Time-Dependent Changes in Matrix Metalloproteinase Activity and Expression During the Progression of Congestive Heart Failure

Relation to Ventricular and Myocyte Function

Francis G. Spinale, Mytsi L. Coker, Chadwick V. Thomas, Jennifer D. Walker, Rupak Mukherjee, Latha Hebbar

Abstract—The development of congestive heart failure (CHF) is associated with left ventricular (LV) dilation and myocardial remodeling. However, fundamental mechanisms that contribute to this remodeling process with the progression of CHF remain unclear. The matrix metalloproteinases (MMPs) have been demonstrated to play a significant role in tissue remodeling in a number of pathological processes. The present project tested the hypothesis that the LV dilation and remodeling during the progression of CHF is associated with early changes in MMP expression and zymographic activity. LV and myocyte function, collagen content, and MMP expression and zymographic activity were serially measured during the progression of CHF caused by pacing-induced supraventricular tachycardia (SVT) in pigs. After 7 days of SVT, LV end-diastolic dimension and myocyte length both increased by 15% from control values, and LV fractional shortening fell by 20%. At the level of the myocyte, percent shortening fell by 16% after 7 days of SVT, with no change in the steady-state velocity of shortening. Longer durations of SVT caused progressive LV dilation, LV pump failure, and myocyte contractile dysfunction. Specifically, 21 days of SVT resulted in a >50% increase in LV dimension, a 56% fall in LV fractional shortening, and a 33% decline in myocyte velocity of shortening. The decline in LV and myocyte function with 21 days of SVT was accompanied by signs and symptoms of CHF. Thus, SVT causes time-dependent changes in LV geometry and function and the subsequent development of CHF. LV myocardial collagen content and confluence fell by 25% after 7 days of SVT and were accompanied by an 80% increase in LV myocardial MMP zymographic activity against the substrate gelatin. After 14 days of SVT, total LV myocardial collagen content was reduced by 24%, and LV myocardial MMP zymographic activity increased by >100% from control values. Interstitial collagenase (MMP-1), stromelysin (MMP-3), and 72-kD gelatinase (MMP-2) were increased by ≈2-fold after 7 days of SVT. LV MMP zymographic activity and abundance remained elevated with longer durations of SVT. The results of the present study demonstrated that in this model of CHF, early changes in LV myocardial MMP zymographic activity and protein levels occurred with the initiation and progression of LV dilation and dysfunction. These findings suggest that an early contributory mechanism for the initiation of LV remodeling that occurred in this model of developing CHF is enhanced expression and potentially increased activity of LV myocardial MMPs. (Circ Res. 1998;82:482-495.)

Key Words: heart failure ■ metalloproteinase ■ myocardial remodeling

A n important event in the progression to CHF is LV dilation and subsequent pump dysfunction.1–3 These past clinical observations suggest that LV remodeling is an important initiating event in the transition to severe CHF. However, the cellular and molecular mechanisms that play a direct contributory role in the initiation and progression of changes in LV geometry and function during the development of the CHF process are unknown. Chronic pacing-induced tachycardia in animals causes well-defined, predictable, and progressive LV dilation, contractile dysfunction, and neurohormonal system activation.4–15 These functional and neurohormonal changes are similar to the clinical spectrum of CHF.1–3,16,17 Therefore, this chronic pacing model may provide an opportunity to identify the early and contributory events responsible for the progression of LV dilation and dysfunction that occurs with CHF. It has been demonstrated that the structure and composition of the fibrillar collagen matrix, which provides structural integrity of adjoining myocytes,18–21 are significantly altered in pacing-induced CHF.7,12–15,22 Furthermore, we have recently reported that concomitant ACE inhibition with chronic tachycardia preserved myocardial collagen content and reduced the LV dilation associated with this disease process.9 These past studies provide indirect evidence that changes in the myocardial collagen matrix contribute to

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From the Division of Cardiothoracic Surgery, Medical University of South Carolina, Charleston, SC.
Correspondence to Francis G. Spinale, MD, PhD, Division of Cardiothoracic Surgery, Room 418 CSB, 171 Ashley Ave, Medical University of South Carolina, Charleston, SC 29425.
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the LV dilation and subsequent dysfunction that occur in this rapid-pacing model of CHF. Accordingly, the overall goal of this project was to define the time-dependent structural and molecular events that occur within the extracellular matrix during the progression of CHF.

The MMPs constitute an important enzyme system that has been demonstrated to contribute to the tissue remodeling process. The MMPs are a class of zinc-dependent enzymes that have a high specificity for components of the extracellular matrix. Increased expression and activity of MMPs have been identified in pathological processes such as tumor metastases and rheumatoid arthritis. Increased MMP expression and activity have been identified in atherosclerotic lesions and have been implicated in atheroma formation and plaque rupture. Increased myocardial MMP activity has also been reported with the development of severe CHF, such as in cardiomyopathic disease. However, whether increased MMP zymographic activity and abundance are early events in the evolution of the CHF process remains unexplored. Accordingly, the present study was designed to determine the relationship of time-dependent changes in MMP expression and zymographic activity to LV and myocyte function and geometry during the progression of CHF.

Materials and Methods
The present project used a model of chronic SVT in pigs that has been shown clearly in previous studies to produce signs and symptoms of CHF after 3 weeks of SVT. LV geometry and function, neurohormonal system activity, myocyte contractility, myocardial collagen content and structure, and MMP expression and activity were measured with each week of SVT.

