Patients With End-Stage Congestive Heart Failure Treated With β-Adrenergic Receptor Antagonists Have Improved Ventricular Myocyte Calcium Regulatory Protein Abundance

Hajime Kubo, PhD; Kenneth B. Margulies, MD; Valentino Piacentino III, BA; John P. Gaughan, PhD; Steven R. Houser, PhD

Background—Alterations in Ca\(^{2+}\)-handling proteins are thought to underlie the deranged Ca\(^{2+}\) transients that contribute to deterioration of cardiac function in congestive heart failure (CHF). Clinical trials in CHF patients have shown that treatment with β-adrenergic receptor antagonists (βB) improves cardiac performance. The present study determined whether the abundance of Ca\(^{2+}\)-handling proteins is different in failing hearts from patients treated or untreated with βB.

Methods and Results—Ca\(^{2+}\) regulatory protein abundance was compared in LV myocardium of 10 nonfailing hearts (NF group) and 44 failing hearts (CHF group) removed at transplantation. Analysis was performed in βB-treated (βB-CHF) and non–βB treated (non–βB-CHF) patients and in 4 subgroups: ischemic cardiomyopathy (ICM, n = 10), nonischemic dilated cardiomyopathy (DCM, n = 10), ICM with βB therapy (βB-ICM, n = 12), and DCM with βB therapy (βB-DCM, n = 12). Sarcoplasmic reticulum Ca\(^{2+}\) ATPase, phospholamban, and Na\(^+\)-Ca\(^{2+}\) exchanger protein abundance were determined by use of Western blot analysis. Ca\(^{2+}\) transients were measured with fluo-3. Sarcoplasmic reticulum Ca\(^{2+}\) ATPase was significantly less abundant whereas phospholamban and Na\(^+\)-Ca\(^{2+}\) exchanger were not significantly altered in non–βB-CHF versus NF. Sarcoplasmic reticulum Ca\(^{2+}\) ATPase in the βB-ICM and βB-DCM was greater than in non–βB-CHF and were not different than in NF. Ca\(^{2+}\) transients in non–βB-CHF myocytes had significantly smaller peaks and were prolonged versus NF myocytes. Ca\(^{2+}\) transients from βB-CHF myocytes had shorter durations than in βB-CHF myocytes.

Conclusions—βB treatment in CHF patients can normalize the abundance of myocyte Ca\(^{2+}\) regulatory proteins and improve Ca\(^{2+}\)-handling. (Circulation. 2001;104:1012-1018.)

Key Words: heart failure • sarcoplasmic reticulum • Ca\(^{2+}\)-transporting ATPase • sodium-calcium exchanger • proteins, calcium regulatory

Progressive deterioration of cardiac pump function is a central feature of congestive heart failure (CHF). Depression of myocyte contractility is thought to underlie much of the poor pump performance of the failing human heart. A recent study has shown that treatment of CHF patients with β-adrenergic receptor antagonists (β-blockers [βB]) improves cardiac function and ventricular geometry and thereby slows (or in some cases may reverse) progression of CHF. Cellular and molecular bases for these βB effects are not well understood. Our working hypothesis is that βB slow (or reverse) progression of myocyte contractile defects in failing human heart.

Abnormal Ca\(^{2+}\) homeostasis appears to be primarily responsible for depression of myocyte contractility in CHF. Peak systolic Ca\(^{2+}\) is smaller, rate of decay of the Ca\(^{2+}\) transient is slower, and diastolic Ca\(^{2+}\) can be elevated in failing versus normal ventricular myocytes, especially at faster heart rates. These observations support the hypothesis that alterations in abundance or activity of molecules that regulate systolic and diastolic Ca\(^{2+}\) are centrally involved in depressed contractility of failing heart.

