Restoration of Diastolic Function in Senescent Rat Hearts Through Adenoviral Gene Transfer of Sarcoplasmic Reticulum Ca\textsuperscript{2+}-ATPase

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Background—Senescent hearts are characterized by diastolic dysfunction and a decrease in sarcoplasmic reticulum (SR) Ca\textsuperscript{2+}-ATPase protein (SERCA2a).

Methods and Results—To test the hypothesis that an increase in SERCA2a could improve cardiac function in senescent rats (age 26 months), we used a catheter-based technique of adenoviral gene transfer to achieve global myocardial transduction of SERCA2a in vivo. Adult rat hearts aged 6 months and senescent rat hearts infected with an adenovirus containing the reporter gene β-galactosidase were used as controls. Two days after infection, parameters of systolic and diastolic function were measured in open-chest rats. Cardiac SERCA2a protein and ATPase activity were significantly decreased in senescent hearts compared with adult rats (Δ−30±4% and −49±5%) and were restored to adult levels after infection with Ad.SERCA2a. At baseline, left ventricular systolic pressure and +dP/dt were unaltered in senescent hearts; however, diastolic parameters were adversely affected with an increase in the left ventricular time constant of isovolumic relaxation and diastolic pressure (Δ+29±9% and +38±12%) and a decrease in −dP/dt (Δ−26±11%). Overexpression of SERCA2a did not significantly affect left ventricular systolic pressure but did increase +dP/dt (Δ+28±10%) in the senescent heart. Overexpression of SERCA2a restored the left ventricular time constant of isovolumic relaxation and −dP/dt to adult levels. Infection of senescent hearts with Ad.SERCA2a markedly improved rate-dependent contractility and diastolic function in senescent hearts.

Conclusions—These results support the hypothesis that increased Ca\textsuperscript{2+}-ATPase activity contributes to the functional abnormalities observed in senescent hearts and demonstrates that Ca\textsuperscript{2+} cycling proteins can be targeted in the senescent heart to improve cardiac function. (Circulation. 2000;101:790-796.)

Key Words: aging • gene therapy • heart failure • sarcoplasmic reticulum • diastole

Congestive heart failure is becoming an increasingly prevalent clinical problem in the United States and now is the most frequent cause of hospitalization in people older than 65 years.\textsuperscript{1} Indeed, mortality rates from congestive heart failure continue to increase in elderly populations.\textsuperscript{2} Interestingly, up to 40% of patients older than 60 years who have symptoms of congestive heart failure have normal systolic function and abnormal diastolic function. In mammalian hearts, aging is associated with impaired cardiac relaxation.\textsuperscript{2,3} Senescent myocytes are characterized by prolonged relaxation, diminished contraction velocity, a decrease in β-adrenergic response, and increased myocardial stiffness.\textsuperscript{4} This impairment in diastolic function contributes to the increased incidence in congestive heart failure in the elderly.\textsuperscript{5} A number of cellular and molecular mechanisms may contribute to the age-related defects. The abnormalities in cardiac relaxation have been attributed to a defect in sarcoplasmic reticulum (SR) Ca\textsuperscript{2+}-ATPase pump activity, which is mainly responsible for controlling the rate at which Ca\textsuperscript{2+} is taken up into the SR during relaxation.\textsuperscript{6–8} The decrease in SR Ca\textsuperscript{2+} uptake during relaxation, which results in prolonged contraction, has been associated with a decrease in the content and activity of the SR Ca\textsuperscript{2+}-ATPase pump in experimental models of senescence.\textsuperscript{9} More recently, SERCA2a protein levels were found to be significantly decreased in senescent human myocardium.\textsuperscript{10} This decrease in SERCA2a levels was associated with impaired myocardial function at baseline and was further accentuated by hypoxic conditions.\textsuperscript{10}

Because a number of key steps of excitation-contraction coupling are altered during normal aging, it is not clear whether a decrease in the number of SR Ca\textsuperscript{2+} pumps constitutes an adaptive or a pathological process. We have

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recently shown that the overexpression of SERCA2a in vitro through adenoviral gene transfer results in increased contractility and a faster relaxation rate of the Ca\(^{2+}\) transient.\(^{1,12}\) These results were further confirmed in transgenic animals with SERCA2a overexpression.\(^{13}\) More recently, we developed a catheter-based technique to deliver adenoviruses to the heart in a homogeneous fashion.\(^{14}\) In addition, we used this approach to demonstrate profound physiological effects on cardiac function specifically mediated by the transduced gene.