Model of Pacing CHF and Experimental Design
Thirty Yorkshire pigs (20 to 25 kg, male) were chronically instrumented in order to measure LV function and arterial blood pressure while they were conscious. The pigs were sedated with diazepam (20 mg PO, Valium, Hoffmann-La Roche) and placed in a custom-designed sling that allowed the animal to rest comfortably. The pacemaker was deactivated (SVT groups only). All studies were performed after a 30-minute acclimation period with the animals in the conscious state and without additional use of sedation. The vascular access port was entered using a 12-gauge Huber needle (Access Technologies), and basal resting arterial pressure and heart rate were recorded. Pressures from the fluid-filled aortic catheter were obtained using an externally calibrated transducer (Statham P23ID, Gould). Two-dimensional and M-mode echocardiographic studies (ATL Ultramark 7, 3.5-MHz transducer) were used to image the LV from a right parasternal approach. Echocardiographic data were measured with the use of American Society of Echocardiography criteria, including the leading-edge convention. The two-dimensional parasternal long-axis view of the LV was first recorded in order to precisely define the LV long axis and papillary muscles. A perpendicular view with respect to the LV long axis was then obtained in order to obtain the two-dimensional parasternal short-axis view. LV short-axis two-dimensional and M-mode echocardiographic recordings were then obtained. The LV dimensions were performed from the septum to the posterior LV free wall with the cursor directed between the papillary muscles. LV end-diastolic and end-systolic dimension, LV end-systolic and end-diastolic wall thickness, and fractional shortening were computed from the two-dimensional targeted M-mode recordings. Peak circumferential global average wall stress was computed using a spherical model of reference with the relationship: $\sigma (\text{g/cm}^2) = \text{PD} \times \text{D} \times 1.36$, where $P$ is aortic systolic pressure, $\text{D}$ is minor-axis dimension at end diastole, and $h$ is wall thickness. After steady-state LV function measurements, 35 mL of blood was drawn from the arterial access port into chilled tubes containing EDTA (1.5 mg/mL). The blood samples were immediately centrifuged (2000g, 10 min, 4°C), and the plasma was decanted into separate tubes, frozen in a dry ice/methanol bath, and stored at −80°C until the time of assay.

LV fractional shortening was measured after incremental increases in LV myocardial wall stress in five pigs in the normal state and with each week of SVT. Simultaneous recordings of two-dimensional targeted M-mode echocardiograms and aortic pressure were obtained by a phenylephrine infusion started at a rate of 1.3 mg·kg⁻¹·min⁻¹ and increased in such a manner as to obtain three to six isochronal LV peak circumferential wall stress versus shortening points for each pig with each week of SVT. This approach for measuring the LV shortening-stress relation has been described previously by this laboratory and others. Determination of the LV shortening-afterload relation was chosen, since it provides a relative index of LV contractile performance and does not require theoretical muscle models and development of LV pressure-volume loops. However, this approach may not completely describe in vivo LV myocardial contractile performance; accordingly, the LV systolic stiffness constant was determined using methods described previously. Briefly, isochronal LV wall stress-strain values were obtained under ambient conditions and after phenylephrine infusion. The LV myocardial stiffness constant $k$, was determined from the following exponential relationship: $\sigma = C_0 e^{\sigma/k}$, where $\sigma$ is wall stress and $ln(1/h)$ is the natural logarithm of the reciprocal of LV wall thickness. This in vivo index of LV contractile performance was thus obtained.
myocardial performance has been shown previously to be unaffected by changes in LV loading conditions and volumes.41,42

Terminal Study: Myocardial Sampling and Myocyte Isolation
After the final set of LV function measurements and plasma collection, the animals were anesthetized as described in the preceding section, a sternotomy was performed, and the heart was quickly excised and placed in a phosphate-buffered ice slush. The great vessels, atria, and right ventricle were carefully trimmed away, and the LV was weighed. The region of the LV free wall incorporating the circumflex artery (5×5 cm) was excised and prepared for myocyte isolation. The region of the LV free wall composing the left anterior descending artery (3×3 cm) was cannulated and prepared for perfusion fixation. The posterior region of the LV free wall (3×3 cm) was snap-frozen in liquid nitrogen for subsequent biochemical analysis of collagen content and MMP zymographic activity and expression.

Myocytes were isolated from the LV free wall using methods described by this laboratory previously.5-7 Briefly, the left circumflex coronary artery was perfused with a collagenase solution (0.5 mg/mL; Worthington, type II; 146 U/mg) for 35 minutes. The tissue was then minced into 2-mm sections and gently agitated. After 15 minutes, the supernatant was removed and filtered, and the cells were allowed to settle. The myocyte pellet was then resuspended in DMEM/F-12 (GIBCO Laboratories). The viability of the isolated myocytes was confirmed at ×100 magnification using a hemocytometer (Reichert-Jung, Cambridge Instruments Inc) and resuspended to a final concentration of (5×10^7) cells/mL. Viable myocytes were defined as those cells that maintained a rod shape and were quiescent in culture. By use of this myocyte isolation method, a high yield (85±5%) of viable myocytes was obtained in all LV preparations used in the present study. Viable myocytes were defined as those cells that retained a rod shape, were calcium tolerant, responded to electrical stimulation, and excluded trypan blue.

Neurohormonal Measurements
The plasma samples were assayed for renin activity, endothelin concentration, and catecholamine levels. Plasma renin activity was determined by computing angiotensin I production using a radioimmunoassay for angiotensin I (BioChem, Inc). The number of viable myocytes was counted with a hemocytometer (Reichert-Jung, Cam-484 Metalloproteinases and Heart Failure

LV Myocardial Function
Isolated myocyte function was examined as previously reported by this laboratory.5-7 Briefly, a thermostatically controlled chamber (37°C) containing a volume of 2.5 mL and two stimulating platinum electrodes was used to image the isolated myocytes on an inverted microscope (Axioskop IM35, Zeiss Inc). A ×20 long-working-distance Hoffmann modulation contrast objective (Modulation Optics Inc) was used to image the myocytes. Myocyte contractions were elicited by field-stimulating the tissue chamber at 1 Hz (S11 stimula-

LV Zymographic MMP Activity
After a stringent washing in ice-cold saline, the LV myocardial samples were homogenized (three 30-second bursts) in 5 mL of an ice-cold extraction buffer (1:3 wt/vol) containing cacodylic acid (10 mmol/L), NaCl (0.15 mol/L), ZnCl (20 mmol/L), NaHCO (5 mmol/L), and 0.01% Triton X-100 (pH 5.0). The maintenance of a low pH and temperature prevented proteolytic activation during the extraction process. The homogenate was then centrifuged (4°C, 10 minutes,
1200g), and the supernatant was decanted and saved on ice. The pellet was resuspended in extraction buffer, and the procedure was repeated in triplicate. The samples were then raised to a pH of 7.6 using Tris buffer and concentrated using an Amicon B-15 concentrator (Amicon Inc) at 4°C. Final protein concentration of the myocardial extracts was determined using a standard colorimetric assay (Bio-Rad protein assay). These extracts were aliquoted, immediately flash-frozen using liquid nitrogen, and stored at −80°C until the time of assay.

