Abnormal cardiac wall motion and early matrix metalloproteinase activity

Running Head: García et al., Cardiac wall motion and MMP activity

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Abstract

Activation of matrix metalloproteinases (MMPs) in the heart is known to facilitate cardiac remodeling and progression to failure. We hypothesized that regional dyskinetic wall motion of the left ventricle would stimulate activation of MMPs. Abnormal wall motion at a target site on the anterior lateral wall of the left ventricle was induced by pacing atrial and ventricular sites of five open-chest anesthetized dogs. Changes in shortening at the left ventricular (LV) pacing site and at a remote site at the anterior base of the left ventricle were monitored with piezoelectric crystals. Simultaneous atrial and ventricular pacing resulted in abnormal motion at the LV pacing site yielding early shortening and late systolic lengthening, while the shortening pattern at the remote site remained unaffected. Assessment of global myocardial MMP activity showed a seven-fold increase in substrate cleavage (p<0.02) at the LV pacing site relative to the remote site. Gelatin zymography revealed increases in 92 kDa MMP-9 activity and 86 kDa MMP-9 activity at the LV pacing site relative to the remote site, whereas MMP-2 activity was unaffected. Abnormal wall motion was associated with increases in collagen degradation (~2 fold; p<0.03), plasmin activity (~1.5 fold; p<0.05), nitrotyrosine levels (~20 fold; p=0.05), and inflammatory infiltrate (~2 fold; p<0.02) relative to the remote site. Results indicate that regional dyskinesis induced by epicardial activation is sufficient to stimulate significant MMP activity in the heart, suggesting that abnormal wall motion is a stimulus for MMP activation.

Key Words: dyskinesis, stretch, remodeling, collagen, metalloproteinases
Activation of matrix metalloproteinases (MMPs) is known to contribute to the degradation of extracellular matrix (ECM), myocardial wall thinning, and left ventricular (LV) dilatation (23). MMPs are a family of enzymes that mediate ECM breakdown and are thought to be involved in the development of pathological LV remodeling (29). Despite the prevalence of reports detailing the activities of MMPs in cardiovascular disease, the exact mechanism(s) by which these enzymes are activated remains unclear.

Activation of MMPs can occur in the setting of myocardial ischemia/infarction (9). Ischemic insult to the myocardium gives rise to increased levels of oxygen radicals, inflammation, and abnormal motion of the dysfunctional myocardium by passive systolic stretch (i.e., dyskinesia) (1, 10, 11). Activation of MMPs by oxygen radicals has been implicated in myocardial contractile dysfunction (10) and activation of MMPs by inflammatory infiltrates is linked to the development of ventricular remodeling (1). Serine proteases such as plasmin have also been suggested as important activators of MMPs during post-infarction ECM remodeling (5).

In vitro studies using cardiac fibroblasts (27) and vascular smooth muscle cells (13) have shown that cyclic stretching stimulates increases in MMP activity. However, nothing is known about the effects of abnormal LV wall motion on in vivo activation of MMPs. In the present study, epicardial ventricular pacing was used to induce abnormal wall motion. Epicardial ventricular pacing is associated with late systolic lengthening (i.e. dyskinesis). Unlike dyskinesia induced by ischemia, this dyskinesia is induced in the absence of myocardial under-perfusion. The data provided herein demonstrate that short-term (< 4h) dyskinesia is sufficient to selectively stimulate high levels of MMP-9 activity at the pacing site. Furthermore, we
provide evidence for possible roles for plasmin, protein nitrosylation, and inflammatory infiltrates as potential activators of myocardial MMPs.
Materials and Methods

Animal Preparation. Animal studies were performed by guidelines described by the AAALAC, and protocols were approved by the UCSD Animal Subjects Committee. Nine mongrel dogs (eight male; one female) weighing between 15 and 28 kg were sedated with thiopental (25-50 mg/kg IV). Subjects were ventilated using a Harvard respirator, anesthetized by mask with 5% isofluorane, and maintained with 1-2% isofluorane for the rest of the study. The femoral veins were catheterized and used as infusion lines. The heart was exposed via a medial sternotomy and supported in a pericardial cradle. Arterial pressure was measured with a fluid-filled catheter placed into the aortic arch via the brachiocephalic or subclavian artery and LV pressure was measured with a second matched fluid-filled catheter introduced through the apex of the heart. One pair of sonomicrometer crystals was placed near the center of the anterior LV wall (LV pacing site, LVP), and a second pair was placed near the base of the anterior wall (remote site, LVR; Figure 1). Crystals were inserted into the midwall of the myocardium, perpendicular to the longitudinal axis of the heart. Epicardial pacing electrodes were sutured to the left or right atrium and a second set was sutured between the crystal pair at the LVP site (Figure 1). Arterial pressure, LV pressure, rate of change of LV pressure (LV dP/dt), sonomicrometer segment lengths, and electrical activity were recorded.

