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Human SERCA2a Levels Correlate Inversely With Age in Senescent Human Myocardium

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Objectives. **This study sought to characterize functional impairment after simulated ischemia-reperfusion (I/R) or Ca^{2+} bolus in senescent human myocardium and to determine if age-related alterations in myocardial concentrations of SERCA2a, phospholamban, or calsequestrin participate in senescent myocardial dysfunction.**

Background. **Candidates for elective cardiac interventions are aging, and an association between age and impairment of relaxation has been reported in experimental animals. Function of the sarcoplasmic reticulum resulting in diastolic dysfunction could be dysregulated at the level of cytosolic Ca^{2+} uptake by SERCA2a, its inhibitory subunit (phospholamban), or at the level of Ca^{2+} binding by calsequestrin.**

Methods. **Human atrial trabeculae from 17 patients (45–75 years old) were suspended in organ baths, field simulated at 1 Hz, and force development was recorded during I/R (45/120 min). Trabeculae from an additional 12 patients (53–73 years old) were exposed to Ca^{2+} bolus (2–3 mmol/L bath concentration). Maximum ± dF/dt and the time constant of force decay (tau) were measured before and after I/R or Ca^{2+} bolus and related to age. SERCA2a, phospholamban, and calsequestrin from 12 patients (39–77 years old) were assessed by immunoblot.**

Results. **Functional results indicated that maximum ±dF/dt and tau were prolonged in senescent (>60 years) human myocardium after I/R ($p < 0.05$). Calcium bolus increased the maximum ±dF/dt and decreased *tau* in younger, but not older patients ($p < 0.05$). SERCA2a and the ratio of SERCA2a to either phospholamban or calsequestrin were decreased in senescent human myocardium ($p < 0.05$).**

Conclusions. **Senescent human myocardium exhibits decreased myocardial SERCA2a content with age, which may, in part, explain impaired myocardial function after either I/R or Ca^{2+} exposure.**

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As an increasing number of aged patients become candidates for elective cardiac procedures, it is important to determine whether adult and senescent myocardium respond differently to common clinical stress (ischemia-reperfusion [IR] injury) or exogenous calcium (Ca^{2+}). Although a decrease in beta-adrenergic receptors with age may, in part, explain some senescent myocardial dysfunction (1), many reports have implicated diastolic dysfunction as the most proximal cause of cardiac failure in humans (2,3). Diastolic dysfunction has been studied in mouse (4), rat (5–7), and sheep (8). These studies demonstrated a relationship between age and impairment of ventricular relaxation (both static and dynamic compliance). Diastolic dysfunction in humans with heart failure has been related to age by echocardiography (9,10) and cineangiography (11).

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Calcium dyshomeostasis and I/R have many features in common (12–14). Intramyocellular calcium ($[Ca^{2+}]_i$) overload due to I/R insult results from ionic dyshomeostasis, substrate deprivation, and impaired energy production processes (5,12). Increased $[Ca^{2+}]_i$ associated with dysregulated Ca^{2+} handling proteins should exacerbate diastolic, and even systolic, dysfunction. Function of the sarcoplasmic reticulum may be dysregulated at the level of the sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA), its regulatory subunit (phospholamban), or at the level of Ca^{2+} binding by calsequestrin (15,16).

Recently, our laboratory (17–25) and others (26–28) have studied the functioning human myocardial syncitium in vitro. We hypothesized that systolic and diastolic function are impaired in senescent human myocardium exposed to either I/R or increased extracellular Ca^{2+} due to alterations of Ca^{2+} handling proteins. The purposes of this study were to determine the relationships between maximum rates of both contraction and relaxation before and after I/R or exogenous Ca^{2+}, and relate these parameters to age. Additionally, we desired to quantify the Ca^{2+} handling proteins most likely to participate in diastolic dysfunction in the human heart and to relate their concentrations to age.

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Abbreviations and Acronyms

SERCA2a = sarcoendoplasmic reticulum Ca^{2+}-ATPase
I/R = ischemia-reperfusion
T = tau, the time constant of force decay
SI = simulated ischemia
$[Ca^{2+}]_i$ = intramyocellular calcium
CAD = coronary artery disease
HTN = hypertension
IDDM = insulin-dependent diabetes mellitus
NIDDM = non–insulin-dependent diabetes mellitus
GERD = gastro-esophageal reflux disease
COPD = chronic obstructive pulmonary disease
CHF = congestive heart failure
LAD = left anterior descending coronary artery
RCA = right coronary artery
Cx = circumflex coronary artery
LVEF = left ventricular ejection fraction

Methods

Isolated atrial trabeculae. Right atrial appendages were obtained from patients undergoing cardiac surgery for the first time. All trabeculae and appendages were from patients with stable cardiac disease. The indication for surgery in all patients evaluated in this study was ischemic coronary artery disease. Patients were excluded from the study if they had hemodynamic instability (mean arterial pressure <80 mm Hg) within 48 h of cardiopulmonary bypass, atrial dysrhythmias, or right atrial pressures greater than 10 mm Hg, or evidence of right atrial hypertrophy. Informed consent was obtained from all subjects, and the study was approved by the University of Colorado Health Sciences Center.

Each appendage was placed in oxygenated, modified Tyrode's solution at 4°C. Three to four trabeculae (diameter <1.0 mm, and length 4–7 mm) were obtained from each appendage, and suspended vertically in an organ bath and attached to a force transducer. Each organ bath contained 30 mL modified Tyrode's solution, which was bubbled (40 mL/min) with a 92.5% O_2-7.5% CO_2 gas mixture during normoxia. This mixture provided for a pO_2 > 360 mm Hg, a pCO_2 of 38–42 mm Hg, and a buffer pH of 7.35–7.45, which were checked routinely with an automated blood gas analyzer (ABL Instruments). Temperature in the organ baths was maintained at 37.0°C. During the simulated ischemic (SI) period (SI: consisting of hypoxic, substrate free Tyrode's solution with pacing at 3 Hz), which was used only for the I/R trabeculae, the buffer was changed, the baths were covered to prevent atmospheric gas exchange, and the gas mixture was switched to 92.5% N_2-7.5% CO_2, which produced a pO_2 <50 mm Hg. Except during the period of SI, the Tyrode's buffer was replaced at 20-minute intervals throughout the experiment. Trabeculae interrogated using Ca^{2+} bolus alone remained oxygenated throughout the experiment.

