Gene Delivery of Sarcoplasmic Reticulum Calcium ATPase Inhibits Ventricular Remodeling in Ischemic Mitral Regurgitation

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Background—Mitral regurgitation (MR) doubles mortality after myocardial infarction (MI). We have demonstrated that MR worsens remodeling after MI and that early correction reverses remodeling. Sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2a) is downregulated in this process. We hypothesized that upregulating SERCA2a might inhibit remodeling in a surgical model of apical MI (no intrinsic MR) with independent MR-type flow.

Methods and Results—In 12 sheep, percutaneous gene delivery was performed by using a validated protocol to perfuse both the left anterior descending and circumflex coronary arteries with occlusion of venous drainage. We administered adeno-associated virus 6 (AAV6) carrying SERCA2a under a Cytomegalovirus promoter control in 6 sheep and a reporter gene in 6 controls. After 2 weeks, a standardized apical MI was created, and a shunt was implanted between the left ventricle and left atrium, producing regurgitant fractions of \(\approx 30\%\). Animals were compared at baseline and 1 and 3 months by 3D echocardiography, Millar hemodynamics, and biopsies. The SERCA2a group had a well-maintained preload-recruitable stroke work at 3 months (decrease by 8\% vs 42\% with reporter gene controls; \(P<0.001\)). Left ventricular dP/dt followed the same pattern (no change vs 55\% decrease; \(P<0.001\)). Left ventricular end-systolic volume was lower with SERCA2a (82.6\% vs 99.4\% mL; \(P=0.03\)); left ventricular end-diastolic volume, reflecting volume overload, was not significantly different (127.8\% vs 134.3\% mL). SERCA2a sheep showed a 15\% rise in antiapoptotic pAkt versus a 30\% reduction with the reporter gene (\(P<0.001\)). Prohypertrophic activated STAT3 was also 41\% higher with SERCA2a than in controls (\(P<0.001\)). Proapoptotic activated caspase-3 rose 5-fold during 1 month in both SERCA2a and control animals (\(P=NS\)) and decreased by 19\% at 3 months, remaining elevated in both groups.

Conclusions—In this controlled model, upregulating SERCA2a induced better function and lesser remodeling, with improved contractility, smaller volume, and activation of prohypertrophic/antiapoptotic pathways. Although caspase-3 remained activated in both groups, SERCA2a sheep had increased molecular antiremodeling “tone.” We therefore conclude that upregulating SERCA2a inhibits MR-induced post-MI remodeling in this model and thus may constitute a useful approach to reduce the vicious circle of remodeling in ischemic MR. (Circ Heart Fail. 2010;3:627-634.)

Key Words: mitral regurgitation ■ valvular heart disease ■ echocardiography ■ remodeling

Expansion of infarcted tissue begins shortly after myocardial infarction (MI), but a more gradual remodeling process also involves noninfarcted areas; initially compensatory, this process becomes maladaptive as the ventricle enlarges and contracts poorly, with subsequent reduced survival.

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MI also causes ischemic mitral regurgitation (MR) by altering ventricular geometry and function, thus doubling the risk of death. Severe nonischemic MR has been shown to promote left ventricular (LV) remodeling and reduce survival. \(^6\) We have previously demonstrated\(^7\) that moderate MR, simulated by an LV-to-left atrial (LA) shunt, added to a small, anteropapical MI (causes no intrinsic MR), causing greater ventricular remodeling than a comparable infarction alone, with an earlier transition to a failure phenotype. We have also shown that repairing the regurgitant-type flow at an early stage after MI reverses the remodeling-related processes.\(^10\)
Whole-heart changes parallel cellular and molecular abnormalities in the noninfarcted myocardium that reflect the complex remodeling process. These molecular events also progress differently with MR than with comparable infarction alone, with an initial rise in prohypertrophic and antiapoptotic signals followed by their exhaustion.

Most experimental models of post-MI remodeling use inferoposterior Mls,11,12 but this necessarily links the MI-induced remodeling to the development of MR. The shunt model allows MR to be varied independently in the presence of MI and without interventions, such as infarct patching,13 that might themselves influence remodeling.

