Targeted SERCA2a Gene Expression Identifies Molecular Mechanism and Therapeutic Target for Arrhythmogenic Cardiac Alternans

Michael J. Cutler, DO, PhD; Xiaoping Wan, MD, PhD; Kenneth R. Laurita, PhD; Roger J. Hajjar, MD; David S. Rosenbaum, MD

Background—Beat-to-beat alternans of cellular repolarization is closely linked to ventricular arrhythmias in humans. We hypothesized that sarcoplasmic reticulum calcium reuptake by SERCA2a plays a central role in the mechanism of cellular alternans and that increasing SERCA2a gene expression will retard the development of cellular alternans.

Methods and Results—In vivo gene transfer of a recombinant adenoviral vector with the transgene for SERCA2a (Ad.SERCA2a) was performed in young guinea pigs. Isolated myocytes transduced with Ad.SERCA2a exhibited improved sarcoplasmic reticulum Ca2+ reuptake (P<0.05) and were markedly resistant to cytosolic calcium alternans (P<0.05) under repetitive constant action potential clamp conditions (ie, when alternation of action potential duration was prevented), proving that sarcoplasmic reticulum Ca2+ cycling is an important mechanism in the development of cellular alternans. Similarly, SERCA2a overexpression in the intact heart demonstrated significant resistance to alternation of action potential duration when compared with control hearts (heart rate threshold, 484±25 bpm versus 396±11 bpm, P<0.01), with no change in action potential duration restitution slope. Importantly, SERCA2a overexpression produced a 4-fold reduction in susceptibility to alternans-mediated ventricular arrhythmias (P<0.05).

Conclusions—These data provide new evidence that sarcoplasmic reticulum Ca2+ reuptake directly modulates susceptibility to cellular alternans. Moreover, SERCA2a overexpression suppresses cellular alternans, interrupting an important pathway to cardiac fibrillation in the intact heart. (Circ Arrhythm Electrophysiol. 2009;2:686-694.)

Key Words: alternans ■ action potentials ■ intracellular calcium ■ adenoviral gene transfer ■ repolarization ■ arrhythmia

Although ventricular arrhythmias are the most common cause of cardiovascular mortality, the mechanisms responsible for triggering electric instability in the heart are poorly understood. Cardiac alternans is a repetitive beat-to-beat fluctuation of cellular repolarization that is closely associated with ventricular arrhythmias and sudden cardiac death in a wide variety of experimental23,36,43 and clinical33,40 conditions. It is clear that alternans arises from alternation of action potential duration (APD-ALT) at the level of the single cell,12,17 and it has been proposed that APD-ALT arises secondarily from an effect on calcium-sensitive electrogentic sarcolemmal currents during cytosolic calcium alternans (Ca-ALT)9,42,7,13. Therefore, understanding the cellular and molecular basis for Ca-ALT can provide important and novel insights into mechanisms of ventricular arrhythmias.

Clinical Perspective on p 694

It is generally accepted that cellular alternans occurs when the heart rate (HR) exceeds capabilities of the cardiac myocyte to cycle calcium on a beat-by-beat basis. This hypothesis predicts that during steady state (ie, resting HR), the amount of Ca2+ released from the sarcoplasmic reticulum (SR) to initiate cardiac contraction must be matched by the Ca2+ reclaimed from the cytoplasm, primarily by SERCA2a. Any sustained disturbance in the myocyte’s ability to load or release SR Ca2+ presumably leads to the development of Ca2+ alternans. For example, instabilities of SR Ca2+ release through the RyR release channel either with7 or without13,27 fluctuations of SR Ca2+ content has been argued as a mechanism for cellular alternans. An alternative and equally compelling hypothesis implicates impaired SR reuptake in the mechanism of Ca-ALT.43 However, the specific SR Ca2+ cycling proteins underlying the molecular basis for cellular alternans are unknown. Recent data from our laboratory provide important insight into the molecular basis for cellular alternans. In particular, cardiac myocytes that are most susceptible to APD-ALT exhibit reduced expression of the SERCA2a and delayed SR Ca2+ reuptake42.19 These findings led us to hypo-
esize in the present study that SERCA2a function plays a critical role in the initiation of cellular alternans.

Identification of a molecular basis for cardiac alternans has been challenging because of difficulties in distinguishing experimentally the complex interactions between sarcolemmal and sarcomeric ionic fluxes using relatively nonspecific pharmacological probes. However, gene transfer techniques can be used to alter the expression of single proteins, and, in this way, disease mechanisms can be elucidated and potential therapeutic targets identified. Therefore, to test our hypothesis that SERCA2a function is an important mechanism in the initiation of cellular alternans, we performed in vivo gene transfer of Ad.SERCA2a.GFP in the guinea pig heart. Overexpression of SERCA2a significantly inhibited cellular alternans and susceptibility to ventricular arrhythmias in the intact beating guinea pig heart. These data establish a molecular mechanism for arrhythmogenic cardiac alternans. Specifically, enhancement of SERCA2a gene expression will diminish susceptibility to cellular alternans, thereby interrupting an important pathway to cardiac fibrillation in the intact heart.

