Gene Transfer of Parvalbumin Improves Diastolic Dysfunction in Senescent Myocytes

Fawzia Huq, MD; Djamel Lebeche, PhD; Vivek Iyer, MSE; Ronglih Liao, PhD; Roger J. Hajjar, MD

Background—Impaired relaxation is a cardinal feature of senescent myocardial dysfunction. Recently, adenoviral gene transfer of parvalbumin, a small calcium-buffering protein found exclusively in skeletal muscle and neurons, has been shown to improve cardiomyocyte relaxation in disease models of diastolic dysfunction. The goal of this study was to investigate whether parvalbumin gene transfer could reverse diastolic dysfunction in senescent cardiomyocytes.

Methods and Results—Myocytes were isolated from senescent (26 months) and adult (6 months) F344/BN hybrid rats and were infected with Ad.Parv.GFP (where GFP is green fluorescent protein) or Ad.βgal.GFP at a multiplicity of infection of 250 for 48 hours. Uninfected senescent and adult myocytes served as controls. After stimulation at a frequency of 0.5 Hz, intracellular calcium transients and myocyte contractility were measured using dual excitation spectrofluorometry and video-edge detection system (Ionoptix). Parvalbumin significantly improved relaxation parameters in senescent myocytes: Both the rate of calcium transient decay and the rate of myocyte relengthening were dramatically increased in senescent cardiac myocytes transduced with parvalbumin compared with nontransduced and GFP-expressing controls, with no effect on myocyte shortening.

Conclusions—Parvalbumin expression corrects impaired relaxation in aging myocytes. Given that abnormalities of myocyte relaxation underlie diastolic dysfunction in a large proportion of elderly patients with heart failure, gene transfer of parvalbumin may thus be a novel approach to target diastolic dysfunction in senescent myocardium.

Key Words: parvalbumin ■ aging ■ gene therapy ■ myocytes ■ diastole

Heart failure is a problem of epidemic proportions, especially in the elderly population. Diastolic heart failure is prevalent in this age group, and it is estimated that over the next 3 decades, as the population ages, half of the individuals who develop heart failure will have normal left ventricular (LV) systolic function but impaired diastolic function.1 In aging cardiac myocytes, diastolic dysfunction is characterized by prolonged relaxation, decreased contraction velocity, decreased responsiveness to β-adrenergic stimulation, and increased myocardial stiffness.2 Prolongation of the calcium transient, which underlies impaired relaxation of the senescent myocyte, is explained by a number of changes that occur in important regulatory calcium-cycling proteins. Levels of the sarcoplasmic reticulum (SR) ATPase (SERCA 2a) are decreased in senescent rat myocytes,3 reducing the rate at which calcium is resequestered back into the myocardium. In addition, the expression of the sodium-calcium exchanger is increased with age, which may partially compensate for the decreased activity of SERCA 2a.4 Changes in the density and function of sarcolemmal calcium channels are also seen, with an increase in the number and activity of the L-type calcium channel. This is accompanied by a slower inactivation of these channels, contributing further to the prolonged calcium transient seen in aging myocytes.5

Parvalbumin is a small (11-kDa), intracellular, soluble calcium-binding protein found exclusively in fast-twitch muscle fibers. It has a calcium affinity that is intermediate between those of troponin and SERCA, allowing it to act as a calcium sink to temporarily bind calcium before SR uptake.6–8 Parvalbumin gene transfer is one approach that has recently been used successfully to target prolonged relaxation in a rodent model of diastolic dysfunction.9 Adenoviral transduction of parvalbumin into the LV free wall has been shown to significantly accelerate LV isovolumic relaxation times in normal animals and also to restore normal relaxation performance in hypothyroidism-induced diastolic dysfunction.10 In addition, adenoviral gene transfer of parvalbumin into adult myocytes in vitro has been shown to significantly increase the rate of myocyte relaxation (∂dL/∂tmax, 1/s) and to decrease the rate of decay of the intracellular calcium transient. Not only were relaxation parameters in normal adult myocytes improved, but also, in hypothyroid animals, parvalbumin gene delivery fully corrected diastolic dysfunction by improving myocyte relaxation to levels seen in control animals.

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Given that impaired relaxation is a key feature of age-related cardiac dysfunction, the goal of the present study was to determine whether (1) senescent cardiac myocytes could be successfully transduced with parvalbumin and (2) parvalbumin could reverse the diastolic dysfunction characteristic of senescent cardiac myocytes.

