Viscoelastic properties of pressure overload hypertrophied myocardium: effect of serine protease treatment

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Viscoelastic properties of pressure overload hypertrophied myocardium: effects of treatment with a serine protease. Am J Physiol Heart Circ Physiol 282: H2324–H2335, 2002; 10.1152/ajpheart.00711.2001.—To determine whether and to what extent one component of the extracellular matrix, fibrillar collagen, contributes causally to abnormalities in viscoelasticity, collagen was acutely degraded by activation of endogenous matrix metalloproteinases (MMPs) with the serine protease plasmin. Papillary muscles were isolated from normal cats and cats with right ventricular pressure overload hypertrophy (POH) induced by pulmonary artery banding. Plasmin treatment caused MMP activation, collagen degradation, decreased the elastic stiffness constant, and decreased the viscosity constant in both normal and POH muscles. Thus, whereas many mechanisms may contribute to the abnormalities in myocardial viscoelasticity in the POH myocardium, changes in fibrillar collagen appear to play a predominant role.

matrix metalloproteinases; muscle; stiffness; viscosity

DIASTOLIC HEART FAILURE (DHF) develops when pressure overload hypertrophy (POH) alters the viscoelastic properties of the composite myocardium. These myocardial viscoelastic properties can be altered by changes in any of the components of the composite myocardium, such as cardiac muscle cells, fibroblast, blood vessels, and the extracellular matrix (ECM) that surrounds these structures. In addition, myocardial material properties can be altered by factors extrinsic to the myocardium such as hemodynamic load, heterogeneity, and neurohumoral activation. Changes in any one of these myocardial and extramyocardial determinants, either singly or in combination, may alter cardiac muscle material properties and may change diastolic function. It is important to recognize that the disease processes that cause POH may alter any number of these determinants individually or simultaneously. Therefore, to understand the basic underlying mechanisms by which POH alters myocardial viscoelastic properties, whether and to what extent each of these potential determinants contributes to myocardial material properties must be defined.

On the basis of experimental and clinical studies (3, 8, 43), it has been hypothesized that changes within the ECM play a predominant role in causing the alterations in viscoelastic properties of POH myocardium. Among the proteins within the ECM, fibrillar protein such as collagen and elastin, proteoglycans, and basement membrane proteins each may play a role in determining the constitutive properties of the myocardium. However, studies (4, 7, 16, 21, 41, 42, 44) suggest that fibrillar collagen, because of its three-dimensional configuration, its widespread distribution, and its plasticity in diseases causing POH, may contribute predominantly to the abnormalities in material properties of POH myocardium. To prove this hypothesis, however, collagen must be degraded in an experimental study in which the other myocardial and extramyocardial determinants are not simultaneously altered. Attempts to accomplish this goal in an acute experimental design have been difficult because the methods used to alter collagen such as ischemia, treatment with disulfide reagents, or treatment with exogenous collagenase have simultaneously altered other determinants, such as intracellular mechanisms or extracellular edema (5, 23, 26, 36, 38, 46). An alternate approach would be to activate a protease system normally present within the myocardium. One example is the zinc-dependent matrix metalloproteinases (MMPs) present in both normal myocardium and in cardiac disease states (19, 22, 27, 28, 30, 31, 37). Under normal conditions, MMPs are synthesized and released into the ECM as a propeptide orzymogen, which requires proteolytic processing to become an active MMP (2, 20, 45). One upstream mechanism for MMP activation is through the ECM matrix metalloproteinases; muscle; stiffness; viscosity

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cardiac determinants of viscoelasticity. This experimental design would allow definition of whether and to what extent this one myocardial determinant contributes to the alterations in myocardial viscoelastic properties caused by POH. Accordingly, the purpose of this study was to treat normal and POH myocardium with the serine protease plasmin, activate endogenous MMPs, degrade collagen, and determine the relative contribution that collagen makes to DHF in POH.

METHODS

Ventricular papillary muscles were isolated from six normal rats, eight normal cats, and eight cats with chronic right ventricular (RV) POH (RVPOH) induced by pulmonary artery banding (PAB) for 4 wk. Initial pilot studies were performed in rats to establish protocol parameters. Protocols were then applied to normal cats and PAB cats. This feline model was used because both cardiac tissue and cardiocytes from this preparation have been fully characterized in terms of morphology, function, and energetics. The extent of POH was determined by catheterization just before isolation of the papillary muscles. A computer-controlled servo motor system was used to mechanically test the papillary muscles. In each animal, a pair of papillary muscles was studied: one muscle was treated with plasmin, and the other muscle was not treated and served as the same animal control. The current study examined the effect of POH and treatment with plasmin on myocardial constitutive viscoelastic properties.

All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Revised 1996).

Pulmonary Artery Banding

RVPOH was induced by partially occluding the pulmonary artery with a 2.9-mm ID band, using previously described methods (47). Briefly, adult cats weighing 1.9–2.7 kg were first anesthetized with ketamine hydrochloride (15 mg/kg im), meperidine (2.2 mg/kg im), and acepromazine maleate (0.25 mg/kg im), and then intubated and placed on a respirator. A left thoracotomy was performed and a band was placed around the proximal pulmonary artery. Eight cats underwent PAB and then recovered for 4 wk. Four normal adult cats and four sham-operated cats served as controls.