Although the idea that abnormal Ca\(^{2+}\) regulation contributes to depressed cardiac performance in human CHF is well established, cellular and molecular bases of aberrant Ca\(^{2+}\) homeostasis have not been identified clearly. The most frequently studied Ca\(^{2+}\)-regulatory molecules have been sarcoplasmic reticulum (SR) Ca\(^{2+}\) ATPase (SERCA), SERCA regulatory protein phospholamban (PLB), sarcolemmal Na\(^+\)/Ca\(^{2+}\) exchanger (NCX), sarcolemmal L-type Ca\(^{2+}\) channels, and SR Ca\(^{2+}\) release channel (ryanodine receptor). Changes in

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the abundance or function of these molecules in human heart failure have been variable in previous studies. In this regard, SERCA mRNA and protein abundance have been reported to be smaller in failing versus nonfailing human ventricular muscle in some studies, whereas others have found no significant differences.

The present research examined the hypothesis that chronic βB therapy in CHF patients improves myocyte Ca\(^{2+}\) regulation and contractility by normalizing expression of myocyte Ca\(^{2+}\) regulatory proteins. The aim of the present research was to measure and to compare the protein abundance of SERCA, PLB, and NCX in nonfailing and failing human ventricular muscle (obtained at time of cardiac transplantation) from patients that either were or were not being treated with βB.

### Methods

#### Patients and Tissue Preparation

Protein was isolated from transmural LV myocardium from 10 nonfailing (NF group) unused donor hearts and 44 failing hearts. A total of 20 CHF patients were not (non-βB-CHF) and 24 were receiving βB therapy (βB-CHF). Ten patients had ischemic dilated cardiomyopathy (ICM) and were not receiving βB therapy (ICM group); 12 had ICM and were receiving βB therapy (βB-ICM group). Ten had nonischemic dilated cardiomyopathies (DCM) and were not receiving βB therapy (DCM group); 12 had DCM and were receiving βB therapy (βB-DCM group).

#### Protein Assay

Tissue was homogenized in PBS lysis buffer containing 2% SDS (Fisher Biotech), 1% Igepal CA-630 (Sigma Chemical Co), 0.5% deoxycholate (Sigma), 5 mM EDTA, and proteinase inhibitors (10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, 5 μg/mL pepstatin A, 8 μg/mL calpain inhibitor I and II, and 200 μg/mL benzamidine). Supernatant was assayed for total protein, mixed with SDS sample buffer (2% SDS, 0.1 mol/L tris-Cl (pH 6.8), 20% glycerol, 100 mM/L DTT, and 0.02% bromphenol blue) and stored at −80°C. Studies that used isolated human myocytes and nonmyocytes used identical techniques.

#### Western Blot Analysis

Total protein was separated by SDS-PAGE in a buffer that contained 25 mM/L tris-base, 190 mM/L glycine, and 0.1% SDS and transferred to nitrocellulose in a buffer that contained 25 mM/L tris-base, 190 mM/L glycine, and 20% methanol. Nonspecific binding was blocked with TBST buffer (0.1% Tween 20, 137 mM/L NaCl, 2.7 mM/L KCl, and 25 mM/L tris base, pH 7.6) that contained 5% nonfat milk. Targeted antigens were probed with monoclonal antibodies; SERCA (gift from Dr Kevin P. Campbell, University of Iowa, Iowa City), PLB (Upstate Biotechnology), NCX (Swant), cardiac α-sarcomeric actin (Sigma Immuno Chemicals), and GAPDH (Biogenesis). Antibodies were labeled with horseradish peroxidase by anti-mouse Ig secondary antibody, 1:2000 dilution (Amersham Life Science). Targeted antibodies were visualized with enhanced chemiluminescence assay (NEL Life Science). Cellular specificity of all antibodies was tested in LV tissue, isolated myocytes, and isolated ventricular nonmyocytes. SERCA, PLB, NCX, and cardiac actin could not be detected in nonmyocytes (data not shown). GAPDH was observed in every sample.