Thirty minutes were allowed after suspension of each trabecula for recovery. After this time, the trabeculae were gradually stretched to a resting force of 1 g, which was determined to be the optimal length-tension for human atrial trabeculae in our laboratory (17–25), and field stimulated. Field stimulation occurred with platinum electrodes (Radnoti Glass, Inc.) at a frequency of 1 Hz. The platinum electrodes were positioned on either side of each trabecula, and were driven with stimulators (Grass SD9 stimulator) with 5-msec pulses at a voltage of 10% above threshold. Isometric contractile responses were detected by force-displacement transducers (Grass FT03), and recorded with a computerized preamplifier/digitizer (MacLab 8; AD Instruments) and a Macintosh computer (Apple Computer). The indices of contractile function assessed were developed force (DF, g) and resting force (g). Trabeculae that failed to generate at least 0.25 g of DF were excluded from the study.

Materials. The modified Tyrode's solution was prepared daily with de-ionized distilled water (ddH_2O) and consisted of (in mmol/L): D-glucose, 5.0; $CaCl_2$, 2.0; NaCl, 118.0; KCl, 4.0; $MgSO_4 \cdot 7\ H_2O$, 1.2; $NaHCO_3$, 25.0; and NaH_2PO_4, 1.2. All reagents were from Sigma Chemical Company. In the substrate-free Tyrode's solution used during simulated ischemia, choline (7.0 mmol/L) was added to maintain constant osmolarity.

Experimental design. To investigate the role of I/R on human myocardial systolic and diastolic function, a total of 17 atrial appendages were obtained from patients with chronic ischemic heart disease (mean age 60.4 ± 2.6 years, 15 males and 2 females). No trabeculae were excluded from analysis. Ischemia reperfusion trabeculae were subjected to a 90-minute equilibration period to allow for plateau and stabilization of developed force, and subsequently these experiments were conducted for 165 minutes. I/R trabeculae were challenged with a 45-minute period of SI, which consisted of hypoxic, substrate-free Tyrode's solution with pacing at 3 Hz, followed by 120 minutes of reoxygenation with normoxic Tyrode's solution with pacing at 1 Hz.

Twelve patients (mean age 61.2 ± 2.0 years, 9 males and 3 females), also with chronic ischemic heart disease, provided atrial appendages to investigate the effect of Ca^{2+} bolus on cardiac function. Again, no trabeculae were excluded from analysis. Calcium-challenged trabeculae were also equilibrated for 90 minutes, then bath Ca^{2+} concentration was increased to 3.0 mmol/L from 2.0 mmol/L. At end of either protocol, all trabeculae were removed from the organ baths, weighed, and measured.

Calcium dose-response data previously determined in our laboratory indicated that 3.0 mmol/L represented approximately the inotropic mid-point, corresponding to one-half maximal inotropic response to Ca^{2+} (20). However, we also wanted to determine if senior myocardium responded any differently to the 3.0 mmol/L Ca^{2+} dose than junior myocardium, in terms of absolute developed force. Data were stratified by age and revealed no difference in myocardial sensitivity to exogenous Ca^{2+}. These Ca^{2+} dose-response data (n = 6 each junior [age ≤60 years] and senior [age >60 years] myocardium) were obtained by determining the change in developed force (in mg) relative to the baseline-developed

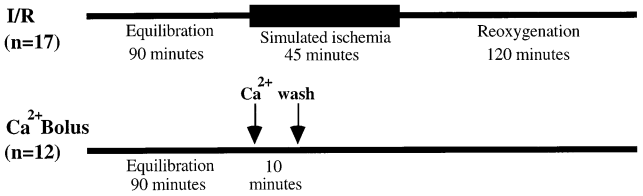


Figure 1. Experimental protocols. All experiments were preceded by a 90-min period that allowed for stabilization of developed force. Simulated ischemia refers to incubation of trabeculae in substrate free, hypoxic Tyrode's solution while pacing at 3 Hz. Normoxic perfusion and reperfusion refer to incubation of trabeculae in oxygenated Tyrode's with substrate and pacing at 1 Hz. Ca^{2+} and wash indicate Ca^{2+} concentration changes to from 2.0 to 3.0 mmol/L and back to 2.0 mmol/L, respectively.

force (%BDF). The experimental protocols are depicted in Figure 1. In addition, control trabeculae were equilibrated for 90 minutes and perfused with normoxic Tyrode's with pacing at 1 Hz for 180 minutes to ensure model stability. Each trabeculae was used in only a single experiment and multiple trabeculae from the same patient were each used in a different protocol. Trabecular baseline tissue characteristics are presented in Table 1.

We measured maximum +dF/dt during contraction and −dF/dt during relaxation before and after I/R injury or Ca^{2+} bolus for each respective trabeculae. The maximum slope of the contraction or the relaxation phase was located and averaged over approximately 120 beats using Matlab software (The Math Works, Inc.). This data analysis resulted in an averaged maximum slope, which was compared with the initial averaged maximum slope. The functional results are expressed as fraction of baseline value for the I/R trabeculae, or fraction over baseline for the Ca^{2+}-challenged trabeculae. Also, we computed T (tau), which is the time constant of force decay (8,29–31) before and after I/R injury or Ca^{2+} bolus for each respective trabeculae at the same time intervals used in the calculation of ±dF/dt using Matlab software. We determined tau according to the method of Weiss et al. (31), who determined the time constant by fitting the monoexponential model to force decay data after the time of maximum negative −dF/dt to a point 5% above the minimum diastolic developed force. Relaxation force data were fit to the following exponential equation: $F(t) = a \cdot e^{(-t/T)}$, where a is a constant, t is the time during the peak −dF/dt to a point 5% above the minimum diastolic force development, and T is tau, the time constant of force decay.