Six dogs were subjected to electrical pacing of the heart. In one animal, post hoc review showed that the left ventricle was not captured after 60 minutes of atrial-ventricular pacing: this animal was excluded from analyses. Three dogs served as shams that were instrumented but not paced. Zetabradine (0.5 mg/kg) was administered to dogs with heart rates >110 beats per minute (bpm) to capture the ventricle at 100–140 bpm. Control measurements were obtained during atrial electrical stimulation. Induction of dyskinesis at the LV site was accomplished by a
second stimulus 0–80 ms after the atrial stimulus directed to LV electrodes. Dyskinesis was verified by the contraction pattern observed at the LVP site (Figure 2B). Regional dyskinesis was maintained for 3.5–4 hours. The ventricular stimulator was turned off and a final reading of physiological parameters with atrial pacing was taken. Animals were euthanized with pentobarbital (200 mg/kg), hearts excised, tissue removed and frozen on dry ice, and stored at -80°C.

MMP and Plasmin Activity. MMP activity was measured using Omni-MMP fluorogenic substrate (P126, BIOMOL Research Inc.). Heart samples were taken from the right ventricle and from paced and non-paced regions of the left ventricle adjacent to the crystal insertion sites: tissues at the crystal insertion sites were not used. Tissue (~50 mg) was homogenized in ice-cold buffer (50 mM Tris pH 7.8, 150 mM NaCl, 5mM CaCl₂, 0.2 mM NaN₃). Samples containing 50 µg protein and 10 µM of substrate were mixed in buffer. Selected samples were supplemented with the MMP inhibitor phenanthroline (1 mM), or the serine protease inhibitor aprotinin (50 kallikrein inhibitory units/ml). Kinetic fluorescence measurements were performed using a microplate reader (excitation 340 nm/emission 405 nm) at room temperature. Fluorescence emission was normalized to moles of cleaved peptide using the analogous unquenched fluorescent peptide of known concentration (P127, BIOMOL Research Inc.). Substrate cleavage rates were determined from the linear regions of the kinetic curves. Data are reported as percent ratios of rate of substrate cleavage with respect to the remote site.

Plasmin activity was measured with N-(p-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt, a plasmin-specific chromogenic substrate (T6140,Sigma-Aldrich). Tissues (~50 mg) were
homogenized in 50 mM Tris pH 7.7, 150 mM NaCl, 2.5 mM, 6-amino-n-hexanoic acid. Samples (100 µg) were incubated with substrate (700 µM) and activity was measured by absorbance (405 nm) at room temperature. Selected samples were supplemented with the serine protease inhibitor aprotinin (50 kallikrein inhibitory units/ml).

**Gelatin Zymography.** Heart samples (~50 mg) were homogenized in 10 mM HEPES pH 7.5, 150 mM NaCl, 0.2 mM EDTA, 25% glycerol, 100 µg/ml phenylmethylsulfonyl fluoride, aprotinin 0.2 kallikrein inhibitory units/ml. Samples (10 µg protein) were analyzed by SDS-PAGE as described (30). MMP-2/-9 protein standards were from Chemicon.

**Immunoblotting.** Protein levels were as assessed by immunoblotting (28) on polyvinylidene difluoride membranes using an anti-MMP-9 antibody (PC309, Calbiochem).

**Myeloperoxidase Activity.** The MPO assay was carried out as previously described (20) with modifications. Tissue was homogenized in ice-cold buffer (50 mM KH$_2$PO$_4$, 0.5% hexadecyltrimethylammonium bromide, pH 6.0) as described above. Homogenates were incubated on ice for 30 minutes, centrifuged at 4 °C and the supernatant was removed. Substrate solution (2 mg/ml tetramethylbenzidine dissolved in dimethyl sulfoxide) was diluted 1:10 in reaction buffer (50 mM KH$_2$PO$_4$, pH 6.0, supplemented with 4 µl of 30 % hydrogen peroxide per 10 ml). Homogenates were diluted 1:10 in reaction buffer (100 µl final volume) and placed into a 96-well microplate. Substrate solution (100 µl) was added to the wells and kinetic absorbance measurements of myeloperoxidase activity were immediately monitored at 655 nm (readings every 40 seconds for about 20 minutes). Substrate cleavage rates were determined from the
linear regions of the kinetic curves. Data were normalized to protein concentrations determined using the BCA Protein Assay Kit (Pierce Inc., Rockford, IL).

