these strands. Magnification, ×100. Lower panel: Subendocardium, where muscle fibers appear in near cross section and thin collagen septae are seen. Magnification, ×100.
and branched collagen fibers coursed through these spaces in a direction perpendicular to muscle fibers. These strands were entwined by colocalized thick and thin collagen fibers that ran in a direction coincident with the long axis of muscle fibers. Together, the perpendicular and longitudinal fibers formed a loosely organized latticework between muscle fibers.

The collagen matrix seen with the hypertrophy that accompanies 8 weeks of perinephritis (see Figure 2) resembled the normal structure except that an increased number of intermuscular spaces, previously free of collagen, were filled with collagen fibers. In addition, colocalized thick and thin collagen fibers of corkscrew configuration were coursed over muscle fibers at various angles to the long axis of muscle. No evidence of myocyte necrosis or muscle fiber disorganization was detected, and there was no preferential distribution of collagen within the subendocardium. The pattern of collagen accumulation and remodeling seen after 8 weeks of abdominal aorta banding was similar to that seen with perinephritis.

The fibrosis seen after isoproterenol treatment alone or after pressure overload consisted of a dense crisscrossing meshwork of thin and thick collagen fibers that resembled a tightly woven wicker basket. Thick collagen fibers were the major component of this meshwork (see Figure 3). Thin collagen fibers extended across the long axis of muscle. Interdigitating through these thin fibers, at right angles, were thick collagen fibers. This accumulation of collagen, extending outward from intermuscular spaces to encircle muscle, was found primarily within the endomyocardium of the left ventricle and interventricular septum although collagenous septae radiated from this region into the midmyocardium, creating a pattern of streaky fibrosis. Isolated patches of fibrosis were also seen in the midmyocardium and epicardium. On cross section, muscle fibers of the subendocardium were encircled by these collagen fibers.

**Active and Passive Stiffness**

The developed systolic stress-strain relation was highly linear ($r=0.82-0.97$) for all hearts. An analysis of covariance resulted in a highly significant ($p<0.0001$) $F$ value (58.87), indicating significant differences in active stiffness between groups (see Table 2). In the pressure overload and isoproterenol groups, active stiffness was significantly greater than control while in the pressure overload plus isoproterenol groups, the force-generating capacity of the myocardium was significantly reduced. Developed systolic stress at $V_0$ or 0% strain was not significantly different between groups.

Highly linear relations ($r=0.87-0.97$) were also found for the diastolic stress-strain relation for each experimental group. Significant differences were observed (see Table 2) compared with control rats ($F=55.97; p<0.0001$) between passive stiffness in groups II, III, IV, V, and VI. Passive stiffness rose disproportionately when more than 15% of the myocardium was occupied by collagen. In group VI, where a marked fibrosis and thinning of the interventricular septum was seen, the diastolic stress-strain relation was significantly less than in controls.

**Discussion**

A disequilibrium of the muscular and collagenous compartments may be the setting that leads to abnormal myocardial stiffness and pathological hypertrophy. In this connection, several pathogenetic mechanisms that disrupt the normal balanced equilibrium between compartments, and which originate in the remodeled collagen matrix of the pressure-overloaded myocardium, have been proposed. One such mechanism is an increase in collagen volume fraction that raises the collagen to muscle mass ratio disproportionately. Another relates to a structural realignment of collagen fibrils and muscle fibers. The purpose of this study was 1) to examine the role of collagen volume fraction on the active and passive stiffness of the intact myocardium, and 2) to assess the importance of collagen matrix-muscle fiber alignment and the importance of fibrous tissue distribution on myocardial mechanics.