Hemodynamic Studies

Four weeks after PAB or sham operation, catheterization was performed with the use of previously described methods (47). Cats were anesthetized with ketamine hydrochloride (25 mg/kg im). Right heart pressures were obtained using a fluid-filled catheter inserted through the right external jugular vein and advanced into the right atrium and ventricle. Arterial pressure was monitored using a second fluid-filled catheter, which was positioned in the proximal left common carotid artery. Each catheter was attached to a strain gauge, and the midchest position was taken as a zero reference point for pressure measurements. Arteriovenous oxygen content was used as a measure of cardiac output and was determined in duplicate by measuring simultaneous blood samples obtained from the carotid artery and the right ventricle.

Papillary Muscle Isolation

Left ventricular (LV) papillary muscles were isolated from normal rats, and RV papillary muscles were isolated from normal and PAB cats using previously described methods (47). A median sternotomy was performed, the pericardium was bluntly dissected away from the heart, and the animal was heparinized intravenously (1,000 units). A perfusion cannula was placed in the proximal aorta. The inferior and superior vena cava were ligated, the aorta was cross-clamped, and the heart was perfused antegrade with a Krebs-Henseleit “cardioplegia solution” composed of (in mM) 98.0 NaCl, 4.7 KCl, 1.2 MgSO4, 1.1 KH2PO4, 24.0 NaHCO3, 20.0 NaAc, 2.5 CaCl2, 11.2 glucose, 30.0 2,3-butanedione monoxime, and 10 U/l insulin. The cardioplegia solution, which was continuously bubbled with 95% O2-5% CO2 at room temperature and pH 7.38, was initially infused as a 50-ml bolus over a 1-min period, causing cardiac standstill, and then continuously infused at a slower rate of 5–10 ml/min while the papillary muscles were excised. During this continuous administration of the cardioplegia solution, the heart was removed and two papillary muscles were dissected free. A 6-0 silk suture was tied to the top of each papillary muscle at the junction of the chordae tendineae with the papillary muscle. The base was attached to a spring clip. Once isolated, the papillary muscles underwent the experimental treatment protocols described below. First, the isolated muscles were immediately placed in a 50-ml container and superfused with the “experimental treatment buffer,” which consisted of the cardioplegia solution described above together either with plasmin (plasmin-treated group) or without plasmin (untreated control group). Muscles remained in the experimental treatment buffer for 240 min and were bubbled continuously with 95% O2-5% CO2 (pH 7.38, 37°C). Second, after 240 min of treatment, the muscles were removed from the experimental treatment buffer and placed vertically in a 250-ml acrylic isolated muscle study chamber and superfused with Krebs-Henseleit “mechanical testing buffer” consisting of (in mM) 98.0 NaCl, 4.7 KCl, 1.2 MgSO4, 1.1 KH2PO4, 24.0 NaHCO3, 20.0 NaAc, 2.5 CaCl2, 11.2 glucose, and 10 U/l insulin, but without 2,3-butanedione monoxime or plasmin (95% O2-5% CO2, pH 7.38, 29°C) to undergo mechanical testing to characterize myocardial viscoelastic properties.

Experimental Treatment Protocols

Plasmin is a serine protease that degrades specific ECM proteins and activates endogenous pro-MMPs. Activated MMPs have the ability to cleave specific ECM proteins, including fibrillar collagen. Myocardial constitutive viscoelastic properties were assessed in both untreated muscles and after treatment with plasmin. Paired muscles were randomly assigned to the untreated control protocol versus the plasmin-treated protocol.

Plasmin treatment. After isolation, papillary muscles assigned to the plasmin-treated group were placed in a 50-ml bath and superfused with experimental treatment buffer containing a 0.64 U/ml concentration of plasmin. This bath was bubbled continuously with 95% O2-5% CO2. Initial studies to determine optimal plasmin concentration and length of treatment with plasmin were performed in normal rats. Dose and time titration studies were done from 0.16 to 0.64 U/ml and from 0 to 240 min. An example of the effects of plasmin treatment in rat papillary muscles on hydroxyproline concentration in the superfusate buffer is shown in Fig. 1. These studies showed that the amount of hydroxyproline liberated.
into the bath reached a plateau at a plasmin dose of 0.64 U/ml for 240 min. In addition, these dose and time ranges resulted in a significant effect in myocardial viscoelasticity in normal muscles. After plasmin treatment, the muscle was placed in the isolated muscle chamber and subjected to the mechanical testing protocol described below.

Untreated control. These muscles were treated in a fashion identical to that described above except that the experimental treatment buffer did not contain plasmin. Values of stiffness and viscosity in these untreated control muscles were comparable to the values obtained in previous studies.

MMP Activation

In a subset of studies (n = 3), the experimental treatment buffer from the untreated control and plasmin-treated normal rat papillary muscles were rapidly decanted and flash-frozen. These samples were subjected to substrate-specific MMP zymographic analysis, as described previously (27, 30, 37). The buffer was initially lyophilized and then reconstituted in zymographic buffer solution. Purified MMP-9 and MMP-2 standards (Calbiochem; La Jolla, CA) were included in all zymograms. Following electrophoresis and incubation, the zymograms (Fig. 2) were stained and digitally quantitated by image analysis (Gel Pro Analyzer, Media Cybernetics; Silver Spring, MD) (27, 30, 37).

Hydroxyproline Assay

Collagen degradation was quantified by measuring the amount of hydroxyproline present in the experimental treatment buffer of both untreated control and plasmin-treated normal rat papillary muscles. Hydroxyproline concentration was determined by using a modified method of Stegemann and Stalder (33). Hydroxyproline standard solutions of 0–10 μg/ml were made, and a standard curve was obtained from these solutions. Two milliliters of the superfusate were lyophilized for 12 h and then resuspended with the assay buffer. Superfusate samples were mixed with 500 μl of chloramine-T reagent and incubated at room temperature for 20 min. Aldehyde-perchloric acid solution (500 μl) was then added, and the samples were immersed in a 60°C bath for 15 min. Samples were then cooled in tap water, and the absorbency of the samples was obtained within 45 min at 550 nm. The hydroxyproline concentration of the superfusate was assessed using the curve obtained from the standard solutions.

