Reduced Myocardial Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase mRNA Expression and Biphasic Force-Frequency Relations in Patients With Hypertrophic Cardiomyopathy

Fuji Somura, MD; Hideo Izawa, MD, PhD; Mitsunori Iwase, MD, PhD; Yasushi Takeichi, MD; Ryoji Ishiki, MD, PhD; Takao Nishizawa, MD; Akiko Noda, PhD; Kohzo Nagata, MD, PhD; Yoshiji Yamada, MD, PhD; Mitsuhiro Yokota, MD, PhD

**Background**—The relationship between left ventricular (LV) contractile functional reserve and gene expression of Ca\(^{2+}\)-handling proteins in patients with hypertrophic cardiomyopathy (HCM) remains to be clarified.

**Methods and Results**—We calculated the maximum first derivative of LV pressure (LV dP/dt max) and the LV pressure half-time (T\(_{1/2}\)) during pacing in 14 patients with nonobstructive HCM (LV ejection fraction >55%) and 7 control subjects. Endomyocardial tissue was obtained, and mRNA levels of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2), ryanodine receptor-2, phospholamban, calsequestrin, and Na\(^+\)/Ca\(^{2+}\) exchanger were quantified by use of a real-time quantitative reverse transcription–polymerase chain reaction method. Group A consisted of 7 HCM patients who showed a progressive rise in the LV dP/dt\(_{max}\) with increased heart rate. Group B consisted of 7 HCM patients in whom the heart rate–LV dP/dt\(_{max}\) relation was biphasic at physiological pacing rates. Both the mean maximal wall thickness and the LV hypertrophy score in group B were greater than in group A (20±5 versus 15±3 mm and 7±1 versus 5±2 points, respectively). SERCA2 mRNA levels were significantly lower in group B (SERCA2/GAPDH ratio 0.34±0.15) compared with group A (0.72±0.27) and control subjects (0.85±0.47), whereas the mRNA expression of ryanodine receptor-2, phospholamban, calsequestrin, and Na\(^+\)/Ca\(^{2+}\) exchanger were similar in all groups.

**Conclusions**—These results suggest that downregulation of SERCA2 mRNA, resulting in altered Ca\(^{2+}\) handling, may contribute to impaired LV contractile reserve in HCM patients with severe hypertrophy, even in the absence of detectable baseline systolic dysfunction. *(Circulation. 2001;104:658-663.)*

**Key Words:** hypertrophy • cardiomyopathy • myocardial contraction • sarcoplasmic reticulum • biopsy

Hypertrophic cardiomyopathy (HCM) is characterized by left ventricular (LV) and/or right ventricular hypertrophy, which is usually asymmetrical and involves the interventricular septum. Typically, patients have reduced chamber compliance, delayed relaxation, and normal to supernormal resting systolic function.1 These characteristic features of HCM can occur without symptoms, but many individuals experience some dyspnea, angina, and palpitations despite overall preserved LV systolic function. Although such symptoms have been linked to subendomyocardial hypoperfusion with worsened diastolic failure,2,3 limited systolic functional reserve may also contribute to these symptoms of HCM patients.

The relationship between force and frequency of stimulation is an important determinant of myocardial contractility; the positive inotropic effect of increasing beat frequency has been demonstrated in a variety of preparations and intact animals as well as normal human subjects.4–9 Conversely, in the failing human heart, a flat or even negative force-frequency relation (FFR) has been observed and is considered to represent limited functional reserve of the heart.10,11 It is not clear, however, whether FFR is preserved in HCM patients whose resting systolic LV function is still preserved.

Interval-dependent changes in the amplitude of intracellular Ca\(^{2+}\) transients have been shown to be responsible for interval-dependent changes in the force of contraction.11 The negative FFR in human heart failure has been suggested to reflect disturbances of Ca\(^{2+}\) handling in terminally failing human myocardium.11,12 Unlike studies on altered Ca\(^{2+}\) handling in end-stage failing myocardium, however, which can be performed on explanted hearts obtained after cardiac transplantation, studies that address the subcellular mechanisms of altered myocardial properties in human HCM are rare, probably because of the limited access to adequate human tissue.

