Heart Failure

Targeted Sarcoplasmic Reticulum Ca\(^{2+}\) ATPase 2a Gene Delivery to Restore Electrical Stability in the Failing Heart

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Background—Recently, we reported that sarcoplasmic reticulum Ca\(^{2+}\) ATPase 2a (SERCA2a), the pump responsible for reuptake of cytosolic calcium during diastole, plays a central role in the molecular mechanism of cardiac alternans. Heart failure (HF) is associated with impaired myocardial calcium handling, deficient SERCA2a, and increased susceptibility to cardiac alternans. Therefore, we hypothesized that restoring deficient SERCA2a by gene transfer will significantly reduce arrhythmogenic cardiac alternans in the failing heart.

Methods and Results—Adult guinea pigs were divided into 3 groups: control, HF, and HF+AAV9.SERCA2a gene transfer. HF resulted in a decrease in left ventricular fractional shortening compared with controls (P<0.001). As expected, isolated HF myocytes demonstrated slower sarcoplasmic reticulum calcium uptake, decreased Ca\(^{2+}\) release, and increased diastolic Ca\(^{2+}\) (P<0.05) compared with controls. Moreover, SERCA2a, cardiac ryanodine receptor 2, and sodium-calcium exchanger protein expression was decreased in HF compared with control (P<0.05). As predicted, HF increased susceptibility to cardiac alternans, as evidenced by decreased heart rate thresholds for both V\(\text{m}\) alternans and Ca alternans compared with controls (P<0.01). Interestingly, in vivo gene transfer of AAV9.SERCA2a in the failing heart improved left ventricular contractile function (P<0.01), suppressed cardiac alternans (P<0.01), and reduced ryanodine receptor 2 P\(\text{S}\)\(_{2814}\) secondary to reduction of ryanodine receptor 2–P\(\text{S}\)\(_{2814}\) (P<0.01). This ultimately resulted in a decreased incidence of inducible ventricular arrhythmias (P=0.05).

Conclusions—These data show that SERCA2a gene transfer in the failing heart not only improves contractile function but also directly restores electric stability through the amelioration of key arrhythmogenic substrate (ie, cardiac alternans) and triggers (ie, sarcoplasmic reticulum Ca\(^{2+}\) leak). (Circulation. 2012;126:2095-2104.)

Key Words: alternans ■ arrhythmias, cardiac ■ calcium ■ electrophysiology ■ gene therapy

Sudden arrhythmic death is one of the most devastating manifestations of heart failure (HF), yet the complex sequence of events responsible for the development of fatal ventricular arrhythmias in HF is poorly understood. The majority of sudden arrhythmic deaths occur in the setting of contractile dysfunction, yet, the precise mechanistic relationship linking mechanical cardiac failure and electric instability remains elusive. Consequently, current antiarrhythmic strategies are palliative, temporizing, or completely ineffective, in part because they fail to target specific arrhythmia mechanisms. One postulated mechanism for arrhythmogenesis in HF is repolarization alternans. There is compelling evidence that subtle beat-to-beat alternation of repolarization (ie, T-wave alternans) is an important marker of arrhythmic risk in HF. Moreover, the absence of T-wave alternans in patients with HF appears to protect against ventricular arrhythmias. At the tissue level, repolarization alternans serves to amplify heterogeneities of repolarization required for conduction block and ventricular fibrillation. At the cellular level, T-wave alternans arises from alternation of action potential duration (APD-ALT) secondary to alternation in cellular calcium cycling (Ca-Alt). It is postulated that abnormal myocardial calcium cycling is a common mechanism linking contractile and electrophysiological dysfunction. For example, HF is associated with significant blunting of sarcoplasmic reticulum (SR) Ca\(^{2+}\) reuptake secondary to decreased sarcoplasmic reticulum Ca\(^{2+}\) ATPase 2a (SERCA2a) expression/function. Our laboratory and others have shown that SERCA2a plays a central role in the molecular mechanism of cardiac alternans in the normal heart. Moreover, we recently demonstrated that impaired SR Ca\(^{2+}\) reuptake in a rapid pacing model of HF was associated with enhanced susceptibility to cardiac alternans. Therefore, it would be predicted that therapies targeting improved SERCA2a expression/function could improve...
both mechanical and electrophysiological function in HF. This possibility is particularly attractive because current therapies designed to enhance contractile function have been associated with increased arrhythmogenesis.