The myocardial extracts were directly loaded onto electrophoretic gels (SDS-PAGE) containing gelatin (0.5 mg/mL, Sigma Chemical Co.), 7.3–314.46 A homogeneous impregnation of this MMP substrate into the gels was facilitated by constant stirring and heating to 45°C before casting. The myocardial extracts at a final protein content of 4 μg were loaded onto the gels using a 3:1 sample buffer (10% SDS, 4% sucrose, 0.25 mol/L Tris-Cl, and 0.1% bromophenol blue, pH 6.8). The gels were run at 15 mA/gel through the stacking phase (4%) and at 20 mA/gel for the separating phase (10%), maintaining a running buffer temperature of 4°C. After SDS-PAGE, the gels were washed twice in 2.5% Triton X-100 for 30 minutes each, rinsed twice in PBS, and incubated for 3 hours in a substrate buffer at 37°C (50 mmol/L Tris-Cl, 5 mmol/L CaCl2, 0.5% Brij-35, and 0.02% NaN3, pH 8). After incubation, the gels were stained using 0.1% amido black, destained in water, digitized, and analyzed as described in the following paragraph. In order to provide a means of comparison with respect to the zymographic activity obtained from the present study, samples were collected from the cell culture medium of the human fibrosarcoma HT 1080 cell line (American Type Culture Collection) as described previously.45–46 Briefly, HT 1080 cells were grown to confluence in DMEM with 10% fetal calf serum and then incubated for 24 hours in serum-free medium containing 1 mmol/L insulin and 5 mg/L transferrin. After which, the cell cultures were incubated for 24 hours in the presence and absence of 100 ng/mL of PMA (Sigma).45 Treatment of this cell culture system with a phorbol ester has been demonstrated previously to increase MMP zymographic activity.28,45 After this incubation period, the cell culture medium was drawn off, and the extracts were isolated by immunoblotting studies. The LV myocardial extracts were purified from the conditioned media of rheumatoid synovial fibroblasts. The primary antisera were diluted in 0.2 mol/L Tris-base and 0.1% Tween 20, 0.08% BSA, 13% DMEM/F-12 cell culture medium (GIBCO Life Technologies), and 0.02% NaN3. After a stringent washing, the membranes were incubated for 1 hour in horseradish peroxidase–conjugated goat anti–mouse antibody (1:5000 dilution, Bio-Rad Laboratories). The membranes were washed again, and the horseradish peroxidase–conjugated secondary antibody was activated with peracrid and luminol (ECL Western blotting detection reagents, Amersham Life Science). The luminescent signal was detected by exposure to x-ray film (Eastman Kodak Co) for exactly 5 minutes. Positive controls for MMP-2 and -3 were included in all immunoblots and were obtained from human epithelial and fibroblast cell lines (AG771 and AG770, respectively, Chemicon International Inc). Cell culture medium from a PMA-stimulated HT 1080 fibrosarcoma cell line4,45 was also used as a positive control for immunoblotting. Prestained molecular weight markers (Bio-Rad Laboratories) were used to ensure adequate protein separation and transfer. The intensity of the signal was analyzed as described in the previous paragraph and normalized to control values.

**Data Analysis**

Indices of LV and myocyte function, collagen structure and composition, and MMP zymographic activity and expression were compared with each week of SVT using multivariate ANOVA. If the ANOVA revealed significant differences, pairwise tests of individual group means were compared using Bonferroni probabilities. The LV shortening-stress data obtained at each week of pacing were fit to a polynomial regression model. Comparisons of the coefficients obtained from the LV shortening-stress relation were compared using the t distribution. For comparisons of neuronormonal profiles, the Student-Newman-Keuls test was used. With respect to the myocyte function data, each pig was considered a complete block. Thus, the numbers of myocytes studied from each pig were considered repeated observations within each block. The summary statistics include the number of myocytes studied from each group, however, all statistical comparisons were performed on a per pig (block) basis. The categorical scores obtained from the scanning electron micrographs were compared between groups using χ² analysis. All statistical procedures were performed using the BMJP statistical software package (BMJP Statistical Software Inc). Results are presented as mean ±SEM. Values of P<.05 were considered to be statistically significant.

**Results**

All of the pigs that were assigned to undergo chronic SVT successfully completed the protocol. The animals that underwent 3 weeks of chronic SVT exhibited signs and symptoms of CHF, which included ascites, tachypnea, and hepatomegaly.

**LV Function and Neurohormonal Profiles With the Progression of SVT-Induced CHF**

Weekly changes in LV function with SVT are summarized in Table 1. All measurements were performed with the pace-
maker deactivated and with the animals in the conscious state. After 7 days of chronic SVT, LV end-diastolic dimension increased, wall thickness was reduced, and LV peak systolic wall stress was increased. These changes in LV geometry after 7 days of SVT were associated with a decline in LV fractional shortening. After 14 days of SVT, the basal resting heart rate was increased from control levels. After 14 days of SVT, LV end-diastolic dimension and peak wall stress were significantly increased, and wall thickness was significantly reduced from both control and 7-day SVT values. The changes in LV geometry and wall stress that occurred after 14 days of SVT were associated with a significant decline in LV fractional shortening compared with control and 7-day SVT values. After 21 days of SVT, a further increase in LV end-diastolic dimension and wall stress was observed, along with a continued decline in LV fractional shortening. After 21 days of SVT, mean aortic pressure was significantly reduced. LV mass did not significantly change at any time point with chronic SVT. Thus, time-dependent changes in LV pump function and geometry occurred during the development of SVT-induced CHF.