Upregulating genes encoding proteins of interest has been demonstrated to be an effective approach to modulate and treat heart failure. One candidate for such gene therapy is the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2a), which is downregulated in that model and plays a pivotal role in the regulation of intracellular Ca\(^{2+}\) in cardiomyocytes.14 Calcium entry into the cytosol during systole induces Ca\(^{2+}\) release from the sarcoplasmic reticulum through the ryanodine receptor, coupling excitation and contraction. During relaxation, Ca\(^{2+}\) is returned to the sarcoplasmic reticulum by SERCA2a. Some is also extruded by the sarcotubular Na\(^+\)/Ca\(^{2+}\) exchanger (NCX), which is upregulated in cardiac hypertrophy and failure.15,16 Because SERCA2a is the major determinant of the amount of Ca\(^{2+}\) available to be released during the upcoming systole, changes in SERCA function significantly affect cardiac excitation-contraction coupling. SERCA2a activity also has a major influence on myocardial relaxation,17,18 Ca\(^{2+}\) extrusion via SERCA2a being more efficient energetically than the alternative NCX pathway.19 SERCA2a mRNA levels are reduced in failing hearts.20 With a gene therapy approach, upregulating SERCA2a levels in different models of heart failure resulted in improvement in systolic21–23 and diastolic17 function, as well as improving metabolism,24,25 potentially reducing arrhythmias,26,27 and improving survival.25 We have demonstrated that SERCA2a is downregulated in the remote zones of post-MI remodeling ventricles, significantly more so when MR was also present, accompanied by a reduction in contractility of the whole ventricle and of isolated cells and reduction in single-cell calcium transients.9 Pathways involved in the compensatory hypertrophic response in remodeling were initially upregulated, only to fall below baseline at 3 months, when severe dilatation and failure were present. Because repairing MR in the early phase, before these processes have been activated, induces reversal of remodeling.10 SERCA2a may have a unique role in determining this reversibility. This is emphasized by the recently reported effects of SERCA2a upregulation in an MR-only pig model of heart failure,8,9 where SERCA2a it induced inhibition of ventricular enlargement and myocardial dysfunction apparent in the control animals.

This study aimed to apply a gene therapy approach in a clinically relevant large-animal model of actively evolving remodeling induced by the combination of ischemic and valvular lesions, in which a biphasic pattern of compensatory and decompensatory changes has been demonstrated. An intriguing question to address in this model is whether the potentially beneficial effects of SERCA2a gene therapy are accompanied by molecular changes typical of compensated hypertrophy,9 as seen early in the course of MR-augmented remodeling, or only by measurable reductions in ventricular volumes and improvements in contractile dysfunction without molecular changes in other aspects of the remodeling processes. Genetic modification of such a key pathway can thereby help dissect its contribution to the entire disease process.

Thus, we hypothesized that upregulating SERCA2a levels by gene delivery with a viral vector might reverse the remodeling process in our model of “ischemic-type” MR, that is, MR associated with MI.9 We also hypothesized that this reversal would be manifest in both ventricular volumes and function and in the persistent activation of prohypertrophic and antiapoptotic pathways.

In this context, prolonged and sustained expression of the transgene is critical, as is the lack of host immune response to the vector. Adeno-associated vector (AAV) has been demonstrated to confer prolonged and sustained expression of myocardial transgenes while lacking immunogenic and cardiotoxic effects,29 and it was therefore used in this study.

Methods

Animal Studies
A total of 12 male Dorsett hybrid sheep (20 to 30 kg) were included. Our established model of independent MI and MR-type flow9,10 was implemented with an 8-cm long, 8-mm-diameter reinforced polytetrafluoroethylene (Teflon) graft (Edwards; cross-sectional area, 0.50 cm\(^2\)) implanted under sterile conditions into the midlateral LV and LA appendage with intramuscular portions stiffened with epoxy resin (Figure 1). The regurgitant flow was confirmed during each thoracotomy with a Transonic flow probe and color Doppler. The standardized shunt diameter and length consistently produced moderate MR (regurgitant fractions of \(\approx 30\%\)10). Animals were treated with heparin (3 days) and then oral aspirin.