**Nitrotyrosine Analyses.** Tissue samples were homogenized as described above and gelatinases were affinity purified with gelatin-agarose resin (Sigma). Briefly, gelatin-agarose resin was washed with 10 bed volumes of buffer (50 mM Tris-HCl, pH 7.5, 400 mM NaCl, 10 mM CaCl$_2$). Supernatants from homogenized samples were incubated with resin for 30 minutes, washed with 4 bed volumes of buffer and dissociated from resin with 2% dimethylsulfoxide (21). Samples were analyzed for nitrotyrosine by slot blot (3) and western blot analyses using a mouse anti-nitrotyrosine antibody (189542, Cayman Chemicals).

**Hydroxyproline Analysis.** Hydroxyproline experiments were performed by the method of Edwards and O’Brien (8), with minor modifications. Transmural tissue samples, typically 300 mg wet weight, were trimmed of their inner and outer 1 mm layers, placed into 20 ml uncapped glass scintillation vials, and dried at 50 °C for about 6-8 hours. The dry weight of each sample was measured, 6 ml of 6M HCl was added to each sample, and the vials were sealed with plastic caps. Samples were hydrolyzed for 36 hours at 80 °C with occasional mixing. The vials were uncapped and the liquid was allowed to evaporate overnight at 80 °C. The dried hydrolyzate film was suspended in 2 ml of de-ionized water and stored at –20 °C. Hydroxyproline standards (8) were prepared in assay buffer (172 mM citric acid, 139 mM glacial acetic acid, 975 mM sodium acetate, 570 mM sodium hydroxide, 0.1 % toluene, 20% isopropanol, pH 6.5). About 10 µl of hydrolyzate was mixed with 190 µl of assay buffer, 100 µl of a hydroxyproline standard, and 150 µl of freshly prepared chloramine-T reagent (0.141g chloramine-trihydrate in 10 ml de-ionized water) in plastic 1
ml micro-centrifuge tubes. The samples were incubated at room temperature for 20 - 25 minutes. Ehrlich’s reagent (1 M p-dimethylaminobenzaldehyde, 60 % isopropanol, 18.2 % perchloric acid) was prepared within 5 minutes of use, 150 µl was added to each sample, and the tubes were incubated at 60 ºC for 15 minutes. The absorbance of each sample was read at 550 nm and concentrations of hydroxyproline were determined from a standard addition curve (8). Collagen content was calculated using the assumption that the average hydroxyproline content of collagen is 10 %, and thus 1 µmol of hydroxyproline is equivalent to 1 mg of collagen (25). Collagen content in each tissue sample was expressed as the percentage of the total dry weight of sample.

**Collagen Dansylation.** Myocardial collagen was purified (22) and labeled with the fluorescent molecule dansyl (“dansylation”) (12). Purified collagen was suspended in 0.5 M sodium bicarbonate. A volume of 0.5 ml of dansyl chloride (20 mg/ml in acetone) was added to collagen samples and incubated for 18 hours in the dark at room temperature. Dansylated collagen was pelleted by centrifugation, supernatants were removed, and pellets were washed with three volumes of acetone. Samples were mixed in buffer (10 mM Tris, pH 7.5, 2% Triton X-100) and dansyl fluorescence was measured using an excitation wavelength of 360 nm and monitored at 516 nm. Fluorescence emission was normalized to collagen content as determined by hydroxyproline analyses and expressed as fluorescence emission/mg collagen (4).

**Data Analyses and Statistics.** Standardized measurements at end diastole (ED) were taken at the time of the initial rise in +dP/dt and LV pressure, and measurements at end systole (ES) were taken at the time of the dicrotic notch from arterial pressure. Sonomicrometer segment lengths at LVP and LVR sites were measured at times corresponding to ED and ES. Zymographs and
immunoblots were analyzed by densitometry. Bands of MMP-9 from tissue samples were normalized to the intensity of an internal MMP-2/9 standard (Chemicon). Statistical analyses were performed with either a Student’s $t$-test or repeated-measures ANOVA. Results were considered statistically significant at $p<0.05$. All data are expressed as mean ± SEM.
Results

Hemodynamics and Abnormal Wall Motion

Averaged hemodynamics measurements are shown in Table 1. Comparisons of data measured during atrial pacing and after starting atrial-ventricular (AV) pacing did not reveal significant changes in heart rate, LV systolic pressure, or LV end-diastolic pressure. By contrast, peak $+dP/dt$ was significantly depressed upon initiation of AV pacing ($p<0.05$, paired t-test). No significant changes occurred in LV pressure, heart rate, or peak $(+/-) dP/dt$ of shams (data not shown).