The first goal of this study was to characterize chronotropic regulation of myocardial contraction and relaxation in HCM
patients with normal baseline LV systolic function. The second goal was to examine the relationship between LV contractile functional reserve and myocardial mRNA expression of Ca\(^{2+}\)-handling proteins in patients with HCM by use of a novel real-time quantitative reverse transcription (RT)–polymerase chain reaction (PCR).

**Methods**

**Patients**

We studied 14 patients with nonobstructive HCM (mean age, 52 years) (Table 1). All patients were suspected of having LV hypertrophy (LVH) on the basis of the ECG and/or echocardiography and had occasional mild breathlessness or atypical chest pain. Of the 14 patients, 5 had an identifiable family history of HCM in a second-degree relative. All patients had normal sinus rhythm on the ECG and normal ejection fraction on left ventriculograms (mean, 71%) at baseline. HCM was diagnosed by established clinical, hemodynamic, and echocardiographic criteria.\(^{13}\) No significant intraventricular pressure gradient was detected at rest or during provocative maneuvers (the Valsalva maneuver and isoproterenol infusion) after completion of the study protocol in any patient with HCM. The control group consisted of 7 patients undergoing diagnostic cardiac catheterization to evaluate atypical chest pain. All control subjects had normal ECGs, echocardiograms, coronary arteriograms, and contrast ventriculograms. None of the patients had valvular heart disease or \(>50\%\) narrowing of the coronary arteries as determined by coronary arteriography. The study protocol was approved by the appropriate institutional review committee. Written informed consent was obtained from all subjects.

**2D echocardiographic measurements** were made from recordings of \(\geq 5\) consecutive cardiac cycles by 2 observers who were unaware of the patients’ clinical status. The degree of LVH was evaluated semiquantitatively by use of the scoring system proposed by Wigle et al.\(^{14}\)

**Table 1. Baseline Clinical, Echocardiographic, and Ventriculographic Characteristics of Patient Groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>Case</th>
<th>Age, y</th>
<th>VST, mm</th>
<th>PWT, mm</th>
<th>LVH Score</th>
<th>LVEF, %</th>
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VST indicates interventricular septum thickness; PWT, posterior wall thickness; and LVEF, LV ejection fraction. All patients were male.

\(^{P}<0.05\) vs control group; \(^{P}<0.05\) vs group A.

**Cardiac Catheterization Procedures**

All cardiovascular agents were discontinued \(\geq 4\) days before cardiac catheterization. A 6F fluid-filled pigtail catheter with a high-fidelity micromanometer (model SPC–464D, Millar Instruments) was advanced into the LV through the right brachial vein and positioned in the right atrium. Right atrial pacing was initiated at 80 bpm and increased in increments of 10 bpm. Micromanometer pressure signals and standard ECGs were recorded with a multichannel recorder (MR–40, TEAC Co) throughout the study. LV pressure signals were digitized at 3-nis intervals and analyzed with software developed in our laboratory with a 32-bit microcomputer system (PC–9821-ST20, NEC Co). We selected steady-state LV pressure data at baseline and at each pacing rate for analysis. We calculated the maximum first derivative of LV pressure (LV dp/dt\(_{max}\)) as an index of contractility. To evaluate LV isovolumic relaxation, the pressure half-time (T\(_{1/2}\)) was computed directly, according to the method of Mirdky.\(^{15}\) We defined the critical heart rate (HR) as the HR at which LV dp/dt\(_{max}\) reached the maximum value during a progressive increase in HR. Thus, the value beyond which LV dp/dt\(_{max}\) declined by 5% was the critical HR for isovolumic contraction. The peak pacing rates were defined as the HR at which either second-degree atrioventricular block or pulsus alternans occurred. After completion of the pacing study, selective coronary angiography, left ventriculography, and endomyocardial biopsy were performed. Several (\(\geq 5\)) endomyocardial biopsy samples were obtained from the right side of the interventricular septum. Biopsy samples for mRNA analysis were frozen immediately in liquid nitrogen and stored at \(-80°\)C until use.