In order to more carefully examine LV myocardial performance, the LV ejection-afterload relation was serially measured under normal conditions and with each week of SVT, and the results from these studies are shown in Fig 1. In normal conscious pigs, the LV fractional shortening fell in a proportional manner with increased LV wall stress, and this curvilinear relation is consistent with the force-velocity relation obtained in human and animal subjects.36–40 Since this relation was curvilinear, the isochronal LV fractional shortening–wall stress points were subjected to polynomial regression. The results from the regression analysis with each week of SVT are shown in Table 2. After 7 days of SVT, the isochronal LV fractional shortening–wall stress points fell below the control curvilinear relation, but the regression coefficients were not different from control values. However, with longer durations of SVT, this relation shifted down and to the right, with a significant change in the regression coefficients computed from this relationship. In addition to the LV shortening-stress relation, LV myocardial performance was also examined through computing the LV systolic stiffness constant with each week of SVT (Fig 2). After 7 days of SVT, the LV systolic stiffness constant was similar to control values. However, after 2 and 3 weeks of SVT, the LV systolic stiffness constant was significantly lower than control values. Thus, using either the

| TABLE 1. Serial Changes in LV Function and Neurohormonal Profiles With Chronic SVT |
|---------------------------------------------|------------------|----------------|----------------|----------------|
| LV function                                | Nonpaced Control | 7 Days         | 14 Days        | 21 Days        |
| Heart rate, bpm                            | 114±2            | 140±4          | 159±4*†        | 171±4*†        |
| End-diastolic dimension, cm                | 3.3±0.1          | 3.9±0.1*       | 4.4±0.2†       | 5.3±0.1†       |
| Fractional shortening, %                   | 44±2             | 35±2*          | 25±2†          | 14±2†          |
| LV ED wall thickness, mm                   | 7.9±0.1          | 7.3±0.3*       | 6.3±0.4†       | 4.9±0.2†       |
| Mean aortic pressure, mm Hg                | 94±2             | 93±2           | 91±2           | 77±4†          |
| LV peak stress, g/cm²                      | 64±7             | 103±9*         | 162±17†        | 279±27†        |
| LV/BW, g/kg                                | 3.2±0.2          | 3.0±0.1        | 3.2±0.1        | 3.1±0.2        |
| Plasma neurohormones                       |                  |                |                |                |
| Norepinephrine, pg/mL                      | 327±107          | 1240±522*      | 1864±460†      | 3281±574†      |
| Endothelin, fmol/mL                        | 2.6±0.2          | 5.9±1.5        | 7.9±0.7*       | 10.1±1.3†      |
| Renin, ng·mL⁻¹·h⁻¹                         | 3.7±0.5          | 6.6±1.4        | 8.2±1.6*       | 16.2±1.3†      |
| Sample size, n                              | 12               | 12             | 12             | 12             |

LVED indicates LV end-diastolic; LV/BW, ratio of LV mass to body weight. Values are mean±SEM. *P<.05 vs nonpaced control; †P<.05 vs SVT 7-day value.

Figure 1. The LV ejection-afterload relation was serially measured under normal conditions and with each week of SVT through a graded phenylephrine infusion.39 In normal conscious pigs (control), LV fractional shortening fell in a proportional manner with increased LV wall stress, and this curvilinear relation is consistent with past reports.36–40 The isochronal LV fractional shortening–wall stress points were subjected to polynomial regression, and the results from this analysis are summarized in Table 2. The polynomial regression line (solid line) was plotted for each week of SVT as well as the 95% confidence interval for the control state (dashed lines). After 1 week of SVT, the isochronal LV fractional shortening–wall stress points fell within the normal confidence interval, and the regression coefficients were not different from control values. However, with longer durations of SVT, this relation shifted down and to the right, with a significant change in the regression coefficients computed from this relationship. These findings suggest that a significant fall in LV contractile performance occurred after 2 weeks of SVT.
stress-shortening relation or the systolic stiffness constant as indices of LV myocardial performance, a significant and time-dependent fall in LV myocardial function was observed during the progression of SVT-induced CHF.

In light of the fact that activation of several neurohumoral systems commonly occurs during the development of CHF, weekly changes in neurohormonal profiles with each week of SVT were determined and are summarized in Table 1. Plasma norepinephrine increased by 3-fold from control values after 7 days of SVT and increased another 2-fold from these elevated values after 21 days of SVT. Plasma endothelin levels significantly increased after 14 days of SVT and increased further after 21 days of SVT. Similarly, plasma renin activity significantly increased by over 120% after 14 days of SVT and by over 250% after 21 days of SVT compared with control values. Thus, consistent with clinical forms of severe CHF, the development and progression of SVT-induced CHF was associated with increased neurohumoral system activity.

**LV Myocyte Geometry and Contractile Performance With Progression of SVT-Induced CHF**

Since LV dilation occurred in the absence of hypertrophy with the development of SVT-induced CHF, significant myocardial remodeling must have occurred. Accordingly, isolated LV myocyte geometry was examined with each week of SVT. Myocyte cross-sectional area was determined from >700 myocyte profiles from each group, and this analysis resulted in an approximate gaussian distribution for this parameter. After 7 days of chronic SVT, myocyte cross-sectional area was unchanged from control values (356±4 versus 363±5 μm², P>0.70). However, after 14 and 21 days of SVT, myocyte cross-sectional area significantly decreased (305±4 and 282±4 μm², respectively) from control values (P<0.05).

Steady-state myocyte contractile function for controls and after each week of SVT are shown in Table 3. Resting myocyte length was increased after 7 days of SVT and significantly increased from this value after 21 days of SVT. After 7 days of SVT, myocyte steady-state percent shortening was reduced from control values, but shortening velocity was unchanged. After 14 and 21 days of SVT, steady-state myocyte contractile performance was significantly reduced from both control and 7-day SVT values. Specifically, after 14 days of SVT, steady-state percentage and velocity of shortening fell by 25% from control values. After 21 days of chronic SVT, myocyte percentage and velocity of shortening was reduced by over 30% from control values. Myocyte contractile function was also examined after β-adrenergic receptor stimulation with the nonselective β-receptor agonist isoproterenol. The results from this portion of the study are summarized in Table 3. Myocyte β-adrenergic responsiveness was reduced after 7 days of SVT. The reduced myocyte β-adrenergic response continued to deteriorate with longer durations of SVT. For example, compared with control values, myocyte velocity of shortening was 15% lower after 7 days of SVT and 28% lower after 14 days of SVT and was reduced by >50% after 21 days of SVT. Thus, the progression of SVT-induced CHF was accompanied by significant changes in isolated LV myocyte geometry, contractility, and inotropic responsiveness.