Vector Design
Vector production, harvest, purification, and testing were done as previously described.31 The recombinant AAV6.SERCA2a vector used in this study contains an AAV serotype 6 viral capsid and a single-strand, \(\approx 4.5\)-kb DNA containing the human SERCA2a cDNA driven by a Cytomegalovirus immediate-early promoter/enhancer, a hybrid intron, and a bovine growth hormone polyadenylation signal, all flanked by 145-nucleotide AAV2 inverted terminal repeat sequences necessary for replication and packaging of the vector DNA in the capsid. The vector was manufactured by standard calcium phosphate transfection methods in adherent 293 cells. Three plasmids were used, 1 containing helper functions from adenovirus, 1 containing the AAV rep2 and cap1 genes, and the third containing the vector genome. Final vector preparations were \(\approx 95\%\) pure, as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Invitrogen, Carlsbad, Calif).

Gene Delivery
Two weeks before the first thoracotomy (to obtain significant gene expression at model creation), antegrade coronary arterial injection with concomitant great cardiac vein blockade was performed with AAV6 as a vehicle for the reporter gene β-galactosidase (control) and SERCA2a, each at a titer of \(5 \times 10^{14}\) genomes/mL. The great cardiac vein was cannulated via internal jugular access and occluded with a standard balloon-tipped catheter. The left anterior descending coronary artery was cannulated via the femoral artery and occluded with a standard angioplasty balloon before the first diagonal branch. With both arterial and venous balloons transiently inflated for 2 minutes, intracoronary adenosine was administered to increase per-
meability and prolong dwell time,22 followed by 5×10^{12} genomes of either AAV6-β-galactosidase or AAV6.SERCA2a (6 sheep each). This sequence was repeated for the left circumflex artery. Previous work has shown increased tissue expression in the whole adult heart by this delivery method.28

**Model Creation**

Sheep were loaded for 3 days with amiodarone (200 mg PO BID), anesthetized with thiopentotal (0.5 mL/kg), intubated, ventilated at 15 mL/kg with 2% isoflurane-oxygen, and administered glycopyrrolate (0.4 mg IV), prophylactic vancomycin (0.5 g IV), and amiodarone (150 mg IV drip). Surface ECG was monitored, and a sterile left thoracotomy was performed with pericardial cradle creation. A high-fidelity, micromanometer-tipped catheter (Millar, Houston, Tex) was placed into the left ventricle. After baseline 2-dimensional and 3-dimensional echocardiography (3D echo) imaging, a septal MI was produced by ligating the mid and distal left anterior descending coronary artery, a technique that is known to produce substantial MIs without MR.33 2D echo confirmed that the wall-motion abnormality involved approximately one third of the anteroseptum from the apex to base for standardization. In addition to analgesia, propranolol, 1 mg IV in 2 doses, was given for evident stress and tachycardia (>150) on extubation. Antibiotics (cephapirin, 0.5 g IV) and analgesics (buprenorphine, 0.3 mg BID) were administered for 5 days and oral amiodarone (200 mg BID) for 3 days.

During repeat sterile thoracotomy at day 30, 3D echo evaluated LV remodeling and function, with directed TruCut needle biopsies of the noninfarcted myocardium near and remote from the border zone. LV remodeling and function, with directed TruCut needle biopsies of the noninfarcted myocardium near and remote from the border zone.

**3D Echo and LV Function**

For 3D echo and assessment of LV function,34 rotated apical images were obtained at 10° intervals with an epicardial 5-MHz transesophageal echo probe (Sonos 7500, Philips, Andover, Mass), rotated by software and gated to the ECG and respiration. Digital images were analyzed on a workstation with custom programs by an operator blinded to treatment assignment. Endocardial surfaces were traced to calculate LV volumes validated against a 36-crystal sonomicrometer array. Remodeling was quantified in terms of increasing LV volumes in our laboratory were 3.5%, as previously reported.9 Inter- and intraobserver variability for 3D echo–measured LV volumes were 3%. ANOVA (JMP 8, SAS Institute).

