Adenoviral Gene Transfer of Mutant Phospholamban Rescues Contractile Dysfunction in Failing Rabbit Myocytes With Relatively Preserved SERCA Function

Mark T. Ziolo, Jody L. Martin, Julie Bossuyt, Donald M. Bers, Steven M. Pogwizd

In heart failure (HF), a main factor in reduced contractility is reduced SERCA function and reversed force-frequency response (FFR), ie, from positive to negative. Our arhythmogenic rabbit HF model exhibits decreased contractility mainly due to an increase in Na/Ca exchange (NCX) activity (with only modest decrease in SR Ca\(^{2+}\)-ATPase (SERCA) function), similar to many end-stage HF patients. Here we test whether phospholamban (PLB) inhibition using a dominant-negative mutant PLB adenovirus (K3E/R14E, AdPLB-dn, with β-galactosidase adenovirus as control) could enhance SERCA function and restore Ca\(^{2+}\) transients and positive FFR in ventricular myocytes from these HF rabbits. HF myocytes infected with AdPLB-dn (versus control) had enhanced Ca\(^{2+}\) transient amplitude (2.0±0.1 versus 1.6±0.05 F/F\(_{0}\) at 0.5 Hz, \(P<0.05\)) and had a positive FFR, whereas acutely isolated HF myocytes or those infected with Adβgal had negative FFR. Ca\(^{2+}\) transients declined faster in AdPLB-dn versus Adβgal myocytes (RT50%: 317±29 versus 551±90 ms at 0.5 Hz, \(P<0.05\)) and had an increased SR Ca\(^{2+}\) load (3.5±0.3 versus 2.6±0.2 F/F\(_{0}\) at 0.5 Hz, \(P<0.05\)), indicative of increased SERCA function. Furthermore, this restoration of function was not due to changes in NCX or SERCA expression. Thus, increasing SERCA activity in failing myocytes by AdPLB-dn gene transfer reversed the contractile dysfunction (and restored positive FFR) by increasing SR Ca\(^{2+}\) load. This approach could enhance contractile function in failing hearts of various etiologies, even here where reduced SERCA activity is not the main dysfunction.

Heart failure (HF) is characterized by a reversed force frequency response (FFR), ie, positive to negative. This has been attributed to alterations in the expression and/or function of the sarcoplasmic reticulum (SR) Ca\(^{2+}\) ATPase (SERCA) and reduced SR Ca\(^{2+}\) content.

Transgenic and adenoviral gene transfer approaches to increase SERCA activity, either by increasing SERCA levels or decreasing inhibition by phospholamban (PLB; by PLB knockout, PLB antisense, or a pseudophosphorylated PLB mutant) can prevent the development of HF and increase contractility, although mechanistic information was limited.

Not all HF exhibits decreased SERCA expression and/or function, and Hasenfuss et al showed that nearly half of HF patients exhibit relatively preserved SERCA function but greatly increased Na\(^{+}/Ca\(^{2+}\) exchanger (NCX) function. This is analogous to our nonischemic rabbit HF model, where contractile function and SR Ca\(^{2+}\) load are reduced mainly because of increased NCX function rather than depressed SERCA function or SR Ca\(^{2+}\) leak.

It is unclear whether enhancement of SERCA function would be beneficial in myocytes during chronic HF and where SERCA is not the main dysfunction. Thus, the purpose of this study was to determine whether (and how) increasing SERCA function, via ectopic expression of a PLB dominant-negative mutant, can reverse contractile dysfunction.

Materials and Methods

Rabbit Heart Failure Model and Cardiac Myocyte Isolation

In New Zealand White rabbits, HF was induced by aortic insufficiency and 2 to 4 weeks later by aortic constriction. HF progression was assessed by echocardiography and rabbits were studied 7.1±1.9 months after aortic constriction when LV end-systolic dimension exceeded 1.4 cm. Left ventricular (LV) myocytes were isolated as previously described.