Table 1. Human Myocardial Baseline Tissue Characteristics

Group	Systolic Force (mg)	Resting Force (mg)	Cross-Sectional Area (mm^2)
I/R (n = 17)	897 ± 113	1,008 ± 7	0.49 ± 0.10
Ca^{2+} (n = 12)	661 ± 143	1,016 ± 11	0.53 ± 0.12

Values are mean ± SEM.

Preparation of myocardial tissue homogenates. Approximately 250 mg of human atrial appendage, from which a trabeculae was harvested for use in the I/R, Ca^{2+} challenge experiments, or in some cases other protocols, was homogenized in a tenfold volume of 20 mmol/L Na-Hepes, pH 7.4, for 4 × 15 s using the Tekmar tissumizer (Tekmar Company), at 4°C. The protein concentration was determined in triplicate according to Lowry et al. (32). The protein per gram net weight was calculated from the protein concentration of the homogenates. The yield of protein per gram wet weight of tissue was 191.5 ± 29.7 mg/g in the youngest four patients, 176.0 ± 6.7 mg/g in the middle four patients, and 160.7 ± 18.7 mg/g in the oldest four patients. Aliquots of the homogenates were frozen in liquid nitrogen and stored at −70°C until use.

Western blot analysis. Tissue homogenates were solubilized in 2% sodium dodecyl sulfate (SDS), 5% 2-beta-mercaptoethanol, 10% glycerol, 0.00125% bromophenol blue, and 0.0625 mol/L Tris-Cl, pH 6.8. Lysis was performed for 5 min at 95°C to dissociate the pentameric form of phospholamban into subunits and de-aggregate myocellular proteins. Samples were subjected to SDS-PAGE using the Laemmli buffer system (33) in a Mini-Protean II dual slab cell (Bio-Rad Ltd.). Electrophoresis was performed until complete elution of the dye front. Proteins were transferred to nitrocellulose in a mini Trans-Blot Transfer Cell (Bio-Rad Ltd.) according to the procedure of Towbin with the modification that SDS was included in the transfer buffer (25 mmol/L Tris, 192 mmol/L glycine, 0.0375% SDS, and 20% [vol/vol] methanol, pH 8.3). The transfer was carried out at room temperature for 1 h at a constant voltage setting of 100 V. Transfer was checked by staining the blots in Ponceau S solution (Sigma Ltd.) and staining of the remaining polyacrylamide gels in Coomassie brilliant blue G (Sigma Ltd.). The blots were blocked in 5% nonfat milk diluted in phosphate-buffered saline (PBS) (40 mmol/L Na_2HPO_4, 8 mmol/L NaH_2PO_4, 154 mmol/L NaCl, pH 7.4) for 1 h at room temperature. The blots were washed three times for 10 min with fresh PBS. The blots were incubated with the appropriate primary antibody to SERCA2a, phospholamban, or calreticulin (Affinity Bioreagents) in solution, diluted in TPBS (40 mmol/L Na_2HPO_4, 8 mmol/L NaH_2PO_4, 154 mmol/L NaCl, 0.1% Tween-20, pH 7.4) and 5% nonfat milk overnight at 4°C. The blots were washed three times for 10 min in TPBS and then incubated in the secondary anti–mouse horseradish peroxidase–conjugated antibody solution (Amersham Life Science) diluted in the same buffer as above for 1 h at room temperature. The blots were again washed two times for 10 min in TPBS and two times for 10 min in PBS, incubated in enhanced chemiluminescence (ECL)-detection reagents (Bio-Rad Ltd.) for 1 min, and exposed to an X-OMAT AR X-ray film (Kodak, Inc.) for 30 s to 10 min. Western blot analysis of SERCA2a, monomeric phospholamban, pentameric phospholamban, and calsequestrin were each performed using trabeculae from 12 patients (aged 39–77 years). Patient demographic data for all myocardium evaluated in the functional studies as well as the protein quantification study are provided in Table 2.

