Prevention of Ventricular Arrhythmias With Sarcoplasmic Reticulum Ca\(^{2+}\) ATPase Pump Overexpression in a Porcine Model of Ischemia Reperfusion

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Background—Ventricular arrhythmias are life-threatening complications of heart failure and myocardial ischemia. Increased diastolic Ca\(^{2+}\) overload occurring in ischemia leads to afterdepolarizations and aftercontractions that are responsible for cellular electric instability. We inquired whether sarcoplasmic reticulum Ca\(^{2+}\) ATPase pump (SERCA2a) overexpression could reduce ischemic ventricular arrhythmias by modulating Ca\(^{2+}\) overload.

Methods and Results—SERCA2a overexpression in pig hearts was achieved by intracoronary gene delivery of adenovirus in the 3 main coronary arteries. Homogeneous distribution of the gene was obtained through the left ventricle. After gene delivery, the left anterior descending coronary artery was occluded for 30 minutes to induce myocardial ischemia followed by reperfusion. We compared this model with a model of permanent coronary artery occlusion. Twenty-four-hour ECG Holter recordings showed that SERCA2a overexpression significantly reduced the number of episodes of ventricular tachycardia after reperfusion, whereas no significant difference was found in the occurrence of sustained or nonsustained ventricular tachycardia and ventricular fibrillation in pigs undergoing permanent occlusion.

Conclusions—We show that Ca\(^{2+}\) cycling modulation using SERCA2a overexpression reduces ventricular arrhythmias after ischemia-reperfusion. Strategies that modulate postischemic Ca\(^{2+}\) overload may have clinical promise for the treatment of ventricular arrhythmias. (Circulation. 2008;118:614-624.)

Key Words: arrhythmia · calcium · gene therapy · infarction · ischemia · reperfusion · sarcoplasmic reticulum

Two common patterns in the initiation of fatal arrhythmias have been recognized in patients with ischemic heart disease: ventricular tachyarrhythmia triggered by acute myocardial ischemia and during reperfusion in patients with or without preexisting myocardial scarring and ventricular tachyarrhythmia related to an anatomic scarring from previous myocardial infarctions without active myocardial ischemia.

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At the molecular level, the mechanisms of ventricular arrhythmias are heterogeneous, but common mechanisms have been identified in triggering arrhythmias as changes in the membrane potential, ion transporters, and intracellular Ca\(^{2+}\) handling.

The role of abnormal Ca\(^{2+}\) signaling in the genesis of cardiac arrhythmias has been known for many years through various mechanisms. Ca\(^{2+}\) overload of the sarcoplasmic reticulum (SR) generates spontaneous release of Ca\(^{2+}\) by the ryanodine receptors and a depolarizing inward current mediated by the sodium-calcium exchanger. These spontaneous events, known as delayed afterdepolarizations, underlie triggered arrhythmias. Early afterdepolarizations, another source of arrhythmias occurring during reperfusion injury, are caused by prolonged action potentials allowing excessive Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels. In addition, a reperfusion-induced rise in [Ca\(^{2+}\)]\(_{i}\) induces heterogeneity of repolarization. Abnormal Ca\(^{2+}\) cycling by the SR also has been implicated in the pathogenesis of action potential alternans, spiral wave breakup, and ventricular fibrillation (VF). Thus, many aspects of Ca\(^{2+}\) cycling are inviting targets for antiarrhythmic strategies. In this study, we inquired whether SR Ca\(^{2+}\) ATPase pump (SERCA2a) overexpression could protect against ventricular arrhythmias by modulating Ca\(^{2+}\) overload.
Study Flow Chart

Catheter-Based Intracoronary Adeno-Viral Gene delivery Virus injection:

Ad. β-galactosidase (n=17) or Ad. Serca2a (n=17)

Day 0

2 exclusions (1 tamponade, 1 congenital ventricular septal defect)

Day 7

Basal cardiac function measurements (echocardiography, thermodilution catheter, left ventricular pressure catheter)

Permanent occlusion:
- Ad.βgal (n=6)
- Ad.Serca2a (n=9)

30 min occlusion:
- Ad.βgal (n=7)
- Ad.Serca2a (n=6)

Sham procedure:
- Ad.βgal (n=2)
- Ad.Serca2a (n=2)

1 exclusion (thrombus in LAD)

Day 8

Basal cardiac function measurements (echocardiography, thermodilution catheter, left ventricular pressure catheter)

Sacrifice (Area at risk and MI size assessment, tissue collection)

24 HOURS ECG HOLTER

Animal Studies

All procedures were performed under anesthesia (isoflurane), and the Institutional Animal Care Committee approved the experiment protocol. All pigs were female of the same body weight. In this set of experiments, 34 pigs were randomized to receive either the control adenovirus carrying β-galactosidase (n=17) or the SERCA2a gene (n=17) (Figure 1).