Methods

Animals

Adult (6 months old, 28 animals) and senescent (26 months old, 34 animals) male F344/BN rats were obtained from the National Institute on Aging, Bethesda, Md. This strain has become a popular model of aging because of its decreased incidence of diseases and increased longevity compared with other strains. Animals were anesthetized with pentobarbital, and their hearts were rapidly excised in preparation for the isolation of ventricular myocytes.

Preparation of Cardiac Myocytes

Calcium-tolerant rat ventricular myocytes were isolated via enzymatic dissociation, as described previously. Briefly, the heart was retrogradely perfused for 5 minutes with oxygenated Krebs-Henseleit buffer containing (in mmol/L) 2 L-carnitine, 5 creatine, and 5 taurine, plus 100 IU/mL penicillin, and plated at a density of 104 cells/mL onto laminin-precoated coverslips (2/H9252). Myocytes were filtered through a nylon mesh, collected, and made quiescent in the absence of electrical stimulation. Only myocytes with a biphasic pulse (0.5 Hz, 50% above threshold) were included in the study. Myocytes were stimulated with injected current of magnitude 100 pA/pF for a duration of 0.1 ms. Pacing was performed to steady state for all simulations, usually on the order of 20 beats.

Intracellular Calcium and Contractility Measurements

Cultured myocytes 48 hours after transfection were loaded with fura 2-AM (Molecular Probes), superfused with 1.2 mmol/L Ca®-Tyrode’s solution, pH 7.40 at 37°C, and electrically stimulated with biphasic pulse (0.5 Hz, 50% above threshold). Cells included in the study were rod-shaped with a clear striation pattern and were quiescent in the absence of electrical stimulation. Only myocytes expressing GFP (evidence of successful transduction) were examined. Contraction amplitude, rates of contraction and relaxation, and intracellular calcium levels were recorded online using a dual excitation spectrophotometer and video-edge detection system (Ionoptix), as described previously.

Immunoblotting

Senescent cardiac myocytes were homogenized, and protein concentration was determined by the Bradford Assay. Proteins were then transferred to a Hybond-ECL nitrocellulose and blocked in 5% nonfat milk. For immunoreactions, the blot was incubated with anti-parvalbumin antibody at a dilution of 1:500. The densities of the bands were detected by a chemiluminescence system.

Computer Modeling

A previously developed model of the canine action potential and calcium transient was used to study the effects of parvalbumin. The calcium-handling subsystem of the model was composed of 4 compartments in which intracellular calcium can locally achieve different concentrations: (1) The bulk myoplasm, denoted by Ca®; (2) a network sarcoplasmic reticulum (NSR) uptake compartment, CaNSR; (3) a junctional sarcoplasmic reticulum (JSR) release compartment, CaJSR; and (4) an excluded subspace (SS) compartment CaSS, which consists of the pooled dyadic spaces. The resulting differential formulation for Ca® is

\[
\frac{d[Ca^{2+}]}{dt} = \beta(J_{\text{diff}} - J_{\text{upt}} - J_{\text{parv}} - J_{\text{CaSS}}),
\]

where \( \beta \) is a scaling factor reflecting fast calcium buffering by calmodulin. \( J_{\text{diff}} \) is the diffusional flux from subspace to the bulk myoplasm compartment, \( J_{\text{upt}} \) is the uptake flux into the NSR through the SR Ca® ATPase, \( J_{\text{parv}} \) is the calcium flux representing buffering by high- and low-affinity troponin sites, and \( J_{\text{CaSS}} \) represents the summed sarcoplasmic membrane currents carried by Ca® (which are scaled by \( k_{\text{Ca}} \), which factors in physical constants, membrane capacitance, ionic valence, and cell volume to convert membrane current into units of calcium flux). Full details on the computer model and calcium handling equations can be found in Winslow et al. Jparv, the calcium flux representing binding to calcium sites on parvalbumin, was formulated according to a previously developed mathematical model of parvalbumin buffering describing competition between magnesium and calcium for each divalent cation-binding site on parvalbumin.

\[
J_{\text{parv}} = Parv_{\text{tot}} Parv_{\text{Ca}},
\]

where each \( k \) is an on or off rate constant for each ion, and \( Parv_{\text{Ca}}, Parv_{\text{Mag}}, \) and \( Parv_{\text{tot}} \) are the calcium-bound, magnesium-bound, and free states of parvalbumin occupancy, respectively. ParvCa, the total intracellular concentration of parvalbumin-binding sites, is set to 0.1 mmol/L. Values of rate constants and a complete description of parvalbumin-buffering equations can be found in Robertson et al. A minimal model of the senescent myocyte was simulated by incorporating several changes to the model, reducing the magnitude of SR ATPase flux (encoded by SERCA) by 25% and reducing the amplitude of \( I_{\text{Ca}} \) current (encoded by Kv4.3) by 25%. Myocytes were stimulated with injected current of magnitude ~100 pA/pF for a duration of 0.1 ms. Pacing was performed to steady state for all simulations, usually on the order of 20 beats.