Films were scanned (Epson Expression 636), and band intensities were quantified with densitometric analysis by use of the NIH Image 1.62f program. Targeted bands were normalized to cardiac actin and GAPDH.

#### Intracellular Ca\(^{2+}\) Measurements

Fluo-3 was used to measure changes in cytosolic free Ca\(^{2+}\) (37°C) induced by 500-ms voltage-clamp steps through patch pipettes as described previously.

### Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>Age, y (n)</th>
<th>HW, g</th>
<th>BW, kg</th>
<th>HW/BW</th>
<th>CHF Duration, mo</th>
<th>HR, bpm</th>
<th>EF, %</th>
<th>CI, L ⋅ m (^{-2}) ⋅ min (^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF</td>
<td>56.3 ± 4.4 (10)</td>
<td>340 ± 38 (8)</td>
<td>68.0 ± 4.3 (10)</td>
<td>4.90 ± 0.4 (8)</td>
<td>NA</td>
<td>89 ± 15 (5)</td>
<td>55.0 ± 6.5 (5)</td>
<td>NA</td>
</tr>
<tr>
<td>ICM</td>
<td>56.4 ± 3.7 (10)</td>
<td>473 ± 32 (10)</td>
<td>68.8 ± 3.3 (10)</td>
<td>6.90 ± 0.4 (10)</td>
<td>29.6 ± 10.5 (10)</td>
<td>90 ± 7 (8)</td>
<td>17.8 ± 2.4* (10)</td>
<td>2.6 ± 0.2 (10)</td>
</tr>
<tr>
<td>βB-ICM</td>
<td>57.8 ± 1.6 (12)</td>
<td>526 ± 27.7 (12)</td>
<td>86.9 ± 3.1† (12)</td>
<td>6.13 ± 0.4 (12)</td>
<td>27.0 ± 8.3 (12)</td>
<td>71 ± 5 (8)</td>
<td>22.9 ± 5.0‡ (12)</td>
<td>2.4 ± 0.2 (12)</td>
</tr>
<tr>
<td>DCM</td>
<td>48.6 ± 4.4 (10)</td>
<td>582 ± 55.5 (10)</td>
<td>77.3 ± 5.1 (10)</td>
<td>7.60 ± 0.6* (10)</td>
<td>31.1 ± 6.5 (10)</td>
<td>89 ± 6 (8)</td>
<td>8.3 ± 0.8* (10)</td>
<td>2.4 ± 0.2 (10)</td>
</tr>
<tr>
<td>βB-DCM</td>
<td>50.0 ± 4.9 (12)</td>
<td>493 ± 41.2 (12)</td>
<td>81.5 ± 4.9 (12)</td>
<td>6.16 ± 0.5 (12)</td>
<td>81.1 ± 19.2‡§ (12)</td>
<td>82 ± 9 (8)</td>
<td>12.7 ± 1.4* (12)</td>
<td>2.6 ± 0.2 (11)</td>
</tr>
</tbody>
</table>

HR indicates heart rate; CI, cardiac index. Values in parentheses indicate No. of patients.

*, †, ‡, and § indicate significant difference from NF, ICM, DCM, and βB-ICM group mean, respectively.

### Table 2. Medications

<table>
<thead>
<tr>
<th>Group</th>
<th>Medication, group (n)</th>
<th>Mean Dosage, mg/d</th>
<th>Mean Duration, mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF</td>
<td>Dobutamine (8)</td>
<td>1.2 ± 0.3</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>ICM</td>
<td>Metoprolol (12)</td>
<td>2.5 ± 0.5</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>DCM</td>
<td>Metoprolol (12)</td>
<td>2.5 ± 0.5</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>βB-ICM</td>
<td>Metoprolol (12)</td>
<td>2.5 ± 0.5</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>βB-DCM</td>
<td>Metoprolol (12)</td>
<td>2.5 ± 0.5</td>
<td>2.5 ± 0.5</td>
</tr>
</tbody>
</table>

