

# Overexpression of Catalase Targeted to Mitochondria Attenuates Murine Cardiac Aging

Dao-Fu Dai, MD; Luis F. Santana, PhD; Marc Vermulst, PhD; Daniela M. Tomazela, PhD; Mary J. Emond, PhD; Michael J. MacCoss, PhD; Katherine Gollahon, PhD; George M. Martin, MD; Lawrence A. Loeb, MD, PhD; Warren C. Ladiges, DVM; Peter S. Rabinovitch, MD, PhD

**Background**—Age is a major risk for cardiovascular diseases. Although mitochondrial reactive oxygen species have been proposed as one of the causes of aging, their role in cardiac aging remains unclear. We have previously shown that overexpression of catalase targeted to mitochondria (mCAT) prolongs murine median lifespan by 17% to 21%.

**Methods and Results**—We used echocardiography to study cardiac function in aging cohorts of wild-type and mCAT mice. Changes found in wild-type mice recapitulate human aging: age-dependent increases in left ventricular mass index and left atrial dimension, worsening of the myocardial performance index, and a decline in diastolic function. Cardiac aging in mice is accompanied by accumulation of mitochondrial protein oxidation, increased mitochondrial DNA mutations and deletions and mitochondrial biogenesis, increased ventricular fibrosis, enlarged myocardial fiber size, decreased cardiac SERCA2 protein, and activation of the calcineurin–nuclear factor of activated T-cell pathway. All of these age-related changes were significantly attenuated in mCAT mice. Analysis of survival of 130 mice demonstrated that echocardiographic cardiac aging risk scores were significant predictors of mortality. The estimated attributable risk to mortality for these 2 parameters was 55%.

**Conclusions**—This study shows that cardiac aging in the mouse closely recapitulates human aging and demonstrates the critical role of mitochondrial reactive oxygen species in cardiac aging and the impact of cardiac aging on survival. These findings also support the potential application of mitochondrial antioxidants in reactive oxygen species–related cardiovascular diseases. (*Circulation*. 2009;119:2789-2797.)

**Key Words:** aging ■ diastole ■ mitochondria ■ oxidant stress ■ survival

Cardiovascular diseases are highly prevalent in the geriatric population. Age is a major risk factor for cardiovascular disease, at least in part because it prolongs exposure to hypertension, diabetes mellitus, hypercholesterolemia, and other cardiovascular risks. However, intrinsic cardiac aging, the slowly progressive structural changes and functional declines with age, also makes the heart more susceptible to stress and contributes to increased cardiovascular mortality and morbidity in the elderly.

## Clinical Perspective on p 2797

Epidemiological data from the Framingham Heart Study and the Baltimore Longitudinal Study on Aging showed that in healthy populations there is an age-dependent increase in the prevalence of echocardiographic left ventricular (LV) hypertrophy, a decline in diastolic function, relatively preserved systolic function (ejection fraction) at rest, but a decline in exercise capacity (maximal ejection

fraction) and an increased prevalence of atrial fibrillation.<sup>1</sup> LV hypertrophy is well known to increase the risk of coronary heart disease, stroke, and sudden death. Diastolic heart failure, heart failure with diastolic dysfunction but preserved systolic function, plays a major role in congestive heart failure and exercise intolerance in the elderly population. Given demographic projections, heart failure is likely to become the major cause of hospital admissions and mortality in North America.<sup>2</sup>

According to the mitochondrial variant of the free radical theory of aging, reactive oxygen species (ROS) produced mainly in the mitochondria attack mitochondrial constituents, causing mitochondrial DNA (mtDNA) damage and mitochondrial dysfunction, leading to further production of ROS, increases in oxidative damage to lipids and proteins, and declines in cellular and organ function that contribute to death.<sup>3</sup> We have recently shown that although both point mutations and deletions in mtDNA

Received April 22, 2008; accepted March 13, 2009.

From the Departments of Pathology (D.-F.D., M.V., K.G., G.M.M., L.A.L., P.S.R.), Physiology and Biophysics (L.F.S.), Genome Sciences (D.M.T., M.J.M.), Biostatistics (M.J.E.), and Comparative Medicine (W.C.L.), University of Washington, Seattle.

The online-only Data Supplement can be found with this article at <http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.108.822403/DC1>.