To answer these questions, we used various animal models that provided us with a range of collagen volume fraction and a transmural versus subendocardial distribution of collagen similar to that reported in humans. We also desired to examine a model in which the alignment of collagen and muscle fibers had been transformed. Pressure-overload models were selected with the view that they represented reactive fibrosis, or collagen accumulation in the absence of cell loss. We wished to avoid substantial amounts of myocyte necrosis with replacement fibrosis since it alone might independently alter active stiffness. In addition, our previous studies had shown that with necrosis there would also be a structural realignment of collagen fibers and a consequent disorganization of muscle fibers. Abdominal aorta coarctation, which includes the right renal artery, represents a pressure overload that presumably does not lead to necrosis and is associated with an excess accumulation of collagen mediated by enhanced collagen synthesis in existing fibroblasts and subsequent fibroblast proliferation. Perinephritis also presents a pressure overload model with reactive fibrosis. To create a broader range and distribution of collagen content than was available with pressure-overload hypertrophy alone and in which the influence of subendocardial fibrosis could also be examined, we administered a small dose of isoproterenol.

The results of our study indicate hitherto unrecognized effects of collagen remodeling on the stress-strain relation. Active stiffness, or the developed systolic stress-strain relation of the intact rat left ventricle, is increased with an adaptive remodeling of collagen fibers that includes a moderate ($<15\%$)
FIGURE 2. Polarizing light microscopy of hypertrophied rat myocardium after 8 weeks of perinephritis (group III). Top panel: A greater number of intermuscular spaces are seen to contain fibrillar collagen, including collagen strands (arrow) and colocalized collagen fibers (arrowhead). Twisted or corkscrew appearing fibers are seen crossing over muscle fibers (small arrowheads). Magnification, ×100. Lower panel: Subendocardium, where an increase in collagen fiber diameter and perimuscular collagen is evident. Magnification, ×100.
FIGURE 3. Sirius red stained myocardium and polarizing light microscopy of rat myocardium after 8 weeks of abdominal aorta banding followed by 10 days of isoproterenol (group IV). Top panel: A dense meshwork of thick and thin collagen fibers have formed to encircle muscle fibers. Magnification, ×100. Lower panel: A dense collection of perimyscular collagen is now seen in the subendocardium. Magnification, ×100. The insert is a scanning electron photomicrograph showing crisscrossing network of thick and thin collagen fibers. Magnification, ×1,900.
increment in collagen concentration in a greater proportion of intermuscular spaces. Once the accumulation of collagen exceeds 20% and a realignment of collagen and muscle fibers occurs beyond the boundaries of the intermuscular space to encircle and encase muscle in "cement," myocyte force generation is impaired. Furthermore, this pathological excess collagen may isolate myocytes and disrupt the orderly transmission of force between contracting myocytes. In this connection, Winegrad and Robinson have previously shown that in the absence of intercalated disks, force transmission between cardiac muscle fibers is sustained by connective tissue fibers. The fibrosis, or accumulation of collagen, that develops after pressure overload plus isoproterenol is an example of how fibrous tissue can isolate muscle fibers. In so doing, it may also foster a dyssynchronous contraction of the myocardium. Inelastic collagen that has encharged groups of muscle fibers in the endomyocardium also imposes a physical restraint on muscle distension, and this too will reduce force generation by curtailing the length-dependent property of cardiac muscle.

Our study demonstrates that an increase in passive myocardial stiffness occurs with a rise in collagen volume fraction. Broadly speaking, our findings in the intact left ventricle are in agreement with in vitro studies of cardiac muscle that have been obtained from the endomyocardium of rats with pressure-overload hypertrophy secondary to aortic banding or renovascular or genetic hypertension. Although the concentration of collagen in these muscles was not examined. We also found that when the fibrotic process involved a thinning of the interventricular septum, the expected rise in passive stiffness was not seen. Instead, passive stiffness was lower than normal. This paradoxical reduction in passive stiffness may have been secondary to the disparity in material properties within different regions of the myocardium or to some aspect of the more advanced fibrotic process in group VI. These issues will need to be examined in more detail. Nevertheless, it is clear that the proportion, alignment, distribution, and consequences of collagen remodeling are each important determinants of myocardial stiffness in the intact ventricle.