Table 2. Demographic Characteristics

Age (Years)	Diseases	Medications	Coronary Angio	LVEF
39	CAD	heparin/restoril/vicodin	90% L main	normal
45	CAD/depression/HTN/GERD	benazapril/atenolol/zoloft/clonepine/tagamet	100% LAD/60% RCA/50% Cx	47%
47	CAD/GERD	famotidine/isosorbide	95% LAD/50% RCA/50% Cx	72%
48	CAD/IDDM/HTN/hypercholesterolemia/ hypothyroidism	atenolol/felodipine/insulin/levothyroxine/ isosorbide/pravastatin/cholestyramine	80% LAD/75% RCA/70% Cx	54%
48	CAD/HTN	atenolol/aspirin	100% LAD/70% RCA/40% Cx	45%
49	CAD/HTN/GERD	verapamil/lisinopril/famotidine/zocor lopressor	100% LAD/90% RCA/80% Cx	26%
50	CAD	pepcid/restoril	50% LAD/60% RCA/50% Cx	normal
50	HTN	atenolol/aspirin/heparin/isordil	70% LAD/95% RCA/50% Cx	61%
51	CAD	none	100% LAD/70% RCA/70% Cx	60%
53	CAD/HTN	atenolol	80% LAD/40% RCA/30% Cx	71%
53	CAD/HTN	metaprolol/captopril	80% LAD/100% RCA/30% Cx	45%
55	CAD/NIDDM/GERD	NTG/metaprolol/cimetidine/glyburide	80% LAD/400% RCA/30% Cx	71%
56	CAD/CHF/hypercholesterolemia	HCTZ/benazapril/isorbide/pravastatin	75% LAD/50% RCA/50% Cx	55%
59	CAD/NIDDM/gout/HTN	felodipine/metaprolol/benazapril/ cholchicine/glyburide	70% LAD/100% LAD/50% Cx	normal
60	CAD/Hypothyroidism/NIDDM	glucophage/zestril/synthroid/glucotrolzocor	90% LAD/90% RCA/90% Cx	45%
60	CAD/HTN/NIDDM/GERD	lasix/zocor/heparin/metaprolol/fosinopril/ indur/ASA/axid/glucophage	90% LAD/50% RCA/70% Cx	60%
61	CAD/HTN/GERD	captopril/omeprazole/metaprolol/NTG/sucralafate	90% LAD/50% RCA/70% Cx	normal
62	CAD/HTN	isorbide/vasotec/atenolol/amlodipine	70% LAD/90% RCA/50% Cx	50%
63	CAD/HTN/GERD/COPD	aspirin/amlodipine/axid/isordil/atrovent	50% LAD/100% RCA/30% Cx	60%
63	CAD/hypercholesterolemia	benazapril/pravastatin/nitroglycerin	90% LAD/40% RCA/70% Cx	normal
63	CAD/HTN/hypercholesterolemia	vasotec/zocor/clonidine/ASA/lasix/prednisone	40% LAD/70% RCA/40% Cx	normal
64	CAD/HTN/GERD/hypercholesterolemia	atenolol/isorbide/gemfibrozil/tagamet	90% LAD/100% RCA/50% Cx	normal
65	CAD/HTN/GERD	atenolol/cimetidine	80% LAD/95% RCA/70% Cx	35%
66	CAD/GERD/HTN/CHF	benazapril/omeprazole/diltiazem/prazosin/lasix/Fe	90% LAD/80% RCA/80% Cx	40%
66	CAD/HTN	captopril	80% LAD/60% RCA/80% Cx	69%
69	CAD	none	70% LAD/100% RCA/90% Cx	59%
69	CAD/HTN/hypercholesterolemia	atenolol/pravastatin	95% LAD/70% RCA/100% Cx	normal
71	CAD/HTN	verapamil/testosterone	50% LAD/80% RCA/75% Cx	normal
72	CAD/IDDM/hypercholesterolemia/HTN	pravastatin/desipramine/insulin/atenolol	100% LAD/50% RCA/80% Cx	normal
73	CAD/GERD/HTN/NIDDM	paxil/ticlid/cisapride/pepcid/atrovent/ glucotrol/propranolol/NTG	90% LAD/90% RCA/60% Cx	60%
73	CAD/hypercholesterolemia	NTG/ASA/heparin/zocor	50% LAD/80% RCA/75% Cx	normal
74	CAD/NIDDM	glipizide/isordil	60% LAD/80% RCA/90% Cx	44%
75	CAD/NIDDM/gout	heparin/allopurinol/lasix	80% LAD/60% Cx	55%
77	CAD/HTN	potassium/metaprolol	95% LAD/100% RCA/50% Cx	normal

Quantification of immunoreactive bands. The band densities were evaluated by densitometric scanning using a laser scanning densitometer. Relative protein quantitation was performed by normalization of all patients for a given protein to the absorbance value computed for a defined reference heart (the 39-year old patient) to promote standardization. Results were expressed graphically as linear regression of each protein versus age.

Statistical analysis. Differences in preservation of systolic and diastolic function as well as myocardial protein content were assessed using linear regression versus age (95% confidence bands were computed). Also, differences in myocardial protein content were assessed between junior and senior cohorts by ANOVA (Bonferroni/Dunn). $p < 0.05$ was accepted to represent significance. Regression coefficient (R) was computed for each linear regression. Differences in *tau* between junior and senior myocardium were compared using the paired *t*-test.

Results

Baseline Functional Data. Linear regression of $\pm$dF/dt at baseline for either the I/R trabeculae or the Ca^{2+}-challenged trabeculae revealed no relationship to age (each $p > 0.05$). However, when posttreatment functional results were compared with baseline values, significant relationships to age were demonstrated.

I/R Trabeculae. Systolic function, as assessed by remaining +dF/dt after I/R (fraction of baseline) for each trabeculae versus age using linear regression, revealed the relationship depicted in Figure 2A. The equation of the regression line was $Y = -0.01X + 0.928$. $R = 0.85$, $p < 0.0001$. The negative slope indicated that older patients did not preserve their rates of contraction after I/R as well as younger patients. For diastolic function, remaining −dF/dt after I/R (fraction of baseline) for each trabeculae versus age using linear regression revealed the relationship depicted in Figure 2B. The equation of the

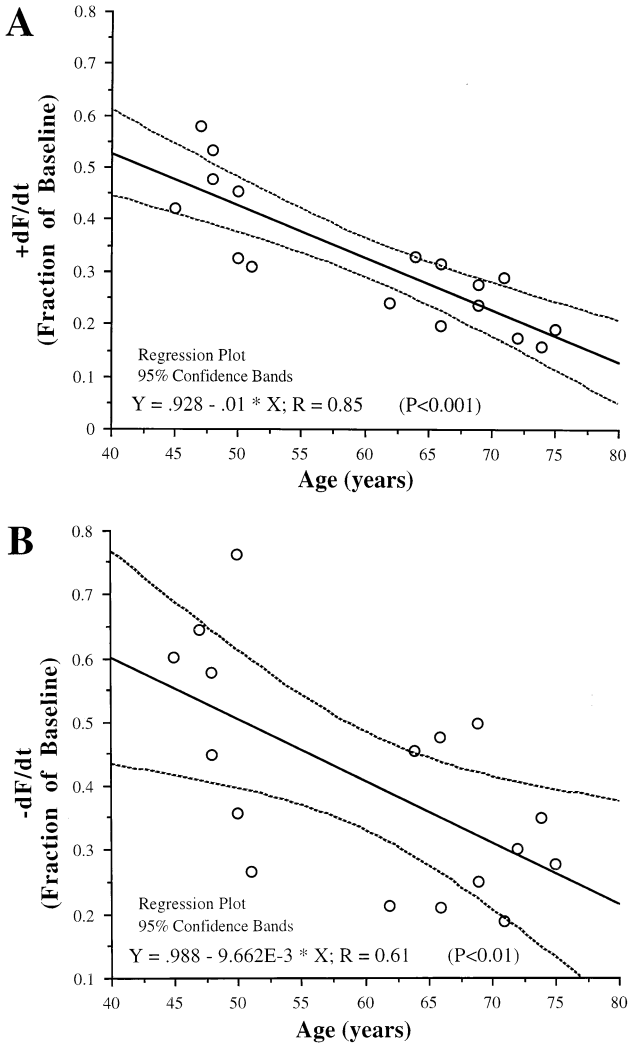


Figure 2. A) Maximum rate of systolic contraction after simulated ischemia reperfusion injury (as measured by the ratio of +dF/dt before and after I/R) was better preserved in the younger versus older patients. **B**) Maximum rate of diastolic relaxation or compliance after simulated ischemia reperfusion injury (as measured by the ratio of −dF/dt before and after I/R) was better preserved in the younger versus the older patients. Dotted lines depict the 95% confidence limits.