**Methods**

**In Vivo Gene Delivery**

Recombinant adenoviral vectors were used with cytomegalovirus-driven expression cassettes for SERCA2a (Ad.SERCA2a) with a second cassette in each adenovirus containing GFP substituted for E1 by means of homologous recombination as previously described. Control adenoviral vectors were used with cytomegalovirus-driven expression cassettes for GFP (Ad.GFP) or β-galactosidase (Ad.β-gal).

Experiments were carried out in accordance with the United States Public Health Service guidelines for the care and use of laboratory animals. In vivo gene transfer was performed using a modified cross-clamping method. Brieﬂy, animals were anesthetized (Ketamine, Xylazine, Acepromazine, and Atropine) and mechanically ventilated (2.0-cm tidal volume at 50 cycles per minute) via a tracheostomy (18-gauge tube). An anterior thoracotomy was performed, and the pulmonary artery and aorta were isolated. A 27-gauge catheter was advanced from the apex to the aortic root. Subsequently, the aorta and pulmonary artery were cross-clamped for 50 to 60 seconds, and the virus solution (1×10¹² particle/mL Ad.SERCA2a.GFP, n = 7; 1×10¹² particle/mL Ad.GFP, n = 5; or 1.2×10¹¹ particle/mL Ad.β-gal, n = 3; plus 75 μg/mL of nitroglycerin) was 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 closely observed until fully awake.

**Efficiency of In Vivo Gene Transfer**

**Qualitative Assessment of Transduction Efficiency**

Seventy-two hours after gene transfer with β-galactosidase, animals (n=3) were euthanized with pentobarbital. The heart was rapidly removed, flushed with phosphate-buffered saline, and transversely sliced into 3 pieces used for x-gal staining. This procedure was performed to assess the regional distribution and homogeneity of gene delivery in the intact heart.

**Quantitative Assessment of Transduction Efficiency**

Seventy-two hours after gene transfer, myocytes isolated from the left ventricular free wall were examined by fluorescence microscopy to calculate transduction efficiency. Efficiency is reported as the percentage of all myocytes in the microscope field that fluoresced green.

**SERCA2a Protein Expression**

Myocytes isolated from the transmural left ventricular free wall of both SERCA2a transduced hearts and age-matched control hearts, as previously described, were used for Western blotting to determine the relative expression levels of SERCA2a.42 Cardiac homogenates (10 μg for SERCA2) were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were probed with rabbit anti-SERCA antibody (Dr Periasamy, Ohio State University). They were then treated with horseradish peroxidase–conjugated anti-rabbit antibody. Protein bands were quantified using ImageQuant software.

**Isolated Myocyte Studies**

Seventy-two hours after in vivo gene transfer, isolated myocyte electrophysiological studies were performed. Membrane voltage and intracellular calcium were measured simultaneously using the perforated patch technique and the fluorescent Ca²⁺ indicator indo-1AM. Myocytes transduced with Ad.SERCA2a and Ad.GFP (ie, control) were confirmed using GFP fluorescence. Because there were no statistical differences in calcium cycling characteristics and alternans susceptibility between myocytes isolated from untreated hearts and Ad.GFP transduced hearts, data are presented as a combined control group.

**Patch-Clamp Recordings**

The amphotericin perforated patch technique was used to obtain whole-cell recordings of membrane voltage under current-clamp conditions as described previously. Brieﬂy, the cells were bathed in a chamber continuously perfused with Tyrode solution composed of (mmol/L) NaCl 137, KCl 5.4, CaCl2 2.0, MgSO4 1.0, glucose 10, HEPES 10, pH to 7.35 with NaOH. Patch pipettes were pulled from borosilicate capillary glass and lightly fire-polished to resistance 0.9 to 1.5 mol/L when ﬁlled with electrode solution composed of (mmol/L) aspartic acid 120, KCl 20, NaCl 40, MgCl2 2, HEPES 5, and 240 μg/mL of amphotericin-B (Sigma, St Louis, Mo), pH 7.3. A gigaseal was rapidly formed. Typically, 10 minutes later, amphotericin pores lowered the resistance sufﬁciently to current-clamp the cells. Myocytes were paced using a 1.5 to 2 diastolic threshold, 5-ms current pulse. Experiments were performed at 30°C. Command and data acquisition were operated with an Axopatch 200B patch-clamp amplifier controlled by a personal computer using a Digidata 1200 acquisition board driven by pCLAMP 7.0 software (Axon Instruments, Foster City, Calif).