Adenoviral Gene Transfer and Cell Culture

HF myocytes were cultured for adenoviral infection in modified M199 medium for 24 to 36 hours after infection. Mocytes were infected with adenovirus (MOI=100 to 200, 1 to 2 hour infection) encoding either β-galactosidase (Adβgal, served as control) or a dominant-negative PLB mutant [AdPLB-dn, with amino acid substitutions at positions 3 and 14 (K3E/R14E), which inhibits PLB function].

Western Blots

Antibodies for PLB (Badrilla), SERCA (Affinity Bioreagents), and NCX (Affinity Bioreagents) were used and data normalized to sarcomeric α-actin (Sigma).

Measurement of Ca\(^{2+}\) Transients

Myocytes were field-stimulated and superfused with (in mmol/L): 140 NaCl, 4 KCl, 1 MgCl\(_{2}\), 2 CaCl\(_{2}\), 10 glucose, and 5 HEPES (pH=7.4). [Ca\(^{2+}\)]\(_{i}\) was measured by Fluo-3 epifluorescence with excitation at 480 nm and emission at 530 nm, with recorded fluorescence (F) normalized to baseline fluorescence (F\(_{0}\)).

Results

HF Rabbits

HF rabbits exhibited significant depression of LV systolic function; with increases in both LV end-diastolic (by 50%) and end-systolic dimension (by 73%; \(P<0.001\)) and a 27%
decrease in LV fractional shortening ($P<0.01$ versus baseline).

**AdPLB-dn Enhances Ca$^{2+}$ Transients, Force-Frequency Response, and SR Ca$^{2+}$ Load**

Cells exposed to AdPLB-dn (versus Adβgal) showed increased PLB expression and the characteristic$^{13}$ decrease in electrophoretic mobility of pentamers in Western blots (Figure 1A). In addition, infection with AdPLB-dn or Adβgal did not alter expression of either NCX or SERCA2a versus acutely isolated HF myocytes (Figure 1B).

Figure 1C and 1D shows that steady-state Ca$^{2+}$ transient amplitude in HF myocytes was increased by 58% after AdPLB-dn versus Adβgal infection. Notably, Adβgal had similar Ca$^{2+}$ transient amplitude compared with acutely isolated noncultured, noninfected HF myocytes (from the same HF hearts used for adenoviral infection). Figure 1E shows that HF myocytes acutely isolated or infected with Adβgal displayed the negative FFR typical of HF, whereas HF myocytes infected with AdPLB-dn had a positive FFR.

We also assessed whether the enhanced function in AdPLB-dn is due to increased SERCA function and SR Ca$^{2+}$ load (despite unaltered SERCA or NCX expression). The rate constant of [Ca$^{2+}$] decline in myocytes infected with AdPLB-dn was 42% faster than that in Adβgal (Figure 2A), consistent with enhanced SERCA function. Likewise, SR Ca$^{2+}$ content was significantly increased in AdPLB-dn versus Adβgal (Figure 2B).

**Discussion**

HF myocytes typically demonstrate decreased SR Ca$^{2+}$ load, Ca$^{2+}$ transient amplitude, and a negative FFR.$^{1,10,12}$ Although this is frequently attributed to decreased SR Ca$^{2+}$ uptake and SERCA function,$^{2,3}$ reduced SR Ca$^{2+}$ load in HF models and human HF can arise from upregulation of the NCX.$^{9,10}$ Moreover, adenoviral overexpression of NCX in normal rabbit ventricular myocytes decreased SR Ca$^{2+}$ content and Ca$^{2+}$ transients.$^{15}$

In this study, we found that, even after HF development, inhibition of PLB function enhanced Ca$^{2+}$ transient amplitude, rate of [Ca$^{2+}$] decline, and SR Ca$^{2+}$ content and restored a positive FFR in LV HF myocytes (without altered NCX or SERCA expression). This HF model exhibits severely depressed LV and myocyte function and reduced SR Ca$^{2+}$ content, 2-fold upregulation of NCX expression and function, enhanced SR Ca$^{2+}$ leak, and unchanged SERCA expression (but detailed quantitative analysis resolved a 24% decrease in SERCA function).$^{10–12}$ The main cause of depressed SR Ca$^{2+}$ content was found to be the increased NCX function (not altered SERCA or SR Ca$^{2+}$ leak).$^{12}$