As shown in Figure 2, atrial pacing produced a normal phasic pattern of segment shortening during ejection and lengthening during diastole at both sites (Figure 2A and 2B, top panels). AV pacing, however, yielded dyskinetic segment motion at the LVP site (Figure 2B bottom panel). As shown in Figure 2B, ventricular activation resulted in early regional shortening at the LV pacing site just prior to end diastole (denoted by “*”) and was followed by early segment lengthening (or bulging) at the onset of systole (denoted by “†”) followed by a second segment lengthening at end systole. The observed pattern of early ventricular activation followed by systolic segment lengthening has been observed in other comparable experimental ventricular pacing preparations (2) (19). Figure 2C shows that at the remote site, myocardial segments shorten (-9%) with atrial pacing. Upon AV pacing, the degree of shortening changes from -9% to -5%. Recovery to approximately -9% shortening occurred upon return to atrial pacing at the end of the study period (Figure 2C). By contrast, the LVP site initially shows normal shortening with atrial pacing (-11%) but becomes positive upon AV pacing reflecting segment lengthening. Recovery to approximately -10% shortening occurs upon return to atrial pacing, demonstrating that the effects of AV pacing at the site of abnormal wall motion are
reversible. Myocardial segment shortening in shams (from –9% to -13% at mock LVP site, and
–10% to -14% at LVR site) did not exhibit significant changes from initial to final measurements
(p>0.5, paired t-test).

**MMP and Plasmin Activity**

Representative gelatin zymography analysis of MMP activity is shown in Figure 3A.
The LVR site shows a moderate level of MMP-9 and MMP-2 activity of similar intensity whose
molecular weights correspond to the pro forms of each enzyme. At the LVP site, there is a
robust level of activity of 92 kDa MMP-9 that is ~forty-fold greater than MMP-9 activity levels
observed at the remote site. A band directly below the 92 kDa MMP-9 band is visible in the
LVP lane having a molecular weight of approximately ~86 kDa, which is consistent with the
active form of MMP-9. The 86 kDa MMP-9 activity at the LVP site is ~ten-fold greater than 92
kDa MMP-9 activity levels at the LVR site (p<0.01), and ~one hundred fold greater than 86 kDa
MMP-9 levels at the LVR site (p<0.001). The percent ratio of 86 kDa MMP-9 to 92 kDa MMP-9
is approximately 27%, as determined by densitometry. Ratios of 86 kDa to 92 kDa MMP-9
measured from zymograms developed at different time points ensured that zymographic activity
was in the linear range. MMP activity from the right ventricle (RV) is of moderate intensity and
resembles basal activity levels found at the LVR site. No significant differences in MMP-2
between LVR and LVP were measured (p>0.2). Average MMP-9 activity for all pacing subjects
is summarized in Figure 3B.

A representative kinetic plot (Figure 4A inset) shows the progress of substrate cleavage
with samples obtained from LVP, LVR, and RV sites. These data reveal that MMP activity at
the LVP site is ~seven-fold greater than activity levels at the LVR site, and ~eleven-fold greater
than activity levels at the right ventricle. To establish that substrate cleavage was specifically mediated by MMPs, and not by other endogenous proteases within the tissue, parallel reactions were conducted with the MMP inhibitor phenanthroline. Phenanthroline completely inhibits cleavage of substrate (Figure 4A inset). Fluorescence data obtained with the omni-MMP substrate were converted to moles of cleaved fluorescent product. The bar graph in Figure 4A shows the ratios of percent substrate cleavage normalized to LVR regions in paced and sham animals. MMP activity from the LVP region shows a seven-fold higher activity (p<0.02) than shams (LVP/LVR, 564±122% vs. mock LVP/LVR, 85±5%). Relative activity levels in the LVP region were also significantly higher (P<0.04) than the RV of paced and sham subjects (143±53 vs. 86±16%).

Proteolytic activation of MMPs has been correlated with increased activity of serine proteases such as plasmin (5). Figure 4B shows comparisons of plasmin activity between LVP and mock LVP sites, relative to LVR regions. Data reveal that plasmin activity from the LVP site of paced dogs is ~1.5-fold greater than activity from the mock LVP site of shams (LVP 164±25 vs. mock LVP 115±9%; p<0.05).

**Immunoblotting for MMP-9**

Representative immunoblots against MMP-9 are shown in Figure 5A with values for all subjects in Figure 5B. MMP-9 protein levels from the LVP site are ~1.5-fold greater than the remote site (LVP:400±22; LVR:273±30 arbitrary units; p<0.04). No statistical difference was observed between mock LVP sites and LVR sites of shams (mock LVP:286±9; LVR:298±11 arbitrary units; p>0.3).
Collagen Cleavage Assessment via Amino-Terminal Dansylation

Figure 6 shows the extent of dansyl labeling (“dansylation”) of purified collagen samples. The highest level of fluorescence emission occurs with collagen from the LVP site (24,113±4,094 RFU/mg), while lower levels are found in the LVR site and in both sites of shams (Figure 6: 11,454–12,834 RFU/mg). Fluorescence data were normalized to collagen levels measured by hydroxyproline analyses [LVP 3.8±0.7 vs. LVR 3.0±0.4% collagen (dry weight); p>0.2]. Dansyl fluorescence increased two-fold at the LVP site relative to the other regions (p<0.03) indicating increased collagen degradation.