ECM Characterization

Fibrillar collagen distribution and geometry were characterized using scanning electron microscopy, as previously described (17, 31, 32). After the myocardial viscoelastic properties were assessed using the mechanical testing protocol, muscles were held at a constant length equal the length at the peak of the active tension versus length curve (L_{max}). Then the mechanical testing buffer was removed and replaced by 2.5% gluteraldehyde fixative for 3 h. The myocardial samples were critical point dried and then freeze-fractured. The freeze-fractured samples were dehydrated and gold sputter coated (Hummer II, Technics). The sections were examined in a JOEL JSM-25S scanning electron microscope at an accelerating voltage of 15 kV. Myocardial samples were prepared in triplicate and at least 10 photomicrographs of...
the extracellular space were obtained from each sample (Fig. 3).

Papillary Muscle Servo Control System

After the experimental treatment described above, the papillary muscles were placed in the isolated muscle study chamber and superfused with the mechanical testing buffer to undergo mechanical testing to characterize myocardial viscoelastic properties. The silk suture on the upper end of the papillary muscle was attached to a dual-mode Servo Control System (model 300 B, Cambridge), and the lower clip was attached to a semiconductor strain-gauge transducer (model DSC-3, Kistler-Morse). A digital computer with an analog-to-digital interface controlled either tension or length of the preparation. Tension and length data were sampled at a rate of 1 kHz and stored for later analysis. The precision of the force and length settings was 5 mg and 2 μm, respectively. The step response of the system to an imposed length change was 95% complete in 2 ms. Equipment compliance was <1.0 μm/mN (47).

The muscle was electrically stimulated by parallel platinum electrodes delivering 5-ms pulses at a voltage 10% over threshold. Each papillary muscle was allowed to equilibrate in the isolated muscle chamber by contracting isotonically at a light, 0.5-g preload for a period of 120 min. During this preconditioning period, at 15-min intervals, the muscle was first preconditioned, reproducible state (12). The papillary muscle was determined to be at mechanical equilibrium (fully preconditioned) when values for muscle length at $L_{\text{max}}$ shortening extent during isotonic contraction, and active tension during isometric contraction reached a steady state during three consecutive measurements separated by 15-min intervals. Once mechanical equilibrium was achieved, baseline values were obtained. Three determinations of $L_{\text{max}}$ were made. A series of four uniaxial variable rate stretches were then performed.

Both before the performance of the above mechanical testing protocol and at the conclusion of each experiment, muscle length was measured while the muscle was held at a force equal to the passive tension at $L_{\text{max}}$. After muscle length was measured, the muscle was removed from the clips, blotted, and weighed. The muscle was then dried at 110°C for 24 h and weighed again. Muscle cross-sectional area was determined, assuming a uniform cross section, from muscle length at $L_{\text{max}}$, the muscle dry weight, a wet weight-to-dry weight ratio of 4:1, and a specific gravity of 1.0. Muscles with cross-sectional areas $<0.5$ or $>1.5$ mm$^2$ were excluded from further analysis. Previous studies (6, 11, 29) have clearly shown that if muscle cross-sectional area is $<1.5$ mm$^2$, there is no central core hypoxia in the isolated muscle preparation used under the conditions employed in the present study.

Measurements of Viscoelastic Properties

Myocardial viscoelastic properties were assessed by defining $L_{\text{max}}$ and then by performing uniaxial variable rate stretches. $L_{\text{max}}$ was determined by stretching the muscle at a slow rate of 0.1 mm/min to the peak of the active tension versus muscle length relationship. $L_{\text{max}}$ was defined as that resting muscle length resulting in peak active tension generation.

Uniaxial variable rate stretches were performed by increasing muscle length at 0.1 mm/min and 0.1, 1.0, and 10 mm/s, respectively. Muscles were stretched under length...
control over a range of passive tension from a minimum length at 0.2 g passive tension to a maximum length at 20% above the passive tension at $L_{\text{max}}$. An example of passive muscle stress ($\sigma$), muscle strain ($\varepsilon$), and strain rate ($\dot{\varepsilon}$) data obtained using these methods is shown in Fig. 4.

$\sigma$ was calculated from force measurements as

$$\sigma = \text{force/CSA} \quad (1)$$

where CSA is the muscle cross-sectional area. $\varepsilon$ was calculated from length measurements as

$$\varepsilon = (L_0 - L_N)/L_0 \quad (2)$$

where $L_0$ is the muscle length at 0.2 g preload and $L_N$ is the muscle length during the uniaxial stretches. $\dot{\varepsilon}$ was calculated from lengthening rate measurements as

$$\dot{\varepsilon} = 1/L_0(dL/dt) \quad (3)$$

where $dL/dt$ is the muscle lengthening rate.

The myocardial $\sigma$ versus $\varepsilon$ relationship at any $\dot{\varepsilon}$ can be affected by a number of factors which may alter the material properties of the myocardium. These factors include passive elastic stiffness and viscous damping. Changes in both of these determinants acting individually or in concert can alter the $\sigma$ versus $\varepsilon$ relationship. The experimental methods described above and the analytic model described below were used to examine the effects of POH and the effects of an acute change in cardiocyte constitutive properties on elastic stiffness and viscous damping separately.