Statistics

Data are presented as mean±SEM and were analyzed using a 1-way ANOVA. A probability value <0.05 was considered statistically significant.
myocytes express GFP. At 48 hours after transfection, >75% of transduced myocytes express GFP.

Results

Five groups of myocytes were examined: Control (nontransduced) senescent (CS), Ad.Parv.GFP senescent (PS), Ad.βgal.GFP senescent (GS), control (nontransduced) adult myocytes (CA), and control myocytes infected with Ad.βgal.GFP. Myocytes isolated from senescent (26 months) rats were infected with Ad.Parv.GFP and Ad.βgal.GFP at an MOI of 250. Optimal expression of parvalbumin and GFP occurred 48 hours after infection. Homogeneous expression of GFP was demonstrated in >75% of senescent myocytes 48 hours after isolation in both the βgal and parvalbumin-transduced groups (Figure 1).

As shown in Figure 2 and Table 1, contraction in senescent cardiomyocytes was significantly prolonged in the relaxation phase, as evidenced by an increase in $t_{50\%}$ and $t_{90\%}$. The systolic phase was not affected in the senescent cardiomyocytes, and shortening was also the same among the different groups. Overexpression of parvalbumin restored the relaxation parameters to levels comparable to adult control levels, whereas infection with the reporter adenovirus did not affect these parameters. The relaxation velocity was greatly increased in parvalbumin-expressing senescent myocytes compared with GFP-expressing and control senescent myocytes. In fact, the introduction of parvalbumin normalized the rate of myocyte relaxation in senescent myocytes to that of the adult myocyte. However, parvalbumin expression also had no significant effect on the rate of myocyte shortening in senescent myocytes compared with both the control and GFP-expressing groups.

Calcium handling was impaired in the senescent group, as shown in Figure 3 and Table 2. There was an increase in the relaxation phase of the calcium transient and an increase in diastolic calcium. Representative tracings of calcium transients from adult control, senescent control, GFP-expressing, and parvalbumin-expressing senescent myocytes are depicted in Figure 3 and show how parvalbumin markedly increases the rate of calcium transient decay compared with control and GFP-transduced senescent myocytes. The calcium transient decay was significantly slower in senescent compared with control adult myocytes, and the introduction of parvalbumin significantly reduced the relaxation time of calcium and restored it to control adult levels. In addition, the introduction of parvalbumin decreased diastolic calcium levels but had no effect on systolic calcium levels.

To examine whether there is a dose effect of parvalbumin on physiological parameters in the isolated cardiomyocytes, we increased stepwise the concentration of Ad.Parv.GFP. We found a gradual decline in the time course of relaxation of the senescent cardiac myocytes, as shown in Figure 4.

Parvalbumin is a small (11-kDa) protein with 2 divalent cation binding sites with different affinities for calcium and magnesium as first described by Rhyner et al.19 and Heizmann et al.20 Because of the unique kinetics of calcium and magnesium binding and unbinding to the buffer, parvalbumin

![Figure 1. Epifluorescent images showing successful transduction of senescent myocytes with GFP and parvalbumin. Myocytes were isolated via enzymatic dissociation and were infected with either Ad.βgal.GFP or Ad.Parvalbumin.GFP at an MOI of 250. At 48 hours after transfection, >75% of transduced myocytes express GFP.](http://circ.ahajournals.org/)

![Figure 2. Myocyte contraction recordings (normalized to peak contraction) from adult control, senescent control, GFP-transduced, and parvalbumin-transduced myocytes, stimulated at 0.5 Hz at 37°C. Introduction of parvalbumin significantly accelerates myocyte relaxation in senescent myocytes.](http://circ.ahajournals.org/)