Myeloperoxidase Activity

LV homogenates were analyzed for inflammatory infiltrates by measuring myeloperoxidase (MPO) activity (18, 20). MPO activity from the LVP site shows a two-fold increase in activity relative to the LVR region (LVR 16.1±1.6 vs. LVP 33.1±6.1 abs.units x10^-3/min; p<0.02; Figure 7), whereas MPO activities in tissue from shams were comparable (LVR 21.8±5.2 vs. mock LVP 23.8±8.4 abs.units x10^-3/min).

Nitrotyrosine Levels

Tissue homogenates were enriched for gelatinases by affinity purification with gelatin-agarose (21). Purified samples were analyzed by slot blot immunostaining for nitrotyrosine, as protein nitrosylation is known to activate latent MMPs (14). Figure 8A shows comparisons of nitrotyrosine levels between LVP and LVR sites from a single animal with averaged values for all paced dogs in Figure 8B. Nitrotyrosine levels at the LVP site shows an average twenty-fold increase in nitrotyrosine relative to the LVR site (LVP:125±53; LVR:6±1 arbitrary units;
p=0.05). Western analyses against nitrotyrosine reveal bands that approximate 92 kDa and 86 kDa MMP-9 (Fig. 8C). Reblotting against MMP-9 verified that the amounts of MMP-9 in each lane were equivalent (Fig. 8D).
Discussion

Results demonstrate that short-term abnormal wall motion induced by epicardial ventricular activation is associated with enhanced MMP activity. Significant changes in activity were observed with MMP-9. Increases in MMP activity were concomitant with enhanced collagen degradation. These data are consistent with results from in vitro studies that correlate stretching of myocardial fibroblasts and vascular smooth muscle cells with increases in MMP levels and activity (13, 27). However, several alternate explanations for MMP activation described in this report must be considered including i) regional variations in LV MMP activity, and ii) potential for ischemia during ventricular pacing.

Regional variation in LV MMP levels could account for the observed differences in MMP activity. To our knowledge, no detailed studies have been reported that describe normal regional levels of MMPs present in the left ventricle. Wilson and coworkers describe the regional LV distribution of MMPs after myocardial infarction (29). In their study, no significant differences in regional MMP levels of normal LV samples were reported. In our studies, no regional differences (apex to base) in constitutive MMP activity within the LV were observed with sham subjects by zymography or by substrate cleavage analyses (data not shown). These observations indicate that increases in MMP activity at the LVP are not due to regional differences in MMP levels.

Systolic stretch of myocardium has been shown to increase oxygen demand at the site of regional dyskinesis (11). This point is critical since early ventricular activation by epicardial stimulation can reduce regional oxygen uptake at sites of LV pacing (7). This brings into question whether systolic stretching at the LVP site induces some degree of ischemia and/or oxidative stress and thus stimulates MMP-9 activity: increases in MMP activity are known to
result from oxidative damage (6) (14). Figure 2C shows that myocardial LVP segments initially shorten by -11±2% with atrial pacing. Upon cessation of ventricular pacing, shortening instantly returns to -10±3% with atrial pacing. These data reveal that ventricular pacing does not result in significant changes in myocardial shortening from initial to final readings with atrial pacing (p>0.7, paired t-test), thus demonstrating that ventricular pacing does not impair subsequent contractile function (i.e., no stunning). However, assessments of nitrotyrosine, a marker for oxidative stress, revealed increases in nitrotyrosine levels at the site of dyskinesis (p<0.04). The increase in nitrotyrosine was not influenced by changes in pacing rate, voltage applied to the pacing site, or pacing duration (data not shown), making it unlikely that pacing, per se, stimulated protein nitrosylation. Because the increase in nitrotyrosine does not occur concomitant with myocardial contractile dysfunction or as a function of pacing parameters - even after at least 3.5 hours of epicardial pacing - it is reasonable to infer that activation of MMPs at the LVP site by ischemia seems unlikely. The increase in nitrotyrosine, however, may be associated with activation of MMPs (6, 14).