**TaqMan Real-Time Quantitative RT-PCR Analysis**

The respective primers and TaqMan probes were designed with a software program from Perkin-Elmer according to the sequences available in GenBank (Table 2). TaqMan GAPDH control reagents (Perkin-Elmer) were used for fluorogenic detection of human GAPDH transcript as an internal standard.

The total RNA was isolated from 1 to 2.5 mg of frozen samples (RNasey Mini Kit, Qiagen). cDNA was synthesized from 300 ng of total RNA and human heart total RNA (Clontech) at 42°C for 50 minutes with oligo(dT)\(_{18}\) and Moloney murine leukemia virus RT (Super-ScriptII, Gibco BRL). The DNAse I treatment was carried out in the laboratory with a 32-bit microcomputer system (PC–9821-ST20, NEC Co). We selected steady-state LV pressure data at baseline and at each pacing rate for analysis. We calculated the maximum first derivative of LV pressure (LV dp/dt\(_{max}\)) as an index of contractility. To evaluate LV isovolumic relaxation, the pressure half-time (T\(_{1/2}\)) was computed directly, according to the method of Mirdky.\(^{15}\) We defined the critical heart rate (HR) as the HR at which LV dp/dt\(_{max}\) reached the maximum value during a progressive increase in HR. Thus, the value beyond which LV dp/dt\(_{max}\) declined by 5% was the critical HR for isovolumic contraction. The peak pacing rates were defined as the HR at which either second-degree atrioventricular block or pulsus alternans occurred. After completion of the pacing study, selective coronary angiography, left ventriculography, and endomyocardial biopsy were performed. Several (\(\geq 5\)) endomyocardial biopsy samples were obtained from the right side of the interventricular septum. Biopsy samples for mRNA analysis were frozen immediately in liquid nitrogen and stored at \(-80°\)C until use.

The mRNA expression of Ca\(^{2+}\)-handling proteins was analyzed by a fluorogenic 5’-nucleotide PCR assay using an ABI PRISM 7700 sequence detector (Perkin-Elmer).\(^{18}\) The cDNA products were amplified with an initial denaturation at 95°C for 10 minutes and 50 cycles of PCR, with each cycle consisting of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 60 seconds (TaqMan PCR core kit, Perkin-Elmer). All PCR assays were performed in triplicate. Serial dilutions of cDNA from human heart total RNA were analyzed for each target gene. These served as standard curves for quantitative analysis.
Statistical Analysis

Results are expressed as mean±SD. One-way factorial ANOVA was used to compare baseline characteristics and hemodynamic variables at peak HR during pacing among groups. Within-group comparisons were performed for the hemodynamic changes during pacing by 2-way repeated-measures ANOVA. When a significant difference was present, intergroup comparisons were made by Fisher’s multiple comparison test. The FFR and relaxation-frequency relation were assessed by the nonlinear least-squares fitting technique, as appropriate. Between-group comparisons of the regression curves were determined statistically significant.

Results

Subgroup Classification

We divided the HCM patients into 2 groups on the basis of FFRs during atrial pacing. Group A consisted of 7 patients in whom LV dP/dt max increased progressively with increases in HR up to the peak pacing rate (the positive FFR). Group B consisted of 7 patients in whom FFRs at physiological pacing rates were biphasic, with an initial positive slope (ascending limb) and a subsequent negative slope (descending limb).

Histological analysis revealed mild to moderate interstitial fibrosis, myocyte hypertrophy, and characteristic cell-to-cell and/or myofibrillar disarray in all HCM patients. The severity of histological abnormalities was not different between the 2 HCM groups. Of 5 cases of familial HCM, 1 case (case 13) belonged to group A and the other 4 cases (cases 15, 17, 19, and 20) belonged to group B. Two cases (cases 9 and 10) in group A and the other 2 cases (cases 20 and 21) in group B had hypertension.

Baseline Data

Both groups A and B had increased LV wall thickness, with the greater wall thickness present in group B (Table 1). The LVH score was significantly higher in group B than in group A. There was no difference in the LV ejection fraction among the 3 groups. LV end-diastolic pressure at baseline was significantly higher in groups A and B than in the control group. There was no difference in LV dP/dt max at baseline among the 3 groups, but T 1/2 was significantly prolonged in groups A and B compared with the control group (Table 3).