DOB indicates Dobutamine; MIL, Milrinone; ACE, angiotensin-converting enzyme inhibitor; AIIIB, angiotensin II receptor blocker; CaB, calcium channel blocker; NIT, nitrate; HYD, hydralazine; DIG, digoxin; DIU, diuretic; and AMI, amiodarone.
Analysis of NF versus CHF samples was with an unpaired 2-tailed Student t test. Follow-up subgroup analyses (3 group, 5 group) was by ANOVA with pairwise analysis. A Bonferroni multiple comparison was used to locate significant differences among groups. A value of $P < 0.05$ was set for statistical significance.

**Results**

**Patient Characteristics**

CHF duration was greatest in $\beta B$-DCM patients (see Table 1). Heart weight/body weight ratio (HW/BW) was significantly greater in CHF ($n=44$) than NF ($n=10$; $6.7 \pm 0.25$ versus $4.9 \pm 0.4$; $P<0.01$). When patients were sorted by disease and treatment, HW/BW was greater in non-$\beta B$-treated CHF patients than in those with NF hearts. HW/BW of $\beta B$-treated CHF hearts were also greater than in NF hearts and less than in non-$\beta B$-treated CHF hearts. However, these differences were not significant. Ejection fraction (EF) was significantly smaller in all CHF groups versus NF patients.

Average $\beta B$ dosages and other medications are listed in Table 2. Of the CHF patients, $>90\%$ required intravenous inotropic agents at time of transplantation, and 5 of 10 NF patients were receiving dopamine for blood pressure support.

**SERCA, PLB, and NCX in NF Versus CHF LV Samples**

Ca$^{2+}$ regulatory protein abundance was first compared in NF ($n=10$) versus all CHF ($n=44$) samples (Figure 1). Substantial patient-to-patient variability occurred in abundance of SERCA, PLB, and NCX within the CHF group. Despite this variability, SERCA (SERCA/actin) was significantly less abundant in CHF versus NF muscles (Figure 2A), whereas PLB (Figure 2B) and NCX (Figure 2C) were not significantly different.

PLB interacts with SERCA and inhibits its Ca$^{2+}$ transport rate. Phosphorylation of PLB relieves this inhibition. The relative abundance of these 2 proteins and level of PLB phosphorylation influences Ca$^{2+}$ homeostasis and cardiac myocyte contractility. Therefore, SERCA/PLB ratio should reflect Ca$^{2+}$ transport capacity of the SR. SERCA/PLB was significantly smaller in CHF versus NF samples (Figure 2D).

SERCA and NCX compete for cytoplasmic Ca$^{2+}$ during the Ca$^{2+}$ transient and a high SERCA/NCX activity is associated with rapid decay of the Ca$^{2+}$ transient. Decreases in SERCA/NCX activity should reduce the size and slow the decay rate of the Ca$^{2+}$ transient. SERCA/NCX protein abundance of CHF samples was smaller but not significantly different from NF samples (Figure 2E).

CHF samples were grouped with respect to absence or presence of $\beta B$ treatment and compared with NF samples. Representative data are shown in Figures 3A and 3B and averages in Figure 4. SERCA was significantly less abundant in non-$\beta B$-CHF than in NF but there was no difference
Significantly smaller in non-
NF and samples, and no significant differences were seen between NF and non-
NF muscles (Figure 5A). Among DCM patients who received 
B treatment was less than but not significantly differ-
CHF versus NF myocytes (Figure 6). Ca
transients in failing human ventricular myocytes through the NCX.17 Therefore, the faster rate of Ca
transient decay in 
B versus non-
B CHF myocytes probably reflects enhanced SR Ca
transport.