**LV Myocardial Collagen With Progression of SVT-Induced CHF**

Fibrillar collagen structure and composition were examined during the development of SVT-induced CHF, and the results from this analysis are summarized in Fig 3. Morphometric analysis of picrosirius-stained LV myocardial sections revealed a significant reduction in the confluence, or connectivity, of the collagen matrix after 7 days of SVT. The confluent nature of the collagen weave continued to decline with longer durations of SVT. Representative scanning electron micrographs taken of control myocardium and of myocardial samples taken after 7, 14, and 21 days of SVT are shown in Fig 4.
TABLE 3. Serial Changes in Isolated Myocyte Contractile Performance With Chronic SVT

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Isoproterenol (25 nmol/L)</th>
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<tr>
<td><strong>Resting length, μm</strong></td>
<td></td>
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<tr>
<td>Nonpaced control</td>
<td>137±2</td>
<td>135±3</td>
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<tr>
<td>SVT</td>
<td></td>
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<tr>
<td>7 Days</td>
<td>160±8*</td>
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<tr>
<td>14 Days</td>
<td>169±2*</td>
<td>162±2†</td>
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<tr>
<td>21 Days</td>
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<td>173±6†</td>
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<td><strong>Percent shortening, %</strong></td>
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<td>4.4±0.2</td>
<td>10.6±0.8††</td>
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<tr>
<td>SVT</td>
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<tr>
<td>7 Days</td>
<td>3.7±0.1*</td>
<td>7.9±0.3†</td>
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<td>14 Days</td>
<td>2.6±0.2†</td>
<td>6.0±0.6†††</td>
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<td>21 Days</td>
<td>2.3±0.2†</td>
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<td><strong>Shortening velocity, μm/s</strong></td>
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<tr>
<td>Nonpaced control</td>
<td>48.2±2.9</td>
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<tr>
<td>SVT</td>
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<tr>
<td>7 Days</td>
<td>45.9±3.2</td>
<td>145.2±12.7‡†</td>
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<td>14 Days</td>
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<td><strong>Relengthening velocity, μm/s</strong></td>
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<tr>
<td>SVT</td>
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<td>7 Days</td>
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<td>21 Days</td>
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</table>

The number of myocytes studied in each group is shown for reference purposes only. Statistical analysis was performed with each animal serving as the treatment block (n=6 pigs in each group). Values are mean±SEM. *P<.05 vs nonpaced control; †P<.05 vs SVT 7-day value; and ‡P<.05 vs baseline.

In order to more carefully quantify biochemical changes in LV myocardial collagen, analysis of hydroxyproline was performed on LV midmyocardial samples and converted to collagen values (Fig 3). LV myocardial collagen content fell in a time-dependent fashion with SVT, and a significant fall from control values was observed after 7 days of SVT. Therefore, the significant LV dilation that occurred during the progression of SVT-induced CHF was paralleled by changes in LV myocardial collagen structure and content.

LV Myocardial Zymographic Activity and MMP Abundance With Progression of SVT-Induced CHF

In order to examine whether changes in LV myocardial collagen degradative processes occurred during the progression of SVT-induced CHF, MMP zymographic activity and relative abundance were examined from LV myocardial extracts with each week of SVT. A representative zymogram from control LV myocardium and after each week of SVT using gelatin as a proteolytic substrate is shown in Fig 5. In control LV myocardium, zymographic activity could be identified between the 70- and 50-kD region on the basis of calibrated molecular weight markers, which were included in each zymogram. After 7 days of SVT, zymographic activity appeared increased from time 0 control values and remained elevated with longer durations of SVT. The LV myocardial gelatin zymograms were subjected to densitometric analysis in order to determine total proteolytic activity. LV myocardial MMP gelatinolytic activity increased after 7 days of SVT compared with control values (67±5 versus 36±12 pixels, P<.05) and remained increased from control values at 14 days (74±6 pixels, P<.05) and 21 days (63±5 pixels, P<.05) of SVT. In order to provide an internal reference, cell culture medium (2 μg total protein) from HT 1080 cells incubated for 24 hours in the and absence and presence of 100 ng/mL of PMA were included in all zymograms (Fig 5). Incubation with PMA significantly increased zymographic activity in the HT 1080 cell culture medium and is consistent with past reports. From the PMA-treated HT 1080 cell culture...
experiments, a more rapidly migrating band that likely represents the activated form of MMP-2 was observed. In an additional series of studies, MMP zymographic activity was examined in the presence of 10 mmol/L EDTA or 2 mmol/L of PMSF. Incubation with EDTA inhibited all zymographic activity, consistent with past reports (data not shown); however, in the presence of 2 mmol/L PMSF, a serine proteinase inhibitor, zymographic activity was unchanged, consistent with MMP activity.

In order to determine whether the increased MMP zymographic activity observed during the progression of SVT-induced CHF was accompanied by an absolute increase in MMP abundance, immunoblotting was performed for interstitial collagenase (MMP-1), the 72-kD gelatinase (MMP-2), and stromelysin (MMP-3). A representative immunoblot with each week of SVT is shown in Fig 6. In all LV myocardial samples, a distinct immunoreactive band could be localized to the appropriate molecular weight that corresponded to the specific MMP of interest. The internal controls included in each immunoblotting procedure resulted in a strong immunoreactive signal consistent with the molecular weight for these specific MMP species.

After 7 days of SVT, the immunoreactive signals for MMP-1, MMP-2, and MMP-3 were increased from control levels. With longer durations of SVT, the relative abundance of MMP-1 appeared to increase in a time-dependent manner. Densitometry of the immunoblots was performed, and the results were normalized to control values (Fig 7). After 7 days of SVT, the abundance of MMP-1 increased by 150 ± 15% from control values and by 360 ± 30% after 21 days of SVT. The relative abundance of MMP-2 and MMP-3 increased by 2-fold after 7 days of SVT and appeared to plateau with longer durations of SVT. Thus, increased MMP zymographic activity was demonstrable in LV myocardial extracts during the progression of SVT-induced CHF and was accompanied by a relative increase in the abundance of several MMP species.