Correspondence to Peter S. Rabinovitch, MD, PhD, Department of Pathology, University of Washington, 1959 Pacific Ave NE, HSB-K081, Seattle, WA 98195. E-mail [petersr@u.washington.edu](mailto:petersr@u.washington.edu)

© 2009 American Heart Association, Inc.

*Circulation* is available at <http://circ.ahajournals.org>

DOI: 10.1161/CIRCULATIONAHA.108.822403

accumulate with aging, mtDNA deletions are likely to drive the aging process.<sup>4</sup> As a vital organ rich in mitochondria and high in oxygen use, the heart is especially prone to oxidative damage. Together with the fact that cardiovascular diseases are the leading cause of death in humans, we hypothesized that in the absence of other cardiovascular risks, cardiac aging is a predictor of mortality.

In this report, we have made use of a transgenic mouse model in which catalase is overexpressed and targeted to mitochondria (mCAT) to clearly define cardiac aging phenotypes in a murine model of aging and investigate their plausible molecular mechanisms, to investigate the impact of reductions in mitochondrial ROS on cardiac aging, and to investigate the impact of cardiac aging on all-cause mortality.

## Methods

See the online-only Data Supplement for additional details.

### Animal Longevity Cohort and Echocardiography

C57Bl6 mice in the longevity cohort were maintained as described.<sup>5</sup> This study included a cross-sectional sample of 170 mice from this cohort, with 20 to 30 mice in each group. Mice were not subjected to any invasive experimentation other than echocardiography. Echocardiography was performed with an Acuson CV-70 (Siemens Medical Systems, Malvern, Pa) using standard imaging planes: M-mode, conventional, and tissue Doppler imaging. All protocols were approved by the University of Washington Institutional Animal Care and Use Committee.

### Biochemical and Molecular Analysis

Gene expression, mtDNA copy number, and mutation frequency assays were performed with quantitative polymerase chain reaction. Mitochondrial protein carbonyl and calcineurin activity assays were determined with commercial kits. Measurement of cardiac angiotensins was performed with a tandem quadrupole mass spectrometry coupled to high-performance liquid chromatography. For study of calcium transients, cardiomyocytes were isolated by an enzymatic method, loaded with Fluo-4, and electrically stimulated at 1 Hz. Calcium transient fluorescence was collected with an IonOptix system (IonOptix, Milton, Mass).<sup>6</sup>

### Statistics and Survival Analysis

Continuous variables with normal distribution are presented as mean±SEM. Those significantly skewed are presented as box plots (median, 25%, 75%) with an x within the box to indicate the mean. Standard *t* tests, ANOVA, and linear regression were applied as appropriate to detect the statistical differences between genotypes or age groups. Probability values are shown uncorrected for multiple testing. To be more conservative, a Bonferroni correction ( $n=2$ ) can be applied to *t* test comparisons between young wild-type (WT) and old WT and between old WT and old mCAT groups, after which  $P<0.025$  would be considered significant. To investigate the effect of cardiac aging on survival, we chose variables representing structure (LV mass index [LVMI]) and function (myocardial performance index [MPI]) a priori to create a cardiac aging risk score. Univariate Cox regression was used to verify a strong association between these variables and mortality. We then included both age-adjusted functional decline (MPI) and structural changes (LVMI) in a multivariate analysis to generate the semiparametric maximum likelihood estimate of the best-fitting linear combination of MPI and LVMI score in predicting subsequent mortality ( $\beta_2$  MPI+ $\beta_3$  LVMI in model 1 in the Table). We define this linear combination as the cardiac aging risk score; Kaplan–Meier curves stratified on the risk score tertiles are used to illustrate the differences in risk as the score increases. Log-rank test probability values serve