**Figure 1.** AdPLB-dn and Ca$^{2+}$ handling in HF myocytes. A, Western blot of HF rabbit myocytes infected with AdPLB-dn or Adβgal (MOI 100 or 200), showing heteropentamer shift due to mutant PLB, as in ref 13. B, Pooled data of NCX and SERCA expression after infection with AdPLBdn or Adβgal (vs acutely isolated HF myocytes). C, Ca$^{2+}$ transients in HF myocytes infected with Adβgal or AdPLBdn (0.5 Hz). D, Pooled data for Ca$^{2+}$ transient amplitude and (E) force-frequency relationship (FFR). Data are mean±SEM. *$P<0.05$ vs acute and Adβgal (n=6 to 10 cells from 4 HF hearts).

**Figure 2.** AdPLBdn: Ca$^{2+}$ transient kinetics and SR Ca content in HF myocytes. A, Half-time of [Ca$^{2+}$] decline during twitches and (B) SR Ca$^{2+}$ load assessed by rapid application of 10 mmol/L caffeine. Pooled data (mean±SEM; *$P<0.05$ vs Adβgal; n=8 to 10 cells from 4 HF hearts).
We show, for the first time in HF myocytes, that PLB inhibition increased SR Ca\textsuperscript{2+} load and restored a positive FFR, which can explain most of the beneficial effects observed by us and others.\textsuperscript{4–8} This indicates that enhanced SERCA function can overcome the NCX upregulation and functional normalization of Ca\textsuperscript{2+} transients.

As in HF myocytes, nonfailing rabbit ventricular myocytes also showed significantly enhanced Ca\textsuperscript{2+} transient amplitude (by 43\%) and rate of [Ca\textsubscript{i}] decline (by 20\%), as previously reported.\textsuperscript{13} Precisely how this dominant-negative PLB effect is mediated is unclear, but it may either sequester endogenous PLB in pentameric form (Figure 1A) relieving SERCA of inhibition, or displace endogenous PLB from SERCA2 (without inhibiting the pump itself). A quantitative limitation is that we cannot assess the effect of AdPLB-dn on endogenous PLB expression because the antibody used recognizes both endogenous and mutant PLB.

SERCA function in HF can be stimulated by either SERCA overexpression or interfering with the inhibition of SERCA by PLB (PLB knockout or by viral gene transfer of antisense PLB RNA or of a pseudophosphorylated form of PLB mutant). These can all improve myocyte and cardiac contractile function and prevent the development or progression of HF, including in some animal models of HF.\textsuperscript{4–8} Our data indicates that we are able to restore contractility even long after the onset of HF. In addition, it may be more advantageous to inhibit PLB function versus SERCA overexpression because too much SERCA may increase cytosolic Ca\textsuperscript{2+} buffering and accelerate [Ca\textsubscript{i}] decline in a manner that limits myofilament activation.\textsuperscript{16} Inhibition of the NCX may also be beneficial,\textsuperscript{17} but excessive NCX inhibition may prevent necessary Ca\textsuperscript{2+} removal from the cell. Our results attest to the potential of PLB as a therapeutic target for the treatment of HF even when reduced SERCA activity is not the major abnormality.

In conclusion, enhanced SERCA function via gene transfer of a dominant-negative PLB mutant reversed the contractile dysfunction in HF myocytes by increasing SR Ca\textsuperscript{2+} load and restoring the positive FFR. This may justify future tests of this rescue approach in vivo.

Acknowledgments
This work was supported by NIH grants HL46929 and HL73966 (S.M.P.) and HL64724 and HL30077 (D.M.B.).

References

Key Words: heart failure ■ gene transfer ■ SERCA ■ phospholamban
Adenoviral Gene Transfer of Mutant Phospholamban Rescues Contractile Dysfunction in Failing Rabbit Myocytes With Relatively Preserved SERCA Function
Mark T. Ziolo, Jody L. Martin, Julie Bossuyt, Donald M. Bers and Steven M. Pogwizd

Circ Res. 2005;96:815-817; originally published online March 24, 2005;
doi: 10.1161/01.RES.0000163981.97262.3b

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/96/8/815

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/