The goal of the present study was to test the hypothesis that the restoration of the SR Ca\(^{2+}\)-ATPase through adenoviral gene transfer in the aging heart will normalize diastolic function in vivo.

**Methods**

**Construction of Recombinant Adenoviruses**

Two first-generation type 5 recombinant adenoviruses were used in these studies: Ad.\(\beta\)-galactosidase (Ad.\(\beta\)-Gal) and Ad.SERCA2a. Ad.\(\beta\)-Gal was generously provided by Dr David Dichek (Gladstone Institute for Cardiovascular Disease).\(^{15}\) The construction of Ad.SERCA2a has been described previously, and the recombinant viruses were prepared as high titer stocks through the propagation in 293 cells as described previously.\(^{11,16}\) The stocks used for the present study measured with the use of plaque assays was 9\(\times\)10\(^{11}\) plaque-forming units/mL (pfu/mL) for Ad.\(\beta\)-Gal and 10\(\times\)10\(^{11}\) pfu/mL for Ad.SERCA2a with a particle-to-pfu ratio of 25:1 and 40:1, respectively (viral particles/mL were determined with the relation of 1 absorbance unit at 260 nm being equal to 10\(^{12}\) viral particles/mL).

**Experimental Protocol**

Both 26-month old (senescent) and 6-month old (adult) male Fisher 344 rats obtained from the National Aging Institute were studied. Animals were divided into 4 groups: (1) 8 uninfected, sham-operated senescent rats, (2) 8 senescent rats infected with 10\(^{11}\) pfu Ad.\(\beta\)-Gal, (3) 8 senescent rats infected with 10\(^8\) pfu Ad.SERCA2a, and (4) 8 uninfected sham-operated adult rats.

**Adenoviral Delivery Protocol**

The delivery of adenovirus was described previously by our group in detail.\(^{14}\) Briefly, rats were anesthetized with intraperitoneal pentobarbital (60 mg/kg) and placed on a ventilator. The chest was entered from the left side through the third intercostal space. A 22-gauge catheter containing 200 mL adenovirus was advanced from the apex of the left ventricle (LV) to the aortic root. The aorta and pulmonary arteries were clamped distal to the site of the catheter, and the solution was injected. The clamp was maintained for 10 seconds while the heart pumped against a closed system (isovolumically). After 10 seconds, the clamp on the aorta and pulmonary artery was released, the chest was closed, and the animals were extubated and transferred back to their cages.

**Pressure Measurements**

Rats in the different treatment groups were anesthetized with 60 mg/kg pentobarbital and mechanically ventilated. The chest was then opened, and a 18F high-fidelity pressure transducer (Millar Instruments) was introduced into the LV. LV systolic pressure (LVSP), LV end-diastolic pressure, the maximal rates of pressure rise (+dP/dt), and of pressure fall (−dP/dt), and the time constant of isovolumic relaxation (τ) were measured or derived in the different groups. The time course of isovolumic relaxation was measured with the following equation: P = P\(_e\) e\(^{-t/\tau}\) + P\(_b\), where P is LV isovolumic pressure, P\(_e\) is pressure at the time of peak +dP/dt, and P\(_b\) is residual pressure. A pair of 0.7-mm piezoelectric crystals (Sonometrics Co) were attached to the LV epicardium across the short axis of the ventricle at the level of the mitral valve to obtain intercrystal distances. LV pressure–dimension loops were generated under different loading conditions by transient occlusions of the inferior vena cava. The end-systolic pressure–dimension relation was obtained through the production of a series of pressure–dimension loops during transient inferior vena cava occlusion and through linear regression analysis of the end-systolic points of the individual pressure–dimension loops to generate the end-systolic elastance.