**Table. Cox Regression Models Predicting Mortality**

| Predictors               | Hazard Ratio | <i>P</i> | 95% CI    |
|--------------------------|--------------|----------|-----------|
| Univariate Cox model     |              |          |           |
| Age, mo                  | 1.18         | <0.001   | 1.11–1.26 |
| Men vs women             | 1.20         | 0.46     | 0.74–1.94 |
| MPI* (0.1 unit)          | 1.35         | <0.001   | 1.16–1.58 |
| LVMI,† mg/g              | 1.44         | 0.002    | 1.15–1.80 |
| Multivariate Cox model 1 |              |          |           |
| Age, mo                  | 1.15         | <0.001   | 1.07–1.23 |
| MPI* (0.1 unit)          | 1.26         | 0.005    | 1.07–1.48 |
| LVMI,† mg/g              | 1.12         | 0.41     | 0.86–1.45 |
| Multivariate Cox model 2 |              |          |           |
| Age, mo                  | 1.16         | <0.001   | 1.09–1.24 |
| Cardiac aging score T2‡  | 1.80         | 0.067    | 0.96–3.39 |
| Cardiac aging score T3‡  | 2.88         | 0.003    | 1.43–5.82 |

\*The hazard ratio rises by a factor of 1.35 for every 0.1-unit difference in MPI.

†Hazard ratios associated with LVMI are calculated as for MPI.

‡Cardiac aging risk score= $\beta_2$  MPI+ $\beta_3$  LVMI, calculated from the multivariate Cox model 1, and presented as the middle (T2) or high (T3) vs lower (T1) tertile.

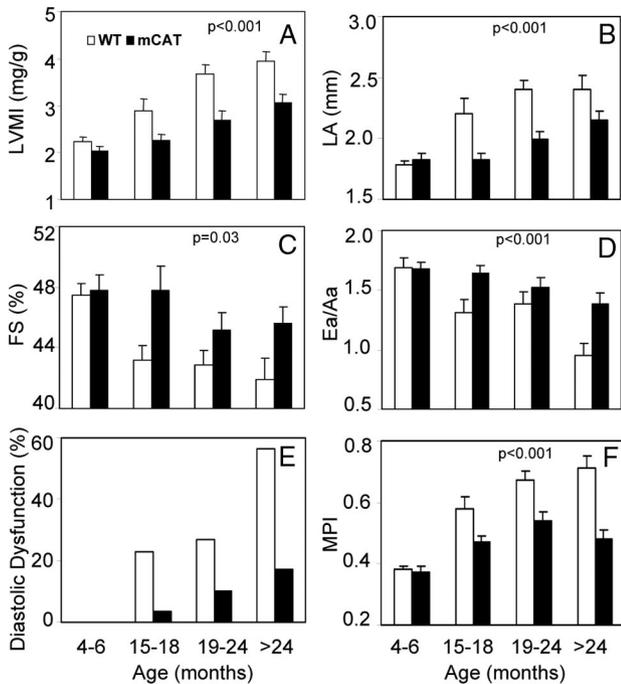
as descriptive statistics for the strength of the differences in survival between tertiles. To approximate the attributable risk fraction of cardiac aging on mortality, we calculated the difference between the incidence of the all-cause mortality rate of mice with risk scores in the highest tertile and the incidence in those with scores in the middle and lowest tertiles.<sup>7</sup>

## Results

### MCAT Attenuates the Cardiac Aging Phenotype

There were no differences in body weight or food consumption between mCAT and WT mice.<sup>5</sup> There were no significant differences with age or genotype in blood pressure, heart rate, pulse pressure, fasting plasma glucose, or total cholesterol in the mouse longevity cohort (Table I of the online-only Data Supplement). This is consistent with a low incidence of conventional cardiovascular risks in these C57Bl6 mice.<sup>8</sup>

Echocardiography was performed in 90 mCAT and 80 WT littermate mice across a wide range of ages in a mouse longevity cohort. Despite the absence of cardiovascular risk factors, we found significant age-dependent linear trends for all parameters ( $P<0.001$  for all except fractional shortening,  $P=0.005$ ). LVMI (Figure 1A) was 76% higher in the oldest group compared with the young adult group. Left atrial dimension was significantly increased by 35% with age (Figure 1B). Systolic function measured by fractional shortening showed a 12% decline from young adult to the oldest group (Figure 1C). Tissue Doppler imaging revealed an age-dependent decline in Ea/Aa, from  $1.69\pm 0.3$  in young adult to  $0.95\pm 0.4$  in the oldest (Figure 1D). The prevalence of diastolic dysfunction, defined as Ea/Aa  $<1$ ,<sup>9</sup> was dramatically increased to 55% in the oldest age group (Figure 1E). The MPI was significantly increased (worsened) with age (Figure 1F), consistent with the age-related declines in systolic and diastolic function. These abnormalities closely mimic the age-related echo-