Our observation that abnormal wall motion is associated with MMP activation is supported by studies that correlate mechanical stimulation of isolated cells with increases in MMP activity (13, 27). Tyagi and coworkers demonstrated that MMP activity levels from infarcted human heart fibroblasts are identical to MMP activity levels achieved after 24h of cyclic stretching of fibroblasts from normal tissue, suggesting that activation during ischemia may be due in part to deformation of the myocardium (27). In our studies, the magnitude of systolic bulging achieved during AV pacing at the LVP site ranged from 89–97% of the segment length at ED during atrial pacing. These data imply that the magnitude of myocardial stretching is not as important as is the timing of stretching (systolic bulging during dyskinesis) for MMP-9.
activation. It is possible that the frequency of myocardial segment shortening and lengthening that occurs during a single beat (approximately 3 cycles: Figure 2B) might contribute to MMP activation at the site of abnormal wall motion. In addition, MMP activity was shown to be independent of pacing rate as comparisons of substrate cleavage data from subjects paced at different heart rates did not reveal a correlation between pacing rate and the degree of MMP activity observed at the LV pacing or remote sites (data not shown).

The preponderance of 92 kDa MMP-9 in the zymograms suggests that abnormal wall motion stimulates de novo MMP-9 expression and/or triggers localization of enzyme to the dyskinetic site from pre-existing reserves. Western blot analyses of MMP-9 showed moderate increases in MMP-9 levels at the LVP site (~1.5 fold), suggesting that zymographic activity of 92 kDa MMP-9 increases in part by an alternative manner. Recent evidence suggests that reserves of MMP-9 are stored in cytoplasmic secretory granules in endothelial cells (26). Shedding of pro-MMP–2 and MMP–9 from endothelial cells occurs during angiogenesis (26). Secretory granule release occurs rapidly (within hours), and maximal release occurs by 4 hours (26). Our studies are consistent with the time frame of MMP-9 release by shedding. More extensive studies to probe this issue may be warranted.

To investigate biochemical mechanisms of MMP activation, we examined possible involvement of serine proteases, inflammatory infiltrates, and protein nitrosylation. Activation of pro-MMPs has been correlated with activity of the serine protease plasmin (5). Plasmin activity was significantly increased by ~1.5 fold in the LVP site of paced animals (Fig. 4B). It is possible that increased plasmin activity at the LVP site might account for the enhanced presence of 86 kDa MMP-9 activity detected by zymography via proteolytic processing of 92 kDa MMP-9. Infiltration of inflammatory cells such as neutrophils into myocardium has been shown to
activate MMP-9 in a canine ischemia/reperfusion model, where MMP-9 activity increased
during the first hours of reperfusion (17). Increased myeloperoxidase activity was observed at
the LVP site relative to the LVR site of paced dogs (Fig. 7). The two-fold increase in
myeloperoxidase activity at the LVP site was similar in magnitude to the increase in MMP-9
protein levels observed by immunoblotting (~1.5 fold; Fig. 5), suggesting that protein expression
levels do not rise during induced dyskinesis, but rather increase as a consequence of
inflammatory cell infiltration. Protein nitrosylation of latent MMPs is known to stimulate MMP
activity without proteolytic removal of the enzyme’s pro-domain (6, 14). Nitrotyrosine and
MMP-9 immunoblotting of tissue homogenates affinity-purified for gelatinases showed bands
close to 92 kDa and 86 kDa (Fig. 8C-D) in the LVP region. These data indicate that tyrosine
residues of MMP-9 are specifically nitrosylated. Gu and coworkers demonstrated that thiol-
nitrosylation of pro-MMP-9 in vitro directly activates the enzyme (14). Although the exact
mechanism by which nitrotyrosine levels increase is unclear, specific nitrosylation of 92 kDa
MMP-9 may be involved in activation of its catalytic activity. Overall, our data imply that
biochemical mediators such as plasmin, protein nitrosylation, and inflammatory infiltrates may
be involved in activating MMPs within 4 hours after the onset of abnormal wall motion.

Regional damage to collagen resulting from MMP-mediated proteolysis can yield a loss
of ECM superstructure that leads to myocyte slippage and LV dilatation (15). Our data reveal
that short-term dyskinesis stimulates a two-fold increase in regional cleavage of collagen (Fig.
6). This increase in collagen degradation, however, does not result in significant changes in
collagen content as revealed by hydroxyproline analyses. The cessation of LV pacing allowed
LV systolic function to return to normal. This observation indicates that the changes in ECM
during this duration of pacing were not associated with changes in LV function. Under
conditions of chronic rapid atrial pacing, Spinale and coworkers found that LV collagen content dropped by approximately 16% after 3 weeks of pacing tachycardia, and was accompanied by LV systolic and diastolic dysfunction (24). The decrease in collagen was concomitant with the disruption of collagen fibrils, or “struts,” that connect adjacent myocytes (24). The recovery of contractile function we observe at the LVP site upon termination of ventricular pacing suggests that myocardial ECM remained functionally intact after 4 hours of pacing-induced abnormal wall motion, despite increases in MMP activity. Longer durations of ventricular pacing, however, could lead to further changes in ECM structure via MMP-9 activation and eventually lead to LV dysfunction. Although not directly determined, increased MMP-9 activity by abnormal wall motion and the concomitant increase in collagen degradation suggest coordinate increases in collagenase activity. Early activation of the LV myocardium by ventricular pacing can also pre-stretch passive myocardium of later-activated regions distal to the ventricular pacing site (19). Thus, other regions of the ventricle may also be subjected to stimulation of MMP activity by passive deformation of myocardium.