**Calcium Transient Recordings**

Intracellular Ca²⁺ transients were measured simultaneously using the fluorescent Ca²⁺ indicator indo-1AM as described previously. Cells were loaded with indo-1AM by incubating them in Tyrode containing indo-1AM (2 μmol/L) (Molecular Probes) and 0.025% (wt/wt) Pluronic F-127 (Molecular Probes) for 30 minutes at room temperature. The intracellular indo-1 was excited at 385 nm. Fluorescence emitted at 405 and 485 nm was collected by 2 matched photomultiplier tubes. Data were ﬁltered at 200 Hz and sampled at 1 kHz. The ratio of the intensity of ﬂuorescence emitted at 405 nm over that at 485 nm was calculated after subtraction of background ﬂuorescence as described previously. The emission ﬁeld was restricted to a single cell with the aid of an adjustable window. To determine intracellular calcium concentration, the ratiometric calcium transients were calibrated using the techniques developed by Grynkiewicz et al. The calibration parameters, Rmax and Rmin, were obtained from isolated myocytes with either a modiﬁed calcium-free, Rmin (n = 8) or a calcium-saturated, Rmax (n = 8) Tyrode solution. The modiﬁed Rmin solution contained (mm) 132 KCl, 1.0 MgCl2, 10 EGTA, 10 HEPES, 0.05 4-bromo-A-23187; pH 7.05. The modiﬁed Rmax solution contained (mm) 132 KCl, 1.0 MgCl2, 2 CaCl2, 10 HEPES, and 10 BDM; pH 7.05. The dissociation constant (Kd) was 250 nm and β was 2.5(8). Calcium concentrations were calculated using the following standard calibration equations: 

**Stimulation Protocol**

Myocytes were stimulated at a baseline stimulation rate of 150 beats per minute (bpm). After a period of stimulation to establish steady state, measurements were made for the subsequent 20 beats. This protocol was repeated at progressively faster rates until 1:1 capture was lost.
APD-ALT was measured by calculating the difference in action potential duration (APD₀) on 2 consecutive beats and was defined to be present when APD-ALT exceeded 10 ms, as previously described.²⁶ Ca-ALT was measured by calculating the difference in amplitude on 2 consecutive beats and was defined to be present when Ca-ALT exceeded 10% of Ca²⁺ transient amplitude, as described previously.¹⁹

**Whole Heart**

APD-ALT was defined as the difference in APD between 2 consecutive beats.²⁰,²⁵ The alternans-HR relation was plotted as the magnitude of APD-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 HR that induces alternans was defined as the threshold HR for alternans.²⁰,²⁵,²⁸ Activation time, repolarization time, and APD were measured from optically recorded action potentials using automated algorithms that have been validated extensively.²⁵,²⁶,³² Dynamic APD restitution was measured by plotting APD as a function of DI measured during periods of rapid pacing used to promote alternans.²⁹ Arrhythmia susceptibility was determined using a standardized ramp pacing protocol starting at 300 ms (200 bpm) with stepwise 10-ms decrements in pacing CL until failure of 1/1 capture or the induction of a ventricular arrhythmia.²⁹ Arrhythmias were defined as a tachyarrhythmia sustained for >30 seconds after pacing was halted.

**Statistical Analysis**

Statistical analyses of data were performed using Sigmastat (SPSS, Inc, Chicago, Ill). Statistical differences were assessed with Student t test and Fisher exact test as appropriate. When unequal variabilities was detected during normality testing, the Wilcoxon rank-sum test was used. Results are expressed as mean±SEM.

**Results**

**Efficiency of Transgene Expression**

Figure 1 shows transduction efficiency 72 hours after in vivo gene transfer. Panel A illustrates a representative example of an X-gal–stained cross section of guinea pig ventricles after Ad.β-gal gene transfer. The blue stain demonstrates relatively homogeneous gene transfer throughout the epicardium and extending through approximately two thirds of the transmural wall, leaving the endocardial-most myocardium unstained. Panel B illustrates myocytes isolated from the left ventricular free wall of a guinea pig heart transduced with Ad.SERCA2a. The fluorescent image shows GFP transduction efficiency (green cells), which averaged 29±2%. Panel C demonstrates that SERCA2a (n=3) gene transfer significantly increased SERCA2a protein expression in the left ventricular free wall by 37±7% (P<0.05), compared with control (n=3).