Construction of Adenovirus

To construct the adenoviruses, we used the method described by He et al.10 SERCA2a cDNA was subcloned into the adenoviral shuttle vector (pAd.TRACK), which uses the cytomegalovirus long terminal repeat as a promoter. The shuttle vector used also has a concomitant green fluorescent protein under the control of a separate cytomegalovirus promoter. An adenovirus containing both β-galactosidase and green fluorescent protein controlled by separate cytomegalovirus promoters (Ad.β-gal-GFP) was used as control.

Gene Delivery

The gene was delivered to the myocardium using percutaneous anterograde myocardial gene transfer (PAMGT) as previously described.11 Using this technique, we injected viral vectors directly into the coronary artery distal to an angioplasty balloon occluding the vessel proximally while the venous drainage was occluded thought the coronary sinus. For the LAD, left circumflex artery, and right coronary artery territories, the myocardium was preconditioned with a 1-minute arterial balloon occlusion. With both the arterial and venous balloons inflated (total, 3 minutes) and after an intracoronary adenosine (25 μg) injection to increase cellular permeability, PAMGT was performed by anterograde injection through the lumen of the angioplasty balloon with either an adenoviral solution (1 mL of 1011 plaque-forming units in each coronary) carrying β-galactosidase or SERCA2a. Arterial blood pressure was monitored continuously.

Procedures Protocol

Seven days after gene delivery, pigs from each group (SERCA2a and β-galactosidase) were assigned to undergo ischemia-reperfusion (I/R) using a 30-minute balloon occlusion (n=13), permanent occlusion (PO) using embolic coils (n=16) in the LAD, or a sham procedure without LAD occlusion (n=4) (Figure 1). Aortic pressure, ECG, and oxygen blood saturation were monitored throughout all procedures. An ECG Holter device was connected to the pigs at the beginning of I/R, PO, or the sham procedure. Echocardiography and invasive hemodynamic parameters were measured at baseline before I/R, PO, or the sham procedure. Twenty-four hours later, the Holter recording was stopped, and the device was removed for analysis. The echocardiography parameters and invasive hemodynamic parameters were repeated at this time.

Echocardiography

Transhoracic 2-dimensional and M-mode echocardiography images were obtained in anesthetized animals with a 3.4-MHz probe (Vivid 7, GE Healthcare, Waukesha, Wis). A midpapillary-level left ventricular (LV) short-axis view was used to measure anterior wall over the wire and the proximal site of the left anterior descending artery (LAD), and the coronary balloon was inflated incrementally until complete occlusion was confirmed by angiography. Similarly, an angioplasty balloon was placed proximal to the left circumflex and right coronary arteries. For the LAD, left circumflex artery, and right coronary artery territories, the myocardium was preconditioned with a 1-minute arterial balloon occlusion. With both the arterial and venous balloons inflated (total, 3 minutes) and after an intracoronary adenosine (25 μg) injection to increase cellular permeability, PAMGT was performed by anterograde injection through the lumen of the angioplasty balloon with either an adenoviral solution (1 mL of 1011 plaque-forming units in each coronary) carrying β-galactosidase or SERCA2a. Arterial blood pressure was monitored continuously.
thickness, LV systolic and diastolic dimensions, and fractional shortening.

Thermodilution Catheter
A Swan-Ganz catheter was inserted through the femoral vein to measure pressure in the right atria, right ventricle, pulmonary artery, and pulmonary capillary wedge. The catheter was then positioned in the pulmonary artery to measure cardiac output by the thermodilution method.

LV Pressure Measurement
A Millar pigtail catheter (Millar Instruments Inc, Houston, Tex) was introduced through the femoral artery in the LV cavity. Pressure measurements were digitized at 1 kHz. LV systolic pressure, LV end-diastolic pressure, and the maximal rates of pressure rise and fall were measured offline.