**Isolation of Single Cardiac Myocytes**

Ca\(^{2+}\)-tolerant adult rat ventricular myocytes were isolated as described previously.\(^{17}\) The isolated myocytes were then fixed with 0.5% glutaraldehyde for 15 minutes and stained with a solution containing 5-bromo-4-chloro-3-indolyl-\(\beta\)-D-galactopyranoside (X-Gal) for 2 hours. To quantify the percentage of cells with blue nuclei, we counted 100 cells from each heart preparation.

**Preparation of SR Membranes**

We isolated SR membranes from the LVs of hearts as described previously.\(^{14}\) Briefly, LV myocardium was suspended in a buffer containing 300 mmol/L sucrose, 1 mmol/L PMSF, and 20 mmol/L PIPES, at pH 7.4, that was homogenized and centrifuged at 25 000 g for 60 minutes. The pellet was resuspended in a buffer containing 600 mmol/L KCl, 30 mmol/L sucrose, and 20 mmol/L PIPES; frozen in liquid nitrogen; and stored at −70°C. Protein concentration was determined in these preparations according to a modification of the method of Bradford (Bio-Rad).

**Western Blot Analysis**

SDS-PAGE was performed on the isolated membranes from cell cultures under reducing conditions on a 7.5% separation gel with a 4% stacking gel in a Miniprotein II cell (Bio-Rad). For immunoreaction, the blot was incubated with 1:2500 diluted monoclonal antibodies to SERCA2a (MA3-919), Na\(^+\)/Ca\(^{2+}\) exchanger (MA3-926), or ryanodine receptors (MA3-925; Affinity BioReagents) or with 1:2500 diluted anti-cardiac phospholamban monoclonal IgG (Upstate Biotechnology) for 90 minutes at room temperature. The densities of the immunoreactive bands were evaluated with the use of NIH Image. Normalization was performed by dividing densitometric units of each membrane preparation by the protein amounts in each of the preparations.

**SR Ca\(^{2+}\)-ATPase Activity**

SR Ca\(^{2+}\)-ATPase activity assays were carried out based on a pyruvate/NADH coupled reactions as described previously.\(^{11,12}\) With a photometer (DU 640; Beckman) adjusted at a wavelength of 340 nm, oxidation of NADH (which is coupled to the SR Ca\(^{2+}\)-ATPase) was assessed at 37°C in the membrane preparations according to the difference between the total absorbance and basal absorbance. The activity of the Ca\(^{2+}\)-ATPase was calculated as ΔAbsorbance/6.22×protein×time (in nmol ATP·mg protein\(^{-1}\)·min\(^{-1}\)).

**Statistical Analysis**

All values are presented as mean±SD. ANOVA was used to calculate statistical differences among the different groups, and ANOVA for repeated measures was used where appropriate. Statistical significance was accepted at the level of \(P<0.05\).

**Results**

**Cardiac Gene Transfer**

We have previously reported that the catheter-based adenoviral technique induces global gene transfer in rat hearts with an expression pattern that is grossly homogeneous throughout the ventricles. Because these results were obtained in adult rats, we quantified the efficiency of gene transfer in senescent rat hearts. We isolated single cardiomyocytes from adult and senescent rat hearts after in vivo transduction with Ad.\(\beta\)-Gal. As shown in Figure 1, the majority of cardiomyocytes...
demonstrate specific nuclear β-Gal activity, reflecting transgene expression in both adult and senescent rat hearts, 2 days after in vivo transduction with Ad.β-Gal (72±3%, n=3 young adult; 61±2%, n=4 senescent; P<0.05).