**Effect of SERCA2a Gene Transfer on Calcium Transient Characteristics**

Intracellular Ca²⁺ transients and action potentials were induced under current clamp-conditions at a pacing rate of 150 bpm at 30°C and were compared between isolated control myocytes and myocytes transduced with Ad.SERCA2a.²⁸ The fluorescent image shows GFP transduction efficiency (green cells), which averaged 29±2%. Panel C demonstrates that SERCA2a (n=3) gene transfer significantly increased SERCA2a protein expression in the left ventricular free wall by 37±7% (P<0.05), compared with control (n=3).
transient characteristics, APD was not significantly changed by SERCA2a overexpression (Figure 2A). As expected, these data demonstrated that in vivo transduction of Ad.SERCA2a improved SR Ca\(^{2+}\) reuptake accompanied by larger Ca\(^{2+}\) transients and faster rates of \([\text{Ca}^{2+}]_i\) decline, confirming that this model of SERCA2a overexpression produces a meaningful effect on SR calcium cycling.

**Effect of SERCA2a Gene Transfer on Susceptibility to Cellular Alternans in Isolated Myocytes**

APD-ALT and Ca-ALT were measured simultaneously as stimulus rate was progressively increased. Figure 2A shows representative examples of action potential and Ca transient recordings obtained from an Ad.SERCA2a myocyte and a control myocyte. Traces recorded from 2 consecutive beats are superimposed to illustrate alternans. APD-ALT and Ca-ALT were induced as stimulation rate was increased to 200 bpm in the control myocyte, whereas alternans was not initiated until a pacing rate of 375 bpm in the Ad.SERCA2a myocyte. B, SERCA2a overexpression increased alternans threshold and decreased alternans magnitude in isolated myocytes. Plot of pacing rate versus magnitude of APD-ALT from summary data of control myocytes (n=9) and Ad.SERCA2a myocytes (n=11) shows that the magnitude of APD-ALT increased as pacing rate increased, and the magnitude of APD-ALT was consistently greater in control myocytes compared with Ad.SERCA2a myocytes. Inset shows that under current-clamp conditions, the threshold stimulation rate for both APD-ALT and Ca-ALT in control myocytes (n=9) is significantly lower than in Ad.SERCA2a myocytes (n=9) (265 \pm 17 bpm and 349 \pm 22 bpm, respectively, \(P<0.01\)).

Figure 3 illustrates that SERCA2a overexpression imparts significant resistance to cellular alternans even under constant...
action potential (AP) clamp conditions (ie, when APD-ALT is prevented). The top tracing is a representation of the AP clamp protocol (voltage command). In this example, Ca2+ transients recorded under constant AP clamp conditions at a stimulation rate of 200 bpm are shown in the middle (Ad.SERCA2a myocyte) and in the bottom (control myocyte). At this stimulation rate, Ca2+ transients alternate in the control myocyte but not in the Ad.SERCA2a myocyte. B, The differences in threshold for Ca-ALT between control (n=12) and Ad.SERCA2a myocytes (n=4) remained even under constant AP clamp conditions (254±11 bpm and 352±26 bpm, respectively, P<0.01).

Effect of SERCA2a Gene Transfer on Susceptibility to Cellular Alternans in the Intact Beating Heart

APD alternans was measured as pacing rate was progressively increased in the Langendorff-perfused whole heart. Figure 5 demonstrates that overexpression of SERCA2a increased alternans threshold and decreased alternans magnitude in the whole heart. The plot of pacing rate versus magnitude of APD-ALT from Ad.SERCA2a, control Langendorff-perfused whole hearts shows that the magnitude of APD-ALT increased as pacing rate increased, and the magnitude of APD-ALT was consistently greater in control (n=8) hearts compared with Ad.SERCA2a (n=4) hearts. Inset shows that the threshold pacing rate for APD-ALT in control hearts is significantly lower than Ad.SERCA2a hearts (396±11 bpm and 484±25 bpm, respectively, P<0.01). *P<0.01, Ad.SERCA2a versus control.

Figure 4. SERCA2a overexpression increased alternans threshold even under constant AP clamp conditions. A, Ca-ALT occurred under constant AP clamp conditions. The top trace is AP clamp protocol (voltage command). In this example, Ca2+ transients recorded under constant AP clamp conditions at stimulation rate of 200 bpm are shown in the middle (Ad.SERCA2a myocyte) and in the bottom (control myocyte). At this stimulation rate, Ca2+ transients alternate in the control myocyte but not in the Ad.SERCA2a myocyte. B, The differences in threshold for Ca-ALT between control (n=12) and Ad.SERCA2a myocytes (n=4) remained even under constant AP clamp conditions (254±11 bpm and 352±26 bpm, respectively, P<0.01).