Discussion

Structural remodeling of the LV and subsequent changes in LV geometry are common events in the progression to CHF. However, fundamental mechanisms that contribute to the LV remodeling and the temporal relationship that contributes to changes in myocyte contractile performance with developing CHF remain unclear. The LV fibrillar collagen matrix ensures structural integrity of adjoining myocytes, provides the means by which myocyte shortening is translated into overall LV pump function, and has been postulated to be essential for maintaining...
alignment of myofibrils within the myocyte through a collagen-integrin-cytoskeletal-myofibril relation. A number of past reports have demonstrated that abnormalities in LV fibrillar collagen structure and composition occur with changes in LV geometry and function. The MMPs selectively degrade extracellular proteins such as the fibrillar collagens and have been implicated in directly contributing to tissue remodeling in a number of pathological processes. The present study was designed in order to test the hypothesis that an early event in the progression to CHF is LV remodeling and increased MMP zymographic activity. Accordingly, the present study measured time-dependent changes in LV geometry, myocyte contractility, myocardial collagen content, and MMP zymographic activity and expression during the progression of SVT-induced CHF. The significant and unique finding of the present study was that a time course of events in the progression of SVT-induced CHF is LV dilation and myocyte lengthening, alterations in LV myocardial collagen structure, and significantly increased MMP zymographic activity and abundance. These observations suggest that early and dynamic changes in collagen degradative pathways occur within the LV myocardial interstitium during the progression of CHF.

**LV and Myocyte Function During Progression of SVT-Induced CHF**

This study examined time-dependent changes in LV geometry and myocyte contractile processes during the development of SVT-induced CHF. After 1 week of SVT, significant LV dilation and increased LV peak wall stress accompanied by a fall in LV fractional shortening occurred. The diminished LV pump function that occurred early with SVT may be due to differences in loading conditions, chamber geometry, contractile performance, or a combination of these determinants. Accordingly, indices of LV myocardial performance and isolated myocyte contractile function were serially examined during the progression of SVT-induced CHF. After 1 week of SVT, the LV ejection-afterload relation and the LV systolic stiffness constant were not significantly reduced from control values. Using these relatively load-independent indices of LV myocardial function, the results from the present study suggest that the reduction in LV pump function that occurred after 1 week of chronic SVT may have not been solely due to changes in myocardial contractile performance. However, after 1 week of SVT, plasma catecholamines were increased by 4-fold from control values and would suggest that significantly increased LV myocardial sympathetic activation had occurred. Thus, the in vivo measurements of LV myocardial function that were obtained in control conditions and after 1 week of SVT were

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**Figure 6.** Immunoblotting was performed on LV myocardial extracts for interstitial collagenase (MMP-1), the 72-kD gelatinase (MMP-3), and stromelysin (MMP-3) taken under the control condition (time 0) and with each week of SVT. A positive immunoreactive band that corresponds to the proenzyme form of MMP-1 was observed at the 52/57-kD region in LV myocardial extracts. A time-dependent increase in MMP-1 abundance was observed after 7 and 14 days of SVT, with a strong signal observed after 3 weeks of SVT. In addition to substitution of nonimmune anti-sera, which abolished all staining, cell culture medium from a PMA-stimulated HT 1080 fibrosarcoma cell line was used as a positive control for immunoblotting and revealed an immunoreactive band corresponding to MMP-1. Middle, Immunoblotting for MMP-2 revealed a robust increase in this MMP species after 7 days of SVT, which remained elevated for longer durations of SVT. After 7 days of SVT, a positive immunoreactive band for MMP-2 was observed at a lower molecular weight, which likely reflects an intermediate form of this MMP species. In addition to substitution of nonimmune sera that abolished all staining, a positive control for MMP-3 (AG770, Chemicon International Inc) was included in all immunoblotting procedures. A positive immunoreactive signal was obtained in these positive control samples at the 72-kD region that corresponds to MMP-2. Bottom, After 7 days of SVT, an increase in the density of the immunoreactive band corresponding to MMP-3 was readily observed. This immunoreactive signal for MMP-3 remained elevated with longer durations of SVT. A positive control for MMP-3 (+CON) was included in all immunoblots and was obtained from an human epithelial cell line (AG771, Chemicon). A strong immunoreactive signal was detected using this positive control at the 59/57-kD region that corresponds to MMP-3.

**Figure 7.** The relative abundance of specific MMPs was serially quantified in LV myocardial extracts with SVT. The abundance of interstitial collagenase, or MMP-1, in LV myocardial samples increased significantly from control values (time 0) after 7 days of chronic SVT (P < .05) and increased further with longer periods of chronic SVT (P < .05). The relative abundance of the 72-kD gelatinase, MMP-2, increased significantly after 7 days of SVT (P < .05) and appeared to plateau with longer durations of chronic SVT. The abundance stromelysin, or MMP-3, in LV myocardial samples increased significantly from control (time 0) values after 7 days of chronic SVT (P < .05) and appeared to plateau with longer durations chronic SVT. Time 0 represents control values.

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**Figure 7 for METALLOPROTEINASES AND HEART FAILURE**

