Endomyocardial Gene Expression During Development of Pacing Tachycardia–Induced Heart Failure in the Dog

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Abstract Selective and specific changes in gene expression characterize the end-stage failing heart. However, the pattern and relation of these changes to evolving systolic and diastolic dysfunction during development of heart failure remains undefined. In the present study, we assessed steady-state levels of mRNAs encoding a group of cardiac proteins during the early development of left ventricular dysfunction in dogs with pacing-induced cardiomyopathy. Corresponding hemodynamic assessments were made in the conscious state in the same animals and at the same time points at baseline, after 1 week of ventricular pacing, and at the onset of clinical heart failure. Systolic dysfunction dominated after 1 week of pacing, whereas diastolic dysfunction was far more pronounced with the onset of heart failure. Atrial natriuretic factor mRNA was undetectable in 7 of 12 hearts at baseline but was expressed in all hearts at 1 week (P<.01 by x² test), and it increased markedly with progression to failure (P=.05). Creatine kinase-B mRNA also rose markedly with heart failure (P<.01). Levels of mRNA encoding β-myosin heavy chain, mitochondrial creatine kinase, phospholamban, and sarcoplasmic reticulum Ca²⁺-ATPase did not significantly change from baseline, despite development of heart failure. Additional analysis to determine if these mRNA changes were related to the severity of diastolic or systolic dysfunction revealed that phospholamban mRNA decreased in hearts with larger net increases in end-diastolic pressure (+19.2±1.9 mm Hg) compared with those hearts in which it did not change (+4.0±4.9, P<.02). These results suggest that selective alterations in gene expression occurring during early and later development of heart failure in the tachycardia-paced dog may be related to specific hemodynamic abnormalities. (Circ Res. 1994;75:615-623.)

Keywords selector gene expression ▪ heart failure ▪ atrial natriuretic factor

William Osler¹ first observed more than a century ago that the pathophysiology of human heart failure could be divided into three stages: (1) initial damage to the contractile apparatus, resulting in ventricular dysfunction, (2) a phase during which cardiovascular compensatory mechanisms are activated, and (3) loss of compensation with progressive deterioration in both systolic and diastolic function and eventual death. The availability of human myocardium explanted at the time of cardiac transplantation has recently enabled investigation into the molecular pathophysiology of the failing heart during the third and final stage of cardiac decompensation. Such studies have identified several mRNAs that are differentially regulated at the end stage of disease and therefore appear to be markers of the end-stage heart failure phenotype. Decreases in steady-state levels of mRNA encoding sarcoplasmic reticulum Ca²⁺-ATPase,²,³ phospholamban,³⁴ the dihydropyridine receptor,⁵ the Ca²⁺ release channel,⁶ and the β₁-adrenergic receptor⁷ accompany reexpression of atrial natriuretic factor⁴ (ANF) in ventricular myocardium from patients with end-stage heart failure. In some cases, changes in the expression of these heart-failure marker genes have been accompanied by complementary alterations in the levels of the proteins encoded by these mRNAs⁵-⁷ and there is evidence to suggest that changes in gene expression may be reversible with restitution of normal left ventricular function.⁸ However, these results reflect end-stage disease, with tissue obtained from patients having a variable duration of illness and varied pharmacologic therapy. There is virtually no information in either humans or large animals regarding the steady-state levels of similar “marker” mRNAs earlier in the course of heart-failure evolution, specifically during the first two stages delineated by Osler.

Recent investigations suggest that large animals with pacing-induced cardiomyopathy provide a useful model that bears many similarities to human heart failure. Animals develop progressive biventricular dilation and failure after 3 to 5 weeks of pacing without evidence of either hypertrophy or ischemia.⁹-¹² Analogous to humans with congestive failure, diminished ventricular function in this model is associated with abnormal Ca²⁺ handling¹³ and diminished function of the receptor–G protein–adenyl cyclase transmembrane signaling complex.¹⁴,¹⁵ Furthermore, studies of this model in larger animals facilitate repetitive in vivo evaluation of the evolution of failure at both the hemodynamic and molecular levels.