There is accumulating evidence that gene therapy targeting increased expression of SERCA2a improves mechanical function in a variety of animal models of HF. More recently in human clinical trials. Moreover, we recently demonstrated that targeted SERCA2a gene transfer in the normal heart reduces cardiac alternans and alternans-mediated arrhythmogenesis. However, the effect of restoring SERCA2a expression to normal levels on cardiac alternans in the setting of HF is unknown. Therefore, in the present study, we tested the hypothesis that in vivo gene therapy targeting deficient SERCA2a will improve contractile function and will significantly reduce arrhythmogenic cardiac alternans in the failing heart.

Methods

Pressure-Overload Model of HF in the Guinea Pig

Experiments were carried out in accordance with the US Public Health Service guidelines for the care and use of laboratory animals. Chronic pressure overload was produced in young male guinea pigs weighing 225 to 275 g by subtotal descending thoracic aortic banding as previously described. Briefly, animals were anesthetized (ketamine, xylazine, acepromazine, and atropine) and mechanically ventilated (2.0-cm³ tidal volume at 50 cycles per minute) via a tracheostomy (18-gauge tube). A small anterior thoracotomy was performed, exposing the left ventricular apex. A 27-gauge catheter was advanced from the apex to the aortic root. Aortic-bandaged and sham-operated animals were sham operations were performed using a similar procedure except for ligature placement. Aortic-banded and sham-operated animals were withdrawn from the ligature, the chest was closed, and intrathoracic air was evacuated. Animals were extubated on spontaneous breathing and observed closely until fully awake. In a subset of animals, sham operations were performed using a similar procedure except for ligature placement. Aortic-banded and sham-operated animals were housed and fed under identical conditions for an average of 27±2 months. Echocardiography was performed with a Phillips iE33 Ultrasound System with an S12-4 sector array ultrasound probe (Phillips Medical Systems, Andover, MA). Doppler echocardiograms were recorded with computer software. These measurements were made during diastole immediately before terminal electrophysiological studies. The incidence of sudden death, defined as any death that occurred suddenly (ie, <12 hours since last observed alive and in no distress) >2 weeks postoperatively, was monitored in all animals.

In Vivo Gene Delivery

Recombinant adeno-associated virus (serotype 9) vectors carrying SERCA2a cDNA with a cytomegalovirus promoter (AAV9.SERCA2a) were used. In vivo gene transfer was performed with a catheter-based procedure. In particular, animals were anesthetized (ketamine, xylazine, acepromazine, and atropine) and mechanically ventilated (2.0-cm³ tidal volume at 50 cycles per minute) via a tracheostomy (18-gauge tube). A small anterior thoracotomy was performed, exposing the left ventricular apex. A 27-gauge catheter was advanced from the apex to the aortic root. Subsequently, the virus solution (5x10⁴ viral genomes per animal of AAV9.SERCA2a diluted to a total volume of 1 mL) was slowly injected. The animals were placed on a heating pad (42°C), the chest was closed, and intrathoracic air was evacuated. Animals were extubated on spontaneous breathing and observed closely until fully awake. Terminal electrophysiological studies were performed 6 weeks after in vivo gene transfer.

Protein Expression

Tissue was obtained from the left ventricular free wall from control, HF, and HF+AAV9.SERCA2a (SERCA2a expression only). Western blotting was performed to determine the relative expression levels of cardiac ryanodine receptor (RyR2), SR Ca²⁺ ATPase, sarcoplasmic Na/Ca exchanger (NCX), phospholamban, and L-type Ca²⁺ channel. Cardiac homogenates were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were probed with the following primary antibodies: mouse anti-RyR2, mouse anti-NCX, and mouse anti-phospholamban (all 3 from Affinity Bioreagents Inc) and mouse anti-Cav1.2 Ca²⁺ channel clone L57-46 (UC Davis/NIH NeuroMab Facility), rabbit anti-SERCA (Dr Periasamy, Ohio State University), and polyclonal anti-rabbit RyR P2614 (Dr Weihrich, Baylor College of Medicine, respectively). They were then treated with horseradish peroxidase–conjugated anti-mouse or anti-rabbit secondary antibodies (Amersham). Protein bands were quantified with ImageQuant software.

Isolated-Myocyte Studies

In a subset of HF and control animals, isolated-myocyte studies were performed. The amphoterin perforated patch technique was used to obtain whole-cell recordings of membrane voltage under current clamp conditions, and intracellular Ca²⁺ transients were measured simultaneously with the fluorescent Ca²⁺ indicator Indo-1AM, as described previously.