### TABLE 1. Cardiomyocyte Contractility in the Different Groups

<table>
<thead>
<tr>
<th>Contractility Measurements</th>
<th>Control</th>
<th>Adult</th>
<th>Adult + Ad.βgal.GFP</th>
<th>Control Senescent</th>
<th>Senescent + Ad.βgal.GFP</th>
<th>Senescent + Ad.Parv.GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell shortening, %</td>
<td>4.8 ± 0.2</td>
<td>4.6 ± 0.3</td>
<td>4.5 ± 0.3</td>
<td>4.4 ± 0.4</td>
<td>5.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>$t_{50%}$, ms</td>
<td>82 ± 5</td>
<td>92 ± 7</td>
<td>133 ± 11*</td>
<td>145 ± 13†</td>
<td>89 ± 10‡</td>
<td></td>
</tr>
<tr>
<td>$t_{90%}$, ms</td>
<td>160 ± 22</td>
<td>177 ± 28</td>
<td>210 ± 19*</td>
<td>221 ± 25†</td>
<td>158 ± 22‡</td>
<td></td>
</tr>
<tr>
<td>Dev. Vel., μm/s</td>
<td>87 ± 11</td>
<td>77 ± 17</td>
<td>76 ± 20</td>
<td>68 ± 13</td>
<td>91 ± 9</td>
<td></td>
</tr>
<tr>
<td>Rel. Vel., μm/s</td>
<td>66 ± 10</td>
<td>59 ± 11</td>
<td>49 ± 10*</td>
<td>44 ± 8†</td>
<td>70 ± 10‡</td>
<td></td>
</tr>
<tr>
<td>No. of cells</td>
<td>38</td>
<td>25</td>
<td>29*</td>
<td>24</td>
<td>59</td>
<td></td>
</tr>
</tbody>
</table>

Dev. Vel. indicates developed velocity; Rel. Vel., relaxation velocity.

* $P<0.01$ vs control adult group; † $P<0.01$ vs Adult + Ad.βgal.GFP; ‡ $P<0.01$ vs Senescent + Ad.βgal.GFP.
delivery appears to affect the lusitropic state of the heart while minimally affecting the inotropic state. To determine whether these results are generalizable to larger mammals and humans, in which intrinsic pacing rates are much slower and properties of calcium cycling may differ considerably, a computer model of the canine action potential and calcium transient activation is used to determine whether parvalbumin exerts an appreciable effect during systole or whether its effects are limited to diastolic calcium buffering. In the simulation studies, a minimal model of the senescent myocyte was constructed by incorporating downregulation of SR ATPase current (encoded by SERCA) by 25% and downregulation of the transient outward current (encoded by Kv4.3) by 25%. A previously developed mathematical model of the parvalbumin buffer is incorporated at a concentration of 100 μmol/L, and cells are paced at a typical pacing rate for humans and large mammals (1 Hz) until steady state. Figure 5A shows the action potential, of duration 300 ms. On stimulation, the rise in intracellular calcium (Figure 5B) causes a slow dissociation of magnesium (Figure 5C, thin trace) and a corresponding increase in occupancy of calcium-bound parvalbumin (Figure 5C, thick trace). However, because dissociation of magnesium is slow (time constant of 289 ms) relative to activation of the calcium transient (which peaks at 42 ms), the majority of calcium-parvalbumin complexes are formed during the decay phase of the calcium transient. This leads the calcium to decay more rapidly (time to decay to half-maximal calcium of 262 ms in parvalbumin senescent simulation versus 341 ms in control senescent simulation). These changes occur with little change in peak calcium transients (479 nmol/L in parvalbumin senescent simulations versus 478 nmol/L in control senescent simulation), confirming that parvalbumin acts during diastole with minimal effects on systolic contractility. Similar results were observed using short (150-ms) voltage clamps to +10 mV, which were used to approximate the shorter rat calcium transient.

**Discussion**

Impaired relaxation is a cardinal feature of senescent myocyte dysfunction and manifests clinically in a large proportion of elderly patients as diastolic heart failure with relatively preserved systolic function. Despite its high prevalence, there is a paucity of clinical trial data to guide management decisions for diastolic heart failure, with options currently limited to the control of hypertension, ischemia, and tachycardia and judicious use of nitrates, diuretics, and angiotensin-converting enzyme inhibitors. The problem is compounded by the significant overlap between systolic and diastolic heart failure.