Regulation of MMP activity has been linked to the inhibitory action of tissue inhibitor of metalloproteinases (TIMPs) (23). Alterations in the balance of TIMPs/MMPs that favor increased MMP activity have been shown to occur in the failing human heart (16). Our data indicate that increased activation of MMPs at the site of abnormal wall motion is not ostensibly affected by endogenous TIMPs present at the site. However, it is possible that longer durations of induced dyskinesis might allow compensatory regulation of MMP activity by increased TIMP expression.

In conclusion, our data demonstrate that induction of short-term abnormal wall motion can stimulate significant levels of MMP activity in the heart. These results may provide an early
view into the process of collagen degradation caused by abnormal wall motion that precedes pathological ECM remodeling.
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**Figure 1. Pacing preparation.** Sonomicrometer crystals were placed at the LV pacing site (LVP) and at a remote site (LVR) at the base of the heart. Epicardial pacing wires were sutured to the atrium and to the LVP site. Two connected stimulators were used for pacing and LV pressure was measured with a fluid-filled catheter.

**Figure 2. Segment motion and percent changes in segment length at remote and LVP sites.**

Representative recordings of segment motion from a single animal during atrial and AV pacing. **A)** Remote site with atrial pacing only (top panel), and with AV pacing (bottom panel). The solid black tracing shows segment length during contraction. End diastole (ED) and end systole (ES) are shown (broken vertical lines) with respect to LV pressure (dashed line). Both traces show normal phasic movement of the sonomicrometer crystals before and during AV pacing. **B)** LVP site with atrial pacing only (top panel), and with AV pacing (bottom panel). With AV pacing, LVP site shows early shortening that precedes ventricular ED (*) and early systolic bulging (†). Note change in segment length pattern from normal phasic movement (top panel) to abnormal movement (bottom panel). **C)** Average percent changes in segment length at the remote (●) and LVP (■) sites. Negative values reflect segment shortening and positive values lengthening. Percent change = [(ES-ED)/ED X 100]. Data are mean ± SEM of five animals per time point (n=4 at 150 min).

**Figure 3. Gelatin zymography showing activation of MMP-9 by abnormal wall motion.**  **A)** Gelatin zymography bands corresponding to 92 kDa MMP-9, 86 kDa MMP-9, and 72 kDa MMP-2. MMP-2/-9 standards are shown in the left lane (Std). Homogenate from the LVR site shows bands of moderate intensity corresponding to MMP-9 and MMP-2. Increased levels of MMP-9 are
apparent at the LVP site. The 86 kDa band is about 27% as intense as the 92 kDa MMP-9 band. The right ventricle (RV) shows bands for 92 kDa MMP-9 and 72 kDa MMP-2 of similar intensity as those found in the LVR lane. B) Bar graph showing averages of band intensity of MMP-9 activity.

**Figure 4. Relative rate of substrate cleavage by MMPs and plasmin.** A) Tissue homogenates from LVP, LVR, and RV sites were exposed to a fluorogenic substrate specific for MMPs. Rate of substrate cleavage was determined for each sample and is reported as a ratio of basal MMP activity found at the LVR site. LVP shows approximately seven-fold more activity than LVR. **Inset** shows a representative fluorescence assay with tissue from the LVP (solid triangles), LVR (open triangles), and RV (open circles) sites that were exposed to a fluorogenic substrate specific for MMPs. MMP activity was specifically blocked by 1 mM phenanthroline (solid diamonds). B) LVP site of paced dogs shows a 1.5-fold increase in plasmin activity than activity levels found at the mock “LVP” site of shams (p<0.05) using a chromogenic substrate for plasmin. Rate of plasmin-mediated substrate cleavage was determined for each sample and is reported as a ratio of basal plasmin activity found at the LVR site.