Arrhythmia Recording
The Holter device (Del Mar Reynolds, Spacelabs Healthcare, Issaquah, Wash) was attached to the pigs with a protective bandage around the chest to record the ECG over a 24-hour period. Three adhesive leads were placed at the fifth intercostal space on the left and right anterior axillary lines, with the reference electrode on the manubrium of the sternum. The ECG was recorded continuously (Figure 2).

To classify ventricular arrhythmias, we defined 3 periods of time in the I/R groups: first, ischemia, corresponding to the 30 minutes of LAD occlusion (ischemia); second, the reperfusion period, corresponding to the first 10 minutes immediately after balloon deflation (early reperfusion); and third, the follow-up period, corresponding to the time from the 11th minute after balloon deflation to the end of Holter recording, ie, 24 hours later or to the last ventricular beat in case of death (late reperfusion). In the PO groups, the follow-up period started from the occurrence of ST elevation approximately 1 minute after coil insertion. The ECG was monitored continuously in all groups for 90 minutes from balloon inflation or coil insertion. Sustained ventricular tachycardia (VT) or VF was treated by defibrillation during these 90 minutes. VT was defined by 3 consecutive ventricular extra beats with heart rate ≥120 bpm. Sustained VT was defined by VT duration over 30 seconds.

Ischemia-Reperfusion
Seven days after gene delivery in the I/R groups, a coronary balloon was inflated in the mean LAD beyond the takeoff of the first diagonal branch for 30 minutes, inducing transmural ischemia, and then deflated to reperfuse the LAD territory. Specifically, the LAD was cannulated with a 7F hockey stick guiding catheter; 100 μg nitroglycerin was injected; and baseline coronary angiography was performed. A 3.5-mm over-the-wire balloon catheter was deployed in the LAD beyond the takeoff of the first diagonal branch to induce transmural ischemia. Coronary angiography was performed to confirm total occlusion with the balloon. After 30 minutes of occlusion, the balloon catheter was deflated to reperfuse the LAD. The duration of the ischemic time was previously shown to be critical for the induction of arrhythmia on reperfusion, with an occlusion time <2 minutes and >45 minutes no longer inducing the vulnerability to VF.12–14 The duration of the occlusion time also determines the incidence of reperfusion arrhythmias as a function of the extent of the ischemic injury,15 arguing for the importance of the release of metabolic products from the injured myocytes during the occlusion.

Figure 2. Representative ECG tracing of the types of ventricular arrhythmias from the Holter recording. A, Baseline recording; B, Recording during balloon occlusion; C, VT; D, VF; E, Idioventricular accelerated rhythm.
time. We therefore limited the occlusion time to 30 minutes. Continuous ECG and aortic pressure were monitored carefully during the procedure.

**Permanent Coronary Occlusion**

As described for the I/R groups, the LAD was cannulated with a 7F hockey stick guiding catheter; 100 μg nitroglycerin was injected; and baseline coronary angiography was performed. An embolic coil (0.018 in, 4 cm length, 4×2-mm diameter, Cook Medical Inc, Bloomington, Ind) was introduced with a 2.6F microcatheter (Excelsior, Boston Scientific/Target, Fremont, Calif) into the LAD beyond the takeoff of the first diagonal branch to completely occlude the mid LAD and to induce myocardial infarction. Coronary angiography was performed to confirm the total occlusion with the coil.

**Gene Distribution**

Intracoronary injection was performed in a spare pig to visualize the distribution area by injecting a near-infrared fluorescent dye (IRDYE 786) (Sigma-Aldrich No. 102185-03-5, Sigma-Aldrich, St Louis, Mo)16 and fluorescent microspheres (Molecular Probes, Carlsbad, Calif) instead of adenoviral solution (Figure 3).

**Detection of Gene Expression by Immunohistochemistry**

Gene infection was detected by expression of the reporter gene β-galactosidase on frozen sections. The staining is based on the hydrolysis of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal), which yields a blue precipitate. β-Galactosidase activity was measured using X-gal 40 mg/mL in dimethylformamide on tissue sections fixed with 0.5% glutaraldehyde.

**Immunoblotting**

Protein concentrations in tissue lysate preparations were measured with the Bradford method.27 Immunoblotting was performed under reducing conditions on a gradient gel. For immunoreactions, the blots were incubated with antibodies for anti-SERCA2a (antibody made in the laboratory); anti-GADPH (Zymed, 39-8600, Zymed Laboratories Inc, South San Francisco, Calif) was used for normalization against total proteins.