The papillary muscle was modeled using two elements in parallel, a nonlinear spring ($e$) where

$$\sigma_e(\varepsilon) = (Ae^{Be} + B) \quad (4)$$

and a nonlinear viscous damper ($v$) where

$$\sigma_v(\varepsilon') = (C - De^{-v\varepsilon'}) \quad (5)$$

where $A-D$ are curve-fitting constants.

The total stress for this two-element parallel model is

$$\sigma(\varepsilon, \dot{\varepsilon}) = \sigma_e(\varepsilon) + \sigma_v(\varepsilon') \quad (6)$$

To calculate elastic stiffness constant ($\beta$) and viscous damping constant ($\eta$), measurements of muscle $\sigma$, $\varepsilon$, and $\dot{\varepsilon}$ were fit by a constitutive equation for a nonlinear viscoelastic composite biomaterial

$$\sigma(\varepsilon, \dot{\varepsilon}) = (Ae^{Be} + B) + (C - De^{-v\varepsilon'}) \quad (7)$$

where $\eta$ was assessed with the use of all four uniaxial variable rate stretches (Fig. 4). From these stretches, the relationship between stress and strain rate was defined at a selected, constant value of strain (Fig. 4). At any selected, constant value of strain, the relationship between stress and strain rate was curvilinear. Under these experimental conditions, strain was constant, the first part of Eq. 7 became constant, and stress became a function of strain rate alone. Therefore, the stress versus strain rate data obtained at a constant strain (0.05) from the variable rate stretches were

Fig. 4. Schematic of methods used to assess myocardial viscoelastic properties: A: stress ($\sigma$) vs. strain ($\varepsilon$) curves derived from variable rate stretches performed in a hypothetical normal (solid line) and abnormal (dashed line) papillary muscle. The vertical dotted line serves to mark a constant value of strain (5%) and indicates the stress vs. strain rate ($\varepsilon'$) points that each stretch taken from each curve to derive stress vs. strain rate relationship at constant strain shown in B. B: data from the slowest lengthening rate (0.1 mm/min) were used to derive the elastic stiffness ($\beta$) constant. These curves were fit by the first part of the 7. When elastic stiffness is increased, as in the abnormal muscle (dashed line), the stress vs. strain relationship is shifted up and $\beta$ is increased. C: stress vs. strain rate data at constant strain derived from the variable rate stretches in A. Each stress vs. strain rate curve was defined from strain rates 0.0002, 0.02, 0.2, and 2.0 s$^{-1}$, respectively, and at a constant value of strain (0.05). These curves were fit by the second part of Eq. 7 (shown in the legend). When viscosity was increased as in the abnormal muscle (dashed line), the stress vs. strain rate relationship shifted up and viscous damping constant ($\eta$) is increased. $A-D$, curve-fitting constants; $v$, viscous damper; $e$, nonlinear spring.
fit to the second part of Eq. 7, and \( \eta \) was determined. We hypothesized that if POH increased viscous damping, the \( \sigma \) versus \( \epsilon' \) relationship would shift up and \( \eta \) would increase (Fig. 4).

**Statistics**

Data are presented as the means ± SE for each data group. Differences between normal and POH groups at baseline and following plasmin treatment were determined using a multifactor analysis of variance and a Newman-Keuls multiple-comparison test and were considered significant at \( P < 0.05 \).

### RESULTS

**Hemodynamic Studies In Vivo**

The effects of PAB on in vivo measurements of pressure, oximetry, and mass are summarized in Table 1. The data for normal and PAB cats were similar to those in our previous studies (47) in that PAB caused significant increases in RV systolic pressure and mass.

### Effects of Plasmin on MMP Activation and Collagen Degradation

Representative zymograms from the experimental treatment buffer from untreated control and plasmin-treated muscles are shown in Fig. 2. In the untreated controls, there was no detectable zymographic activity in the buffer. This absence of MMP zymographic activity suggested that MMPs were not spontaneously activated and released into the buffer during this treatment period. In marked contrast, however, MMP zymographic activity contained within the 50- to 90-kDa region, consistent with proteolytic activity for MMP-9 and MMP-2, respectively (24), was significantly increased with plasmin treatment (Fig. 2). These results indicated that treatment with the serine protease plasmin caused MMP activation and release into the experimental treatment buffer superfavoring the muscle. Moreover, a greater degree of MMP zymographic activity appeared to be contained within the experimental treatment buffer from plasmin-treated muscles from the POH muscle samples compared with the normal muscles treated with plasmin (Fig. 2). Denititomeric analysis of the proteolytic region spanning 50 to 90 kDa revealed a greater lytic region contained within the POH samples compared with normal samples (922 ± 22 \times 10^3 vs. 1,049 ± 33 \times 10^3 pixels, respectively, \( P = 0.033 \)).

Activation of MMPs caused collagen degradation as evidenced biochemically by an increase in the hydroxyproline liberated into the experimental treatment buffer superfavoring the papillary muscle and morphometrically by a change in fibrillar collagen, using scanning electron microscopy (Figs. 1 and 3). Figure 1 shows that the hydroxyproline concentration in the experimental treatment buffer increased from 0.02 ± 0.01 \( \mu \)g/ml for untreated normal muscles to 0.23 ± 0.09 \( \mu \)g/ml for plasmin-treated normal muscles. Collagen degradation was further evidenced with the use of scanning electron microscopy (Fig. 3). Plasmin-treated muscles exhibited a defect in the collagen fascicular weave, which surrounds cardiac myocyte bundles.

