

Clinical Investigation and Reports

Ca\textsuperscript{2+}-Transporting ATPase, Phospholamban, and Calsequestrin Levels in Nonfailing and Failing Human Myocardium

Matthew A. Movsesian, MD; Mohsen Karimi, BS; Karen Green, BS; Larry R. Jones, MD, PhD

Background Observations of abnormalities in the diastolic components of intracellular Ca\textsuperscript{2+} transients in failing human left ventricular myocardium have raised the possibility that reductions in the level or function of sarcoplasmic reticulum proteins involved in Ca\textsuperscript{2+} transport contribute to the pathophysiology of dilated cardiomyopathy in humans. Functional assays, however, have revealed no differences in ATP-dependent Ca\textsuperscript{2+} transport or its modulation by phospholamban in sarcoplasmic reticulum–enriched microsomes prepared from nonfailing and failing human left ventricular myocardium. The purpose of the present study was to quantify protein levels of Ca\textsuperscript{2+}-transporting ATPase, phospholamban, and calsequestrin directly in nonfailing and failing human left ventricular myocardium.

Methods and Results Total protein extracts were prepared from nonfailing left ventricular myocardium from the hearts of unmatched organ donors with normal left ventricular contractility (n=6) and from failing left ventricular myocardium from the excised hearts of transplant recipients with class IV heart failure resulting from idiopathic dilated cardiomyopathy (n=6). Ca\textsuperscript{2+}-transporting ATPase, phospholamban, and calsequestrin contents were determined by quantitative immunoblotting with monoclonal and affinity-purified polyclonal antibodies. The levels of the three proteins were identical in nonfailing and failing human left ventricular myocardium.

Conclusions These results indicate that protein levels of Ca\textsuperscript{2+}-transporting ATPase, phospholamban, and calsequestrin are not diminished in failing human left ventricular myocardium and that downregulation of the Ca\textsuperscript{2+}-transporting ATPase and phospholamban is not part of the molecular pathophysiology of dilated cardiomyopathy in humans. (Circulation. 1994;90:653-657.)

Key Words • cardiomyopathy • Ca\textsuperscript{2+} ATPase • phospholamban • calsequestrin

Observations of abnormalities in the diastolic components of intracellular Ca\textsuperscript{2+} transients in failing human left ventricular myocardium have led to the hypothesis that reductions in the level or function of sarcoplasmic reticulum proteins involved in Ca\textsuperscript{2+} transport contribute to the pathophysiology of dilated cardiomyopathy in humans. Reports of diminished steady-state mRNA levels for the Ca\textsuperscript{2+}-transporting ATPase and phospholamban in homogenates of failing human left ventricular myocardium have been interpreted as being consistent with this hypothesis. In studies of sarcoplasmic reticulum–enriched microsomes prepared from nonfailing and failing human left ventricular myocardium, however, no quantitative differences between the two groups were identified with respect to ATP-dependent Ca\textsuperscript{2+} transport or its modulation by phospholamban.

The latter studies had two limitations. First, the experiments were performed in microsomes prepared from nonfailing and failing left ventricular myocardium by homogenization and differential sedimentation. These procedures might have resulted in the selective recovery of nonpathological sarcoplasmic reticulum from failing myocardium, and the detection of changes in Ca\textsuperscript{2+} transport and its modulation by phospholamban phosphorylation might have been obscured as a result. Second, the experiments were able to provide only functional information on the Ca\textsuperscript{2+}-transporting ATPase and phospholamban, and neither Ca\textsuperscript{2+}-transporting ATPase nor phospholamban protein levels have been quantified directly in nonfailing and failing human myocardium. The recent availability of monoclonal and affinity-purified polyclonal antibodies directed against myocardial sarcoplasmic reticulum proteins has made it possible to circumvent these limitations. In the present study, we used these antibodies to quantify Ca\textsuperscript{2+}-transporting ATPase and phospholamban in total (ie, nonselective) protein extracts of nonfailing and failing human left ventricular myocardium. For comparative purposes, we also used these antibodies to quantify calsequestrin, whose mRNA levels have been reported to be similar in nonfailing and failing human myocardium.