regression line is Y = −0.0096X + 0.988. R = 0.61, $p < 0.01$. Again, the negative slope revealed that older patients did not preserve their rates of relaxation after I/R as well as younger patients. The time constant of force decay (tau) was calculated before and after I/R to further define the diastolic phase of the myocardial cycle. The time constant of force decay was increased, indicating prolonged relaxation, in both junior and senior myocardium (17.7 ± 2.9 vs. 21.7 ± 2.7 ms and 14.7 ± 1.2 vs. 18.8 ± 1.2 ms respectively; both $p < 0.05$ vs. tau pre-I/R). Tau pre-I/R did not differ between junior and senior myocardium ($p > 0.05$) (Table 3).

Ca^{2+}-challenged Trabeculae. To determine if Ca^{2+} sensitivity differed between junior and senior myocardium, inotropic response was measured in terms of increase over baseline-developed force. Junior and senior myocardium did not differ in terms of Ca^{2+} sensitivity (Fig. 3A). Systolic function (+dF/dt) after Ca^{2+} bolus (expressed as increase over baseline) for each trabeculae versus age revealed the relationship depicted in Figure 3B. The equation of the linear regression line is Y = −0.022X + 1.573. R = 0.85, $p < 0.0004$. This regression revealed that younger patients can increase their maximum rates of systolic contraction after Ca^{2+} bolus, in contrast to older patients, whose maximum systolic rates appear nearly fixed. Diastolic function (−dF/dt) after Ca^{2+} bolus for each trabeculae versus age revealed the relationship depicted in Figure 3C. The equation of the regression line is Y = −0.024X + 1.712, R = 0.84, $p < 0.0007$. Younger patients were capable of increasing their maximum rates of relaxation in contrast to older patients, whose relaxation rates were nearly constant. The time constant of force decay was decreased, indicating a shorter relaxation phase, in junior myocardium (15.2 ± 1.1 vs. 18.5 ± 2.1 ms; $p < 0.05$ vs. tau pre-Ca^{2+}), but not senior myocardium (15.8 ± 0.7 vs. 16.7 ± 0.9 ms; $p > 0.05$ vs. tau pre-Ca^{2+}). Tau pre-Ca^{2+} did not differ between junior and senior myocardium ($p > 0.05$) (Table 3). There were no changes in resting force (diastolic tension) before, during, or after Ca^{2+} infusion.

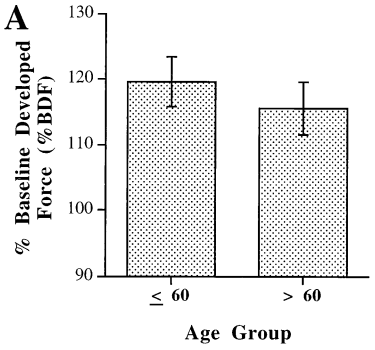
Sarcoplasmic reticulum Ca^{2+} handling proteins. Immunoblot against the sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA2a), its inhibitory subunit phospholamban, and calsequestrin versus increasing age is shown in Figure 4. Protein levels of SERCA2a, pentameric phospholamban, and monomeric phospholamban were decreased ($p < 0.003$, $p < 0.003$, and $p < 0.03$, respectively) in senescent myocardium as assessed by linear regression (Figure 5). Protein levels of calsequestrin were unaffected by age when analyzed by linear regression ($p > 0.05$). Division of the patients into junior and senior cohorts revealed that SERCA2a and pentameric phospholamban were decreased ($p < 0.001$, and $p < 0.002$, respectively) in senior versus junior myocardium (Fig. 6, A and B). Calsequestrin and phospholamban were unaffected by age (both $p > 0.05$, and $p > 0.05$) (Fig. 6, C and D). Calsequestrin has been determined to remain relatively constant over disease states (15), and thus normalization of SERCA2a to calsequestrin was performed to determine if the loss of SERCA2a simply represented a loss of all Ca^{2+} handling proteins. The ratio of SERCA2a to calsequestrin was decreased in senescent myocardium ($p < 0.007$) (Fig. 7A). As phospholamban (mo-

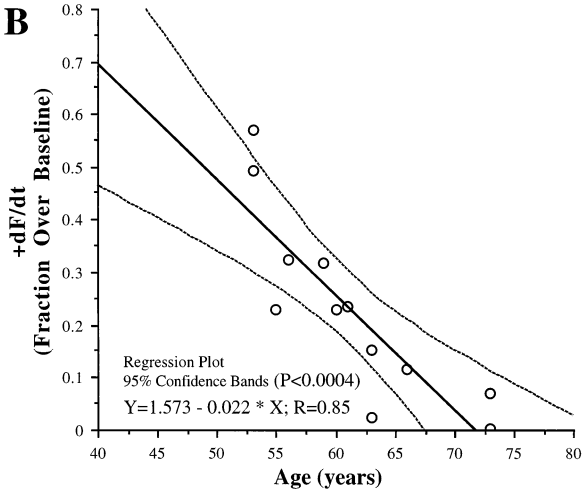
Table 3. Time Constant of Force Decay (Tau)