### Effects of Plasmin on Muscle Geometry

The effect of plasmin on the viscoelastic properties of the papillary muscle must be interpreted in light of simultaneous changes in indices of muscle geometry such as muscle length, width, cross-sectional area, and wet weight-to-dry weight ratio, each of which could potentially alter viscoelasticity. Table 2 shows that plasmin treatment in normal rat, normal cat, and POH cat muscles did not alter muscle length, width, cross-sectional area, or wet weight-to-dry weight ratio.

### Effects of Plasmin on Myocardial Viscoelasticity in Normal Rat

The effects of plasmin treatment on the elastic stiffness and viscous damping in normal rat papillary muscles are shown in Fig. 5. When normal rodent papillary muscles were treated with plasmin, there was a significant decrease in the elastic stiffness constant from 22.2 ± 1.1 in the untreated papillary muscles to 16.4 ± 1.4 in the plasmin-treated papillary muscles (\( P < 0.05 \)). Plasmin caused a significant decrease in the viscous damping constant from 15.3 ± 0.8 in the untreated papillary muscles to 8.6 ± 1.5 in the plasmin-treated papillary muscles (\( P < 0.05 \)).

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**Table 1. Pulmonary artery banding model of pressure overload hypertrophy**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>POH</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV systolic pressure, mmHg</td>
<td>25 ± 2</td>
<td>55 ± 3*</td>
</tr>
<tr>
<td>RV weight/body weight, g/kg</td>
<td>0.57 ± 0.04</td>
<td>1.04 ± 0.05*</td>
</tr>
<tr>
<td>RV weight/tibial length, g/cm</td>
<td>0.12 ± 0.01</td>
<td>0.28 ± 0.02*</td>
</tr>
<tr>
<td>LV weight/body weight, g/kg</td>
<td>2.5 ± 0.2</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>2.2 ± 0.2</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>Arteriovenous O₂ difference, ml/l</td>
<td>35.4 ± 21</td>
<td>32.1 ± 1.0</td>
</tr>
<tr>
<td>RV diastolic pressure, mmHg</td>
<td>1 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Liver weight/body weight, g/kg</td>
<td>30.1 ± 1.0</td>
<td>32.8 ± 1.9</td>
</tr>
</tbody>
</table>

*Values are means ± SE; \( n = 8 \) cats in each group. RV, right ventricle; LV, left ventricle; POH, pressure overload hypertrophy. *\( P < 0.05 \) vs. control.

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**Table 2. Effect of plasmin on muscle geometry**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Plasmin Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle length, mm</td>
<td>5.3 ± 0.5</td>
<td>5.4 ± 0.5</td>
</tr>
<tr>
<td>Muscle width, mm</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Cross-sectional area, mm²</td>
<td>1.2 ± 0.2</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Wet weight/dry weight, g/g</td>
<td>4.3 ± 0.2</td>
<td>4.1 ± 0.2</td>
</tr>
</tbody>
</table>

Normal rat (\( n = 6 \))

| Muscle length, mm         | 6.2 ± 0.5 | 6.2 ± 0.5       |
| Muscle width, mm          | 1.2 ± 0.1 | 1.2 ± 0.1       |
| Cross-sectional area, mm² | 1.2 ± 0.2 | 1.2 ± 0.1       |
| Wet weight/dry weight, g/g | 4.4 ± 0.2 | 4.0 ± 0.2       |

Normal cat (\( n = 8 \))

| Muscle length, mm         | 6.4 ± 0.4 | 6.4 ± 0.6       |
| Muscle width, mm          | 1.4 ± 0.1 | 1.4 ± 0.1       |
| Cross-sectional area, mm² | 1.4 ± 0.1 | 1.4 ± 0.2       |
| Wet weight/dry weight, g/g | 4.4 ± 0.1 | 4.4 ± 0.2       |

Values are means ± SE; \( n = \) no. of cats.
Effects of Plasmin on Myocardial Viscoelasticity in Normal Cat

The effects of plasmin treatment on the elastic stiffness and viscous damping in normal cat papillary muscles are shown in Fig. 6. When normal feline papillary muscles were treated with plasmin, there was a significant decrease in the elastic stiffness constant from $22.1 \pm 1.1$ to $15.4 \pm 0.9$ in the plasmin-treated papillary muscles ($P < 0.05$). Plasmin caused a significant decrease in the viscous damping constant from $20.4 \pm 1.6$ to $15.8 \pm 1.1$ ($P < 0.05$). Plasmin treatment decreased elastic stiffness and viscosity in treated POH muscles to levels comparable to normal untreated muscles but not as low as normal feline muscles treated with plasmin.

DISCUSSION

The acute activation of endogenous MMPs contained within a ventricular papillary muscle was achieved by treatment with the serine protease plasmin. Plasmin has been postulated to be an important upstream activation step for MMP activation (2, 20, 37, 45). With the use of this approach, it was possible to cause a proteolytic disturbance of normal ECM structure and function. Plasmin treatment decreased elastic stiffness and viscosity in treated POH muscles to levels comparable to normal untreated muscles but not as low as normal feline muscles treated with plasmin.
determine whether, and to what extent, changes in ECM structure contribute to the abnormalities in myocardial viscoelastic properties produced by POH. Data from the current study support several conclusions. Treatment with plasmin caused activation of endogenous MMPs and degradation of fibrillar collagen. Plasmin decreased elastic stiffness and viscous damping in normal and POH myocardium. Whereas elastic stiffness and viscous damping decreased in plasmin-treated POH myocardium, plasmin treatment did not reduce elastic stiffness and viscous damping to levels equivalent to that seen in normal muscles treated with plasmin. These data support the following concepts: 1) ECM collagen is one determinant of elastic stiffness and viscosity, even in normal muscles, 2) factors in addition to ECM collagen contribute to POH-induced changes in myocardial viscoelastic properties, and 3) a significant portion (perhaps a predominant portion) of the increase in elastic stiffness and viscosity in POH is dependent on changes in the ECM collagen.