Whole-Heart Studies

Whole-heart electrophysiological studies were performed in control, HF alone, and HF+AAV9.SERCA2a hearts. Isolated hearts were Langendorff perfused with oxygenated (95% O₂/5% CO₂) Tyrode solution (in mmol/L: NaCl 130, NaHCO₃ 25.0, MgSO₄ 1.2, KCl 4.75, dextrose 5.0, and CaCl₂ 1.25; pH 7.40; 32°C). Dual calcium and voltage mapping was performed by use of high-resolution optical mapping. Spontaneously, optical action potentials and calcium transients were measured simultaneously from the anterior surface of the left ventricle using the voltage-sensitive dye di-4-ANEPPS (15 μmol/L) and the calcium-sensitive dye Indo-1AM, as previously described. Cardiac alternans was induced by stepwise decrements (10 milliseconds) in pacing cycle length (CL) but was not measured until 30 seconds after the decrement in rate to ensure its stability. CL was decreased until failure to capture the preparation or the development of a ventricular arrhythmia. Spontaneous diastolic SR Ca²⁺ release (a marker of RyR₂) in the whole heart was measured as the maximum derivative of the optical Ca²⁺ tracing (dCa/dtmax) with computer software. These measurements were made during diastole immediately after the last paced beat (CL, 130–150 milliseconds). For each heart studied, the slope of the optical Ca²⁺ tracing was calculated from 256 separate myocyte clusters.

Data Analysis

Isolated Myocytes

APD was measured at 90% repolarization. Ca²⁺ transient parameters were defined as described previously. Diastolic Ca²⁺ was defined as cytosolic Ca²⁺ level just before the onset of the Ca²⁺ transient or just before the action potential upstroke when there was no obvious Ca²⁺ transient. Amplitude of intracellular Ca²⁺ transient was calculated from the difference between peak and diastolic Ca²⁺. The rate of reuptake of intracellular Ca²⁺+, the decay portion of the Ca²⁺ transient (from 30%–100% of decline phase), was fit to a single exponential function with a time constant, τ, that was used to measure Ca²⁺ decay.

Whole Heart

APD-ALT was defined as the difference in APD between 2 consecutive beats. Similarly, Ca-ALT was defined as the difference in Ca²⁺ transient amplitude between 2 consecutive beats. The alternans–heart rate (HR) relation was plotted as the magnitude of APD-ALT or Ca-ALT as a function of HR. A leftward or rightward shift in this relation (ie, development of alternans at lower or higher HRs) indicates greater or reduced susceptibility to alternans, respectively. The slowest HRs that induce stable APD-ALT of >10 milliseconds and stable Ca-ALT of >10% were defined as the threshold HRs for alternans.
Table. Pressure-Overload Heart Failure: Model Characteristics

<table>
<thead>
<tr>
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<th>Control (n=7)</th>
<th>Heart Failure (n=12)</th>
<th>(P)</th>
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<tr>
<td>Male, %</td>
<td>100</td>
<td>100</td>
<td>NS</td>
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<td>Body weight, g</td>
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<tr>
<td>End-diastolic diameter, mm</td>
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<td>End-systolic diameter, mm</td>
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<td>Fractional shortening, %</td>
<td>50±2</td>
<td>24±2</td>
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<tr>
<td>Action potential duration, ms</td>
<td>176±4</td>
<td>188±4</td>
<td>0.04</td>
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</table>

Data are presented as mean±SEM.

transient amplitude were measured from optically recorded action potentials and \(Ca^{2+}\) transients with the use of methods that have been validated extensively. Arrhythmia susceptibility was determined by use of a ramp pacing protocol starting at 300 milliseconds (200 bpm) with stepwise 10-millisecond decrements in pacing CL until failure of 1/1 capture or the induction of a ventricular arrhythmia. This arrhythmia induction protocol has previously been validated in our laboratory to assess alternans-mediated arrhythmogenesis and has similarities to the clinical test for alternans (T-wave alternans test), which increases HR in a ramp-like fashion by exercise and thus is a good correlate to our ramp pacing protocol. Pacing was performed at each CL for 1 to 2 minutes to ensure steady state. Arrhythmias were defined as a tachyarrhythmia that is sustained for >30 seconds after pacing was halted. The incidence of sudden death was monitored throughout the duration of the study. Sudden death was defined as an unexpected death of an animal that had been observed to be clinically stable within 12 hours of being found dead. Because continuous telemetry monitoring was not performed, it is not possible to define the exact mode of death (ie, tachyarrhythmia) in these animals.