Figure 5. SERCA2a overexpression increased alternans threshold and decreased alternans magnitude in the whole heart. Plot of pacing rate versus magnitude of APD-ALT from Ad.SERCA2a, control Langendorff-perfused whole hearts shows that the magnitude of APD-ALT increased as pacing rate increased, and the magnitude of APD-ALT was consistently greater in control (n=8) hearts compared with Ad.SERCA2a (n=4) hearts. Inset shows that the threshold pacing rate for APD-ALT in control hearts is significantly lower than Ad.SERCA2a hearts (396±11 bpm and 484±25 bpm, respectively, P<0.01). *P<0.01, Ad.SERCA2a versus control.

In addition to calcium cycling mechanisms, APD restitution has been implicated in the mechanism of APD-ALT. However, as shown in Figure 6, there was no difference in APD restitution (average APD restitution from all experi-
ments are plotted) between control hearts and Ad.SERCA2a hearts. Moreover, derived metrics of restitution properties such as maximum restitution curve slope and predicted onset HR for APD-ALT failed to predict the marked reduction in susceptibility to APD-ALT seen SERCA2a-treated hearts.

Effect of SERCA2a Gene Transfer on Susceptibility to Ventricular Arrhythmia

Figure 7A shows representative optical action potential tracings from control and Ad.SERCA2a transduced hearts. In the control heart, beat-to-beat alternation of APD is seen immediately preceding the onset of ventricular fibrillation. This was a consistent finding in all control preparations. Conversely, in the heart transduced with Ad.SERCA2a, the identical stimulation sequence fails to induce cellular alternans or ventricular fibrillation. Figure 7B demonstrates that SERCA2a overexpression reduced susceptibility to alternans-mediated ventricular arrhythmias compared with control ($P<0.05$).

In summary, these data show that overexpression of SERCA2a inhibited cellular alternans in both isolated myocytes and the whole heart, suggesting that SERCA2a function plays an important role in the initiation of cellular alternans. Furthermore, SERCA2a overexpression suppresses alternans-mediated ventricular arrhythmias in the intact beating heart.

Discussion

Previously, we and others demonstrated that cellular alternans is a mechanistic precursor to ventricular fibrillation in the mammalian heart. Presumably in diseased hearts, by lowering the HR threshold for T-wave alternans, vulnerability to ventricular fibrillation increases. Therefore, identifying molecular mechanisms that modulate susceptibility to alternans could provide important clues to novel triggers of electric instability in the heart. The primary findings of this investigation are (1) overexpression of SERCA2a significantly inhibited cellular alternans in both isolated myocytes and the whole heart, and (2) SERCA2a overexpression suppresses alternans-mediated ventricular arrhythmias in the intact beating heart. These data support our hypotheses that SR calcium cycling plays a causative role in cellular alternans, SERCA2a function specifically plays an important role in the initiation of cellular alternans, and SERCA2a is a potentially novel molecular therapeutic target for the treatment of ventricular arrhythmias.

Sarcoplasmic Reticulum Ca$^{2+}$ Cycling Underlies Mechanism of Cellular Alternans

There are 2 major hypotheses that have been proposed to explain the development of cellular alternans: (1) the APD restitution hypothesis, largely secondary to alternating sarcolemmal currents, and (2) the calcium cycling hypothesis, which states that alternans occurs when HR exceeds the capacity of the myocyte to cycle calcium. Theoretical models suggest that APD alternans occurs when the slope of the APD restitution curve exceeds unity. Moreover, a variety of sarcolemmal currents such as $I_{\text{Na}}$, $I_{\text{Ca}}$, and $I_{\text{Kr}}$ can exhibit alternating-type activity. Although the restitution hypothesis provides a very useful theoretical framework for understanding the dynamics of APD alternans, there is also considerable experimental evidence that does not support the restitution...
hypothesis.\textsuperscript{29,9} By using in vivo gene transfer to selectively increase SERCA2a expression, the present study shows that selective enhancement of SR Ca\textsuperscript{2+} reuptake significantly inhibits both Ca-AlT and V\textsubscript{m}-ALT. These findings indicate a causative role of SR Ca\textsuperscript{2+} cycling in the mechanism of V\textsubscript{m}-ALT. However, it is also possible, based on theoretical predictions, that SERCA2a overexpression could have altered sarcolemmal ionic currents and that these alterations could alter susceptibility to cellular alternans.\textsuperscript{9} In the present investigation, we distinguished mechanisms arising from intracellular Ca\textsuperscript{2+} handling versus sarcolemmal ionic currents in isolated myocytes by using a nonalternating action potential (AP) clamp. Moreover, because AP clamp is not possible in the intact heart, we compared APD restitution properties of SERCA2a with controls, particularly because APD restitution has been used as an index of the extent to which sarcolemmal ionic currents drive cellular alternans. Interestingly, suppression of Ca-AlT under AP clamp conditions in myocytes (Figure 4) and the lack of a change in APD restitution in whole hearts overexpressing SERCA2a (Figure 6) provide important new evidence that calcium cycling properties can underlie susceptibility to alternans without any involvement of APD restitution. Also, our data are consistent with previous observations that inhibiting Ca\textsuperscript{2+} cycling by blocking the RyR, I\textsubscript{Ca} or by depleting SR Ca\textsuperscript{2+} stores with caffeine eradicates V\textsubscript{m}-ALT.\textsuperscript{11,34,35} Furthermore, the seminal observations of Chudin et al\textsuperscript{1} and our laboratory\textsuperscript{42} that Ca-AlT is similarly induced under current-clamp (where V\textsubscript{m}-ALT occurs) and voltage-clamp (ie, where V\textsubscript{m}-ALT is prevented) conditions proved that Ca-AlT is not dependent on V\textsubscript{m}-ALT and strongly supported the notion that cellular alternans arises from SR Ca\textsuperscript{2+} cycling.