**Discussion**

The present data support the hypothesis that 
B treatment of CHF patients can improve myocyte Ca
handling by normalizing the abundance of Ca
-regulatory proteins. Major observations are (1) that SERCA protein was significantly less abundant in CHF patients not receiving 
B than in NF patients, (2) that SERCA protein abundance was significantly greater in CHF patients treated with 
B than in CHF patients not receiving 
B, (3) that SERCA/PLB and SERCA/NCX were significantly smaller in non-
B CHF versus NF and 
B CHF versus non-
B CHF patients, (4) that 
Ca
transients were smaller and decayed more slowly in non-
B CHF versus NF myocytes, and (5) that 
Ca
transients in 
B CHF myocytes had significantly faster rates of decay than non-
B CHF myocytes. These results strongly support the idea that deranged 
Ca
homeostasis in human CHF involves alterations in abundance and activity of SR 
Ca
transport proteins and suggests that some of these changes can be reversed with 
B therapy. Our observations also suggest a potential mechanism (improved myocyte Ca
regulation) for the consistent yet unexplained finding that 
B improve LV function in failing human hearts.24

**Ca
-Handling Protein Abundance and 
Ca
Transients in CHF**

Previous studies7–14 have shown marked variability in the abundance of SERCA, PLB, and NCX proteins among patients with CHF. Our findings of more uniform results when 
B treatment is taken into account suggest that some of this variability is related to treatment effects superimposed on disease-related alterations.

Reduction in SERCA/PLB and SERCA/NCX in CHF myocytes can explain the reduced peak and slower decay of

| Protein | NF | ICM | 
|---------|----|-----|---|
| NCX     | 120| 120 | 120|
| SERCA   | 100| 100 | 100|
| Actin   | 45 | 45  | 45 |
| GAPDH   | 38 | 38  | 38 |
| PLB Pentamer | 25 | 25 | 25 |
| PLB Monomer | 6.9| 6.9| 6.9|

**Figure 3.** Representative Western blot of samples (A) from NF, non-
B-ICM, and 
B-ICM patients and (B) from NF, non-
B-DCM and 
B-DCM patients. NCX, SERCA, PLB pentamer and monomer, GAPDH, and cardiac-specific actin are shown.

Ratios of SERCA/PLB and SERCA/NCX protein abundance were significantly smaller in ICM and DCM CHF patients versus NF hearts (Figures 5D and 5E). SERCA/PLB and SERCA/NCX protein abundance in 
B-treated patients was significantly greater than in disease-matched non-
B patients and was not significantly different than in NF hearts (Figures 5D and 5E). Actin/total protein abundance was also significantly smaller in ICM versus NF (see Figure 3).
the Ca\textsuperscript{2+} transient we observed in failing myocytes. Partial normalization of Ca\textsuperscript{2+} regulatory protein abundance in βB-CHF myocytes can also explain the improved rate of decay of their Ca\textsuperscript{2+} transients. These findings support the hypothesis that the reduced abundance of SERCA protein disrupts the balance of Ca\textsuperscript{2+} transport between SERCA and NCX to produce defective Ca\textsuperscript{2+} homeostasis, with an increased reliance on NCX in failing human ventricular myocytes.

How Do βB Work in CHF Patients?
Heightened sympathetic nerve activity in CHF helps maintain cardiac output and mean blood pressure in the face of intrinsic defects in cardiac function. However, sustained sympathetic activity in CHF is thought to produce abnormalities in adrenergic signaling and cell physiology (including abnormal Ca\textsuperscript{2+} handling). The bases of these adrenergic cascade–related myocyte abnormalities are largely unknown. Similarly, although chronic βB agents are effective in the...
treatment of heart failure, cellular mechanisms for their beneficial effects on cardiac function likewise are unexplained. For example, chronic treatment with carvedilol is particularly effective for improvement of LV EF among CHF patients, but this agent does not produce the alterations in β-adrenergic receptor density observed with other βB, which implies modifications in myocyte phenotype at a level beyond the adrenergic receptor.