**Calcium Cycle**

Sarcoplasmic reticulum membrane was obtained by using sucrose gradient centrifugation.36 Proteins were separated and an immunoblot with a monoclonal anti-SERCA2 and anti-phospholamban (Santa Cruz Biotechnology, Santa Cruz, Calif) antibody was performed, normalized to total protein. NCX levels were measured by Western blot, with monoclonal anti-NCX antibodies (Santa Cruz Biotechnology).

**Prohypertrophic and Proapoptotic Cascades**

We measured levels of several molecular species associated with remodeling that modulate cell hypertrophy and death and are responsible for extracellular matrix turnover.9,10 All protein assays were performed for each treatment group and stage on each sheep separately, and the results were averaged.

**Molecular Assays**

We measured levels of several molecular species associated with remodeling that modulate cell hypertrophy and death and are responsible for extracellular matrix turnover.9,10 All protein assays were performed for each treatment group and stage on each sheep separately, and the results were averaged.

**Statistics**

All values are reported as mean±SD. Statistical analysis used 2-tailed Student t test for continuous variables compared at specific time points; the Bonferroni correction was applied when appropriate. Repeated measures over time were analyzed with repeated-measures ANOVA (JMP 8, SAS Institute). P<0.05 was considered significant. Inter- and intraobserver variability for 3D echo–measured LV volumes in our laboratory were 3.5%, as previously reported.9

**Results**

Infarct size, traced and integrated by 3D echo, was 12% to 22% of the endocardial surface area, with a mean of 17±3% (n=12).

**Function and Volumes**

The SERCA2a group had well-maintained preload-recruitable stroke work at 3 months (decrease by 8±10%) versus a 42±12% decrease with reporter gene controls (P<0.001, Figure 2). Peak systolic LV dP/dt followed the same pattern (no change vs 55% decrease, P<0.001; Figure 2). Although 3D echo–derived LV ejection fraction was decreased in both groups, beginning with the post-MI base-
line, it was better maintained with SERCA2a (35.2±4.0% vs 26.1±3.5%, P=0.01, Figure 2) at sacrifice. This was accompanied by a lower LV end-systolic volume with SERCA2a (82.6±9.6 vs 99.4±9.7 mL, P=0.03; Figure 3); LV end-diastolic volume, reflecting the volume overload, was not significantly different at sacrifice (127.8±6.2 vs 134.3±9.4 mL, P=NS; Figure 3). Although no quantitative assessment of animal well-being could be performed, the animals in the control group were less active and seemed more short of breath.

Molecular Pathways of Remodeling
As expected, in the SERCA group, there was a very significant increase in SERCA2a protein levels in both remote and border zones at sacrifice, which was already apparent at 1-month follow up, whereas control sheep demonstrated a sharp reduction in SERCA2a levels at 1-month follow-up (average integrated density, 85.6±15.2 vs 54.2±10.8, P<0.001; Figure 4) and even more so at sacrifice (average integrated density, 93.6±20.1 vs 34.2±6.3, P<0.001; Figure 4). Of note, no significant change was noted in regulatory phospholamban levels (Figure 4). NCX levels were significantly more elevated in the control sheep compared with the SERCA sheep at sacrifice, consistent with a more active remodeling process (P=0.025, Figure 5).

SERCA2a sheep at 3-months sacrifice showed a 15% rise in antiapoptotic phospho-Akt versus a 30% reduction with the reporter gene (P=0.001, Figure 5). The sample mean of STAT3 was also 41% higher at sacrifice with SERCA2a than with the reporter gene (P=0.001, Figure 5). In contrast, gp130 fell by 25% to 26% in both groups (P=NS by repeated-measures ANOVA), raising the possibility that improved contractility blunted the stimulus for prohypertrophic compensation or alternatively, that SERCA2a overexpression compensated for but did not entirely eliminate the remodeling drive. Proapoptotic activated caspase-3 rose 5-fold and to a comparable extent at 1 month in both SERCA2a and reporter sheep.
gene animals (P=NS, Figure 5) and decreased by only 19% from 1 to 3 months, remaining elevated in both groups at sacrifice.