**RNA Isolation and Retrotranscription**

We quantified the levels of human SERCA2a present in the anterior wall samples from 4 animals from the SERCA2a and β-galactosidase I/R groups and 1 sample from a sham pig by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Tissue (100 mg) was homogenized in TRIzol (Invitrogen, Carlsbad, Calif) and processed according to the manufacturer’s guidelines.

RNA was retrotranscribed with the Omniscript RT kit (Qiagen, Valencia, Calif) following the manufacturer’s protocol. qRT-PCR was performed on the cDNA obtained from the reverse transcription using RT² SYBR Green/ROX PCR Master Mix (Superarray, Frederick, Md) with the following conditions: 15 minutes at 95°C for 40 cycles (95°C for 15 seconds, 64°C for 30 seconds, 72°C for 30 seconds). The following primers were used to evaluate the content of human SERCA2a var2 mRNA: forward, 5'-CCTCCACAG-TCTAAAATC-3' and reverse, 5'-AGCAATGCCAATCTCGGT-3'.

To differentiate the endogenous SERCA2a from the exogenous human SERCA2a injected into the tissue, we compared the human and the swine sequences of SERCA2a and identified the regions characterized by the higher interspecific variability. These primers were manually selected to interact with these regions. Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used to identify the primer combinations characterized by lower self-dimerization and 3' compatibility.

Because of the high conservation of the nucleotide sequence of SERCA2a, the choice of primers was limited, and the primers selected were characterized by 2 and 5 nucleotide mismatches between the 2 sequences. As expected from the design process, the selected primers were affected by high 3' complementarities, producing a background in the blank. The combination of the use of these primers and elevated annealing temperature produced a negligible amplification of the swine SERCA2a DNA.

The content of the transcript in each sample was standardized to the level of swine 18S RNA using the following primers: forward, 5'-AGACAAATTCGCTCACCACAC-3' and reverse, 5'-GACT-CACAACGGGAAACCTC-3'. The data were analyzed with the 7000 SDS 1.1 RQ application (Applied Biosystem, Foster City, Calif). The software automatically detected the C₅ threshold for both transcripts analyzed.

**Figure 3.** Images showing the distribution of IRDYE 786 and fluorescent beads as a representation of the distribution of the viral particles using PAMGT. A solution with red fluorescent beads mixed with fluorescent dye (NIR786 dye, 2 mg/mL) is injected in place of the viral solution during PAMGT in the 3 coronary arteries. Top, Front view of the entire heart; bottom, heart sections. Left, Direct light; middle, red fluorescent beads distribution; right, IRDYE 786 distribution.
Table 1. Baseline Echocardiography and Hemodynamic Data

<table>
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<th>β-Gal–Coil (n=6)</th>
<th>SERCA2a-Coil (n=9)</th>
<th>β-Gal–IR (n=6)</th>
<th>SERCA2a-IR (n=6)</th>
<th>β-Gal–Sham (n=2)</th>
<th>SERCA2a-Sham (n=2)</th>
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<td>BW, kg</td>
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<td>AWd, cm</td>
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<td>HR, bpm</td>
<td>80±16</td>
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<td>91±17</td>
<td>100±7</td>
<td>100±15</td>
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<td>SAP, mm Hg</td>
<td>89±9</td>
<td>100±11</td>
<td>106±17</td>
<td>89±6</td>
<td>111±18</td>
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<td>CO, L/min</td>
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<td>5.00±0.65</td>
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<td>5.46±0.11</td>
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<td>LVEDP, mm Hg</td>
<td>5.83±2.32</td>
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<td>dP/dt, mm Hg·s⁻¹</td>
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<td>1393±258</td>
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<td>1669±423</td>
<td>1432±143</td>
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<td>1415±105</td>
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</table>

β-Gal indicates pigs receiving β-galactosidase gene; SERCA2a, pigs receiving SERCA2a gene; CoiI, permanent coronary artery occlusion; BW, body weight; AW, anterior wall thickness in diastole (d) and systole (s); LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; FS, fractional shortening; HR, heart rate; SAP, systolic aortic pressure; CO, cardiac output; LVDPD, LV end-diastolic pressure; and dP/dt, first derivative of LV pressure. Values are mean±SD.