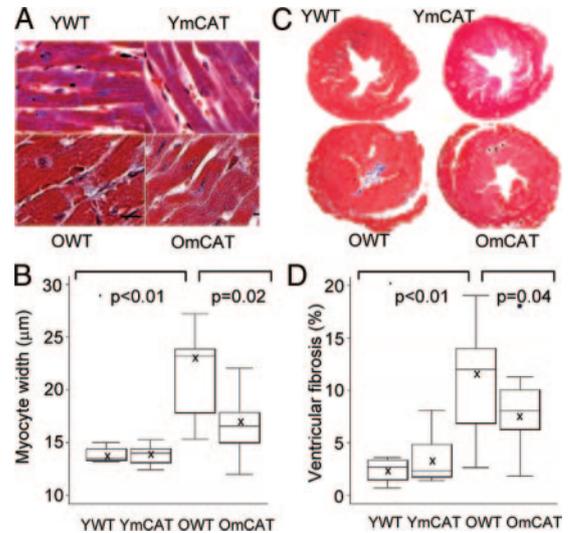
**Figure 1.** Echocardiography of mice from different age groups in the longevity cohort. Age-related changes in (A) LVMI (mg/g body weight); (B) left atrial (LA) dimension (mm); (C) fractional shortening (FS; %); (D) Ea/Aa measured by tissue Doppler imaging of the mitral annulus; (E) proportion of mice with diastolic dysfunction, defined as Ea/Aa <1; and (F) MPI. There was a significant linear increase across ages for all outcomes ( $P < 0.001$  for all except for FS,  $P = 0.005$ , fit linearly to a continuous age function). mCAT mice had a significantly lower rate of progression with age in all of the above measurements vs WT ( $P < 0.001$  for all except for FS,  $P = 0.03$ , for an interaction between age-slope and genotype). Ninety mCAT and 80 littermate WT mice were examined, with  $\approx 20$  mice in each age and genotype group.

cardiographic changes previously reported in healthy human populations.<sup>1</sup>

The age-related changes in all of the above echocardiographic findings were significantly delayed and attenuated in age-matched mCAT littermates. In the oldest group, mCAT significantly reduced the age-related increase in LVMI by 39%, the percent of mice with diastolic dysfunction by 69%, the MPI by 67%, and the age-related decrease in Ea/Aa by 59% (Figure 1). The genotype difference in the linear rate of change with age (the genotype-by-slope interaction) was highly significant for all outcomes ( $P < 0.001$  for all except fractional shortening,  $P = 0.03$ ).

To confirm the protective effects of mCAT on cardiac aging, we performed a cross-sectional study of 42 additional old WT and mCAT C57BL6 mice (age, 27 to 29 months) in which cardiac tissue harvest was also performed within 24 hours after echocardiography. The protective effects of mCAT on echocardiographic parameters were fully reproduced in this independent data set (online-only Data Supplement Table II).

Histological analysis of tissue showed that compared with young WT (4 months of age), old WT mice had significantly larger myocardial fiber width (Figure 2A and 2B) and a higher cross-sectional area with fibrosis (Figure