Quantification of Ca\(^{2+}\) Regulatory Proteins
To assess SERCA2a protein content in the different groups, we performed quantitative immunoblotting. The intensity of the labeled SERCA2a band was proportional to the amount of cardiac membrane protein electrophoresed in the range of 5 to 50 μg (Figures 2A and 2B). The protein content of SERCA2a was significantly decreased in 26-month-old rats compared with 6-month-old rats as shown in Figures 2C and 2D. On the other hand, other Ca\(^{2+}\) regulatory proteins, such as phospholamban, Na\(^+\)/Ca\(^{2+}\) exchanger, and ryanodine receptors, were unchanged in the senescent ventricles. Adenoviral gene transfer of Ad.SERCA2a induced a significant increase in SERCA2a content in the senescent hearts restoring its expression level to adult levels.

SR Ca\(^{2+}\)-ATPase Activity
Senescent hearts had a significant decrease in Ca\(^{2+}\)-ATPase activity compared with adult hearts (Figure 3). Adenoviral gene transfer with Ad.β-Gal did not alter Ca\(^{2+}\)-ATPase activity.
activity in the senescent heart. However, adenoviral gene transfer of Ad.SERCA2a induced a significant increase in $\text{Ca}^{2+}$-ATPase activity in the senescent hearts, increasing it by nearly 2-fold to levels comparable to those seen in adult animals.

**Hemodynamic Effects of SERCA2a Overexpression in Senescent Hearts**

As shown in Figures 4 and 5, the systolic parameters were not altered in the senescent rat hearts compared with adult hearts. However, overexpression of SERCA2a increased the rate of rise of pressure but did not alter systolic pressure. Diastolic parameters were significantly altered in the senescent rat hearts compared with adult hearts as demonstrated by a decrease in the maximal rate of decline of LVSP, an increase in diastolic pressure, and a significantly prolonged time course of pressure decline ($\tau$) (Figures 4 and 5). The overexpression of SERCA2a normalized both the maximal rate of decline of LVSP, and the time course of pressure decline ($\tau$); however, it did not restore diastolic pressure back to adult levels.

**Pressure-Dimension Relationship**

To further assess systolic function, pressure-dimension analysis was performed in a subset of animals. Figure 6A shows pressure-dimension relations in an adult heart compared with a senescent heart. Even though the slope of the end-systolic pressure-dimension relation was similar in adult and senescent rat hearts (137±21 versus 118±31 mm Hg/mm, n=4, $P>0.1$), the pressure-dimension relations were shifted toward higher ventricular dimensions. However, as shown in Figure 6B, the slope of the end-systolic pressure-dimension relation was higher in senescent rat hearts overexpressing SERCA2a compared with senescent rat hearts infected with Ad.β-Gal (154±24 versus 112±28 mm Hg/mm, n=4, $P<0.03$).

**Effect on Contractile Reserve**

To test whether SERCA2a overexpression augmented inotropic reserve, we studied the effect of incremental atrial pacing on hemodynamic parameters in adult and senescent hearts. As shown in Figure 7, $+dP/dt$ normalized to end-diastolic dimension ($+dP/dt$/LVEDD) increased with a rise in heart rate, but this rate-dependent response was significantly blunted in the senescent hearts. Overexpression of SERCA2a in the senescent hearts induced an increase in $+dP/dt$/LVEDD at baseline, and with increasing frequency, there was a further increase in the maximal rate of pressure rise that paralleled the adult situation. Atrial pacing resulted in a significantly higher increase in diastolic pressure in senescent hearts compared with adult hearts. Overexpression of SERCA2a attenuated the rate-dependent increase in diastolic pressure but did not restore it to adult levels.