### Table 2. Calcium Measurements in the Different Groups

<table>
<thead>
<tr>
<th>Calcium Measurements</th>
<th>Control Adult</th>
<th>Adult + Ad.Parv.GFP</th>
<th>Control Senescent</th>
<th>Senescent + Ad.Parv.GFP</th>
<th>Senescent + Ad.Parv.GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diastolic [Ca2+] , nmol/L</td>
<td>190 ± 21</td>
<td>210 ± 31</td>
<td>265 ± 12*</td>
<td>257 ± 25†</td>
<td>201 ± 20†</td>
</tr>
<tr>
<td>Systolic [Ca2+] , nmol/L</td>
<td>687 ± 44</td>
<td>665 ± 54</td>
<td>634 ± 43</td>
<td>701 ± 69</td>
<td>713 ± 76</td>
</tr>
<tr>
<td>t90%, ms</td>
<td>28 ± 5</td>
<td>26 ± 5</td>
<td>27 ± 4</td>
<td>27 ± 3</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>t50%, ms</td>
<td>75 ± 4</td>
<td>81 ± 6</td>
<td>95 ± 6*</td>
<td>98 ± 7</td>
<td>74 ± 6‡</td>
</tr>
<tr>
<td>t90%, ms</td>
<td>221 ± 11</td>
<td>232 ± 8</td>
<td>310 ± 13*</td>
<td>331 ± 10†</td>
<td>240 ± 11‡</td>
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<td>No. of cells</td>
<td>36</td>
<td>25</td>
<td>28</td>
<td>24</td>
<td>61</td>
</tr>
</tbody>
</table>

*P<0.01 vs control adult group; †P<0.01 vs Adult + Ad.Parv.GFP; ‡P<0.01 vs Senescent + Ad.Parv.GFP.
diastolic dysfunction and the lack of availability of any agent with proven, direct lusitropic properties. Gene transfer in senescent cardiac myocytes is a relatively novel approach that offers the ability to explore and selectively target pathophysiological mechanisms that cause impaired relaxation and diastolic dysfunction.

Because prolongation of the action potential and the consequent calcium transient occurs with age, adeno-virus-mediated gene transfer of calcium-handling proteins has been one approach that has been used successfully to reverse this phenomenon. Both protein levels and gene expression of SERCA 2a (which resequesters calcium back into the SR during relaxation) are decreased with aging, and it has been demonstrated that adeno-viral transfer of SERCA2a into senescent hearts in vivo improves rate-dependent contractility and diastolic function (time constant of isovolumic relaxation and \(-\frac{dP}{dt}\)) to adult levels. The force-frequency response is also impaired in senescent myocardium, and gene transfer of SERCA 2a restores this blunted frequency response to a great degree.

Adenoviral gene transfer of parvalbumin into cardiac myocytes has shown that both the rate of calcium transient decay and the rate of myocyte relaxation are increased by the introduction of parvalbumin, with no decrease in contraction amplitude. This effect is observed both in healthy adult myocytes and in a disease model of diastolic dysfunction (hypothyroidism) in which expression of parvalbumin fully corrected relaxation parameters \(t_{1/2}\) and \(-\frac{dL}{dT}\) in myocytes from diseased animals back to normal (euthyroid) levels. Further confirmation of the ability of parvalbumin to restore diastolic parameters to normal was obtained in an in vivo setting, in which injection of parvalbumin into the LV free wall both accelerated myocardial relaxation under physiological conditions and improved myocardial twitch relaxation rates and \(-\frac{dP}{dt}\) in hypothyroid hearts to normal.

In this study, we demonstrate that adenoviral gene transfer of parvalbumin, a protein affecting solely the lusitropic properties of myocardium, can be used to successfully transduce senescent myocytes and significantly improves myocardial relaxation in healthy senescent cardiac myocytes. The calcium transient decay rate is significantly improved in senescent parvalbumin-transduced myocytes compared with GFP-expressing and control myocytes, and the time from peak to 50% calcium in senescent myocytes transduced with parvalbumin is comparable to that of adult controls. In parallel with the improvement in calcium decay rate, we also found that the rate of myocyte relengthening is dramatically increased in senescent myocytes expressing parvalbumin and in fact slightly exceeds the relaxation rate of adult control myocytes. These results are consistent with the findings of Wahr et al. who demonstrated that relaxation parameters in both hypothyroid and healthy myocytes were significantly ameliorated with parvalbumin, with no effect on the rate of cell shortening or the amplitude of peak contraction.

Our results suggest that in a rodent model of aging, adenoviral gene transfer of parvalbumin can be used to successfully transduce senescent myocardium in vitro and improves both the rate of calcium decay and the rate of myocyte relengthening in transduced myocytes. They also show that in aging myocytes, parvalbumin expression does not affect systolic function. Interestingly, agents that increase contractility have been associated with worsening morbidity in clinical trials. Therefore, the fact that parvalbumin does not enhance contractility but improves relaxation may prove beneficial in the long term.

Finally, abnormalities of calcium handling play a major role in the diastolic dysfunction seen with aging, and gene transfer offers a distinct opportunity to target these age-related pathophysiological changes. Parvalbumin is unique because it selectively targets and improves the lusitropic arm of the contractile response, which is impaired in senescent myocardium. Gene transfer of parvalbumin may thus be one approach that can be developed to target senescent cardiac dysfunction.

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References
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