Statistical Analysis
Statistical analyses of data were performed with Sigmastat (SPSS Inc, Chicago, IL). Normality was tested with the Shapiro-Wilk test and by visual inspection of the distribution of all data sets. Homogeneity of variance was assessed with the Levene test. When data were found to have failed the homogeneity of variance assumption, the Wilcoxon rank-sum test was performed in place of the Student \(t\) test or the Kruskal-Wallis test was performed in place of ANOVA. Statistical differences were assessed with ANOVA, Student \(t\) test, Wilcoxon rank-sum test, Kruskal-Wallis test, and \(\chi^2\) test as appropriate. All data are expressed as mean±SEM.

Results

Guinea Pig Model of Pressure-Overload HF

Model Characteristics
The Table demonstrates the characteristics of the guinea pig thoracic aortic-banded model of HF. Consistent with the development of systolic HF, aortic banding (n=12) increased heart weight, ratio of body weight to heart weight, and end-diastolic diameter compared with sham controls (n=7; \(P<0.01\)). In addition, APD in the intact heart was prolonged in HF compared with sham control (\(P<0.05\)). Figure 1A (top) illustrates representative M-mode echocardiographic tracings comparing sham control and aortic-banded animals. Fractional shortening was decreased 27±2 weeks after aortic banding compared with sham controls (\(P<0.001\)), reaffirming the development of HF.

Isolated Myocytes
HF was further confirmed in isolated myocytes measuring intracellular \(Ca^{2+}\) transients. As expected, isolated HF myocytes (n=5) demonstrated slower SR calcium uptake (361±30 versus 243±21 milliseconds; \(P=0.05\)), decreased \(Ca^{2+}\) release (182±20 versus 368±73 nmol/L; \(P<0.05\)), and increased diastolic \(Ca^{2+}\) (333±37 versus 229±26 nmol/L; \(P<0.01\)) compared with controls (n=5).

Protein Expression
Figure 1B shows Western blots of key \(Ca^{2+}\) handling proteins from aortic-banded (n=4) and sham control (n=4) hearts. As shown, HF was associated with decreased SERCA2a, L-type \(Ca^{2+}\) channel (data not shown), RyR2, and NCX protein expression compared with control (\(P<0.02\)). Phospholamban protein expression was unchanged by HF (\(P=0.65\)).

Effect of SERCA2a Gene Transfer on Contractile Function and \(Ca^{2+}\) Cycling Proteins in Pressure-Overload HF
In vivo gene transfer of AAV9.SERCA was performed with an intracoronary delivery method and produced relatively homogeneous whole-heart gene transfer. For example, Figure 1A (middle) shows an X-gal–stained cross section of guinea pig ventricles excised 6 weeks after AAV9.\(\beta\)-gal exposure compared with an X-gal–stained untreated, control heart. Figure 1A (bottom) illustrates that AAV9.SERCA2a gene transfer in HF improved left ventricular fractional shortening compared with HF before undergoing in vivo gene transfer (n=4; \(P<0.01\)). In addition, Figure 1C shows protein expression of key \(Ca^{2+}\) cycling proteins 6 weeks after in vivo gene transfer of AAV9.SERCA2a in HF animals and HF animals that did not undergo in vivo gene transfer. Our results demonstrate that AAV9.SERCA2a (n=4) gene transfer in HF significantly increased SERCA2a protein expression but did not change the expression of NCX, RyR2, phospholamban, or L-type \(Ca^{2+}\) channel (data not shown) in the left ventricular free wall compared with HF alone (n=4).