The relative abundance of specific MMPs was serially quantified in LV myocardial extracts with SVT. The abundance of interstitial collagenase, or MMP-1, in LV myocardial samples increased significantly from control values (time 0) after 7 days of chronic SVT (P < .05) and increased further with longer periods of chronic SVT (P < .05). The relative abundance of the 72-kD gelatinase, MMP-2, increased significantly after 7 days of SVT (P < .05) and appeared to plateau with longer durations of chronic SVT. The abundance stromelysin, or MMP-3, in LV myocardial samples increased significantly from control (time 0) values after 7 days of chronic SVT (P < .05) and appeared to plateau with longer durations chronic SVT. Time 0 represents control values.
likely performed under different inotropic states. As a result, inherent defects in LV myocardial contractile function that may have occurred early in the progression of SVT-induced CHF would have been difficult to detect. Accordingly, the present study examined LV isolated myocyte contractile function after each week of SVT. Through this approach, differences in external loading conditions and neurohormonal influences that occurred during the progression of SVT-induced CHF were removed. After 1 week of SVT, isolated LV myocyte length was increased and paralleled the LV dilation that had occurred. The increased LV myocyte length after 1 week of SVT was accompanied by a significant reduction in myocyte percent shortening. This reduction in myocyte percent shortening was similar to the relative reduction in LV fractional shortening that occurred after 1 week of SVT. Although myocyte percent shortening was reduced after 1 week of SVT, myocyte velocity of shortening, which reflects the rate of actin-myosin crossbridge formation, was not changed from control values. Taken together, these findings would suggest that contributory mechanisms for the reduction in LV pump function that occurred early in the progression of SVT-induced CHF include changes in both LV and myocyte geometry and shortening characteristics. However, after 2 weeks of SVT, the continued LV dilation and reduction in LV pump function were paralleled by diminished capacity of the LV myocardium to generate contractile force. Furthermore, these changes in LV geometry and function after 2 weeks of SVT were associated with diminished steady-state myocyte contractile function. Consistent with past reports, 260 bpm) was not associated with reduced indices of LV contractile function with more prolonged durations of chronic pacing in dogs. In their study, rapid atrial pacing caused signs and symptoms consistent with severe CHF. For example, defects in Ca2+-adrenergic receptor stimulation. Myocyte β-adrenergic responsiveness was significantly reduced after 1 week of SVT. In the present study and consistent with past reports, chronic rapid pacing causes an early and sustained increase in plasma catecholamines.9,10,56 The early increase in plasma catecholamines with chronic rapid pacing has been reported to cause defects in β-adrenergic–mediated phosphorylation and transduction.56,57 Thus, an early defect in the progression of pacing-induced CHF appears to be diminished β-receptor transduction and myocyte inotropic responsiveness. After 2 weeks of SVT, steady-state myocyte function was significantly reduced. Abnormalities in a number of processes responsible for myocyte excitation–contraction have been identified with the development of pacing-induced CHF. For example, defects in Ca2+ homeostatic mechanisms have been identified to occur with the development of tachycardia-induced CHF.58,59 Taken together, these past reports suggest that a number of cellular and intracellular processes likely contribute to the progression of SVT-induced CHF. Thus, the present study demonstrated that early events in the progression of this CHF process include LV and myocyte remodeling and inherent defects in the capacity of the myocyte to respond to an inotropic stimulus.

LV Collagen Matrix Remodeling During Progression of SVT-Induced CHF

In the present study, early changes in fibrillar collagen structure were observed to occur with chronic SVT and paralleled changes in LV geometry. The early fall in LV pump performance with SVT was accompanied by LV dilation and wall thinning and by myocyte lengthening. A contributory factor in these changes in LV and myocyte geometry may have been a loss of myocardial fibrillar collagen support. Furthermore, the changes in the myocardial fibrillar collagen matrix that were observed early in the development of SVT-induced CHF may have contributed to a loss in the coordination between myocyte contractile performance and an effective LV ejection. The collagen matrix has been proposed to provide the support essential for maintaining alignment of myofibrils within the myocyte as well as for maintaining myocyte alignment within the LV free wall.18–22 This laboratory and others have demonstrated previously that significant alterations in extracellular myocyte support and basement membrane adhesion capacity occur with pacing-induced CHF.7,12–15,22 The loss of fibrillar...
collagen tethering of the myocyte that occurred during the development of SVT-induced CHF could potentially result in myocyte lengthening, LV wall thinning, and dilation. In the present study, the first time point chosen for LV myocardial collagen and MMP studies was after 1 week of SVT. This time point was selected since global changes in LV geometry and pump function had been clearly documented to occur after this period of chronic rapid pacing.26,49 However, Weber et al14 have reported changes in LV myocardial collagen structure after 24 hours of pacing tachycardia in dogs. In light of the findings from the present study in which changes in LV collagen content and structure were temporally related to the onset of LV dilation and pump dysfunction, future studies examining LV myocardial collagen structure and MMP activity at earlier time points in the progression of this CHF process would be appropriate.

This laboratory has demonstrated previously that concomitant ACE inhibition with chronic rapid pacing reduced the degree of LV dilation and improved LV myocardial collagen structure compared with that of untreated animals undergoing chronic rapid pacing.49 Thus, the reduced LV dilation that was observed with concomitant ACE inhibition during rapid pacing may have been due, at least in part, to a preservation of myocardial collagen-mediated extracellular support. Cleavage of fibrillar collagen molecules by MMPs occurs at specific peptide lengths and sequences.25,29 The remaining collagen fragments would not be reflected in the total LV myocardial hydroxyproline pool but would be evident on structural analysis. Thus, the present study coupled LV myocardial hydroxyproline measurements with quantitative histomorphometry in order to determine LV fibrillar collagen structure as well as total abundance. In the present study, early LV dilatation was paralleled by changes in both LV myocardial structure and hydroxyproline content. These findings suggest that changes in fibrillar collagen support is an early contributory mechanism responsible for the LV dilation and diminished pump function with chronic SVT. However, the present study did not address whether possible changes in collagen phenotypes or stability occurred during the development of SVT-induced CHF. It has been clearly demonstrated that alterations in myocardial collagen phenotype and cross-linking can occur in different cardiac pathologies,34,60 which would influence steady-state myocardial collagen content. Thus, appropriate future studies would include examination of potential changes in collagen synthetic pathways, both transcriptional and post-translational, which occur during the development of SVT-induced CHF.

LV MMPs During Progression of SVT-Induced CHF

An important determinant of collagen degradation is through the activation of the MMPs, which have high selectivity and affinity for components of the extracellular matrix.23–30 MMPs are secreted in a proenzyme form and require proteolytic cleavage for activation.23,26,27,29,32,53 Studies have provided evidence that an important MMP activation process occurs through a proteolytic cascade that can be initiated by serine proteases.25–30,52,53 One approach for measuring relative MMP activity in tissue extracts is through the use of zymographic assays.26–28,31,33,34,45–47,50–53 A significant and sustained increase in MMP zymographic activity against the proteolytic substrate gelatin was observed early in the progression of SVT-induced CHF. Through the use of in vitro assay systems, several past reports have provided evidence to support the concept that increased MMP activity may contribute to the development of LV remodeling.19,32–34,61 After 3 hours of coronary occlusion in the rat, a 2-fold increase in LV collagen protease activity occurred and was associated with a loss in the fibrillar collagen weave and transmural LV wall thinning.51 Increased MMP zymographic activity has been reported to occur as a function of age in the Syrian cardiomyopathic hamster model.25 More recently, MMP zymographic activity has been demonstrated to be significantly increased in human end-stage cardiomyopathic disease.31 The present study builds on these past reports by demonstrating that a potential contributory mechanism for the LV myocardial remodeling that occurs during the progression of a CHF process may be due to increased MMP activity.