In the present study, we combined hemodynamic and molecular analysis in dogs during development of pacing-induced cardiomyopathy. The steady-state levels of the mRNAs encoding six cardiac proteins were assessed in endomyocardial biopsies obtained sequentially dur-
ing the development of congestive failure, and the measurements were correlated with simultaneously measured longitudinal assessments of ventricular systolic and diastolic dysfunction in these animals.

Materials and Methods

Animal Model

Twelve adult mongrel dogs (40 to 50 lb) were sedated with sodium thiamylal, intubated, and anesthetized with 1% to 2% halothane with continuous volume ventilation. Oxygen saturation and expired CO₂ were maintained within the physiological range throughout surgery. After a left lateral thoracotomy incision, an arterial catheter was inserted into the descending thoracic aorta through a purse-string suture, and a venous catheter was placed in the right atrium via the right atrial appendage. A flexible tube (internal diameter, 1.5 mm) was advanced through an apical stab and secured within the left ventricular chamber, providing a conduit through which a micrometerometer catheter (model SPC-350, Millar) could be advanced for pressure recording. A pair of sonomicrometer crystals were introduced into the endomyocardial surface of the mid left ventricle to provide anteroposterior (AP) dimension; a second pair of crystals were positioned within the midanteriowall to measure wall thickness. A pneumatic cuff occluder was placed around the thoracic inferior vena cava. Transient inflation of this cuff impeded vena caval inflow, thereby lowering preload volume and facilitating measurement of pressure-dimension relations. A programmable pacemaker (Activitrax or Spectract, Medtronic) with a bipolar pacing wire adaptor was placed in a subcutaneous pocket, and the two ventricular pacing leads were sewn to the right ventricular free wall. To enable programming at faster rates, pacemakers were modified by permanently attaching a small flat magnet to the posterior surface of the pacemaker canister.

The vascular and ventricular catheters, cuff occluder line, and sonomicrometer and pacing wires were exteriorized at two midscapular sites. Four separate pacing wires, two from the heart and two from the pacemaker, were exposed on the dorsal surface of the animal and could be attached or detached as required during data recording. Fluid catheters were flushed and filled with heparinized saline. The incision was closed in three layers, and chest air was evacuated. Animals were allowed to fully recover from surgery for at least 11 days before study. During recovery, animals were also acclimated to standing quietly in a slinging apparatus for data recording. Antibiotics were administered for the first 72 hours after surgery.

Experimental Protocol

All hemodynamic assessments were obtained with animals in the conscious state. Baseline data were measured, and on the following morning, pacing was begun at 250 beats per minute. Hemodynamic measurements were repeated at 1 week and at the occurrence of clinical signs of heart failure, consisting of lethargy, anorexia, ascites, and dyspnea. Longitudinal evaluations included measurement of steady-state left ventricular pressures, dimensions, and wall thickness, with pressure-dimension relations obtained from multiple beats at varying preload during gradual inflation of the inferior vena caval occluder. All measurements were made in normal sinus rhythm with the pacemaker turned off for at least 30 minutes. Data were displayed on custom-designed software and digitized at 200 Hz for later analysis.

Left ventricular endomyocardial biopsies were obtained after hemodynamic assessment at the same three time points. Dogs were sedated with intramuscular butorphanol tartrate (4 mg) and intramuscular xylazine (1 mg/lb) and placed on a fluoroscopy table, and the right or left groin was prepped and draped for sterile instrumentation. Lidocaine (1%) was administered at the surgical site for additional analgesia. The femoral artery was isolated and cannulated using a 7F long sheath. An endomyocardial biopsy (Cordis) was introduced retrograde into the left ventricle through the sheath. Five to six endomyocardial biopsy samples were obtained, immediately frozen in liquid nitrogen (within 5 to 10 seconds), and stored at -70°C until subsequent analysis.