Effect of SERCA2a Gene Transfer on Susceptibility to Cellular Alternans in Pressure-Overload HF
Ca-ALT and APD-ALT were measured as the pacing rate was progressively increased in the Langendorff-perfused whole heart. Figures 2A and 3A show representative examples of optical calcium transients and action potential tracings from control, HF, and HF+AAV9.SERCA2a hearts over a range of pacing rates. As illustrated, HF substantially enhanced susceptibility to alternans in that the Ca-ALT and APD-ALT appeared at slower HRs in HF compared with sham control hearts. Moreover, treatment with SERCA2a overexpression in the failing heart markedly reduced both Ca-ALT and APD-ALT compared with HF and was similar or even slightly improved compared with control hearts. In addition, plots of pacing rate versus magnitude of Ca-ALT (Figure 2B) and APD-ALT (Figure 3B) show that magnitude of alternans increased as pacing rate increased and that the magnitude of APD-ALT and Ca-ALT was consistently greater in HF (n=5) compared with sham control (n=6) hearts. Importantly, SERCA2a overexpression in HF mark-
Figure 1. Pressure-overload heart failure: functional characteristics. A, Top, Representative M-mode echocardiographic tracings comparing sham control (n = 7) and aortic-banded (n = 12) animals. Middle, Example of X-gal-stained cross section of guinea pig ventricles excised 6 weeks after AAV9.β-gal exposure showing relatively homogenous gene delivery. Bottom, Representative M-mode echocardiographic tracings comparing left ventricular fractional shortening (FS) before and 6 weeks after AAV9.SERCA2a gene transfer in heart failure (n = 4). B, Example of protein expression of key Ca$^{2+}$ cycling proteins in heart failure (n = 4) and control (n = 4). P values are derived from the Student t test (sarcoplasmic reticulum Ca$^{2+}$ ATPase 2a [SERCA2a] and phospholamban [PLB]) and Wilcoxon rank-sum test (ryanodine receptor 2 [RyR2] and Na/Ca exchanger [NCX]). C, Example of protein expression of key Ca$^{2+}$ cycling proteins in heart failure (n = 4) and heart failure + AAV9.SERCA2a gene transfer (n = 4). P values are derived from the Student t test (SERCA2a, NCX, and PLB) and Wilcoxon rank-sum test (RyR2).
edly increased the HR threshold (Figure 2B and 3B, inset) of APD-ALT and Ca-ALT compared with HF alone (*P < 0.001). Interestingly, Ca-ALT susceptibility in HF with AAV9.SERCA2a treatment was similar to control, yet APD-ALT susceptibility was modestly decreased even compared with nonfailing control hearts (*P < 0.02). Surprisingly, in control hearts, the HR thresholds for the onset of Ca-ALT and APD-ALT were similar; in HF hearts, however, Ca-ALT developed at a significantly slower HR than APD-ALT (*P < 0.01; Figure 4), and this disparity persisted even after AAV9.SERCA2a treatment (*P < 0.05).

Modulation of Alternans-Mediated Arrhythmogenesis by SERCA2a Gene Transfer

Previously, we showed that SERCA2a gene transfer in young healthy guinea pigs can suppress cardiac alternans and reduce alternans-mediated arrhythmogenesis.11 In that study, we reported a 100% incidence of pacing-induced ventricular arrhythmias in healthy control hearts. In the present study, all control and HF hearts developed pacing-induced ventricular arrhythmias, and there was no clear difference in the pacing rate in which these arrhythmias developed. This is probably in part secondary to the aggressiveness of the ramp pacing protocol used in these studies. However, there was a 22.7% incidence of sudden death in HF animals compared with 0% in control and HF + AAV9.SERCA2a animals (*P < 0.05), suggesting an increase in arrhythmia susceptibility in HF animals.

Figure 5A demonstrates the effect of SERCA2a gene transfer on arrhythmogenesis in the failing heart. Demonstrated is an optically recorded action potential tracing from an HF heart paced at a CL of 150 milliseconds. Notice the marked APD-ALT immediately preceding the onset of ventricular fibrillation. In contrast, in a failing heart treated with SERCA2a gene transfer paced at an even faster CL (110 milliseconds), there was negligible APD-ALT and failure to induce an arrhythmia. On average, SERCA2a overexpression in the failing heart produced a 50% reduction in alternans-mediated ventricular arrhythmias (*P < 0.05).

Figure 5B demonstrates the central role of SERCA2a in modulating alternans-mediated arrhythmogenesis. Plotted is alternans susceptibility as measured by alternans thresholds
Interestingly, SERCA2a gene transfer in HF decreased spontaneous diastolic SR Ca\textsuperscript{2+} (previously reported\textsuperscript{11}) hearts, plotted in order of increasing SERCA2a expression. It is evident that with increasing SERCA2a there is a progressive reduction in cardiac alternans, as demonstrated by an increased alternans HR threshold. Moreover, as the alternans HR threshold increases with increasing SERCA2a, there is a concomitant decrease in arrhythmia susceptibility, highlighting the role of SERCA2a in modulating arrhythmogenic cardiac alternans.