Previous studies (11–16) have attempted to acutely degrade fibrillar collagen and alter myocardial diastolic function. Prolonged ischemia and treatment with disulfide reagents (oxidized glutathione and 5,5′-dithio-2-ninobenzoic acid) have been used to activate endogenous proteases, which in turn degrade collagen (5, 26, 36, 38, 46). Exogenous proteases have been used directly to degrade collagen (23). The two previous studies (23, 28) examining the mechanical effects of these treatments have shown either no change in myocardial stiffness, or paradoxically, an increase in stiffness. These mechanical effects occurred in the face of histomorphometric and biochemical evidence proving collagen degradation. However, the difficulty interpreting these studies centers around the fact that these treatment regimens changed not just one determinant of the myocardial viscoelastic properties, but many. These changes include inducing myocardial edema, which can increase viscoelastic stiffness. The unique approach of the current study is that no changes occurred in muscle length, water content, or geometry, allowing the effects of a selective change in fibrillar collagen on the myocardial viscoelastic properties to be examined in isolation.

Previous studies (1, 13, 14, 35, 48) suggested that POH alters intracellular determinants of diastolic function, such as the extramyocardial cytoskeleton and calcium homeostasis. Alterations in both of these determinants have been shown to contribute to the POH-induced changes in both cellular and myocardial material properties. Acute corrections of these intracellular abnormalities in POH myocardium have resulted in partial but incomplete correction of myocardial elastic stiffness and viscous damping (13). Therefore, both the current and previous studies suggest that the abnormalities in diastolic function, which result from POH, are multifactorial. The current study suggests that a change in fibrillar collagen is one and perhaps a dominant factor.

### ECM as a Determinant of Myocardial Viscoelasticity

The myocardial ECM is composed of three important constituents: 1) fibrillar protein such as collagen type I, collagen type III, and elastin; 2) proteoglycans; and 3) basement membrane proteins, such as collagen type IV, laminin, and fibronectin. It has been hypothesized that the most important component within the ECM contributing to the development of diastolic congestive heart failure (CHF) is fibrillar collagen (3, 8, 43). Within the myocardial ECM, fibrillar collagen exists in a complex, three-dimensional geometric distribution. This distribution is divided into three layers: 1) endomysial collagen, which surrounds and connects individual cardiomyocytes, 2) perimysial collagen, which surrounds and connects bundles of cardiomyocytes, and 3) epimysial collagen, which forms the outermost layer. Because these fibrillar proteins surround and envelope individual and groups of cardiomyocytes, it

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**Fig. 7.** Effect of plasmin treatment on normal and POH cat myocardial elastic stiffness. The elastic stiffness constant $\beta$ was increased significantly in the POH muscles compared with normal muscles. Plasmin treatment caused a significant decrease in the elastic stiffness constant $\beta$ in POH and normal muscles. POH elastic stiffness was decreased to levels comparable with normal baseline measurements but not as low as normal muscles treated with plasmin.

**Fig. 8.** Effect of plasmin treatment on normal and POH cat myocardial viscous damping. The $\eta$ was increased significantly in the POH muscles compared with normal muscles. Plasmin treatment caused a significant decrease in $\eta$ in POH and normal muscles. POH viscous damping was decreased to levels comparable with normal baseline measurements but not as low as normal muscles treated with plasmin.
has been hypothesized that in normal myocardium, they connect cardiomyocytes into fascicular structures, coordinate their mechanical activity, transmit and transduce mechanical and perhaps neurohumoral input, protect against and limit cardiomyocyte strain, and coordinate and recruit preload recruitable work. In pathological myocardium, the evidence which suggests that changes in ECM fibrillar collagen play an important role in the development of diastolic dysfunction and diastolic CHF follows three lines of evidence: 1) disease processes that alter diastolic function, such as POH, also alters ECM fibrillar collagen particularly in terms of its amount, geometry, distribution, degree of cross-linking, and ratio of collagen type I versus collagen type III, 2) treatment of these disease processes, which is successful in correcting diastolic function, is associated with normalization of fibrillar collagen, and 3) experiments in which a chronic alteration in collagen metabolism is performed in normal animals results in an alteration of diastolic function (10, 15, 17, 19, 32, 34, 39–42, 49).

The best-described example demonstrating the association between an increase in myocardial stiffness and alteration in the ECM is POH clinically caused by valvular aortic stenosis and experimentally modeled as aortic banding (41). Clinical studies (41) were performed in patients with critical aortic stenosis, preserved ejection fraction, and significant increases in mass. Patients with moderate diastolic dysfunction had only moderate increases in myocardial stiffness and moderate increases in fibrillar collagen. Patients with severe diastolic dysfunction had severe increases in myocardial stiffness and severe increases in fibrillar collagen. In fact, these studies demonstrated a stepwise, direct correlation between increases in myocardial stiffness and increases in fibrillar collagen. Other clinical studies (10, 32, 34, 39, 41, 42, 49), as well as animal models, have clearly established that there is an association between increases in the ECM fibrillar collagen, increased myocardial stiffness, the development of diastolic dysfunction, and the development of diastolic CHF.