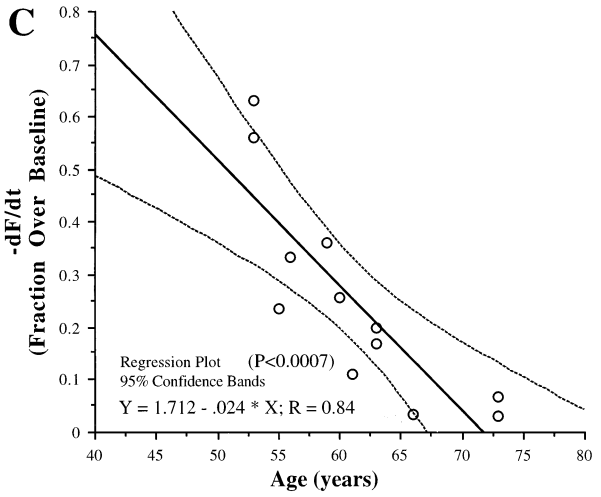
	Tau			
	Junior Myocardium		Senior Myocardium	
Pre I/R	17.7 ± 2.9	n = 7 patients	14.7 ± 1.2	n = 10 patients
Post I/R	21.7 ± 2.7*		18.8 ± 1.2*	
Pre Ca^{2+}	18.5 ± 2.1	n = 6 patients	16.7 ± 0.9	n = 6 patients
Post Ca^{2+}	15.2 ± 1.1†		15.8 ± 0.7‡	

Values are mean ± SEM (ms). *$p < 0.05$ vs. pre-I/R; †$p < 0.05$ vs. pre-Ca^{2+}; ‡$p > 0.05$ vs. pre-Ca^{2+}.

Figure 3. **A**) Inotropic response to 3.0 mmol/L Ca^{2+} concentration. Percent baseline developed force (%BDF) versus age group (senior, >60 years; junior, ≤60 years) is depicted. Increase in developed force after exposure to exogenous Ca^{2+} did not differ between senior and junior myocardium ($p > 0.05$). **B**) Maximum rate of systolic contraction after Ca^{2+} bolus (as measured by the ratio of +dF/dt before and after Ca^{2+} bolus) was increased in the younger patients; however, older patients were not able to increase their rates of systolic contraction. **C**) Maximum rate of diastolic relaxation or compliance after Ca^{2+} bolus (as measured by the ratio of −dF/dt before and after Ca^{2+} bolus) was increased in the younger patients; however, older patients were not able to increase their rates of diastolic relaxation. Dotted lines depict the 95% confidence limits.







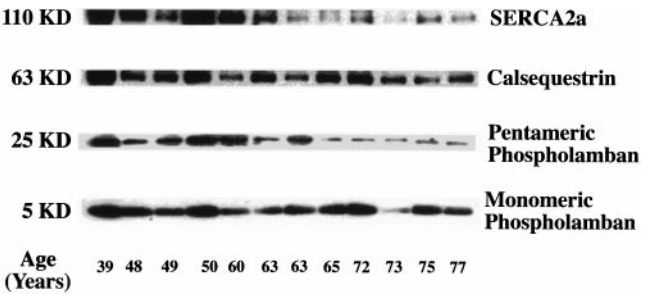


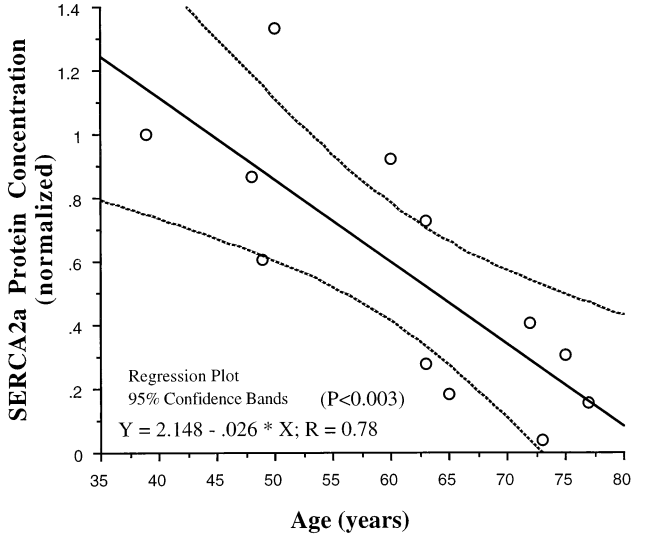
Figure 4. Immunoblot against SERC2a (110 kD), calsequestrin (63 kD), pentameric phospholamban (25 kD), and monomeric phospholamban (25 kD). Samples were each immunoprecipitated with anti-SERC2a, calsequestrin, or phospholamban selective antibodies, separated by SDS-PAGE on a 4–20% acrylamide gel, and then visualized by autoradiography. With increasing age, protein concentration of SERC2a and pentameric phospholamban decrease with age, while calsequestrin and monomeric phospholamban remain relatively constant.

nomeric) inhibits SERCA2a (34,35), perhaps SERCA2a activity is influenced by the ratio of these two proteins (15). Indeed, the ratio of SERCA2a to phospholamban was decreased in the senescent myocardium ($p < 0.035$) (Fig. 7B).

Discussion

The results of the present study demonstrate that: 1) prior to simulated ischemia, systolic and diastolic function in human myocardium are not related to age; 2) simulated ischemia reperfusion causes a decrease in the maximum rate of myocardial contraction and relaxation in all patients; 3) simulated ischemia reperfusion results in a relatively greater impairment of maximum positive and negative dF/dt in senescent human myocardium; 4) senescent myocardium is relatively unrespon-

Figure 5. Protein concentration of SERCA2a (normalized to youngest patient) assessed by Western blot. SERCA2a protein content decreased with increasing age by linear regression ($p < 0.003$). Dotted lines depict the 95% confidence limits.