Hemodynamic Analysis

Five to 10 sequential steady-state beats were signal-averaged, and the result was used to derive end-systolic and end-diastolic pressures, AP dimensions, and percent fractional shortening. Maximal rate of pressure rise (dP/dt max) was digitally derived from a five-point weighted slope, and the isovolumic relaxation time constant was obtained from the left ventricular pressures by the method of Raff and Glantz. End-diastolic wall stress was derived from a thick-walled spherical model: Stress = (EDP × EDD)/h₀(1 − h₀/EDD) × 1.36, where EDP, EDD, and h₀ are end-diastolic pressure, dimension, and thickness, respectively. In addition to these steady-state data, multiple pressure–AP dimension loops measured during transient inferior vena caval occlusion were combined to derive several pump function relations. The end-systolic pressure–dimension point [point of maximal P(D−D₀), where P is pressure, D is dimension, and D₀ is the dimension axis intercept] was determined for each beat to derive the end-systolic pressure-dimension relation. A second index was obtained by regressing stroke work area within each pressure-dimension loop against end-diastolic dimension for that loop. These data yield a linear relation, whose slope provides a measure of the contractile state. An advantage of this latter measure is that it has units in millimeters of mercury and is thus independent of the precise dimension used. Since the AP dimension changes near linearly with chamber volume, the value derived from pressure-dimension data is comparable to that obtained from pressure-volume relations.

Quantification of Steady-State Levels of mRNA

Total RNA was extracted from frozen left ventricular endomyocardial biopsy samples by using acid guanidium thiocyanate/phenol/chloroform (RNAzol B, Tel-Test, Inc). Seque-ntial samples from the same experimental animal were processed at the same time to prevent variations in processing from affecting the experimental results. The concentration of RNA in each sample was assessed spectrophotometrically, and all samples were stored in ethanol at -70°C until use.

Steady-state levels of mRNA were measured in endomyocardial biopsy specimens by using quantitative polymerase chain reaction (PCR) as previously described. In brief, first-strand cDNA was synthesized by reverse transcription of 1 µg of total RNA by using oligo-dT primers according to manufacturer’s instructions (Boehringer Mannheim Biochemicals). The cDNA was then amplified in an OmniGene (Hybaid Limited) with 2.5 U Thermus aquaticus DNA polymerase (Bethesda Research Laboratories) in 100 µL of 10 mmol/L Tris-HCl containing 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.001% (wt/vol) gelatin, 200 µmol/L of each dNTP, and 13 pmol of specific 3′ and 3′ primers. Each reaction also contained varying quantities (5 to 15 pg) of an internal cDNA standard prepared by reverse transcription of the in vitro transcription product of a synthetic DNA template. The 3′ primer of each primer pair was end-labeled with [γ-32P]ATP by using T4 polynucleotide kinase (Pharmacia LKB Biotechnol-ogy), and a trace amount of labeled primer was added to each reaction to provide ~1.5 × 10⁴ cpm. Amplification temperatures were 94°C for 45 seconds, 45°C (42°C for phospholamban) for 30 seconds, and 72°C for 30 seconds.

With the exception of phospholamban, the primer pairs that were previously reported for the amplification of cDNAs from human and rat ventricular myocardium were not applicable to canine myocardium. Therefore, regions from the canine cDNAs of interest were used to design appropriate 3′ and 5′
primers for PCR amplification.\textsuperscript{22,23} Regions of genes of interest were sequenced, and appropriate 3' and 5' primers for amplification of canine cDNAs were identified (Table 1). Amplification reactions with each of these primer pairs yielded a single band in an agarose gel, and the absolute identity of this band was confirmed by sequence analysis. The primer pairs were then incorporated into synthetic DNA constructs containing the bacteriophage T7 promoter at the 5' end and a polyadenine tract at the 3' end that served as templates for in vitro transcribed internal standards as previously described.\textsuperscript{4}

For assessment of steady-state levels of mRNA, 10 μL of each PCR reaction mixture was removed during successive cycles of amplification (12 to 34) and electrophoresed in a 3% (wt/vol) NuSieve GTG 5:1% (wt/vol) SeaKem LE (FMC BioProducts) gel containing Tris-acetate-EDTA and ethidium bromide. Samples were taken every other cycle for a total of nine samples. A Sau3A digest of pBluescript KS\textsuperscript{+} DNA (Bethesda Research Laboratories) was used as a molecular size standard. The gel was visualized with indirect UV radiation and photographed, and appropriate bands representing amplification products of the cDNA of interest and amplification products of the internal cDNA standard were cut out from the gel. Radioactivity in the bands was determined by Cerenkov counting, and the amount of radioactivity in each excised gel band was plotted against the number of PCR cycles.