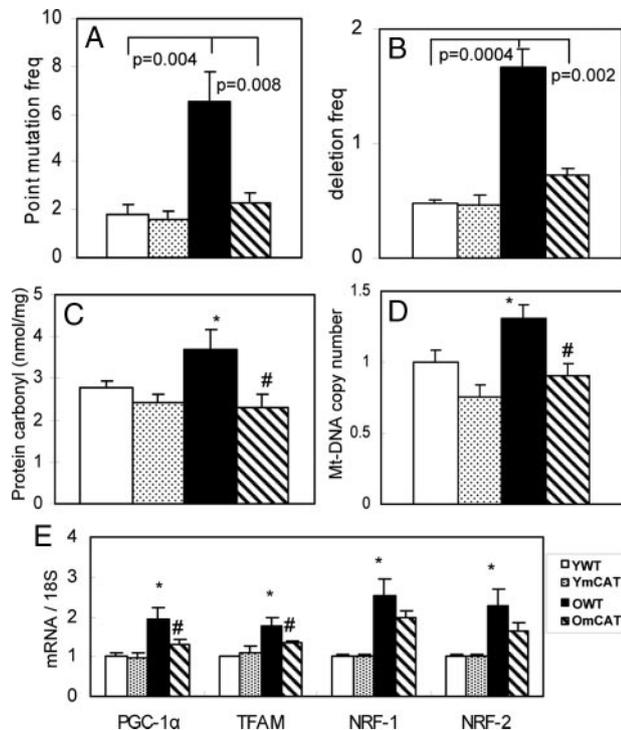


**Figure 2.** Cross-sectional study of cardiac pathology. A, Old WT (OWT) mice had larger myocardial fiber width than old mCAT (OmCAT) mice (trichrome stain,  $\times 400$ ; scale bar =  $10 \mu\text{m}$ ). B, Quantitative analysis showed a significant increase in myocardial fiber width in aged heart, which was significantly attenuated in OmCAT mice. C, OWT mice had more fibrosis (blue; trichrome,  $\times 20$ ) than OmCAT mice, as shown by quantitative image analysis of the percent fibrotic area in aged hearts, which was significantly attenuated in OmCAT mice (D). YWT indicates young WT; YmCAT, young mCAT.

2C and 2D). Both cardiomyocyte hypertrophy and ventricular fibrosis were significantly attenuated in age-matched old mCAT mice. There was no genotype difference in young adults. Moreover, myocardial fiber width by image analysis was highly correlated with LVMI determined by echocardiography ( $R^2 = 0.73$ ,  $P < 0.01$ ; online-only Data Supplement Figure I).

### MCAT Protects Against Mitochondrial Oxidative Damage and DNA Mutations in the Aged Heart

As an extension to our earlier findings that mCAT protected cardiac mitochondrial aconitase enzymes from oxidative damage,<sup>7</sup> we further found that protein carbonyls, an indicator of oxidative damage, increased significantly in cardiac mitochondria with age and was significantly reduced in old mCAT mice (Figure 3C). Aging is associated with increased mtDNA mutations and deletions,<sup>4,10</sup> as well as extensive morphological damage in mitochondria shown by electron micrographs (online-only Data Supplement Figure II). Using the newly developed random mutation capture assay, we found that cardiac mtDNA point mutation and deletion frequencies both significantly increased by  $\approx 3$ -fold with aging.<sup>11</sup> The mCAT genotype significantly reduced DNA point mutation and deletion frequencies to levels more comparable to those of young animals (Figure 3A and 3B). We found that mtDNA copy number increased in the aged heart (Figure 3D) and was accompanied by upregulation of transcription factors involved in mitochondrial biogenesis (Figure 3E), consistent with reports that oxidative stress induces mitochondrial biogenesis.<sup>12</sup> Most of these changes were significantly reduced in old mCAT mice, including an attenuated increase in mtDNA copy number and reduced