It is possible that SERCA2a gene delivery has an influence on calcium-mediated arrhythmia substrates other than decreased cardiac alternans. Therefore, we assessed the effect of SERCA2a gene transfer on spontaneous diastolic SR Ca\textsuperscript{2+} release (a marker of RyR P\textsubscript{o}) in the whole heart.\textsuperscript{22,23} Specifically, we measured the slope of optically recorded Ca\textsuperscript{2+} tracings during diastole immediately after the last paced beat (CL, 130–150 milliseconds). As predicted, we found increased spontaneous diastolic SR Ca\textsuperscript{2+} release (increased RyR P\textsubscript{o}) in HF compared with control (P<0.01; Figure 6A). Interestingly, SERCA2a gene transfer in HF decreased spontaneous diastolic SR Ca\textsuperscript{2+} release compared with HF alone (P<0.01; Figure 6A) and was similar to control. In comparison, in normal hearts, we observed a similar increase and decrease in spontaneous diastolic SR Ca\textsuperscript{2+} in response to treatment with caffeine and ryanodine, respectively (P<0.01; Figure 6B). Finally, Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II–mediated RyR phosphorylation (RyR-P\textsubscript{S2814}) was found to be increased in HF compared with control, and SERCA2a gene transfer in HF reduced RyR-P\textsubscript{S2814} to nearly control levels. These data suggest that SERCA2a gene transfer decreased RyR P\textsubscript{o} secondary to a reduction in RyR-P\textsubscript{S2814}.

**Discussion**

Our studies provide convincing evidence that dysfunctional SR Ca\textsuperscript{2+} cycling is a common mechanism linking contractile and electrophysiological dysfunction in the failing heart and that therapy targeting key SR Ca\textsuperscript{2+} cycling proteins (ie, SERCA2a) can restore both contractile function and electric stability. We show that SERCA2a gene transfer in the failing heart not only improves contractile function but also directly restores electric stability through the amelioration of key arrhythmogenic substrate (ie, cardiac alternans) and triggers (ie, SR Ca\textsuperscript{2+} leak). More specifically, the mitigation of cardiac alternans by selective SERCA2a gene transfer indicates that SERCA2a is linked to this key “mechanism” of electric instability in the failing heart. Additionally, we found that SERCA2a gene transfer reduced RyR2 P\textsubscript{o} (ie, SR Ca\textsuperscript{2+} leak).
leak) secondary to reduced RyR-PS2814, thus suppressing a key trigger of ventricular arrhythmias in HF. The fact that we demonstrated complete recovery of susceptibility to alternans and SR Ca\(^{2+}\)/H\(_{11001}\) leak to pre-HF levels with a concomitant reduction in arrhythmias after restoration of SERCA2a illustrates that regardless of the numerous impacts of HF on various arrhythmia processes, SERCA2a gene delivery is a viable approach for electrically stabilizing the failing heart. Finally, this study also highlights the attractiveness of therapies targeting SERCA2a in HF because of the dual effect of enhanced SERCA2a on both contractile and electric function.

**SERCA2a Underlies Mechanism of Cardiac Alternans in HF**

In the present study, we show that abnormal SR Ca\(^{2+}\) cycling—more specifically, impaired SERCA2a—is a key mechanism of cardiac alternans in HF. This is consistent with the calcium cycling hypothesis, which states that alternans occurs when HR exceeds the capacity of the myocyte to cycle calcium.\(^{10,25,27,28}\) From the findings of the present study, we postulate that reduced SERCA2a protein expression in HF limits the ability of the SR to cycle Ca\(^{2+}\), thus preventing complete SR reclamation of cytosolic Ca\(^{2+}\) above a critical HR threshold. Hence, cytosolic Ca\(^{2+}\) is reclaimed on an every-other-beat basis, resulting in alternating Ca\(^{2+}\) transients. In the present study, this hypothesis is supported by the observation that selective enhancement of SERCA2a expression in HF reduced the susceptibility to alternans and is consistent with earlier findings that SERCA2a is a key molecular mechanism in the development of cardiac alternans in cardiac myocyte monolayers and the intact healthy heart.\(^{11,12}\)

Recently, it was demonstrated that Ca\(^{2+}\) transient restitution is decreased in the spontaneously hypertensive rat model of HF compared with healthy controls.\(^{29,30}\) It was postulated that this change in Ca\(^{2+}\) transient restitution is an important mechanism in increasing Ca-ALT in HF. Because the rate of recovery of SR Ca\(^{2+}\) release is a primary determinant of Ca\(^{2+}\) transient restitution, it is predicted that enhanced SERCA2a expression/function would increase Ca\(^{2+}\) transient restitution.