Amplification of increasing concentrations of both internal DNA standard and sample DNA (reverse transcription products of the original RNA) was linear over several orders of magnitude. Preliminary experiments identified appropriate concentrations of each internal cDNA standard to allow for colinear amplification of the cDNA of interest and the appropriate standard cDNA when the initial reverse transcription reaction contained 1 μg total RNA (ANF, 5 pg; myosin heavy chain-\textbeta; [MHC-\textbeta;], 15 pg; phospholamban, 15 pg; Ca\textsuperscript{2+}-ATPase, 5 pg; creatine kinase-B [CK-B], 5 pg; and mitochondrial creatine kinase [CK-mito], 5 pg). Additionally, a complete amplification curve was plotted for each reaction, and these curves were used to calculate mRNA levels only if regions of linear amplification were identified that spanned at least six amplification cycles. Under these conditions, the sensitivity of the assay for each primer pair was at least 3.4×10\textsuperscript{4} molecules mRNA per microgram total RNA, and the assay was reproducible with a coefficient of variation of 12%.

With the exception of phospholamban, a gene having an intronless coding region, primers were designed to cross-splice junctions to abrogate amplification of contaminating genomic DNA. To exclude the presence of contaminating genomic DNA when assessing the levels of phospholamban mRNA, samples were amplified after deleting reverse transcriptase from the reaction constituents. No bands were detected in these samples, demonstrating the absence of significant genomic contamination.

**Statistical Analysis**

All data are presented as mean±SEM. Hemodynamic and mRNA data were compared by repeated-measures ANOVA using Dunnett's test for multiple comparisons. All data from all time points were entered into a single matrix, in which dummy variables were used to code for individual animals and time-point comparisons. This generated a pooled variance from the full data set and allowed for varying sample size at each time point. A value of \( P < .05 \) was considered to be statistically significant.

**Morphological Assessment**

Frozen specimens obtained at baseline and at the onset of congestive failure were shipped on dry ice and subsequently fixed in formalin, embedded in paraffin, and sectioned. Serial sections were stained with either picrosirius red or hematoxylin and eosin for analysis of collagen content and morphological assessment, respectively, as described previously in detail.\textsuperscript{28,29} Tissue was analyzed on a Quantimet 520 image analysis system. Contrast enhancement of picrosirius red-stained sections was done by 540-nm band-pass filter. Collagen content was assessed and quantified at ×200 magnification. Evaluation was performed with observers blinded as to sample and time-point identity.
TABLE 2. Summary of Hemodynamic Data

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 1</th>
<th>P (Week 1 vs Baseline)</th>
<th>End of Study</th>
<th>P (End of Study vs Baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>12</td>
<td>12</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>126.9±6.9</td>
<td>132.7±7.0</td>
<td>.43</td>
<td>133.9±8.6</td>
<td>.48</td>
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<tr>
<td>End-diastolic dimension, mm</td>
<td>36.5±1.5</td>
<td>36.9±1.7</td>
<td>.72</td>
<td>36.9±2.4</td>
<td>.24</td>
</tr>
<tr>
<td>End-systolic dimension, mm</td>
<td>28.7±1.5</td>
<td>30.8±1.5</td>
<td>.07</td>
<td>32.1±2.5</td>
<td>.03</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>21.6±2.0</td>
<td>16.5±1.4</td>
<td>.006</td>
<td>13.5±1.8</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>End-diastolic pressure, mm Hg</td>
<td>13.0±1.4</td>
<td>17.4±2.3</td>
<td>.09</td>
<td>27.7±2.4</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>r, mm Hg/s</td>
<td>32.2±3.0</td>
<td>35.1±3.1</td>
<td>.47</td>
<td>45.5±5.9</td>
<td>.009</td>
</tr>
<tr>
<td>End-diastolic stress, g/cm²</td>
<td>8.8±1.3</td>
<td>11.1±1.7</td>
<td>.407</td>
<td>25.4±5.2</td>
<td>.001</td>
</tr>
<tr>
<td>End-systolic pressure, mm Hg</td>
<td>133.5±6.0</td>
<td>100.1±4.3</td>
<td>&lt;.001</td>
<td>100.5±3.9</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>dP/dt max, mm Hg/s</td>
<td>3033.8±230.6</td>
<td>1965.2±151.8</td>
<td>&lt;.001</td>
<td>1620.3±218.5</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Slope of ESPDR</td>
<td>9.9±0.72</td>
<td>8.5±1.4</td>
<td>.28</td>
<td>7.6±0.6</td>
<td>.05</td>
</tr>
<tr>
<td>M_sw, mm Hg</td>
<td>78.2±4.2</td>
<td>50.6±4.8</td>
<td>&lt;.001</td>
<td>40.7±2.6</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