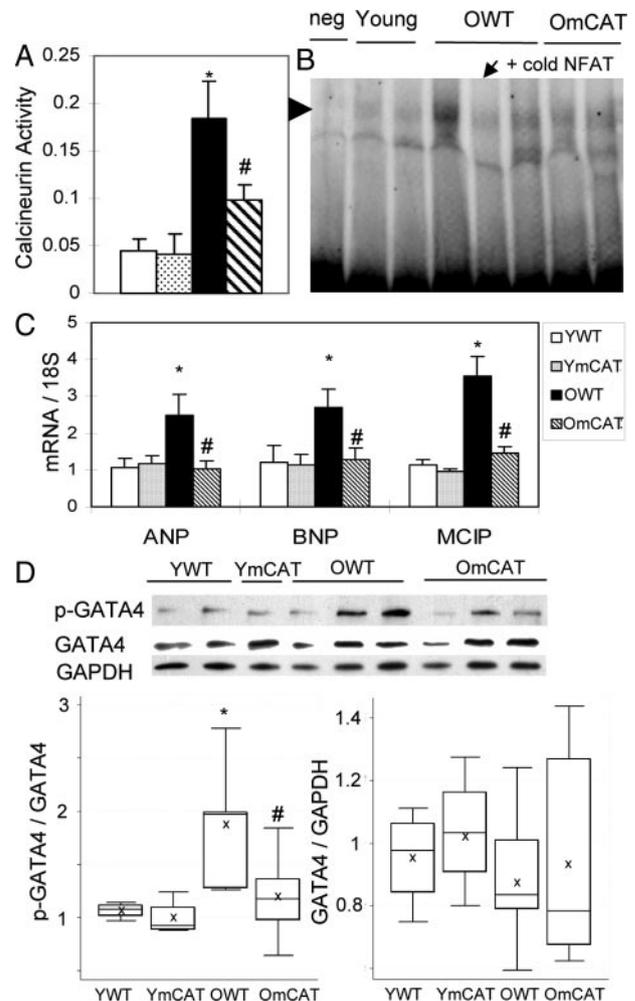


**Figure 3.** mtDNA mutations, oxidative damage, and biogenesis in aged heart and the protective effect of mCAT. mtDNA point mutation (A) and deletion frequencies (B; both per million) increased significantly with age and were better preserved in old mCAT (OmCAT) mouse hearts. C, Mitochondrial protein carbonyls (nmol/mg) increased significantly in old age, whereas OmCAT mice were better protected from oxidative damage to mitochondrial proteins. D, mtDNA copy number (normalized to young WT [YWT]) increased significantly with age, and OmCAT mice had significantly lower mtDNA copy number. E, Genes involved in mitochondrial biogenesis, PGC-1 $\alpha$ , TFAM, NRF-1, and NRF-2, were upregulated in aged heart, and changes in PGC-1 $\alpha$  and TFAM were attenuated in OmCAT mice (\* $P$ <0.025, YWT vs old WT [OWT]; # $P$ <0.025, OWT vs mCAT;  $n$ =9 to 12 each group; old, 26 to 28 months old; young, 4 to 5 months old).

induction of peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 $\alpha$ ) and transcription factor A, mitochondrial (TFAM) but not nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) mRNA (Figure 3D and 3E).

### Plausible Mechanisms of Cardiac Hypertrophy in the Aged Heart and the Effect of mCAT

To investigate the potential mechanisms of aging-associated cardiac hypertrophy, we measured the activation of calcineurin–nuclear factor of activated T-cell (NFAT) and phospho-ERK1/2 pathways, which are known to mediate pathological and compensated hypertrophy, respectively.<sup>13</sup> Both the phosphorylation of ERK1/2 and the total ERK1/2 protein levels did not change significantly with age (data not shown). However, we found that the calcineurin–NFAT pathway was activated in cardiac aging. Activity of calcineurin, a phosphatase that activates NFAT by dephosphorylation, was found to increase significantly with age (Figure 4A). Downstream of calcineurin, NFAT3 activity was increased in aged heart as shown by stronger DNA binding activity (Figure 4B) and increased transcription of NFAT target



**Figure 4.** Activation of calcineurin–NFAT and GATA4 in age-dependent LV hypertrophy and the effect of mCAT. A, Calcineurin phosphatase activity (nmol phosphate/5  $\mu$ g cardiac protein) increased significantly in aged heart and was partially protected in old mCAT (OmCAT) hearts. B, Mobility shift assay showed that NFAT DNA binding activity increased in old WT (OWT) and the increase was less obvious in OmCAT hearts. Competitive DNA binding with excess unlabeled NFAT probe (arrow) using the leftmost OWT sample confirmed the specificity of the bands (arrowhead). C, The relative mRNA levels of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and modulatory calcineurin interacting protein-1 (MCIP), target genes of NFAT, increased significantly in OWT but not OmCAT hearts. D, Age-dependent increased phosphorylation of GATA-4 at Ser105 was reduced in OmCAT heart. \* $P$ <0.025, young WT vs OWT; # $P$ <0.025, OWT vs OmCAT.

genes, including modulatory calcineurin interacting protein-1 and atrial and brain natriuretic peptides (Figure 4C). Furthermore, we found that GATA4, a cofactor of NFAT3,<sup>14</sup> was activated in aged heart, as shown by increased phosphorylation of GATA4 at serine 105 (Figure 4D). The age-dependent activation of calcineurin–NFAT and GATA4 was significantly reduced in old mCAT hearts (Figure 4A through 4D).