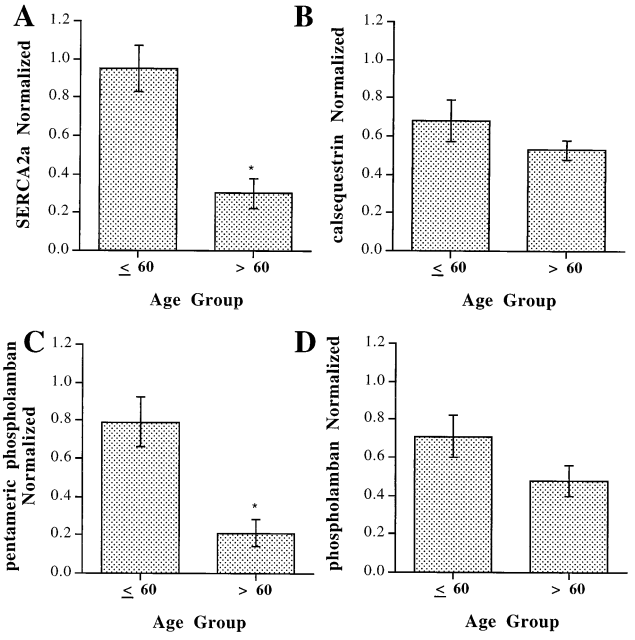


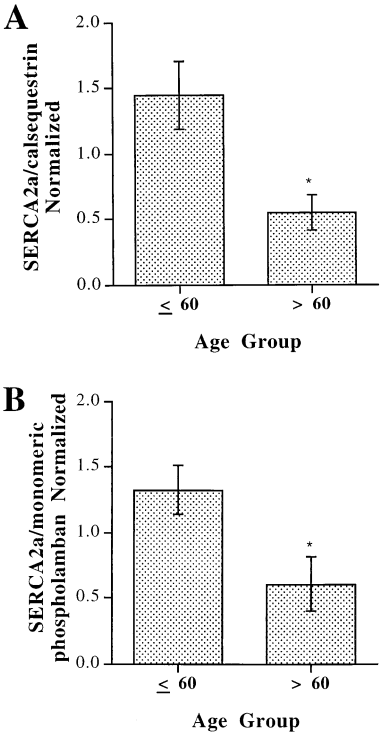
Figure 6. Protein concentration of: **A**) SERCA2a, **B**) calsequestrin, **C**) pentameric phospholamban, and **D**) monomeric phospholamban, between junior (≤60 years) and senior (>60 years). SERCA2a and pentameric phospholamban were both decreased in the senior myocardium (*$p > 0.05$ vs. junior age group). Calsequestrin and monomeric phospholamban did not differ between these two groups ($p > 0.05$).

sive to exogenous Ca^{2+}; 5) SERCA2a, pentameric phospholamban, and monomeric phospholamban protein contents are decreased in senescent human myocardium; and 6) the ratios of SERCA2a to calsequestrin or phospholamban were decreased in senescent human myocardium. These results suggest that the aging impairs Ca^{2+} handling in the human myocardium.

I/R decreases rates of contraction and relaxation. In the present study, no correlation between age and preischemic systolic or diastolic function was observed. This finding is in concert with a study demonstrating normal human myocardial Ca^{2+} uptake in both dilated cardiomyopathy and nonfailing hearts in an unpreturbed state (36). However, a relationship to age was demonstrated when these functional indices were measured after I/R and normalized them to their preischemic values. Systolic function is commonly clinically expressed as ejection fraction calculated from ventriculography, echocardiography, and nuclear medicine studies. Patient morbidity and survival have successfully been predicted based on these parameters (37–40). Diastolic dysfunction as a common cause of heart failure in humans has only recently been appreciated (41,42), and may represent a prevalent early cause of cardiac failure (2,3,43). Diastolic dysfunction has been studied in many species (2–8), and a relationship between age and impairment of relaxation has been suggested. Diastolic dysfunction has also been studied clinically in humans, utilizing Doppler echocardiography and contrast ventriculography (9–11). Perhaps not surprisingly, there was a significant correlation between diastolic relaxation time and age in 75 patients with coronary artery disease (10). In a subsequent study by the same authors, enrolling 85 patients with 56 healthy volunteers as controls, an age-related impairment of relaxation was again reported. However, this same study concluded that Doppler echocardiography may be imprecise when used to diagnose left ventricular diastolic function in patients older than 65 years (9). Ventriculography has recently been used to evaluate systolic and diastolic function in "senior" (mean age = 46 years) and "elderly" (mean age = 70 years) patients with aortic stenosis. The authors found no relationship to age for systolic function, but found a linear correlation between age and myocardial stiffness in their patients with aortic stenosis (11). In the present study, it is likely that the I/R stimulus served to magnify existing differences between patients, resulting in a measurable differential response to I/R in younger versus older patients.

Ischemia reperfusion results in energy substrate deprivation, cellular edema, Ca^{2+} overload, activation of autolytic enzymes, disruption of membranes, and mitochondrial dysfunction (12,22,26–28,44–47). All of the above likely contribute to postischemic Ca^{2+} dyshomeostasis. Indeed, elevated $[Ca^{2+}]_i$ has been related to postischemic mechanical dysfunction in both reversibly and irreversibly injured myocardium (48). The postischemic elevation of $[Ca^{2+}]_i$ is likely due to

Figure 7. A) SERCA2a normalized to calsequestrin protein levels revealed that the decrease in SERCA2a protein did not reflect a general loss of myocardial Ca^{2+} handling proteins with age (*$p < 0.05$ vs. junior age group), and **B**) SERCA2a normalized to monomeric phospholamban protein levels as an assessment of SERCA2a activity revealed that SERCA2a activity was decreased with age (*$p < 0.05$ vs. junior age group).



impaired $[Ca^{2+}]_i$ sequestration and/or extrusion (49,50). Our results suggest that these processes are impaired in senescent myocardium, and perhaps older patients might not be able to tolerate a clinical I/R stimulus as well as younger patients. Since the compromised diastolic function observed in older myocardium in the present study implies reduced myocardial compliance, older patients may require higher filling pressures to optimize cardiac output due to their impaired myocardial pressure-volume relationships.

Exogenous Ca^{2+} and senescent myocardium. In the present study, we found that younger patients were able to increase both their rates of contraction and relaxation after Ca^{2+} bolus. However, senescent patients possessed nearly fixed rates of contraction and relaxation when treated with Ca^{2+}. Precise cardiac myocyte $[Ca^{2+}]_i$ regulation is paramount for efficient systolic and diastolic function. The inotropic effects of extracellular Ca^{2+} are believed to be the result of a "Ca^{2+} trigger" of intracellular sarcoplasmic reticulum (SR) Ca^{2+} release during depolarization (Ca^{2+}-induced Ca^{2+} release) (51). Transient increases in $[Ca^{2+}]_i$ are required for excitation-contraction coupling in the heart (51,52). Calcium binding of troponin C results in a conformational change that enhances actin-myosin cross-linkage. Increased extracellular Ca^{2+} further enhances actin-myosin interaction, resulting in inotropy. Intracellular Ca^{2+} sequestration via the SERCA2a results in a fall in $[Ca^{2+}]_i$, causing dissociation of $[Ca^{2+}]_i$ from the contractile apparatus (12). Thus, the functional responses to increased extracellular Ca^{2+} or I/R observed in the present study in senescent myocardium may be due to impaired or downregulated Ca^{2+} uptake proteins (i.e., SERCA2a).