Area at Risk and Myocardial Infarction Size Assessment
To assess the area at risk (AAR), a solution with red fluorescent microspheres (Molecular Probes) mixed to near-infrared fluorescent dye (IRDYE 780)16 was injected immediately before euthanization into the proximal coronary arteries while a balloon was inflated at the same location used during ischemia in all reperfused pigs and with no balloons in all pigs with PO or the sham procedure. To delineate infarct size, the hearts were sliced into 1-cm-thick slices and stained with triphenyltetrazolium chloride (TTC; Sigma) as previously described.18 The slices were imaged under a near-infrared fluorescent camera to identify the distribution of the beads and dye into the myocardium. The AAR and infarct area were measured from digital micrographs with NIH Image. The AAR was defined by the area delineated by the absence of microspheres. The percentage of myocardial infarction was calculated as the total infarcted area unstained by TTC divided by the total AAR for the heart.

Statistical Analysis
All results are expressed as mean±SD. Between-group differences were compared by use of Student t test or ANOVA for continuous variables and χ² tests for categorical variables. A comparison was considered significant when P<0.05.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Three pigs were excluded because of procedural complications. One pig was excluded because of tamponade at the time of gene delivery; at autopsy, a vein anatomy variation was found. One pig was excluded because of a congenital ventricular septal defect identified at the time of gene delivery. Another pig was excluded at the time of balloon occlusion as a result of the formation of a thrombus occluding the left circumflex artery.

Cardiac Function
As expected, echocardiography and invasive hemodynamic data were similar at baseline in all groups (Table 1).

Twenty-four hours after I/R, PO, or the sham procedure, the echocardiography data, as expected, showed significant LV systolic function impairment in the PO groups compared with the I/R groups. Similarly, the hemodynamic data showed that both PO groups exhibited significantly impaired systolic and diastolic parameters at higher filling pressures compared with the I/R groups. The small amount of muscle necrosis involved in the I/R groups did not significantly affect LV size and function compared with the sham group. In both models, SERCA2a gene transfer did not significantly affect LV hemodynamic and morphological data (Table 2).

Incidence of Ventricular Arrhythmias
In the I/R groups, no significant difference was found between pigs overexpressing SERCA2a and β-galactosidase in VT or VF episodes during ischemia (Figure 4). Similar to the data obtained in a model of I/R in rats,18 SERCA2a overexpression significantly reduced life-threatening arrhythmias after reperfusion, ie, the total number of episodes of VF and VT that occurred from balloon deflation to the end of the follow-up (27±11 episodes in pigs overexpressing SERCA2a versus 226±95 episodes in pigs overexpressing β-galactosidase; P=0.047). Few life-threatening arrhythmias occurred in the early phase of the reperfusion (3±1 episodes in pigs overexpressing SERCA2a versus 7±3 episodes in pigs overexpressing β-galactosidase; P=0.22); therefore, antiarrhythmic effects observed in pigs overexpressing SERCA2a occurred mainly in the late phase of reperfusion (23±12 episodes in pigs overexpressing SERCA2a versus 219±95 episodes in pigs overexpressing β-galactosidase; P=0.05). Detailed results of sustained and nonsustained VT and VF episodes are presented in Figures 4 and 5. No episode of VF was detected in the late reperfusion period in the I/R groups with SERCA2a or the control virus (Figure 5A).
Table 2. Final Echocardiography and Hemodynamic Data

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<th>β-Gal–Coll (n=5)</th>
<th>SERCA2a-Coll (n=5)</th>
<th>β-Gal–I/R (n=6)</th>
<th>SERCA2a-I/R (n=6)</th>
<th>β-Gal–Sham (n=2)</th>
<th>SERCA2a-Sham (n=2)</th>
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<td>AWd, cm</td>
<td>0.78±0.13</td>
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<td>AWs, cm</td>
<td>0.96±0.11</td>
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<td>1.27±0.16*</td>
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<td>LVEDD, cm</td>
<td>4.77±0.65</td>
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<td>LVESD, cm</td>
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<td>FS, %</td>
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<td>Dp/dt, mm Hg · s⁻¹</td>
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<td>1222±236†</td>
<td>1131±195†</td>
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<td>–dP/dt, mm Hg · s⁻¹</td>
<td>1086±147</td>
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<td>1356±264†</td>
<td>1416±108†</td>
<td>1728±229†</td>
<td>1446±210†</td>
</tr>
</tbody>
</table>