n indicates number of dogs; bpm, beats per minute; r, time constant; ESPDR, end-systolic pressure-dimension relation; and M_sw, slope of stroke work–end-diastolic dimension relation. Values are mean±SEM.

Results

Nine of the 12 dogs reached the early heart failure end point and therefore were evaluated at all three time points. In this group of animals, the final biopsy was obtained 25±3 days after pacing was initiated. Three dogs were found dead within 3 to 5 days of the week 1 evaluation, and these animals presumably expired suddenly; one animal expired just before final biopsy tissue was obtained.

Hemodynamic Responses

Substantial abnormalities in systolic dysfunction were observed after only 1 week of tachycardia pacing (Table 2). Fractional shortening and dP/dt max fell significantly, by 25% and 35%, respectively (both P<.01), with relatively little further decline after development of heart failure. In contrast, diastolic dysfunction, manifested by elevation of end-diastolic pressure and wall stress and prolongation of the time constant of relaxation, was not significant at 1 week but became prominent at the heart-failure time point (Table 2).

Fig 1 displays example pressure-dimension loops and relations in one dog. Multiple cardiac cycles are displayed at varying preloads, and the upper and lower boundaries (end-systolic and end-diastolic relations, respectively) are derived from these beats. Similar to the results of the steady-state analysis, the end-systolic pressure-dimension relation displayed a fall in slope and gradual shift rightward with worsening failure, consistent with contractile depression. Both this index and the stroke work–end-diastolic dimension relation index fell significantly early and remained reduced at heart failure (Table 2). All hemodynamic results reported in Table 2 are identical to those obtained when only the animals that developed heart failure (n=8) were used.

Steady-State Levels of mRNA in Endomyocardial Biopsies

Individual mRNA responses for each gene in each animal are provided in Fig 2, and summary results are reported in Table 3. At baseline, ANF mRNA was unde-
tectable in seven dogs. However, by 1 week of pacing, ANF mRNA was abundant in all dogs (P<.01 by χ² test). Steady-state levels of ANF mRNA were further elevated in the final biopsy samples, and these levels were statistically different from those at baseline by ANOVA (P=.05). CK-B mRNA was also barely detectable at baseline and after 1 week of ventricular pacing. However, CK-B mRNA levels were substantially and significantly (P=.01) elevated in the final biopsies. In contrast to both CK-B and ANF, levels of the mRNAs encoding β-MHC, phospholamban, Ca²⁺-ATPase, and CK-mito were not different in left ventricular myocardium, exhibiting early abnormalities in both systolic and diastolic function when compared with baseline values in these animals. Repeated-measures ANOVA using only data from animals that completed the protocol yielded identical quantitative results, but with a P value for ANF increase at heart failure becoming borderline significant (P=.082).