To strengthen this association, investigators (10, 42) examined whether treatment of disease processes, which result in corrections in diastolic function, were associated with a normalization of the ECM fibrillar collagen. There is increasing evidence that supports the concept that in both aortic stenosis and hypertension treatment, which is successful in causing regression of hypertrophy, will also cause significant decreases in the ECM collagen and result in normalization of diastolic function. For example, in patients with aortic stenosis, late after aortic valve replacement, once mass has regressed and ECM collagen is completely normalized, myocardial stiffness also returns to normal (42).

However, none of these experiments prove a clear, causal relationship between changes in the ECM fibrillar collagen and the development of diastolic CHF in POH because within the context of the experiments, many potential determinants of diastolic function change simultaneously. For example, in both the developmental and successful treatment of POH caused by hypertension or aortic valve stenosis, there are simultaneous changes in LV mass, LV systolic wall stress, neurohumoral activation of the renin-angiotensin-aldosterone system, and intracellular processes such as calcium homeostasis as well as changes in the ECM fibrillar collagen (10, 41, 42). Each of these changes may either individually or in concert cause normalizing myocardial diastolic function. Because all of these mechanisms change simultaneously, assigning specificity to any one mechanism for the observed changes in diastolic function remains problematic. In addition, it is likely that it is for these reasons that studies examining treatment of hypertension using drugs that alter neurohumoral activation, alter hemodynamic load, and alter cellular and extracellular determinants do not uniformly result in normalized myocardial stiffness. For example, Brilla et al. (4) treated hypertensive patients with angiotensin-converting enzyme type inhibitor, which caused a reduction in ECM collagen but did not alter myocardial stiffness.

To further support the hypothesis of a cause and effect relationship between changes in the ECM fibrillar collagen and the diastolic function, studies (15, 17, 19, 40) were performed that were able to create a chronic alteration in collagen metabolism in normal animals. For example, in one study, normal pigs were fed β-aminopropionitrile (BAPN) (17). This compound inhibits one posttranslational step in collagen metabolism, that of collagen cross-linking. Treatment of normal adult pigs with BAPN caused no significant changes in LV volume, mass, or systolic function. However, BAPN caused an interruption in the ECM collagen morphology, decreased collagen volume and percent confluence, increased non-cross-linked collagen, and significantly decreased chamber stiffness. These experiments were not designed to examine myocardial stiffness or to selectively examine elasticity versus viscosity. In addition, these studies were limited to normal animals. Nonetheless, these data suggested an association, but were not sufficient to prove a causal relationship, between changes in ECM fibrillar collagen and changes in diastolic function. The current study was designed to address some of these limitations intrinsic to these and other previous studies. However, whereas the current study overcomes some of these limitations, it did not overcome others. Plasmin-induced collagen degradation caused a similar decrease in viscoelasticity in both normal and hypertrophied muscles. This fact limits our ability to be specific about the causal role played by collagen in the “hypertrophy-associated” changes in myocardial viscoelastic properties. To prove that the hypertrophy-associated increase in viscoelasticity was caused by the increased collagen, the amount of residual collagen present in the normal and hypertrophied muscle after plasmin treatment would have to be equal. The resultant differences in viscoelasticity between normal and hypertrophied muscles would then provide evidence concerning the extent to which increased collagen caused the hypertrophy-associated increase in viscoelasticity. This out-
come was not achieved in this study. The electron microscopy studies showed that the hypertrophy muscles have a marked increase in ECM collagen at baseline, plasmin caused collagen degradation, but there was substantially more residual collagen after plasmin treatment in the hypertrophied muscle than the normal muscle. These data support the conclusion that the hypertrophy-associated changes in myocardial viscoelastic properties are multifactorial and include changes in structures and processes within the cardiomyocyte, those in the ECM (including all component proteins of the ECM), and changes in neurohumoral activation. Providing yet a higher degree of specificity for the role of collagen, however, will require further study.

**Regulatory Control of collagen**

The regulatory control of collagen biosynthesis and degradation in POH remains incompletely understood, but has at least two major determinants: transcriptional regulation by physical and chemical factors and posttranslational regulation including collagen cross-linking and degradation (2, 9, 18, 20, 45). Biosynthesis of new collagen and other ECM proteins is under the control of at least three factors: 1) load, including preload and afterload, 2) neurohumoral activation, including the renin-angiotensin-aldosterone system and sympathetic nervous system, and 3) growth factors such as insulin, cytokines, and others. Collagen degradation is under the control of a variety of proteolytic enzymes including a family of zinc-dependent enzymes, the MMPs. The balance among synthesis, cross-linking, and degradation results in the total collagen present in a given pathological state at a specific time.

The MMPs constitute a family of zinc-dependent enzymes that have been shown to contribute to the changes in ECM structure and composition that occur in both normal and abnormal tissue remodeling (2, 9, 18, 20, 45). Furthermore, studies (28, 30, 31, 37) have shown that myocardial MMPs can contribute to the changes in ECM structure that occur in cardiac disease states such as coronary heart disease and dilated cardiomyopathies. However, studies that provide a mechanistic link between changes in MMP expression and/or activity and changes in the mechanical properties of the cardiac muscle itself have been limited.