Role of SERCA2a in Molecular Mechanism of Cellular Alternans
Despite the importance of Ca-AlT on the development of APD-AlT, the specific Ca\textsuperscript{2+} cycling proteins responsible for alternans were based primarily on predictions from theoretical models and experimental evidence relying on associations between impaired SR calcium handling, diminished expression of calcium handling proteins, and susceptibility to cellular alternans.\textsuperscript{32,43,19} The advantage to using in vivo gene transfer targeting a single gene, as in the present study, is the ability to demonstrate a causal relationship between a single protein and the development of cellular alternans. Previously, we suggested that impaired SR Ca\textsuperscript{2+} reuptake may represent a mechanism to initiate alternans.\textsuperscript{42} For example, when compared with epicardial myocytes, endocardial myocytes had reduced SERCA2a expression and reduced ability to reuptake cytosolic Ca\textsuperscript{2+} into the SR. As such, we hypothesized that reduced SERCA2a expression may underlie the increased susceptibility of endocardial myocytes to develop cellular alternans. In the current investigation, we performed in vivo gene transfer using a modified aorto-pulmonary artery cross-clamp technique to achieve a 37% increase in SERCA2a. This resulted in improved SR Ca\textsuperscript{2+} reuptake (ie, accelerated Ca\textsuperscript{2+} transient decay and increased Ca\textsuperscript{2+} transient amplitude) and inhibited cellular alternans in both isolated myocytes and the whole heart. Our results are consistent with the recent observations of Xie et al\textsuperscript{44} that adenoviral-mediated SERCA2a overexpression in cultured rabbit ventricular myocytes suppresses Ca-AlT and the findings that the SERCA2a inhibitor thapsigargin increases susceptibility to cellular alternans.\textsuperscript{15}

Though this investigation demonstrates with a high degree of specificity that the SERCA2a protein directly affects susceptibility to cellular alternans, these data do not rule out other synergistic or complementary molecular mechanisms. For example, observations from both experimental and theoretical models have demonstrated a steep dependence of SR Ca\textsuperscript{2+} release on SR Ca\textsuperscript{2+} load as a mechanism for the development of Ca\textsuperscript{2+} alternans.\textsuperscript{7,43} Diaz et al\textsuperscript{7} used an innovative albeit nonphysiological stimulation protocol to induce Ca-AlT without pharmacological inhibition of RyR (thereby avoiding nonselective drug effects). Ventricular myocytes were repetitively voltage-clamped below the activation voltage for I\textsubscript{Ca}. The resulting weak CICR produced desynchronized RyR release, which dramatically steepened the relationship (feedback gain) between SR Ca\textsuperscript{2+} content (ie, luminal Ca\textsuperscript{2+}) and the subsequent SR Ca\textsuperscript{2+} release. These subcellular conditions highly favored the development of Ca-AlT dynamics that are dependent on beat-to-beat alternation of SR Ca\textsuperscript{2+} content. Moreover, instabilities of SR Ca\textsuperscript{2+} release can also lead to Ca-AlT. For example, using metabolic inhibition in cat atrial and ventricular myocytes to inhibit RyR phosphorylation, Huser et al\textsuperscript{13} reported Ca-AlT without beat-to-beat fluctuations in SR content, suggesting that refractory-like properties of RyR can produce alternating open probabilities of the channel irrespective of SR Ca load. Also, Picht et al\textsuperscript{27} recently demonstrated that beat-to-beat variations in recovery from inactivation of the RyR without variation in SR Ca\textsuperscript{2+} load can produce Ca-AlT. In contrast, Lehnart et al\textsuperscript{20} recently demonstrated that Calstabin (FKBP12.6) deficiency increases susceptibility to the development of APD-AlT by destabilizing RyR.