Although mean changes in CK-mito, Ca²⁺-ATPase, β-MHC, and phospholamban were not significant when compared over time, it remained possible that changes in gene expression depended on the severity of diastolic and/or systolic dysfunction with failure rather than simply the time point of the observation. To test this possibility, we divided dogs into those that demonstrated a decrease in mRNA versus those with either unchanged or increased levels of expression, and we tested whether there were significant differences in end-diastolic pressure or dP/dtmax between these two subgroups. The results for phospholamban were notable. In the five dogs in which phospholamban mRNA fell, the mean change in end-diastolic pressure was +19±1.9 mm Hg as opposed to only +4.0±4.9 mm Hg for those in which mRNA levels did not fall (P=.02). Fig 3 displays the relation between end-diastolic pressure and phospholamban mRNA at baseline and heart failure for each dog demonstrating this dependence. In contrast, there was no significant disparity between dP/dtmax responses in these subgroups. Similar analysis for the other mRNAs revealed no analogous dependencies for either end-diastolic pressure or dP/dtmax.

Morphological Analysis

To evaluate the possibility that changes in steady-state mRNA levels over time were due to or biased by alterations in proportion of the biopsy occupied by myocytes versus other cells containing RNA, six endo-

### TABLE 3. Steady-State mRNA Levels in Endomyocardial Biopsy Samples

<table>
<thead>
<tr>
<th>mRNA Levels</th>
<th>Baseline</th>
<th>Week 1</th>
<th>End of Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANF</td>
<td>2.83±1.3</td>
<td>4.74±0.93</td>
<td>10.7±4.1*</td>
</tr>
<tr>
<td>CK-B</td>
<td>3.62±1.0</td>
<td>3.36±0.85</td>
<td>11.72±3.7†</td>
</tr>
<tr>
<td>Ca²⁺-ATPase</td>
<td>21.26±3.17</td>
<td>18.75±4.51</td>
<td>15.93±4.87</td>
</tr>
<tr>
<td>β-MHC</td>
<td>81.2±10.8</td>
<td>60.1±9.8</td>
<td>64.3±13.1</td>
</tr>
<tr>
<td>Phospholamban</td>
<td>40.0±5.7</td>
<td>40.8±6.1</td>
<td>27.4±5.77</td>
</tr>
<tr>
<td>CK-mito</td>
<td>36.52±4.98</td>
<td>29.26±5.36</td>
<td>24.29±5.62</td>
</tr>
</tbody>
</table>

n indicates number of dogs; ANF, atrial natriuretic factor; CK-B, creatine kinase-B; β-MHC, β-myosin heavy chain; and CK-mito, mitochondrial creatine kinase. Values are mean±SEM. *P<.05 and †P<.01 vs baseline.
myocardial biopsies were randomly selected at baseline and at the onset of heart failure and subjected to histological examination. Three of the specimens displayed normal myocytes, no infiltrates, and a collagen content of ≤2.1%. Of these, two were from hearts exhibiting heart failure, and one was from a baseline control heart. Three additional specimens demonstrated varying increased amounts of collagen (20.1%, 49.2%, and 4%). In the first case, a swirling appearance of the myocytes led us to speculate that the sample was likely obtained at a trabecula insertion site. In the sample with the most collagen, the remaining half of the sample appeared virtually normal, and this likely represented biopsy of a scarred area (due to prior biopsy). In the last case, there was also a small region of myocyte necrosis with cellular infiltrate and some reparative fibrosis. Of the latter three specimens, the first and last were from baseline control hearts. Thus, although there was variability in histological appearance, there was no consistent relation between time point and either collagen content, presence of inflammatory infiltrate, or myocyte dropout. In all cases, at least half the sample contained normal-appearing myocytes.

Discussion

Sequential hemodynamic evaluations in the present study by both standard steady-state measures (ie, dP/dt max) and less load-dependent pressure-dimension relations were consistent with prior reports involving both anesthetized9 and conscious dogs.10 However, these measurements emphasize the finding that left ventricular systolic dysfunction predominates early after the initiation of tachycardia pacing, whereas diastolic dysfunction becomes more pronounced with the onset of clinical signs of congestive failure. Therefore, the tachycardia pacing model of heart failure provides a setting in which predominant early systolic dysfunction can be separated chronologically from later development of diastolic dysfunction. Some prior studies have demonstrated more substantial changes in left ventricular volume with progressive pacing13 when compared with the present data. This suggests that the results of the present study represent time points that are relatively early in the heart-failure phenotype.