**Figure 6.** AAV9.SERCA2a gene transfer reverses increased ryanodine receptor 2 (RyR2) P\(_{o}\) in heart failure. A, Measurement of the slope of optically recorded Ca\(^{2+}\) tracings during diastole immediately after the last paced beat (cycle length, 130–150 milliseconds) as a marker of spontaneous diastolic SR Ca\(^{2+}\) release (ie, RyR P\(_{o}\)). As demonstrated, spontaneous diastolic SR Ca\(^{2+}\) release increased (ie, increased RyR P\(_{o}\)) in heart failure compared with control. Sarcoplasmic reticulum Ca\(^{2+}\)/ATPase 2a (SERCA2a) gene transfer in heart failure (HF) decreased spontaneous diastolic SR Ca\(^{2+}\) release compared with HF alone and was similar to control. B, In normal hearts, we observed a similar increase and decrease in spontaneous diastolic SR Ca\(^{2+}\) in response to treatment with caffeine (increasing RyR P\(_{o}\)) and ryanodine (decreasing RyR P\(_{o}\)), respectively. *P<0.01 by ANOVA. C, Ca\(^{2+}\)/calmodulin-dependent protein kinase II RyR phosphorylation (RyR-PS2814) in control (n=3), HF (n=3), and HF+AAV9.SERCA2a gene transfer (n=4). As shown, RyR-PS2814 increased in HF compared with control, and AAV9.SERCA2a gene transfer in the failing heart decreased RyR-PS2814 (P<0.05 by ANOVA).
and therefore reduce Ca-ALT. Therefore, it is possible that the reduction in Ca-ALT susceptibility reported in the present study after SERCA2a gene transfer is secondary to the effect of increased SERCA2a on Ca$^{2+}$ transient restitution. Further studies are needed to investigate this postulate.

Although we demonstrate with a high degree of specificity that SERCA2a directly affects susceptibility to cardiac alternans in HF, these data do not rule out other synergistic or complementary molecular mechanisms. For example, as demonstrated in the present study, HF is associated with remodeling of multiple Ca$^{2+}$ cycling proteins.\textsuperscript{31-33} It has previously been demonstrated that instabilities of SR Ca$^{2+}$ release can also lead to Ca-ALT. For example, Huser et al\textsuperscript{31} demonstrated that refractory-like properties of RyR can produce alternating RyR P$_o$ and therefore Ca-ALT, regardless of SR Ca load. Interestingly, our studies demonstrate that SERCA2a gene transfer in HF decreased RyR2 P$_o$, secondary to reduced RyR-P$_{52K14}$ without altering RyR2 expression. This implies that in addition to improving SR Ca$^{2+}$ reuptake, SERCA2a improves the stability of RyR2, suggesting a dual mechanism by which SERCA2a gene transfer ameliorates Ca-ALT in the failing heart.

It has been suggested that Ca$^{2+}$ transient amplitude directly affects APD via Ca$^{2+}$-sensitive currents such as NCX and L-type Ca$^{2+}$ current. Thus, it is predicted that a reduction in NCX will decrease the Ca$^{2+}$-to-APD gain, resulting in a disparity between the magnitudes of Ca-ALT and APD-ALT. In the present study, NCX protein expression was consistently reduced in HF and HF+SERCA2a gene transfer, and there was an accompanying disparity between the onset of Ca-ALT and APD-ALT in HF and HF+SERCA2a hearts that was not present in control hearts. For example, in control hearts, the HR thresholds Ca-ALT and APD-ALT were similar (ie, 370±13 versus 388±8 bpm; P=NS). In contrast, in HF and HF+SERCA2a hearts, there was a disparity between the HR thresholds of Ca-ALT and APD-ALT (ie, 230±12 versus 290±12 bpm and 372±20 versus 430±13 bpm; P<0.05) such that CA-ALT developed on average ~60 bpm earlier than APD-ALT. Our data support that NCX current is an important mechanism linking alternans of intracellular calcium cycling and APD. To the best of our knowledge, this is the first experimental evidence supporting a direct mechanistic role for NCX in governing Ca-ALT–to–APD-ALT gain.

$I_{CaL}$ is also postulated to be mechanistically involved in governing Ca-ALT–to–APD-ALT gain. The present study showed a reduction in expression of L-type Ca$^{2+}$ channel, and it has been reported that pressure-overload HF model in the guinea pig is associated with decreased $I_{CaL}$.\textsuperscript{34} In addition, we showed that SERCA2a gene transfer in HF did not alter L-type Ca$^{2+}$ channel expression compared with HF alone, and prior studies in control hearts (rabbit) showed no change in $I_{CaL}$ after SERCA2a gene transfer, but it may alter other inward sarcolemmal Ca$^{2+}$ currents.\textsuperscript{35} Thus, it is possible that the reduction in Ca-ALT–to–APD-ALT gain seen in the present study could be in part secondary to changes in $I_{CaL}$.