SERCA2a is decreased in senescent human myocardium. We observed a relative decrease of the myocardial Ca^{2+} handling protein SERCA2a, monomeric phospholamban, and pentameric phospholamban protein content in older myocardium when analyzed by linear regression. Calsequestrin levels remained relatively unchanged in relation to age. This manuscript represents the first report that SERCA2a is decreased in the aged human heart. Others have previously postulated that the extraordinary $[Ca^{2+}]_i$ load induced by I/R may be improperly handled due to downregulation of the SERCA protein in aged laboratory animal, and in ischemic or failing human myocardium (6,15,53–61). Decreased SERCA2a may result in an inability to sequester cytosolic Ca^{2+} after a depolarization, leading to diastolic dysfunction. We elected to quantify the differences in protein expression between the myocardial proteins SERCA2a, phospholamban, and calsequestrin between younger and older patients for the following reasons: 1) protein expression, rather than mRNA content, likely correlates more closely with function; 2) it is possible that mRNA transcription is independently downregulated with age; 3) studies have demonstrated that in disease, SERCA2a mRNA content and SERCA2a protein content may not always change together or to the same magnitude (62,63); 4) SERCA2a is the predominant isoform expressed in adult myocardium (56); and 5) SERCA2a protein content has been successfully used to differentiate normal from dilated cardiomyopathic human heart (15). Cardiac SERCA2a mRNA was decreased in human myocardium from patients undergoing heart transplant when compared with organ donor heart (15,55,57).

SERCA2a mRNA is decreased in both senescent rat (6) and rat exposed to cardiac overload (54). SERCA2a activity in porcine myocardium was found to be downregulated after the I/R injury of cardiopulmonary bypass, and appeared better preserved using warm blood cardioplegia rather than crystalloid (61). Phospholamban (the inhibitory subunit of SERCA) mRNA expression also appears to be correlated with age in rat (neonatal vs. adult) (64) and heart failure (15). It is the nonphosphorylated monomeric phospholamban that inhibits SERCA2a activity (34,35,65). In the present study, the ratio of SERCA2a to monomeric phospholamban was decreased in senescent myocardium. This index may help estimate the function of SERCA2a in relation to the inhibitory effect of phospholamban. Calsequestrin, a Ca^{2+} binding protein located within the sarcoplasmic reticulum, accounts for the majority of the sarcoplasmic reticulum's Ca^{2+} storage capacity (66,67). In the present study, we found no alteration of calsequestrin protein content related to age. Others have also reported that calsequestrin content remains relatively constant between normal myocardium and dilated cardiomyopathic heart in humans (55,68,69). Due to its constancy over disease states, calsequestrin levels have been used to normalize human myocardial protein levels for purposes of comparison (15). Indeed, in the present study we report that the ratio of SERCA2a to calsequestrin was decreased in senescent human myocardium, which may reflect, in part, the etiology of our functional results. Therapeutic access to impaired SERCA2a function may be on the horizon. Investigators have successfully inserted the gene coding for SERCA2a into rat myocytes via an adenoviral vector. Not only was the SERCA2a content increased after transfection, it enhanced myocyte relaxation and decreased resting $[Ca^{2+}]_i$ levels (70). Similarly, transgenic mice overexpressing a rat SERCA2 transgene were demonstrated to have increased SERCA2a mRNA and protein and accelerated $[Ca^{2+}]_i$ decline and myocyte relengthening (71).

Our computation of T (tau), the time constant of force decay, confirmed our results using $-dF/dt$ to assess diastolic performance. Simulated I/R resulted in prolongation of relaxation in both younger and older myocardium as assessed by $-dF/dt$ or tau. Furthermore, Ca^{2+} challenge decreased relaxation time in younger but not older myocardium. We chose the monoexponential model to fit the force decay tracings because of its excellent fit ($R > 0.98$) to our relaxation data. Others have proposed more complex mathematical models using two-sequential exponential (72) or the logistic model (73) in order to evaluate the isovolumic relaxation pressure time curve.

Error Discussion. This study should be interpreted with several important caveats. First, the use of human atrial tissue as a representative surrogate for myocardial Ca^{2+} handling proteins may differ from results obtained using ventricular tissue. Indeed, there may be considerable subcellular differences between atrial and ventricular myocardium with respect

to electrophysical processes and even differences between the stoichiometry of SERCA2a to phospholamban. We chose atrial tissue because of access. Atrial tissue appears relatively free of ischemic, restrictive, or myopathic disease, in contrast to ventricular tissue. We have previously studied human ventricular muscle derived from cardiac transplant recipients; but were then obligated to concede that we were studying diseased muscle. Using atrial tissue avoids drawing conclusions about the response of healthy human myocardium using explanted cardiomyopathic hearts on high-dose inotropic therapy. We have previously demonstrated that human ventricular tissue can be functionally preconditioned (22) and that the protection is qualitatively similar to atrial preconditioning (23,24). Second, although we have demonstrated in this study that there appeared to be a differential response to I/R and Ca^{2+} handling between younger and older patients, these differences could have resulted from the presence of concurrent long-term illnesses, although we found no age-related differences in concurrent diseases or treatments. Furthermore, hypoxia (simulated ischemia) was used as a surrogate for ischemia (lack of blood flow) in the present study because the atrial trabeculae is not perfused but rather crystalloid superfused during the experiment. Whether the effects reported by this study on rates of myocardial contraction and relaxation are limited in relation to hypoxia versus ischemia remains to be determined.

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