Our present study design was inadequate to prove that βB cause beneficial changes in cardiocyte Ca²⁺ regulatory gene expression. However, our findings support a link between normalization of Ca²⁺ homeostasis and βB treatment in CHF patients. We cannot rule out the possibility that the pattern of Ca²⁺ regulatory gene expression in NYHA class IV CHF patients is highly variable and that only those patients with high SERCA/PLB and SERCA/NCX tolerate βB therapies. Arguing against such a selection bias is the fact that βB-associated increases in SERCA/PLB ratio did not correlate with EF measured before transplantation. In addition, a recent study by Gwathmey et al, in which 1 month of treatment with carvedilol increased SERCA activity and cardiac function in myopathic turkey hearts, supports a causal link between βB therapy and expression of Ca²⁺ regulatory proteins.

We can only speculate about a mechanism for normalization of SERCA protein abundance produced by βB treatment. This mechanism could involve elimination of β-adrenergic receptor–mediated repression of myocyte SERCA expression. βB also could reduce apoptosis, thereby increasing the number of functional myocytes. Still another possibility is that βB could be working through nonmyocytes to eliminate expression of factors that depress myocyte function by decreasing SERCA expression.

Why Are βB-Treated Hearts Still Failing?

Our results suggest that improved heart function in βB clinical trials involves normalization of aberrant Ca²⁺ regulatory protein abundance. We speculate that in early CHF, the beneficial effects of βB on Ca²⁺-handling proteins mediate increased EF and promote beneficial remodeling. However, our data also suggest that CHF still can progress to the point at which transplantation is required in βB-treated patients with normalized (or partially normalized) myocyte Ca²⁺ handling.

Potential explanations as to why patients with βB treatment still required transplantation are as diverse as the mechanisms contributing to the syndrome of advanced CHF. Loss of myocytes through necrosis or apoptosis may overwhelm improvements in Ca²⁺ handling. Similarly, ongoing defects in cardiac perfusion or life-threatening arrhythmias may necessitate transplantation despite improvements in contractile function of remnant myocytes. Within the myocyte, abnormalities of electrophysiology, ultrastructure, metabolism, signaling, phosphorylation, or myofilament properties may persist despite improvements in abundance of dysregulated Ca²⁺-handling proteins. Within and beyond the heart, detrimental neurohormone and cytokine activation, vasoconstriction, and other peripheral abnormalities may drive progression of the syndrome.

Limitations

We could not control the types or concentrations of the medications that the patients were receiving. In this regard, 22 of 24 βB-CHF patients were receiving either metoprolol or carvedilol. Interestingly, normalization of Ca²⁺ regulatory protein abundance was similar in all βB-treated patients, which suggests a common mechanism. Importantly, we were unable to find a correlation between SERCA protein abundance and any other medication, including drugs that interfere with angiotensin signaling. Finally, intravenous inotropic agents, particularly milrinone (in 12 of 24 patients) did not preclude the normalized pattern of SERCA abundance in βB-treated patients.

Summary and Conclusions

CHF patients (NYHA class IV) not treated with βB had less SERCA protein than did those with NF hearts, and their Ca²⁺
transients were abnormal. In CHF patients treated with ββ, SERCA abundance was not different than in the NF hearts, and their Ca\(^{2+}\) transients were improved. These findings suggest that ββ-CHF therapy may cause improved cardiac performance by normalizing expression of Ca\(^{2+}\) regulatory proteins. Our observations also suggest that conventional pharmacotherapy may produce the “molecular remodeling” currently being sought through gene therapy.\(^{12,32,33}\) Potential for long-term improvements in Ca\(^{2+}\) homeostasis in patients with NYHA class IV symptoms of heart failure also supports the use of novel adjuvants, including phosphodiesterase inhibitors\(^{34}\) or mechanical circulatory support\(^{35}\) to help establish ββ therapy in these clinically tenuous patients.

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**References**

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