Final invasive hemodynamic data

| HR, bpm | 101±10 | 94±20 | 88±13 | 83±15 | 89±4 | 87±1 |
| SAP, mm Hg | 81±7 | 64±9 | 93±15 | 89±6 | 83.5±6 | 91±4 |
| CO, L/min | 3.83±0.97 | 3.40±0.59 | 5.19±1.20† | 4.68±0.58† | 5.35±0.18† | 5.40±1.26† |
| dP/dt, mm Hg · s⁻¹ | 14.25±2.22 | 14.40±1.82 | 8.88±3.54† | 7.30±2.74† | 10.25±1.06† | 8.50±2.12† |
| –dP/dt, mm Hg · s⁻¹ | 965±122 | 870±157 | 1222±236† | 1131±195† | 1281±126† | 1157±59 |

Abbreviations as in Table 1. Values are mean±SD.

*p<0.05 vs β-Gal–Coll; †p<0.05 vs SERCA2a-Coll; ‡p<0.05 vs β-Gal–I/R.

Similar to the data obtained in rats with permanent LAD occlusion,19 pigs with PO overexpressing SERCA2a exhibited a tendency toward an increase in fatal arrhythmias: 5 of 9 pigs overexpressing SERCA2a (55.6%) and 2 of 6 pigs overexpressing β-galactosidase (33.3%) required cardiac defibrillation in the first 90 minutes after coil insertion (P=0.40), and 4 pigs overexpressing SERCA2a (44.4%) and 1 pig overexpressing β-galactosidase (16.7%) died because of a VF event during the follow-up (P=0.26). These pigs with late fatal VF after PO were not all the same that have been saved by defibrillation in the first 90 minutes after coil insertion (2 of 4 pigs overexpressing SERCA2a and 0 of 1 pig overexpressing β-galactosidase). No significant difference was found in the occurrence of sustained VT or nonsustained VT and VF episodes between both PO groups, although a tendency toward a reduction in sustained VT and an increase in VF was observed with SERCA2a overexpression (Figure 5B).

Ventricular arrhythmia during 30 min balloon occlusion

![Ventricular arrhythmia during 30 min balloon occlusion](image)

Ventricular arrhythmia during the first 10 min or reperfusion

![Ventricular arrhythmia during the first 10 min or reperfusion](image)

Figure 4. Incidence of ventricular arrhythmias in the model of I/R during ischemia (ie, the 30-minute balloon occlusion; top) and early reperfusion (ie, the first 10 minutes after balloon deflation; bottom). β-Gal indicates pigs receiving the β-galactosidase gene; SERCA2a, pigs receiving the SERCA2a gene.
Gene Expression
The areas of gene distribution identified by IRDYE 786 and fluorescent microspheres exhibited homogeneous distribution of the particles in the left ventricle (Figure 3). The expression efficiency was demonstrated by immunohistochemistry, immunoblotting, and qRT-PCR. The distribution of the blue β-galactosidase expression was homogeneous across the ventricular walls after the expression of the control protein (Figure 6A).

The expression of the SERCA2a protein and the level of human SERCA2a variant gene by qRT-PCR showed increased SERCA2a expression compared with controls, although the difference was statistically significant only in the I/R group (Figure 6B and 6C). It is possible that sampling in the necrotic area would account for the finding.

Quantification of the Ischemic and Necrotic Areas
The AAR was ~30% of the left ventricle in the PO and reperfusion groups (Table 3) and was similar in the β-galactosidase and SERCA2a groups, showing consistency in the level of occlusion among animals (Figure 7).

In the PO groups, no difference in infarct size was observed between the β-galactosidase and SERCA2a groups (Figure 8A and Table 3). Similar to the data obtained in a model of I/R in rats, SERCA2a overexpression reduces the infarcted area in pigs undergoing I/R (Figure 8B and Table 3).