The demonstration of increased ANF mRNA levels during the development of pacing-induced heart failure is not surprising, since reinduction of ANF expression is a ubiquitous finding in cardiac muscle diseases.30 However, the results of the present study are unique in that ANF gene expression was upregulated in a model characterized by an absence of myocardial hypertrophy.9,10,31,32 Reactivation of myocardial ANF expression can be induced by volume overload,33 pressure overload,44 and spontaneous hypertrophy45 and has been documented in end-stage failing human myocardium.4,6 However, ventricular activation of the ANF gene is probably a multifactorial phenomenon,6,30 as recent studies demonstrate that regional histological changes may trigger ANF expression in patients with hypertrophic cardiomyopathy, whereas hemodynamic overload serves as a trigger for reexpression in patients with hypertensive heart disease.37 Similarly, the finding that ANF gene reexpression occurred early after the induction of pacing is consistent with prior studies demonstrating a rapid rise in ANF mRNA levels shortly after initiation of hemodynamic overload.35 Indeed, the ANF gene contains cis-regulatory elements that recognize the immediate-early gene heterodimer c-fos/c-jun.38

Creatine kinase (CK) enzymes exist as several isoforms in both skeletal and cardiac muscle, and these enzymes function in maintaining the proper intracellular ratio of ATP to ADP and the size of the phosphocreatine pool.39 Two CK genes encode the muscle (M) and brain (B) protein subunits that are present as either the homodimeric CK-MM and CK-BB or the heterodimeric CK-MB cytosolic isoenzymes.40 The CK-B–encoded isozymes predominate in fetal tissue; however, the CK-M–encoded isozyme makes up the majority of CK in mature tissues.41 The present study presents the first evidence that CK-B mRNA levels are increased in a model of failing myocardium that lacks myocyte hypertrophy.31 However, like ANF, it appears that CK-B–encoded protein is differentially regulated in many forms of heart muscle disease, since previous studies have demonstrated increases in CK-B–containing isozymes in dogs with cardiac hypertrophy42 and in dogs with coronary artery occlusion.43 In addition, in rats with pressure overload of the left ventricle, the increase in CK-B–encoded protein was associated with a recapitulation of the fetal pattern of gene expression, as there was a substantial increase in CK-B mRNA.44 Increased CK-B mRNA and expressed protein levels may underlie a change to a more efficient energy utilization45 and improved chemomechanical efficiency in dogs with pacing-induced heart failure.12 Therefore, the increase in CK-B mRNA levels may mark adaptive responses in the failing myocardium.

Our inability to detect changes in β-MHC mRNA levels in endomyocardial biopsies obtained from dogs with substantial diminutions in left ventricular function is consistent with earlier studies in failing human myocardium.4,8 However, our inability to demonstrate changes in the steady-state levels of mRNAs encoding phospholamban and sarcoplasmic reticulum Ca2+-ATPase is disparate from previous results in end-stage failing human myocardium.2,4 Nonetheless, it is intriguing that phospholamban mRNA levels were substantially decreased in those hearts with larger net increases in end-diastolic pressure. These results suggest that selective signals modulate the expression of specific genes during the development of congestive failure; however, it remains to be determined whether these
changes in gene expression contribute to or are the cause of the accompanying changes in cardiovascular hemodynamics.

Several notable differences exist between the end-stage human heart and the pacing-induced heart failure model that might explain the absence of a decrease in sarcoplasmic reticulum Ca\(^{2+}\)-ATPase mRNA levels in this model: (1) Patients receive multiple pharmacologic interventions, some of which activate the renin-angiotensin system. (2) Most human samples have been obtained from patients with long-standing chronic disease, whereas dogs were paced only until the first signs of clinical heart failure. (3) End-stage disease in humans is associated with both cardiac dilatation and myocyte hypertrophy, whereas hypertrophy is not evident in dogs with pacing-induced failure. (4) Patients with end-stage disease have marked elevations in plasma catecholamine levels.\(^5,46\)

It is unlikely that the experimental results were artifically influenced by the use of the quantitative PCR technique. The presence of an internal standard in both the reverse transcription and amplification reactions precludes assay-to-assay variations in sample preparation, reverse transcription, and amplification efficiency. This technique has been used previously in studies of endomyocardial biopsies from patients with end-stage heart failure\(^6,8\) and is similar in design to techniques recently validated by other laboratories for quantification of steady-state levels of mRNA in non-cardiac tissues.\(^5,46\) Most important, we have recently demonstrated a close correlation between measurements of mRNA in ventricular myocardium by quantitative PCR and by either traditional Northern blot analysis\(^69\) or by RNase protection.\(^2\) Indeed, the use of more traditional techniques would be problematic with the small size of tissue samples obtained from canine endomyocardial biopsies.