Interestingly, in the present study, inhibition of cellular alternans in the intact heart occurred despite modest adenoviral transduction efficiency on a cellular scale (29±2% of myocytes). Importantly, our method of virus delivery produced spatially homogeneous transgene expression in essentially all regions of the heart that were readily accessible for detailed electrophysiological phenotyping using high-resolution optical mapping of the intact heart. Moreover, this study demonstrates that complete gene transfer is not required to produce an important electrophysiological phenotype. One explanation for this finding is that electrotonic interactions between neighboring cells via gap junctions act to homogenize membrane potential across cells. For example, reduction in Ca-AlT and therefore APD-AlT in a cell transduced with Ad.SERCA2a is expected to attenuate APD-AlT in a nontransduced neighboring cell. This is supported by the observation that the magnitude of SERCA2a suppression of cellular alternans in isolated myocytes (Figure 3B) is greater than in whole hearts (Figure 5). These findings have practical clinical implications, suggesting that strategies designed to target SERCA2a gene expression in patients probably does not need to achieve high transduction efficiency for a desirable clinical benefit to be realized. However, it is likely that...
gene delivery does need to be spatially homogenous throughout the myocardium because lack of homogeneity could produce electrophysiological heterogeneities that are potentially arrhythmogenic. In fact, the completion of a phase 1/2 clinical trial of Myocardial Delivery of AAV1/SERCA2a in Subjects with Advanced Heart Failure has shown to have an acceptable safety profile in the patients.37

Enhanced SERCA2a Gene Expression Interrupts a Pathway to Arrhythmogenesis

The present study demonstrates that targeted overexpression of SERCA2a reduces cellular alternans and susceptibility to inducible arrhythmias in the intact heart. Previously, we demonstrated a mechanistic link between cellular alternans and the genesis of ventricular arrhythmias.25 Specifically, discordant alternans (ie, repolarization alternans occurring with opposite phase between neighboring cells) alters the spatial organization of repolarization across the ventricle by markedly amplifying preexisting heterogeneities of repolarization in the heart, producing a substrate prone to conduction block and reentrant arrhythmogenesis. Therefore, suppression of cellular alternans in the present study decreases the likelihood for amplifying heterogeneity of repolarization, conduction block, and thus, ventricular arrhythmias. This observation is consistent with the clinical observation that patients with heart failure with a negative T-wave alternans test (the surface ECG representation of cellular alternans) are markedly resistant to sudden cardiac death.38 Furthermore, our data are supported by the observations of del Monte et al6 and Prunier et al28 that overexpression of SERCA2a suppressed ventricular alternans in both rat and porcine models of ischemia-reperfusion (Ca\(^{2+}\) overload). Specifically, ischemia-reperfusion increases diastolic calcium and has been linked to delayed afterdepolarizations and triggered arrhythmias. The authors speculate that enhanced SR calcium reuptake with SERCA2a overexpression decreases diastolic Ca\(^{2+}\), thus decreasing the incidence of delayed afterdepolarizations and triggered arrhythmias. Additionally, Prunier et al28 speculated that a possible mechanism by which SERCA2a overexpression suppressed the development of ventricular arrhythmias in ischemia-reperfusion is by inhibiting cellular alternans.

Targeted overexpression of SERCA2a as an antiarrhythmic therapy has a potential advantage in that this approach does not target sarcolemmlal K\(^+\) channels; a strategy known to cause QT interval prolongation and proarrrhythmia. SERCA2a gene transfer did not prolong repolarization in our studies. However, it is possible that overexpression of SERCA2a could be arrhythmogenic because enhanced SR calcium load could increase susceptibility to spontaneous SR calcium release and delayed afterdepolarization–mediated triggered arrhythmias. However, in the present study we saw no evidence of spontaneous arrhythmias or delayed afterdepolarizations. This may be explained by the modest degree of SERCA2a overexpression seen in this study.

Pathophysiological Implications

Our data have important clinical implications. T-wave alternans has been observed in patients with heart failure and is an important marker of risk for sudden cardiac death.24,31,38 Moreover, altered calcium cycling is a common observation in heart failure1 probably caused by reduced SERCA2a expression (impaired SR Ca\(^{2+}\) reuptake), increased phosphorylation of RyR (impaired SR Ca\(^{2+}\) release), and/or altered NCX expression/function. The current investigation suggests that SERCA2a dysfunction can play an important role in modulating susceptibility to cellular alternans, providing a potential link between mechanical and electric dysfunction in the failing heart. To our knowledge, our data are the first to support a direct causal relationship between SERCA2a function and susceptibility to cellular alternans in the intact heart. Importantly, SERCA2a overexpression has been shown to reverse failure-induced changes in contractility.9 Moreover, inhibition of cellular alternans produces a myocardial substrate that is resistant to reentry and fatal arrhythmias. As such, in vivo gene transfer targeting key SR Ca\(^{2+}\) cycling proteins (ie, SERCA2a) provides a novel method for understanding the underlying mechanisms for the development of sudden cardiac death. More importantly, an understanding of these mechanisms combined with improved gene transfer techniques offer a novel strategy for arrhythmia therapy in humans.