There are numerous classes and species of MMPs that have been identified within the myocardium. These MMPs are primarily classified by structure and substrate specificity. For example, the class of MMPs identified as gelatinases, such as MMP-9 and MMP-2, has a common substrate portfolio and gelatin-binding domain within the catalytic region. The most common approach to identifying MMPs in biological samples is through the use of gelatin zymography. This analytic technique can successfully identify relative levels of MMP-9 and MMP-2 in extracted samples. In the present study, the experimental treatment buffer superfusing the untreated muscles did not have any significant MMP gelatinolytic activity. This observation was not unexpected because MMPs are synthesized and released in a proenzyme form and remain bound to the ECM in an inactive state. In vitro studies (2, 45) have demonstrated that MMPs are activated through proteolytic cleavage of the propeptide domain, which can be achieved by serine proteases such as plasmin or trypsin. In the present study, plasmin treatment of papillary muscles resulted in alterations in MMP gelatinolytic activity within the superfusing experimental treatment buffer. The proform of MMP-2 is 72 kDa and the active form is ~68 kDa. The gelatin zymography revealed robust proteolytic activity at ~68 kDa in the experimental treatment buffer following treatment of the muscle with plasmin, which would be indicative of the activation and release of MMP-2.

Previous studies (27, 31, 37) have demonstrated that abundant quantities of MMP-2 exist within normal and hypertrophied myocardium. In contrast, MMP-9 is expressed to a lesser degree in normal myocardium and increases only in cardiac disease states (30, 31, 37). Thus, in the present study, the limited proteolytic activity corresponding to MMP-9 likely reflects a limited endogenous pool of this MMP species contained within these myocardial samples. However, it must be recognized that gelatin zymography is optimized to detect MMP-2 and MMP-9 and therefore the activation and release of other MMPs after plasmin treatment would not be detected by this approach. Evidence to suggest that other MMPs were activated by plasmin treatment is suggested by the hydroxyproline results. Hydroxyproline is an amino acid uniquely contained within collagen protein and the fibrillar collagen types I and III constitute an important set of structural proteins within the myocardial ECM. The primary MMPs responsible for fibrillar collagen degradation are the interstitial collagenases MMP-1 and MMP-13; both of which have been identified in myocardium (2, 20, 37, 45). Thus it is likely that a cascade of MMP activation and subsequent ECM degradation occurred with plasmin treatment of these isolated muscle preparations.

The present study used normal and POH muscle preparations to examine specific determinants of cardiac muscle behavior following plasmin treatment and putative MMP activation. A complete quantification of the endogenous MMP expression and activity in POH muscle preparations was beyond the scope of this report and the alterations in cellular and ECM structure in the POH model have been described previously (24, 25). However, two observations were made in these POH samples with respect to changes in the ECM and MMPs that warrant comment. First, the scanning electron micrographs revealed a thickened fibrillar collagen weave in the POH samples, which is consistent with this form of hypertrophy. Second, a more robust gelatinolytic activity was observed in POH muscles after plasmin treatment compared with normal muscles. This likely reflects a greater quantity of recruitable MMPs in the POH myocardium. Whereas the coexistence of an increased collagen accumulation and an increased MMP abundance in the POH myocardium.
may appear paradoxical, there are several regulatory processes that contribute to MMP activational states. First, MMPs are synthesized in an inactive state and require an exogenous biochemical trigger or physical stimulus to yield a fully active MMP. Second, a set of regulatory proteins identified as the tissue inhibitors of the MMPs (TIMPs) exist within the myocardium, which will rapidly and tightly bind to active MMPs and interrupt proteolytic activity. The development of a pressure overload has been shown to cause a shift in the stoichiometry between MMPs and TIMPs, which would favor reduced MMP proteolytic activity and an increased collagen accumulation (27). The results of the present study suggested that the development of POH was associated with a significant increase in the amount of recruitable MMPs within the ECM.

There are, however, several questions that remain unanswered. First, does POH change other ECM proteins other than collagen? Second, which of the ECM components, fibrillar proteins, proteoglycans, or basement membrane proteins, play a predominant role in diastolic CHF? Third, what are the regulatory mechanisms that control synthesis and degradation of ECM structures in POH? Fourth, what changes in MMP and TIMP expression, abundance and activity occur in POH? Finally, can modulation of these regulatory control mechanisms prevent or reverse diastolic CHF caused by POH?

To prove what role each MMP species and each MMP target protein contributes to changes in the viscoelastic properties produced by POH, additional studies will be required. This is especially important in the context of the current study because of the use of plasmin to activate endogenous MMPs. Plasmin does not directly degrade collagen but can directly alter other ECM proteins. In addition, plasmin does not activate a single MMP species but many. Finally, some MMPs once activated will then activate other MMPs. Therefore, whereas it is clear that collagen was degraded in the current study, determining whether and to what extent other ECM proteins were altered, which specific MMPs were involved in these changes, and whether and how these changes affect myocardial viscoelastic properties was beyond the scope of the current study. This kind of specificity may be possible either by treating with selective recombinant MMPs or by adding selective MMP inhibitors to plasmin treatment. However, the first step in such experimental designs was taken in the current study, providing proof of the concept that activation of endogenous MMPs and degradation of collagen results in the normalization of viscoelastic properties.

In conclusion, treatment with plasmin caused activation of endogenous MMPs and degradation of fibrillar collagen. Plasmin decreased elastic stiffness and viscous damping in normal and POH myocardium. Whereas elastic stiffness and viscous damping decreased in plasmin-treated POH myocardium, plasmin treatment did not reduce elastic stiffness and viscous damping to levels equivalent to that seen in normal muscles treated with plasmin. Thus, while many mechanisms may contribute to the abnormalities in myocardial viscoelasticity in the POH myocardium, changes in fibrillar collagen appear to play a predominant role.

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