There are a number of species of MMPs that have different specificities to the fibrillar collagens.23–30 In the present study, proteolytic banding patterns were observed on the zymograms, suggesting that several species of MMPs likely contributed to the LV myocardial collagen remodeling during the progression of SVT-induced CHF. However, the proteolytic patterns observed with gelatin zymography may not necessarily reflect different species of MMPs, and quantification of MMP species based on zymographic activity can be problematic.29,30,31 For example, Atkinson et al51 demonstrated that in type IV collagen film assays, MMP-9 and MMP-2 migrated to molecular weights that differed from the predicted molecular weight on the basis of primary sequence data. Accordingly, the present study used immunoblotting techniques in order to examine whether the increase in LV myocardial zymographic activity during the progression of SVT-induced CHF was associated with changes in the relative abundance of specific MMPs. The results from this portion of the study demonstrated that after 1 week of SVT, the relative abundance of several species of MMPs was increased. Specifically, a significant increase in LV myocardial content of interstitial collagenase (MMP-1), the 72-kD gelatinase (MMP-2), and stromelysin (MMP-3) occurred after 1 week of chronic SVT and was temporally related to the development of LV dilation and reduced myocardial collagen content.

Although increased MMP-2 abundance was observed in LV myocardial extracts during the progression of SVT-induced CHF, LV myocardial zymographic activity, which would correspond to the activated form of this species of MMP, did not appear increased. There are several problematic issues surrounding the in vitro zymographic assays performed in the present study that prevent direct extrapolation to in vivo LV myocardial MMP activity. First, it is likely that only a relatively small proportion of total myocardial MMPs is active at any one point in time. Second, the zymographic assays were performed under optimal enzymatic conditions and substrate availability. Third, an important control point of MMP activity is the TIMPs.23,24,29,30,45,62,65 These TIMPs form tight complexes with MMPs and therefore play an important role in overall MMP enzymatic activity. The MMP assays performed in the present study could not address whether potential changes in TIMP
abundance and/or the stoichiometric relation to specific MMPs may have occurred during the development of SVT-induced CHF. Moreover, in the absence of activation, the overall increase in MMP abundance that was observed to occur in this model of LV dilation and dysfunction may not necessarily result in increased LV myocardial MMP activity. In light of the findings of the present study in which increased MMP zymographic activity was observed to occur early in the progression of this CHF process, future studies focusing on the determinants that regulate MMP activity in vivo would be appropriate.

Using immunoblotting techniques, the present study demonstrated that a significant increase in MMP-3 abundance occurred early in the progression of SVT-induced CHF. The early increase in MMP-3 abundance has particular relevance with respect to collagen degradation and MMP activation states. MMP-3 has the widest range of substrates and includes all of the fibrillar collagens as well as components of the basement membrane. MMP-3 can activate other MMPs as well as proenzyme and intermediate forms of MMPs (autoactivation). Thus, the increased abundance of MMP-3 that was observed to occur early during the progression of SVT-induced CHF may have had two important consequences. First, early LV myocardial MMP-3 activity with chronic SVT would result in the cleavage of fibrillar collagens with subsequent disruption of the collagen weave surrounding myocytes. Second, the early increase in MMP-3 abundance within the LV myocardium that was observed to occur during the progression of SVT-induced CHF may have induced the proteolytic activation of other MMPs within the LV myocardium.

Chronic pacing-induced tachycardia in animals causes well-defined, predictable, and progressive LV dilation, contractile dysfunction, and neurohormonal activation. Although the etiology of clinical CHF is diverse, a common end point is LV remodeling and pump dysfunction. The SVT model was chosen for the present study since it provides a reliable and practical means for identifying early and contributory events responsible for the LV remodeling and progression of LV dilation and dysfunction that occur with severe CHF. However, there are inherent differences in this specific model of SVT with respect to past studies, which have employed rapid ventricular pacing to induce the CHF phenotype. For example, ventricular pacing in dogs has been reported previously to result in reduced indices of LV contractile function, such as peak rate of LV pressure development, after 1 week of chronic rapid pacing. However, a heterogeneous pattern of LV contractile performance has been reported after 1 week of ventricular pacing in dogs. In a study by Scott et al, differences in the time-dependent changes in LV geometry were reported in which rapid pacing was induced from the atrium versus the ventricle. The present study used SVT, which preserved normal ventricular activation patterns and provided for a homogeneous LV myocardial contraction. Thus, the temporal differences in the onset of LV contractile dysfunction during the progression of pacing-induced CHF that were observed in past reports and the present study were likely due to changes in myocardial activation sequences, ejection patterns, and filling characteristics that occur with rapid ventricular pacing. Although this rapid pacing model may serve as a useful tool for the elucidation of the mechanisms of CHF, it must be recognized that any animal model will not fully represent the complex clinical spectrum of CHF. Specifically, the changes in LV myocardial structure that occur with pacing-induced CHF are not similar to the clinical forms of CHF that are due to chronic ischemia or hypertensive disease. Thus, extrapolation of the findings from this project to clinical forms of CHF should be done with caution. Gunja-Smith et al recently reported that in human idiopathic cardiomyopathic disease, collagen cross-linking was reduced by 50% and MMP zymographic activity was increased by 30-fold. This laboratory has demonstrated previously that SVT-induced CHF was associated with a similar reduction in collagen cross-linking. Therefore, this model of SVT could provide fundamental temporal and mechanistic information on MMP activity and expression in the remodeling myocardium. The findings of the present study provide direct evidence that robust and early changes in LV myocardial MMPs occur in the progression of CHF and provide a potential novel pharmacological target for modulating LV structure and geometry in this pathological process.

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Time-Dependent Changes in Matrix Metalloproteinase Activity and Expression During the Progression of Congestive Heart Failure: Relation to Ventricular and Myocyte Function

Francis G. Spinale, Mytsi L. Coker, Chadwick V. Thomas, Jennifer D. Walker, Rupak Mukherjee and Latha Hebbar

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