**Discussion**

Functionally, aging myocardium is characterized by prolonged relaxation and action potential duration, diminished contraction velocity and $\beta$-adrenergic response, and increased myocardial stiffness.4,18–22 Important cellular and molecular alterations underlie the functional abnormalities of aging myocardium; they include (1) a decrease in SR $\text{Ca}^{2+}$-uptake during relaxation that is associated with a decrease in SERCA2a content, (2) diminished $\beta$-receptor density and uncoupling of the adrenergic receptor from adenyllyl cyclase, resulting in a decrease in cAMP levels, (3) a reduction in the inactivation rate of the $\text{Ca}^{2+}$ current and a decrease in the magnitude of the outwardly directed K$^+$ current, resulting in a prolonged action potential, and (4) an age-associated (and species- dependent [occurring only in rodents]) change from the V$_1$ isoform of the myosin heavy chain (α-MHC) to the V$_3$ isoform (β-MHC), resulting in a decrease in myosin ATPase activity.4,21,22 These age-related changes of the various excitation-contraction coupling steps have been widely implicated in the mechanical alterations of cardiac function in senescence and can individually or in combination contribute to the abnormal relaxation and diastolic function observed in senescent myocardium. Studies have consistently shown that the $\text{Ca}^{2+}$ transient and the simultaneously measured isometric contraction or shortening are significantly prolonged in senescent cardiomyocytes.9 Because SR $\text{Ca}^{2+}$-ATPase controls the rate at which $\text{Ca}^{2+}$ is taken up and is responsible for reuptake of most of the $\text{Ca}^{2+}$ into the SR, these abnormalities in $\text{Ca}^{2+}$ handling in the aged myocardium have led to a close examination of the regulation and function of the SR $\text{Ca}^{2+}$-ATPase in aged myocardium. We found that there was a
40% decrease in SERCA2a protein content in senescent myocardium compared with adult myocardium in this rat model. This decrease in expression was also associated with a decrease in the ATPase activity in the senescent hearts. These results are consistent with prior studies showing that a stable level of SERCA2a mRNA is maintained during adulthood until 20 to 24 months, when a 40% to 50% decrease occurs.23,24 More recently, SERCA2a protein levels were found to be significantly decreased in senescent human myocardium.10 However, our results have also shown that there is no change during the aging process in the content of other key Ca\textsuperscript{2+} cycling proteins: phospholamban, the ryanodine receptors, and calsequestrin.

Recently, we developed a catheter-based technique to deliver adenoviruses to the heart. This results in a homogeneous expression throughout the ventricle. We have used this approach to demonstrate profound physiological effects on cardiac function specifically mediated by the transduced gene.11 With this method, we were able to achieve significant expression as demonstrated by the high percentage of isolated cardiomyocytes being transduced by the reporter adenovirus Ad.β-Gal. Interestingly, we found that the efficiency of adenoviruses at transduction of myocardial cells is lower in senescent rat hearts than in adult rat hearts. Whether this is due to a differential expression of the common receptor for coxsackievirus B and adenoviruses 2 and 525 or to altered viral DNA processing in senescent cardiac cells is unknown. However, even though adenoviral gene transfer is less efficient in senescent hearts, it does achieve a high level of myocardial infectivity, and most importantly, it is sufficient to induce protein expression, resulting in functional

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**Figure 5.** Measurements of systolic parameters LVSP (A) and +dP/dt (B) and diastolic parameters −dP/dt (C), τ (D), and LV diastolic pressure (LVDP) (E) in adult uninfected rat hearts (n=6), senescent uninfected rat hearts (n=8), senescent hearts infected with Ad.β-Gal at day 2 (n=6), and senescent hearts infected with Ad.SERCA2a at day 2 (n=6). *P<0.05 compared with adult. ‡P<0.05 compared with senescent group plus Ad.β-Gal.
changes. The overexpression of SERCA2a in this model of senescence resulted in the restoration of SERCA2a protein to levels comparable to those of adult rats. Furthermore, this increase in SERCA2a content resulted in an enhancement in the SR Ca\textsuperscript{2+}-ATPase activity in the senescent rat hearts. In previous studies, we and others have shown that increasing levels of SERCA2a in isolated cardiomyocytes shortened the relaxation phase of the Ca\textsuperscript{2+} transient, increased Ca\textsuperscript{2+} release, and decreased resting Ca\textsuperscript{2+}.\textsuperscript{12,26,27} However, it was not clear whether these effects could be translated in the in vivo setting. In the present study, overexpression of SERCA2a resulted in an increase in +dP/dt, a decrease in the time constant for isovolumic relaxation, and an increase in both the maximal rate of rise and the maximal rate of fall of LVSP. Isovolumic relaxation, which is an index of active relaxation and reflects the removal of Ca\textsuperscript{2+} from the myofilaments into the SR, was significantly prolonged in the senescent hearts and was restored to adult levels through the overexpression of SERCA2a. The overexpression of SERCA2a did not affect LVSP in the senescent hearts. However, the load-independent parameter of LV contractility, such as the slope of the end-systolic pressure dimension relation, end-systolic elastance, was significantly increased through the overexpression of SERCA2a. Interestingly, diastolic pressure was not decreased in the senescent hearts through the overexpression of SERCA2a.