Methods

Source and Characteristics of Nonfailing and Failing Human Left Ventricular Myocardium

Nonfailing left ventricular myocardium was obtained from six unmatched organ donors with normal left ventricular function. Failing left ventricular myocardium was obtained from the excised hearts of six transplant recipients with class IV heart failure resulting from idiopathic dilated cardiomyop-
Characteristics of Nonfailing and Failing Left Ventricles and Left Ventricular Myocardium

<table>
<thead>
<tr>
<th>Nonfailing (n=6)</th>
<th>Failing (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>42.8±6.2</td>
</tr>
<tr>
<td>Ejection Fraction, %</td>
<td>58±7</td>
</tr>
<tr>
<td>β-Adrenergic Receptor Density, fmol/mg</td>
<td>97.6±15.1</td>
</tr>
</tbody>
</table>

Values are given as mean±SEM.
*P<.001 vs nonfailing, †P=.006 vs nonfailing (unpaired t test).

Preparation of Protein Extracts of Left Ventricular Myocardium

Myocardium from the left ventricular free walls of nonfailing and failing hearts was trimmed of endocardium and epicardium and cut into 0.5×0.5-cm² pieces immediately after removal from organ donors and transplant recipients. Samples of 0.3 to 0.7 g were homogenized at 4°C in a Polytron PT 3000 at 22,000 rpm for 90 seconds in 4 vol (w/v) of 10 mmol/L NaHCO₃. Then, 300 μL of each homogenate was added to 600 μL of 20% sodium dodecyl sulfate (SDS). Mixtures were incubated at 25°C for 20 minutes before sedimentation for 5 minutes at 100,000 rpm in a Beckman TL-100.2 rotor. Supernatants were collected and assayed for protein by the method of Lowry et al.¹³

Quantitative Immunoblotting of Ca²⁺-Transporting ATPase, Phospholamban, and Calsequestrin

SDS–polyacrylamide gel electrophoresis (PAGE) was performed by the method of Porzio and Pearson¹⁴ using 8% polyacrylamide gels. Samples were transferred to 0.2-μm pore size nitrocellulose membranes (Schleicher and Schuell) by electrophoresis at 3.0 amp for 90 minutes in 50 mmol/L NaH₂PO₄ buffer (pH 7.5).¹⁵ Nitrocellulose membranes were stained with Amido black and cut into horizontal strips corresponding to the mobility regions of the Ca²⁺-transporting ATPase, high and low molecular weight forms of phospholamban, and calsequestrin. Immunoblotting was performed using a 1:500 dilution of ascites fluid containing monoclonal antibody 2A7-A1, which reacts exclusively with the SERCA2 (cardiac and slow twitch skeletal muscle) isoform of the Ca²⁺-transporting ATPase (L.R.J., unpublished data); a 1:500 dilution of ascites fluid containing monoclonal antibody 2D12, which reacts with phospholamban;¹⁰,¹¹ and polyclonal rabbit antibodies to canine cardiac calsequestrin that were affinity purified by incubation with canine cardiac calsequestrin bound to nitrocellulose membranes followed by acid extraction.¹²,¹⁶ After blocking with 2% bovine serum albumin, strips were incubated with the appropriate antibodies and then with ¹²⁵I-labeled protein A (New England Nuclear).¹⁰ Protein bands identified by autoradiography were excised, and bound radioactivity was quantified by gamma counting. Background counts, which were ≤10% of total counts for each band, were subtracted from all measurements. Values for Ca²⁺-transporting ATPase, phospholamban, and calsequestrin in nonfailing and failing hearts were treated as continuous variables and compared with the use of an unpaired t test.

Results

As noted, studies in which the steady-state kinetics of sarcoplasmic reticulum Ca²⁺ transport and its modulation by phospholamban phosphorylation were found to be identical in nonfailing and failing human left ventricular myocardium were performed in microsomes prepared by homogenization and differential sedimentation, which could have resulted in the selective recovery of nonpathological sarcoplasmic reticulum from failing myocardium.⁷⁻⁹ For this reason, we chose to quantify Ca²⁺-transporting ATPase, phospholamban, and calsequestrin content in total protein extracts of nonfailing (n=6) and failing (n=6) human left ventricular myocardium. Addition of 13% SDS to tissue homogenates resulted in the solubilization of virtually all of the myocardial protein (removal of the minor amount of cellular debris was achieved by sedimentation). The recovery of protein per wet weight of tissue (mean±SEM) was 124±7 mg/g in nonfailing myocardium and 124±6 mg/g in failing myocardium, indicating that comparable amounts of protein were extracted from nonfailing and failing tissue.

Equal amounts of protein extract from each sample were subjected to SDS-PAGE, electrophoretic transfer to nitrocellulose membranes, and quantitative immunoblotting with monoclonal and affinity-purified polyclonal antibodies to Ca²⁺-transporting ATPase, phospholamban, and calsequestrin.¹⁰-¹² Bound antibody was quantified by incubating immunoblots with ¹²⁵I-labeled protein A, excising the appropriate mobility bands, and measuring bound radioactivity (Fig 1). To ensure the validity of this methodology, aliquots of each sample were applied at amounts that were twice the amounts applied for the other samples (Fig 1, lanes 12 through 14). Bound radioactivity varied linearly with protein over this range (Fig 2).