Responses to Pacing-Induced Tachycardia

There was no difference in peak pacing rate among the groups. Increases in the pacing rate induced progressive increases in the LV dP/dt max in group A and in the control group (Figure 1). HR correlated significantly with LV dP/dt max in group A (r=0.94±0.03) and in the control group (r=0.95±0.09). The slope of the regression curve for the HR-LV dP/dt max relation was similar in the 2 groups. Patients in group A and control subjects showed a similar increase in LV dP/dt max at the peak pacing rate. In contrast, the HR-LV dP/dt max relation was biphasic in group B (Figure 2). The critical HR ranged from 90 to 120 bpm (mean, 106±13 bpm). At the critical HR, LV dP/dt max increased significantly, by 17%, and then decreased by 8% at the peak pacing rate. HR correlated significantly with T 1/2 (r=−0.94±0.07) during pacing in all groups (Figure 1). The slope of the regression curve for the HR-T 1/2 relation was similar in all groups. A pacing-induced increase in HR to ~150 bpm reduced T 1/2 progressively in all groups.

mRNA Expression of Ca2+-Handling Proteins in Endomyocardial Biopsy Samples

RT-PCR of human heart total RNA revealed a single band corresponding to the expected size of each PCR product. No
amplification of genomic DNA was detected in any of these PCR assays (data not shown).

The quantitative data for each target Ca\textsuperscript{2+}-handling protein, normalized to the GAPDH transcript, are shown in Table 4 and Figure 3. No significant difference in the mRNA expression of phospholamban, ryanodine receptor-2, calsequestrin, or Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger were shown to be unchanged in this stage of cardiac hypertrophy.

The most characteristic pathophysiological abnormality in HCM is diastolic rather than systolic dysfunction. Typically, patients have abnormalities in diastolic relaxation and chamber compliance, despite hyperdynamic LV systolic function.\textsuperscript{1} Indeed, in this study, HCM patients showed significantly prolonged $T_{1/2}$ at baseline compared with control subjects, despite normal to supernormal rest LV ejection fraction and LV dP/dt max. Cardiac systolic function is enhanced by mechanisms such as FFR, adrenergic stimulation, and the force-length or Frank-Starling mechanism. In this study, HCM patients were divided into 2 groups on the basis of differences in the FFR. Patients with milder HCM (LVH score, 5\textsuperscript{2}) showed positive FFR, with

### Discussion

The present study presents a novel finding regarding the FFR at physiological pacing rates in patients with HCM. The FFR was biphasic, with an initial positive slope (ascending limb) and a subsequent negative slope (descending limb), in the patients with severe LVH, even in the absence of LV dysfunction. Another important finding is that decreased myocardial SERCA2 expression was present in patients with impaired FFR and severe cardiac hypertrophy before detectable LV systolic dysfunction. Conversely, the myocardial mRNA levels of phospholamban, ryanodine receptor-2, calsequestrin, or Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger were shown to be unchanged in this stage of cardiac hypertrophy.

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the development of LV systolic dysfunction at rest, suggesting reductions in contractile reserve in HCM patients.

In this study, we used real-time quantitative RT-PCR, which allows the measurement of mRNA levels in small quantities of tissue endomyocardial biopsy samples. Thus, in combination with hemodynamic measurements, we may be able to examine the relationship between impaired myocardial properties and their possible molecular mechanisms.