The importance of the structural alignment of collagen to muscle was clearly evidenced by our monitoring the fibrillar nature of fibrous tissue formation. The picrosirius red technique, together with polarization microscopy, provides a unique method to monitor the transformation of the fibrillar collagen matrix and to examine the manner in which these fibers become entwined during collagen accumulation in the hypertrophied myocardium. In 1978, Junqueira et al suggested that thick collagen fibers take on a yellow or yellow-red color while thin fibers are green when viewed with crossed polarization filters at light microscopy. Several years later, having shown the importance of tissue section thickness on collagen fiber birefringence, Junqueira et al underscored the fact that the differential coloration provided by the picrosirius red technique and polarization microscopy was not secondary to a specific chemical interaction between Sirius Red and type I and III collagen but was instead the normal difference in their fiber diameter. Recent findings, using monoclonal antibodies, indicate that type III collagen fibers remain associated with type I collagen to form a copolymer and that this occurs irrespective of collagen fiber diameter. Robinson et al, using antibody labeling to type I and III collagens, suggested that collagen struts between myocytes in the rat and hamster myocardium are type I collagen while their more recent findings indicate that endomysial fibers are composed of both type I and III collagens. Hence, most collagen fibers consist of colocated type I and type III collagens. Our histochemical findings therefore were not used to distinguish or to quantify type I and III collagens in the myocardium. Instead, the nature of fibrillar collagen in perimysial fibers of the normal and hypertrophied myocardium of the rat were characterized by the picrosirius-polarization technique.

Our study indicates the complex nature of the arrangement of collagen fibers in the hypertrophied myocardium with fibrosis. Here, thin collagen fibers can become imbedded in a dense network of thick collagen fibers over time. Collagen strands connecting muscle fibers, located in intermuscular spaces where interfascicular shearing forces would be considerable, are composed of thick collagen fibers and would therefore be expected to have significant tensile strength. Thin collagen fibers are an integral component of most collagen fibers irrespective of fiber size or location. These thin collagen fibers, which normally may serve to provide a resilience to the myocardium, also form the fine reticular net-

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**Table 2. Diastolic and Systolic Stress-Strain Relations of the Intact Left Ventricle**

<table>
<thead>
<tr>
<th>Group</th>
<th>I (Control)</th>
<th>II (Isop)</th>
<th>III (AAB)</th>
<th>IV (AAB+Isop)</th>
<th>V (PN)</th>
<th>VI (PN+Isop)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSm</td>
<td>360±7</td>
<td>446±12*</td>
<td>537±16*</td>
<td>220±23*</td>
<td>495±28*</td>
<td>216±14*</td>
</tr>
<tr>
<td>Intercept</td>
<td>41±13</td>
<td>24±10</td>
<td>45±19</td>
<td>39±32</td>
<td>48±17</td>
<td>28±17</td>
</tr>
<tr>
<td>EDM</td>
<td>86±3</td>
<td>109±7*</td>
<td>110±3*</td>
<td>234±11*</td>
<td>109±4*</td>
<td>55±3*</td>
</tr>
</tbody>
</table>

Isop, isoproterenol; AAB, aortic banding; PN, perinephritis; DSm, slope of the developed systolic stress-strain relation (g/cm²); Intercept, the intercept (g/cm²) of the developed systolic stress-strain relation at 0% strain, EDM, slope of the diastolic stress-strain relation (g/cm²). *p <0.01 vs. control.
work into which thick fibers become entwined to create a crisscrossing latticework of fibrous tissue that can entrap muscle fibers. This sequence and involvement of the fibrillar collagens in myocardial fibrosis requires further investigation and may resemble their response to wound healing in skin.\(^7\)

Eghbali and coworkers,\(^8\) using mRNA probes to type I and III collagens, have clearly shown that fibroblasts are the source of type I and III fibrillar collagens in the heart. Given the adaptive and pathological components of collagen matrix remodeling seen in this study, it would be of interest to determine the signal and transducer of fibroblast collagen biosynthesis that mediates each response. Based on such information, pharmacological methods could be designed to attenuate an unfavorable degree of fibrosis that may accompany pressure-overload hypertrophy. Alternatively, the selective, controlled activation of myocardial collagenase might offer a means to remodel an existing abnormality in the collagen matrix.

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**Key Words** • collagen • collagen birefringence • picrosirius red • isoproterenol • pressure-overload hypertrophy
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