**SERCA2a as a Therapeutic Target in HF**

HF is associated with both mechanical and electrophysiological dysfunction. In the present study, we show that impaired SR Ca$^{2+}$ cycling is associated with contractile dysfunction and increased susceptibility to a known arrhythmia substrate, cardiac alternans. Interestingly, we demonstrate that selective SERCA2a gene transfer in HF, improving SR Ca$^{2+}$ uptake and stabilizing SR Ca$^{2+}$ release, can improve left ventricular contractile function and ameliorate key arrhythmia substrate. These data support the hypothesis that impaired SR Ca$^{2+}$ cycling is a common mechanism for both mechanical and electrophysiological dysfunction in HF. Our findings are consistent with a growing body of literature demonstrating a favorable effect of SERCA2a gene transfer on contractile function.\textsuperscript{14-16} Moreover, previous studies suggested that targeted enhancement of SERCA2a has antiarrhythmic properties, but the mechanisms of these effects were not clear.\textsuperscript{36,37}

The present investigation provides important mechanistic insights into the antiarrhythmic properties of therapies targeting SERCA2a. First, as demonstrated in Figure 5, SERCA2a gene transfer modulates susceptibility to cardiac alternans and correspondingly reduces alternans-mediated ventricular arrhythmias. Additionally, we found that SERCA2a gene transfer may decrease Ca$^{2+}$-mediated triggers of arrhythmias, as evidenced by decreased spontaneous diastolic Ca$^{2+}$ release (ie, RyR P$_o$) resulting from reduced RyR-P$_{52K14}$. This is consistent with the findings of Lyon et al\textsuperscript{38} demonstrating reduced SR Ca$^{2+}$ leak and RyR2 phosphorylation in isolated myocytes. Our studies and that of Lyon et al clearly demonstrate an effect of SERCA2a gene delivery on an important arrhythmogenic trigger (ie, SR Ca$^{2+}$ leak and delayed afterdepolarizations) and an important substrate for ventricular arrhythmias (ie, alternans).

It is possible that therapies that enhance SERCA2a in HF could be arrhythmogenic because enhanced SR calcium load could increase susceptibility to spontaneous SR calcium release and delayed afterdepolarization–mediated arrhythmias. However, in the present study and in our prior investigation of SERCA2a overexpression in healthy hearts, we saw no evidence of delayed afterdepolarizations or delayed afterdepolarization–mediated arrhythmias. In addition, in the recent study by Lyon et al,\textsuperscript{38} SERCA2a overexpression in a chronic myocardial infarction model of HF actually reduced spontaneous catecholamine-induced ventricular arrhythmias. More compelling is the fact that in recent clinical trials there was no reported increase in arrhythmias in patients with severe HF who received AAV.SERCA2a.\textsuperscript{17}

**Clinical Implications**

Our data have important clinical implications in that they highlight the attractiveness of therapies targeting SERCA2a in HF because of the dual effect of enhanced SERCA2a on contractile function and both arrhythmia triggers and substrate. There are clear limitations to inotropic therapies in HF because they increase mortality and have been linked to increased arrhythmogenesis. Furthermore, current antiarrhythmic therapies are palliative, temporizing, or increase sudden death mortality. Therefore, therapies targeting SERCA2a to improve contractile function while altering key arrhythmia mechanisms have the potential to change the paradigm of HF management by offering the opportunity to
genetically induce a heart that is resistance to ventricular fibrillation.

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Disclosures
Dr Hajjar has ownership interest in Celladon and Nanocor. The other authors report no conflicts.

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**CLINICAL PERSPECTIVE**

Sudden arrhythmic death is a major risk in heart failure, yet the mechanisms are incompletely understood. T-wave alternans arises from beat-to-beat alternans of cellular repolarization, is a consistent precursor to ventricular fibrillation in experimental animals, and is a marker of electrical instability in patients. Previously, we reported that sarcoplasmic reticulum Ca\(^{2+}\) ATPase 2a (SERCA2a), the pump responsible for reuptake of cytosolic calcium during diastole, plays a central role in the molecular mechanism of cardiac alternans. In the present study, SERCA2a gene transfer in the failing heart not only improved contractile function but also restored electric stability through the amelioration cardiac alternans and sarcoplasmic reticulum Ca\(^{2+}\) leak. This finding provides evidence that dysfunctional sarcoplasmic reticulum Ca\(^{2+}\) cycling links contractile and electrophysiological dysfunction in the failing heart and that therapy targeting key sarcoplasmic reticulum Ca\(^{2+}\) cycling proteins (ie, SERCA2a) can restore both contractile function and electric stability. Thus, therapies targeting SERCA2a warrant further evaluation in heart failure.
Targeted Sarcoplasmic Reticulum Ca\textsuperscript{2+} ATPase 2a Gene Delivery to Restore Electrical Stability in the Failing Heart

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