The ascending limb of the FFR is caused primarily by increased Ca\(^{2+}\) availability to contractile proteins.\(^{19}\) Although the mechanisms underlying the descending limb of the FFR are poorly understood, they may be related to abnormal Ca\(^{2+}\) handling. Indeed, abnormal FFRs have been shown to be related to impaired Ca\(^{2+}\) handling and/or depressed SERCA2 expression or function in patients with dilated cardiomyopathy,\(^{12,20}\) as well as various experimental animal models.\(^{21,22}\) In addition, several studies of myocardial samples from HCM patients after myectomy have suggested that functional abnormalities in Ca\(^{2+}\)-handling proteins, including SERCA2, might be involved in abnormal intracellular Ca\(^{2+}\) handling and thus, in impaired contractile performance in HCM patients.\(^{23-25}\) SERCA2 is clearly recognized as a major determinant of myocardial contractility.\(^{26}\) In a recent study in genetically engineered mice models, Kadambi et al\(^{22}\) implicated the ratio of SERCA2 to phospholamban as a contributing factor in determining the frequency response of cardiac muscle. Although our measurements of mRNA do not directly reflect the Ca\(^{2+}\) uptake function, our results suggest that decreased expression of SERCA2 and reduced ratio of SERCA2 to phospholamban may lead to a reduced SERCA2 function and could be responsible for the impaired FFR in patients with severe HCM.

Mechanical factors, such as reduced preload and asynchronous atrial contraction, may also contribute to the descending limb of the FFR. LV dP/dt\(_{\text{max}}\) increased progressively in group A, however, despite a similar HR-induced fall in LV end-diastolic pressure in group B. These findings argue against a major contribution of the reduced preload to produce the descending limb of the FFR.

**TABLE 4. Relative Expression Levels of Ca\(^{2+}\)-Handling Protein mRNAs in Ventricular Tissue From Controls and HCM patients**

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Control (n=7)</th>
<th>Group A (n=7)</th>
<th>Group B (n=7)</th>
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<tr>
<td>SERCA2/GAPDH</td>
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<td>0.72±0.27</td>
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<td>Phospholamban/GAPDH</td>
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<td>1.18±0.47</td>
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<td>SERCA2/Phospholamban</td>
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<td>0.65±0.22</td>
<td>0.29±0.11†</td>
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<td>Ryndoline receptor-2/GAPDH</td>
<td>1.07±0.63</td>
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<td>Calsequestrin/GAPDH</td>
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<td>0.53±0.25</td>
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<tr>
<td>Na(^{+})/Ca(^{2+}) exchanger/GAPDH</td>
<td>1.03±0.26</td>
<td>0.90±0.43</td>
<td>0.81±0.32</td>
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*P<0.05 vs control. †P<0.05 vs group A.
Our recent study demonstrated that a biphasic FFR exists at physiological pacing rates in patients with more severe hypertensive LVH and concluded that the biphasic FFR at physiological pacing rates may be one of the earliest markers of the transition from physiological adaptation to the pathological process in LVH patients. In the present study, we again observed similar biphasic FFR in HCM patients, suggesting that a biphasic FFR might be a common phenomenon in patients with severe LVH.

Because SERCA2 activity determines the rate of Ca\(^{2+}\) sequestration from the cytoplasm into the SR, it directly affects the speed of myocardial relaxation. Our findings may suggest, however, that the relaxation-frequency relation is not dependent simply on the rate of relaxation measured at steady state. In fact, \(T_{1/2}\), which was prolonged at rest in HCM, shortened progressively in both HCM and control subjects. Similar findings were observed previously by Liu et al.\(^6\) in patients with LVH. The mechanism of the preserved relaxation-frequency relation despite reduced SERCA2 gene expression in patients with severe HCM is not clear. Pressure decay is influenced by factors other than Ca\(^{2+}\) reuptake, however, such as passive elastic recoil and systolic loading. These factors might counter others related to active Ca\(^{2+}\) cycling. Further studies will be necessary to clarify the precise mechanism involved in this issue.

In conclusion, we found that a biphasic FFR was observed at physiological pacing rates in HCM patients with severe LVH in the absence of baseline LV dysfunction. Myocardial gene expression of SERCA2 was reduced in HCM patients who showed this biphasic FFR. These findings suggest that reduced expression of SERCA2 may play a principal role in the pathogenesis of the biphasic FFR in HCM patients.

References


Figure 3. Results of RT-PCR analysis of SERCA2 and its regulatory protein, phospholamban, mRNA levels in endomyocardial biopsy samples. SERCA2 mRNA expression and ratio of SERCA2 to phospholamban mRNA were significantly reduced in group B compared with group A and control subjects. *P<0.05 vs control; †P<0.05 vs group A.
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