Discussion
Our previous work has shown that targeted gene transfer of SERCA2a to failing myocardium results in sustained im-

Figure 5. Incidence of ventricular arrhythmias during follow-up in pigs undergoing I/R injury or PO. A, Effect of SERCA2a overexpression vs control (β-galactosidase) in the model of I/R showing how SERCA2a protects against sustained and nonsustained VT. The follow-up period (FU) in this model corresponds to late reperfusion (ie, the period of time from the 11th minute after balloon deflation to the end of Holter recording). B, In the model of PO, SERCA2a failed to protect against tachyarrhythmia. The follow-up period in this model corresponds to the period of time from the occurrence of ST elevation to the end of Holter recording. Coil indicates PO model; β-gal, pigs receiving β-galactosidase gene; and SERCA2a, pigs receiving SERCA2a gene.
An important unresolved question about SERCA2a overexpression was whether increasing SR Ca\textsuperscript{2+} load would lead to oscillatory Ca\textsuperscript{2+} release from the SR and worsening arrhythmias. In a rat model in which Ca\textsuperscript{2+} overload was induced by ischemia followed by reperfusion, but not in a model of PO, we showed that SERCA2a overexpression increases afterdepolarization- and inward current with asynchronous spontaneous mechanical activity and afterdepolarizations. In addition, agents that increase SERCA2a activity remove phospholamban inhibition, depolarizations and aftercontractions, and cAMP-dependent protein kinase activity removes phospholamban inhibition, depolarizations and afterdepolarizations.

During ischemia, damage to the sarcolemmal membrane leads to increased influx of Ca\textsuperscript{2+}, which worsens on reperfusion as a result of the higher Ca\textsuperscript{2+} content of the catabolic products of the reperfusing blood flow. The increase in Ca\textsuperscript{2+} ions to the cell in turn overloads the SR, allowing spontaneous Ca\textsuperscript{2+} leakage from the SR and generating a depolarizing inward current with asynchronous spontaneous mechanical activity and afterdepolarizations. In addition, agents that increase cAMP such as catecholamine can induce afterdepolarizations and aftercontractions, and cAMP-dependent protein kinase activity removes phospholamban inhibition, increasing SERCA2a activity. It might therefore be expected that SERCA2a overexpression increases afterdepolarization-induced arrhythmias.

Table 3. AAR and Infarction Size Data

<table>
<thead>
<tr>
<th></th>
<th>β-Gal-Coil (n=5)</th>
<th>SERCA2a-Coil (n=5)</th>
<th>β-Gal-I/R (n=6)</th>
<th>SERCA2a-I/R (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAR/LV, %</td>
<td>26.0±7.6</td>
<td>32.1±5.7</td>
<td>33.5±2.7</td>
<td>31.9±3.3</td>
</tr>
<tr>
<td>MI/AAR, %</td>
<td>97.6±1.3</td>
<td>94.1±9.5</td>
<td>20.3±22.4*†</td>
<td>0.7±1.3*††</td>
</tr>
</tbody>
</table>

MI indicates myocardial infarction. Other abbreviations as in Table 1. Values are mean±SD.

*P<0.001 vs β-Gal-Coil; †P<0.001 vs SERCA2a-Coil; ‡P=0.057 vs β-Gal-I/R.

Critical mechanism for electric instability in ischemic and failing myocardium.

In the present study, ventricular arrhythmias were abrogated in pigs overexpressing SERCA2a after induction of I/R, but SERCA2a failed to abrogate ventricular arrhythmias occurring in pigs with PO of the LAD and in the ischemic phase in both groups. These data support the notion that Ca\textsuperscript{2+} overload to the surviving cells plays a key role in the origin of reperfusion arrhythmia and that SERCA2a overexpression protects against ventricular arrhythmia only when the electric instability is related to Ca\textsuperscript{2+} overload occurring on reperfusion.
On the other hand, SERCA2a favoring sequestration of Ca\(^{2+}\) by the SR and a larger SR Ca\(^{2+}\) store will initially lead to an increase in Ca\(^{2+}\) transient. Autoregulation results from a more rapid inactivation of subsequent Ca\(^{2+}\) currents and reduced Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels. The net effect would be to reduce transsarcolemmal Ca\(^{2+}\) flux while maintaining a normal systolic transient. Thus, we might expect SERCA2a overexpression to reduce L-type current, recapitulating the effects of Ca\(^{2+}\) channel blockade on arrhythmias.6 In addition, SERCA2a, by reducing Ca\(^{2+}\) transient duration, was shown to reduce the occurrence of aftercontractions and afterdepolarization in isolated rabbit cardiomyocytes. The resequestration of Ca\(^{2+}\) in the SR compartment was confirmed by the increase in SR Ca\(^{2+}\) content calculated from the Na\(^{+}/Ca^{2+}\)-exchanger current evoked by rapid caffeine application.23

In addition, Ca\(^{2+}\) dissociation from the myofilaments will initiate Ca\(^{2+}\) waves, trigger propagated contractions, delay afterdepolarizations, and trigger arrhythmic activity. SERCA2a may reduce the occurrence of afterdepolarization, restoring intracellular Ca\(^{2+}\) homeostasis and thus reducing Ca\(^{2+}\) waves.