Discussion

A large number of patients with MI develop MR and progress to congestive heart failure. Our previous results have shown that, for a comparable infarct size, the presence of MR-type volume overload leads to greater LV dilatation and dysfunction and to more severe changes at a cellular and molecular level. Molecular changes are biphasic, with initial upregulation and subsequent exhaustion of prohypertrophic and antiapoptotic pathways that otherwise remain elevated when MI is not accompanied by MR. Maintained elevation of caspase-3 and extracellular matrix turnover lead to a failure phenotype, with abnormal cellular morphology, decreased calcium cycling, and reduced SERCA2a. This reduction was more pronounced in the border zones of the infarction, reflecting a probably larger element of cell loss through ischemic damage, but was also significant in the remote zones, possibly reflecting stretch-induced activation of the fetal program in these myocytes, resulting in diminution of SERCA2a levels. We have also demonstrated the corollary that early repair of such moderate MR-type volume overload reverses these progressive remodeling processes and activates intracellular signals, promoting hypertrophy, opposing apoptosis, and inhibiting matrix proteolysis. Manipulating the expression of key proteins and the activity of specific downstream signaling pathways involved in cardiac hypertrophy and failure will allow us to understand their contribution to the disease process.

AAV is a gene therapy vector that provides gene expression lasting >1 year in muscle and brain with little or no immune reaction. SERCA2a was chosen as the transgene because its expression is reduced in our MI+MR model, and its overexpression improves contractility and inhibiting matrix proteolysis. SERCA2a might not ablate all aspects of the intracellular remodeling cascade. One interesting aspect of the molecular changes was the effect on NCX expression in our model. An increase in NCX expression has been observed in a number of models of heart failure and has been associated with an increased risk of ventricular arrhythmias. In our model, NCX was increased in the control group but remained at baseline levels with overexpression of SERCA2a. Likewise, we did not detect increased levels of gp130, as we have previously seen with MR repair; however, activated STAT3, downstream from gp130, was significantly increased, suggesting the possibility of greater activation of gp130-containing cytokine receptors, STAT3 activation by an alternative pathway, or decreased feedback inhibition. The results suggest that improving contractility and relaxation is insufficient to reverse the remodeling process completely at a molecular level. Nonetheless, the improvement in contraction, volumes, and intracellular prohypertrophic pathways suggests that SERCA2a upregulation does at least strongly inhibit the remodeling process. As SERCA2a upregulation has been demonstrated in different models to improve function and retard progression to heart failure, it is inherently linked to the underlying MI and is not standardized. Based on the study motivation, it was critical to separate the 2 processes of infarction and regurgitation to determine the incremental role of MR and to do so with a standardized
orifice, which provided stable regurgitant fractions of \( \approx 30\% \) throughout the study. In the clinical situation of the tethered mitral valve, SERCA2a may have an even more pronounced effect by reducing the severity of this dynamic MR: increased LV contractility and decreased LV volumes will increase the closing forces and decrease the tethering forces on the mitral valve, thereby improving coaptation and reducing MR.\(^{47}\) This will be examined in a separate study. We performed gene delivery 2 weeks before induction of MR+MI. This was done to have an established upregulation of SERCA2a coincident with the initiation of the remodeling process, which starts immediately after infarction, to provide a proof of concept about the role of SERCA2a in this situation. Variations in timing of SERCA2a therapy relative to MR repair will also be assessed, for example, in the fully dilated remodeling state, as there may be a “point of no return” beyond which these interventions may be ineffective.\(^{51}\)

In conclusion, we have demonstrated that upregulating SERCA2a in a model of MR+MI may inhibit the remodeling process, as manifested by ventricular function, volumes, and intracellular pathways of hypertrophy. This may constitute a potentially useful approach to reduce the vicious circle of remodeling in ischemic MR.

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**Disclosures**

None.
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References


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CLINICAL PERSPECTIVE

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