Acknowledgments

We thank Michelle Jennings for assistance with data analysis.

Sources of Funding

This study was supported by National Institutes of Health grant RO1-HL54807 (D.S.R.) and Fellowship Awards from the Heart Rhythm Society and National Institutes of Health National Research Service (M.J.C.).

Disclosures

Dr Hajjar has significant ownership interest in Celladon and Nanocr.

References

FUNCTIONAL COUPLING BETWEEN GLYCOLYSIS AND EXCITATION-CONTRACTION COUPLING UNDERLIES ALTERNAN IN CARDIAC Myocytes

Mechanism Linking T-Wave Alternans to Cardiac Function

Role of Structural Barriers in the Mechanism of Repolarization Alternans

Possible Contribution of the Sarcoplasmic Reticulum Ca(2+) Pump Function to Electrical and Mechanical Alternans

Electrolyte Inclusions in Intact Guinea Pig Hearts

Myocardial Functional Coupling between Glycolysis and Excitation-Contraction Coupling Underlies Alternans in Cardiac Cells

Differences in the Electrophysiological Function to Electrical and Mechanical Alternans

Circ Arrhythm Electrophysiol December 2009

Clinical Perspective

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 recognized marker of risk for sudden cardiac death in patients. However, the molecular basis for cardiac alternans is poorly understood. Previously, we reported an association between deficient function of the transient outward current and arrhythmias.

However, the molecular basis for cardiac alternans is poorly understood. Previously, we reported an association between deficient function of the transient outward current and arrhythmias. In the present study, we demonstrated that targeted in vivo gene transfer of SERCA2a cassettes with greatly improved fluorescence properties.

In the present study, we demonstrated that targeted in vivo gene transfer of SERCA2a cassettes with greatly improved fluorescence properties. In intact guinea pig hearts.

Recently, we reported an association between deficient function of the transient outward current and arrhythmias. Therefore, we investigated the molecular basis for cardiac alternans.

Recently, we reported an association between deficient function of the transient outward current and arrhythmias. Therefore, we investigated the molecular basis for cardiac alternans. In the present study, we demonstrated that targeted in vivo gene transfer of SERCA2a cassettes with greatly improved fluorescence properties.

In the present study, we demonstrated that targeted in vivo gene transfer of SERCA2a cassettes with greatly improved fluorescence properties. In intact guinea pig hearts.

Recently, we reported an association between deficient function of the transient outward current and arrhythmias. Therefore, we investigated the molecular basis for cardiac alternans.

Recently, we reported an association between deficient function of the transient outward current and arrhythmias. Therefore, we investigated the molecular basis for cardiac alternans. In the present study, we demonstrated that targeted in vivo gene transfer of SERCA2a cassettes with greatly improved fluorescence properties.

In the present study, we demonstrated that targeted in vivo gene transfer of SERCA2a cassettes with greatly improved fluorescence properties. In intact guinea pig hearts.

Recently, we reported an association between deficient function of the transient outward current and arrhythmias. Therefore, we investigated the molecular basis for cardiac alternans.

Recently, we reported an association between deficient function of the transient outward current and arrhythmias. Therefore, we investigated the molecular basis for cardiac alternans. In the present study, we demonstrated that targeted in vivo gene transfer of SERCA2a cassettes with greatly improved fluorescence properties.
Targeted SERCA2a Gene Expression Identifies Molecular Mechanism and Therapeutic Target for Arrhythmogenic Cardiac Alternans
Michael J. Cutler, Xiaoping Wan, Kenneth R. Laurita, Roger J. Hajjar and David S. Rosenbaum

Circ Arrhythm Electrophysiol. 2009;2:686-694; originally published online October 2, 2009; doi: 10.1161/CIRCEP.109.863118
Circulation: Arrhythmia and Electrophysiology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 1941-3149. Online ISSN: 1941-3084

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/2/6/686

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2009/10/06/CIRCEP.109.863118.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation: Arrhythmia and Electrophysiology 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: Arrhythmia and Electrophysiology is online at:
http://circ.ahajournals.org/subscriptions/