Variation in the relative proportion of myocytes in the biopsy samples could influence the mRNA results, since the data are normalized to the total RNA content of the tissue. Indeed, histological analysis revealed variable collagen content and small areas of cellular infiltrate in some samples. However, this variation was randomly distributed between control and experimental time points. It is important to note that altered collagen content itself would not influence the data, since results were normalized to total RNA content, not mass. Finally, to further reduce bias due to varying histology in any one sample, mRNA results were derived from the average analysis of several samples at each time point.

We cannot determine from the present data whether changes in ANF and CK-B gene expression that occur early in the development of pacing-induced heart failure are due to an increase in transcriptional activity or in mRNA stability. In addition, we cannot account for the presence of small but detectable amounts of both ANF and CK-B RNA in several of the baseline samples. Although both ANF and CK-B mRNA levels increased with initiation of pacing in these animals and baseline hemodynamic variables were normal, we cannot exclude the possibility that the earlier surgical instrumentation contributed to these baseline elevations. However, each animal served as its own control, baseline data were obtained nearly 2 weeks after surgery, and further increases in both CK-B and ANF mRNA levels were seen in animals with detectable levels at baseline. Finally, we cannot exclude the possibility that the changes in steady-state mRNA levels reported in the present study are limited to the endomyocardium. Indeed, recent studies suggest that the characteristic decrease in β-adrenergic receptor density in failing human heart predominates in the endocardium and is not obvious in the epicardium.\(^30\) However, despite the endocardial localization of the decrease in β-adrenergic density, there is a consensus that this decrease in β-receptor density accounts in large part for the insensitivity of the failing heart to adrenergic drive.\(^31\)

The increase in ANF mRNA was marked in two dogs; however, five dogs demonstrated at least a 200% rise in ANF mRNA, and four had more than a 10-fold increase. Similarly, CK-B mRNA levels rose by >200% in four dogs. Therefore, the results of our data do not reflect changes in only two dogs. Furthermore, it is interesting that there is no concordance between ANF and CK-B mRNA levels; ie, the dogs with the largest increases in ANF mRNA were not necessarily the same as the dogs with the greatest increase in CK-B mRNA. Finally, it is notable that there were generally consistent increases in ANF mRNA early in the development of heart failure. However, ANF mRNA declined or did not change in four of the eight animals between 1 week and congestive heart failure time points. Thus, there appears to be greater variability in the ANF responses later in heart failure, not with respect to baseline but in terms of the evolution of failure.

In summary, serial measurements of left ventricular function and steady-state levels of mRNA in dogs with pacing-induced heart failure demonstrate substantial abnormalities in systolic and diastolic performance and activation of both ANF and CK-B gene expression early in the development of congestive failure. Phospholamban mRNA levels decreased with the onset of clinical heart failure but only in those hearts demonstrating a substantial increase in left ventricular diastolic dysfunction. However, there was no change in the expression of Ca\(^{2+}\)-ATPase, an mRNA that appears to be downregulated in the end-stage heart-failure phenotype in humans. Therefore, these results have led us to hypothesize that selective changes in gene expression can occur during the early development of heart failure in the tachycardia-paced dog that might not be reflective of the end-stage phenotype. Furthermore, our results demonstrate the importance of simultaneous measurements of gene expression and ventricular hemodynamics in the same animal and the usefulness of quantitative PCR for assessing gene expression in endomyocardial biopsy samples from large animal models of heart failure. Future studies of more chronic heart failure in this model may reveal closer similarities to the end-stage changes in gene expression observed in humans.

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