**Figure 5. Representative immunoblots for MMP-9.** A) Immunoreactive bands for MMP-9 in LVR and LVP samples. B) Bar graph showing averages of MMP-9 band intensities. LVP site shows ~1.5-fold increase in protein levels relative to LVR (p<0.04). Mock LVP and LVR sites of shams were not statistically different (p>0.3).

**Figure 6. Collagen dansylation.** Exposed amino-terminal ends of collagen were labeled with dansyl. Increased dansylation was observed with tissue from the site of abnormal wall motion
(p<0.02; n=5). Fluorescence (relative fluorescence units) was normalized to collagen levels at each site.

Figure 7. Myeloperoxidase activity. Increased myeloperoxidase activity was measured at the LVP site vs. the LVR site of paced animals (p<0.02). No significant differences were measured between LVR and mock LVP sites of shams (p>0.6).

Figure 8. Nitrotyrosine levels. A) Slot blot for one animal showing increased nitrotyrosine at the LVP site of paced animals (p=0.05; n=5). B) Bar graph showing averages of nitrotyrosine band intensities from slot blots. LVP site showed a twenty-fold increase in protein levels relative to LVR. C) Western blot against nitrotyrosine showing bands corresponding to 92 and 86 kDa MMP-9. D) Reblot against MMP-9 shows that MMP-9 protein levels are equivalent in each lane. Positions of molecular weight markers are shown at right.
### Table 1. Hemodynamics Measurements

<table>
<thead>
<tr>
<th></th>
<th>Atrial Pacing</th>
<th>AV Pacing (min)</th>
<th>Atrial Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>120</td>
</tr>
<tr>
<td>HR, (bpm)</td>
<td>122 ± 9</td>
<td>121 ± 9</td>
<td>119 ± 6</td>
</tr>
<tr>
<td>LVSP, (mmHg)</td>
<td>105 ± 7</td>
<td>102 ± 3</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>LVEDP, (mmHg)</td>
<td>10 ± 1</td>
<td>10 ± 1</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>+dP/dt, (mmHg/s)</td>
<td>1318 ± 197*</td>
<td>1256 ± 119*</td>
<td>1308 ± 130</td>
</tr>
<tr>
<td>-dP/dt, (mmHg/s)</td>
<td>-1005 ± 146</td>
<td>-946 ± 73</td>
<td>-964 ± 80</td>
</tr>
</tbody>
</table>

Data were obtained from five dogs. * +dP/dt was significantly depressed upon initiation of atrial-ventricular pacing (p≤0.05, paired t-test). Values are mean ± SEM.
Figure 2

A) Remote Site: Atrial Pacing

B) LVP Site: Atrial Pacing

C) End-Systolic Segment Shortening (%)
Figure 3

A

92kDa MMP-9
86kDa MMP-9
72kDa MMP-2

Std LVR LVP RV

B

Band Intensity (arbitrary units)

LVR 92kDa MMP-9
LVP 92kDa MMP-9
LVR 86kDa MMP-9
LVP 86kDa MMP-9
RV 92kDa MMP-9

4 ± 1
0.43 ± 0.11
45 ± 9
11 ± 4

165 ± 29
Figure 4A

![Graph showing relative rate of substrate cleavage and fluorescence emission over time.](image)
Figure 4B

Relative Rate of Substrate Cleavage (% Remote Site)

- PACED: LVP/LVR
  - 164 ± 25

- SHAM: "LVP"/LVR
  - 115 ± 9

p < 0.05
Figure 5

**A**

Paced Dogs  Shams

LVR  LVP  LVR  “LVP”

**B**

Band Intensity (arbitrary units)

<table>
<thead>
<tr>
<th></th>
<th>LVR</th>
<th>LVP</th>
<th>LVR</th>
<th>“LVP”</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paced Dogs</td>
<td>273 ± 30</td>
<td>401 ± 22</td>
<td>298 ± 11</td>
<td>284 ± 9</td>
</tr>
</tbody>
</table>

p < 0.04
Figure 6

Fluorescence Emission (RFU/mg collagen)

- **LVR**
  - Paced Dogs: 12,834 ± 1,243
  - Shams: 11,753 ± 840

- **LVP**
  - Paced Dogs: 24,113 ± 4,094

*p < 0.03*
Figure 7

MPO Activity (absorbance units x 10^{-3}/min)

p < 0.02

LVR LVP LVR “LVP”

Paced Subjects Shams

16.1 ± 1.6 33.1 ± 6.1 21.8 ± 5.2 23.8 ± 8.4
Figure 8

A. Anti-nitrotyrosine slot blot

B. Band intensity (arbitrary units)

C. Anti-nitrotyrosine

D. Anti-MMP9

LVR LVP

p = 0.05

6 ± 1

LVR LVP

Paced Dogs

125 ± 53

105 kDa

75 kDa

105 kDa

75 kDa