The quantitation of Ca²⁺-transporting ATPase, phospholamban, and calsequestrin in protein extracts of nonfailing and failing human left ventricular myocardium is represented in Fig 3. There were no differences between nonfailing and failing left ventricular myocardium with respect to the levels of any of these proteins. The results in Fig 3 are derived from analysis of 60-μg aliquots; identical results were obtained by analysis of 100- and 200-μg protein aliquots (data not shown).

Discussion

Previous observations that the activity of the Ca²⁺-transporting ATPase and its modulation by phospholamban are quantitatively similar in sarcoplasmic reticulum–enriched microsomes prepared from nonfailing and failing human left ventricular myocardium suggested that abnormalities in the function of these proteins do not contribute to the molecular pathophysiology of dilated cardiomyopathy in humans.⁷⁻⁹ The results of the present experiments, in which protein levels of Ca²⁺-transporting ATPase, phospholamban, and calsequestrin were quantified directly and found to be comparable in nonfailing and failing human left ventricular
myocardium, are further evidence in support of this conclusion.

Although the protein levels of Ca\(^{2+}\)-transporting ATPase and phospholamban are not decreased in failing human left ventricular myocardium, the steady-state levels of their corresponding mRNAs have been reported to be decreased in this tissue.\(^2\)-\(^6\) It appeared reasonable, based on these earlier observations, to predict that protein levels of the Ca\(^{2+}\)-transporting ATPase and phospholamban in failing human myocardium would be diminished in parallel with their mRNA levels. This prediction, however, requires the assumption that protein synthesis and degradation rates are comparable in nonfailing and failing human myocardium, and experimental evidence to the contrary has
emerged. Magid et al \(^1\) have reported that myocardial protein synthesis rates are increased and myocardial protein degradation rates are decreased 3 days after surgery in rabbits with surgically induced aortic insufficiency and that both protein synthesis and protein degradation rates are decreased by the time myocardial hypertrophy develops 1 month later. Protein synthesis and degradation rates thus may be affected in a complex manner in pathological myocardium. In addition, large differences in the ratio of mRNA levels to protein levels for both the SERCA1 and SERCA2 isoforms of the Ca\(^{2+}\)-transporting ATPase have been observed in striated muscle.\(^{18,19}\) For these reasons, steady-state mRNA levels cannot be assumed to be predictive of protein content in comparisons of nonfailing and failing myocardium. Decreases in Ca\(^{2+}\)-transporting ATPase and phospholamban mRNA levels in failing human myocardium might not be accompanied by decreases in the levels of the cognate proteins because of concomitant decreases in their degradation rates. Alternatively, decreases in Ca\(^{2+}\)-transporting ATPase and phospholamban mRNA levels, which could result from either a decrease in gene transcription rates or an increase in mRNA degradation rates, might reflect compensatory responses by cardiac myocytes to decreased rates of degradation of the cognate proteins. Further clarification will ultimately require quantitation of protein synthesis and degradation rates for individual proteins in nonfailing and failing myocardium.

Reductions in the level and activity of the Ca\(^{2+}\)-transporting ATPase of the sarcoplasmic reticulum and phospholamban have been identified in animal models of myocardial hypertrophy and failure.\(^{20-28}\) In a recent study involving surgically induced right ventricular hypertrophy in mice, Ca\(^{2+}\)-transporting ATPase and phospholamban were quantified immunohistochemically using the same antibodies that were used to quantify these proteins in our experiments.\(^{20}\) The contents of both proteins were found to be diminished in hypertrophic murine right ventricular myocardium. The discrepancies between these findings in animal models and the lack of change in the protein levels or activity of the Ca\(^{2+}\)-transporting ATPase and phospholamban in failing human myocardium suggest that observations made in currently available animal models may be limited in their applicability to dilated cardiomyopathy in humans. The development of animal models of dilated cardiomyopathy that more closely mimic the molecular pathophysiology of the human disease may be helpful in future studies.

In summary, our results indicate that protein levels of Ca\(^{2+}\)-transporting ATPase, phospholamban, and calsequestrin are not diminished in failing human left ventricular myocardium and that downregulation of the Ca\(^{2+}\)-transporting ATPase and phospholamban is not part of the molecular pathophysiology of dilated cardiomyopathy in humans.

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References


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