An age-associated decrease in the cardiovascular response to pacing has been well documented in the elderly and in experimental models of senescence.\textsuperscript{5} The response to increased atrial pacing was blunted in the senescent hearts compared with the adult hearts. Overexpression of SERCA2a restored to a great degree the frequency response in these senescent hearts. These results again point to a deficiency in SR Ca\textsuperscript{2+} handling in senescent hearts that is not apparent at rest but becomes relevant at higher frequencies of stimulation.

A transgenic approach to overexpression of SERCA2a has been undertaken in both mice and rats\textsuperscript{13,27,28}; however, adenoviral transduction offers several advantages. In transgenic animals overexpressing SERCA2a, developmental adaptation to higher levels of SERCA2a occurs with upregulation of other important excitation-contraction proteins, such as phospholamban and the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, thereby masking or diluting the effects of transgene overexpression. These compensatory alterations make it difficult to assess the specific effect of increased SERCA2a on cardiac function. In the present study, overexpression of SERCA2a did not significantly alter protein expression of the ryanodine Ca\textsuperscript{2+}-releasing channels, SERCA2a, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, or calsequestrin, all of which are involved in intracellular Ca\textsuperscript{2+} handling. However, adenoviruses have significant disadvantages, including the transient nature of overexpression of the desired gene and the immune/inflammatory response they produce, which were also present in our infected hearts. However, in older rats, there is an overall reduction in the immune response that may prolong gene expression after adenoviral infection, thereby validating the use of gene therapy in the elderly.
There are a number of limitations of the study that must be addressed. The open-chest model that we used is subject to the effects of anesthesia on cardiovascular function, both directly and via reflex responses to stress. 22 Our hemodynamic measurements, however, were well within the range of those observed in previous studies of open-chest rats. For the measurement of cardiac function, LV short-axis epicardial dimensions were used. Although endocardial LV cavity dimensions would have been preferable, given the size of the piezoelectric crystal (0.7 mm diameter) relative to the normal rat LV wall thickness (1 to 2 mm), such measurements are not currently feasible. Our data add to the accumulating body of rodent physiological data and support the feasibility and applicability of LV pressure-dimension analysis in the phenotypic characterization of genetically altered models. 30, 31

Our results suggest that the decrease in SERCA2a content and associated decrease in SR Ca2+ uptake play a major role in the diastolic dysfunction observed in the elderly. Exercise training has also been shown to improve SR Ca2+ uptake and to improve systolic and diastolic parameters in senescent rats. However, an improvement in cardiac function after long-term exercise cannot be entirely ascribed to the observed SERCA2a changes. In the present study, the beneficial cardiac effects resulted from the somatic gene transfer of SERCA2a.

Our physiological data demonstrate the feasibility of achieving important functional cardiac effects through in vivo somatic gene transfer in a rodent model of senescence. This study demonstrates that the overexpression of SERCA2a through adeno viral gene transfer in senescent rat hearts improves diastolic function and restores contractile reserve. Therefore, targeting the SR Ca2+-ATPase pump may be an important strategy to improve diastolic function in the aging myocardium.

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