The effects of ischemia and heart failure on myocardial Ca\(^{2+}\) transient include beat-to-beat Ca\(^{2+}\) transient alternans shown on the ECG as ST-segment and T-wave morphology alternans changes occurring in ischemia just before the onset of VF. ST and T alternans have been attributed to the spatial and temporal heterogeneity in the action potential duration in the myocardium, but heterogeneity of [Ca\(^{2+}\)] within myocytes also can be involved.8,23 SERCA2a, by restoring Ca\(^{2+}\) reuptake and intracellular Ca\(^{2+}\) homeostasis, may reduce the Ca\(^{2+}\) transient alternans and ST-T alternans, triggering arrhythmia.

Furthermore, a mechanism for SERCA2a protection from ventricular arrhythmias can reside in the protection from the mitochondrial permeability transition pore that compromises ATP production after Ca\(^{2+}\) overload at reperfusion.25–27 ATP depletion, in addition to increasing intracellular Na\(^{+}\) and damaging Ca\(^{2+}\) handling proteins, can contribute to Ca\(^{2+}\) oscillation and increased [Ca\(^{2+}\)], that is taken up by mitochondria, leading to further depletion of ATP contributing to ventricular arrhythmias. Improvement in SR Ca\(^{2+}\) handling can mediate protection from reperfusion injury through metabolic preservation.

We also showed that SERCA2a in a rat model and in a pig model of I/R reduces myocardial injury. SERCA2a may protect against ventricular arrhythmia by reducing myocardial scarring from preservation of viable myocytes after ischemic injury. A previous study on isolated myocytes23 provided background support for the notion of a protective effect of SERCA2a on myocardial viability, as SERCA2a gene expression significantly reduces the loss of viable rabbit myocytes over a 48-hour culture. In addition, improving SR Ca\(^{2+}\) handling by protecting against mitochondrial Ca\(^{2+}\) overload may prevent necrotic cell death by preserving ATP production.

On the other hand, cardiac function is deteriorated in both permanent coronary artery occlusion groups. The degree of this impairment is about the same with or without SERCA2a overexpression. The lack of better cardiac function outcome after PO in SERCA2a-overexpressing pigs reflects the inability of SERCA2a overexpression to reduce myocardial infarction necrosis in the context of a definitive coronary occlusion. These data in pigs confirm previous results observed by Chen et al.19 in transgenic rats overexpressing SERCA2a in which scar size and global cardiac function were similar in wild-type rats and transgenic rats overexpressing SERCA2a.

Conclusions
Abnormal Ca\(^{2+}\) cycling and Ca\(^{2+}\) overload are critical in the induction and perpetuation of cardiac arrhythmia in acute and chronic conditions by mechanisms that can be targeted by favoring Ca\(^{2+}\) reuptake. SERCA2a, by reducing Ca\(^{2+}\) overload, improves mechanical and electric stability of the heart and may be a successful therapeutic approach that addresses
the subcellular events critical for initiating and perpetuating arrhythmias.

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Disclosures
Dr Hajjar is a founder of Celladon and Nanocor. Dr Kawase is a consultant for Celladon. The other authors report no conflicts.

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CLINICAL PERSPECTIVE

Cardiac arrhythmia is a potentially life-threatening complication of heart failure and ischemic heart disease. In the past, some therapeutic approaches for both of these conditions were associated with an increased risk of arrhythmia and/or sudden cardiac death. Previous work in a variety of animal models has demonstrated that enhancing sarcoplasmic reticulum calcium uptake through expression of the sarcoplasmic reticulum ATPase (SERCA2a) can improve cardiac contractile function, survival, and the energetic state. Here, we demonstrate in a preclinical large animal model that, in contrast to some pharmacological agents that improve inotropy but increase electric instability, SERCA2a expression actually reduces arrhythmia as a result of calcium overload. These findings support the notion that enhancing sarcoplasmic reticulum calcium uptake may hold promise for the prevention and treatment of arrhythmia for reperfusion and heart failure.
Prevention of Ventricular Arrhythmias With Sarcoplasmic Reticulum Ca\(^{2+}\) ATPase Pump Overexpression in a Porcine Model of Ischemia Reperfusion

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