### Plausible Mechanisms of Diastolic Dysfunction in the Aged Heart and Effect of mCAT

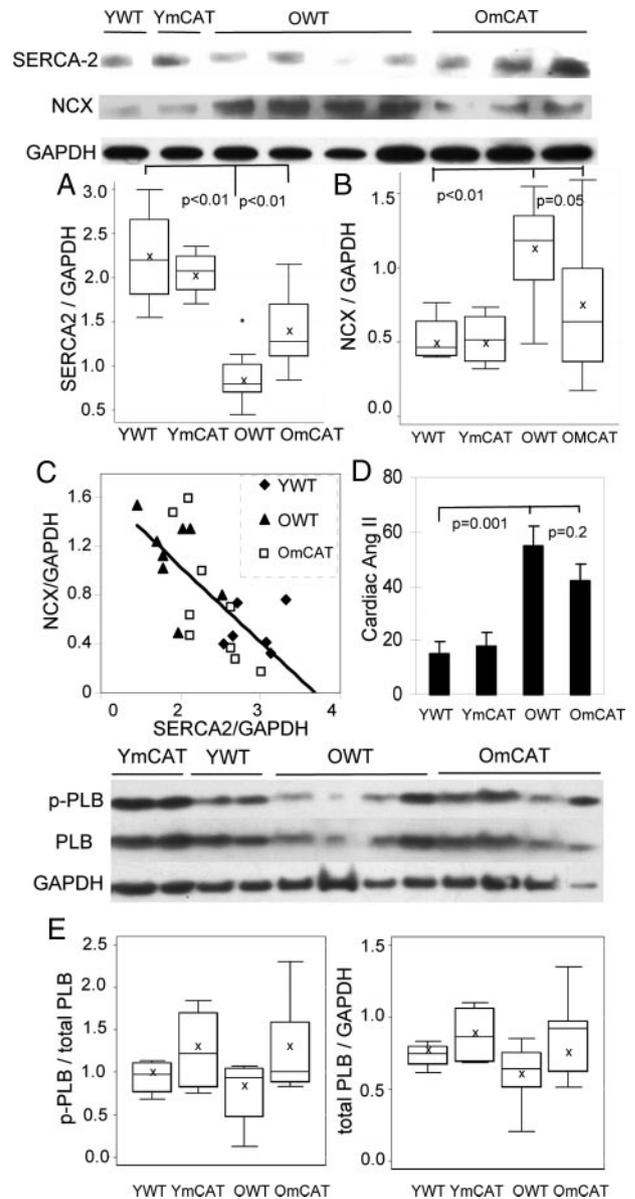
Several factors have been reported to cause diastolic dysfunction, including impaired myocardial relaxation resulting from

decreased calcium reuptake and increased myocardial stiffness.<sup>15</sup> Increased fibrosis in aged heart and amelioration of fibrosis in mCAT mice were described above.

Sarco(endo)plasmic reticulum Ca ATP-ase (SERCA2) and the sodium-calcium exchanger (NCX) are key proteins in calcium reuptake that assist in myocardial relaxation during the diastolic phase. They were examined by Western blots in aged heart. Quantitative analysis revealed that SERCA2 protein decreased by 52% in aged WT heart compared with young WT heart ( $P < 0.01$ ). SERCA2 was much better preserved in old mCAT heart compared with old WT littermates (Figure 5A). NCX protein increased by 103% in the aged WT heart compared with the young WT heart (Figure 5B). The increase was significantly less in old mCAT hearts compared with old WT hearts. Interestingly, we found an inverse correlation between the level of NCX and SERCA2 proteins (Figure 5C). This novel observation suggests that NCX may be upregulated in the aged heart to compensate for the decline in SERCA2. Levels of total and phosphorylated phospholamban, which inhibits SERCA2 activity in its dephosphorylated form, did not change significantly with age (Figure 5E).

To further investigate plausible mechanisms of diastolic dysfunction in the aged heart, we analyzed the relationships of myocardial fiber width, ventricular fibrosis, and SERCA2 protein with diastolic dysfunction. Univariate analysis showed that cardiomyocyte hypertrophy and ventricular fibrosis were significantly associated with the presence of diastolic dysfunction (Figure 6A and 6B). These findings were consistent with the fact that both ventricular hypertrophy and fibrosis would increase myocardial stiffness. The level of SERCA2 protein was dramatically decreased in mice with age-related diastolic dysfunction (Figure 6C). Multivariate regression modeling showed that SERCA2 was the strongest predictor of Ea/Aa, an indicator of diastolic function ( $P = 0.01$ ; online-only Data Supplement Table III).

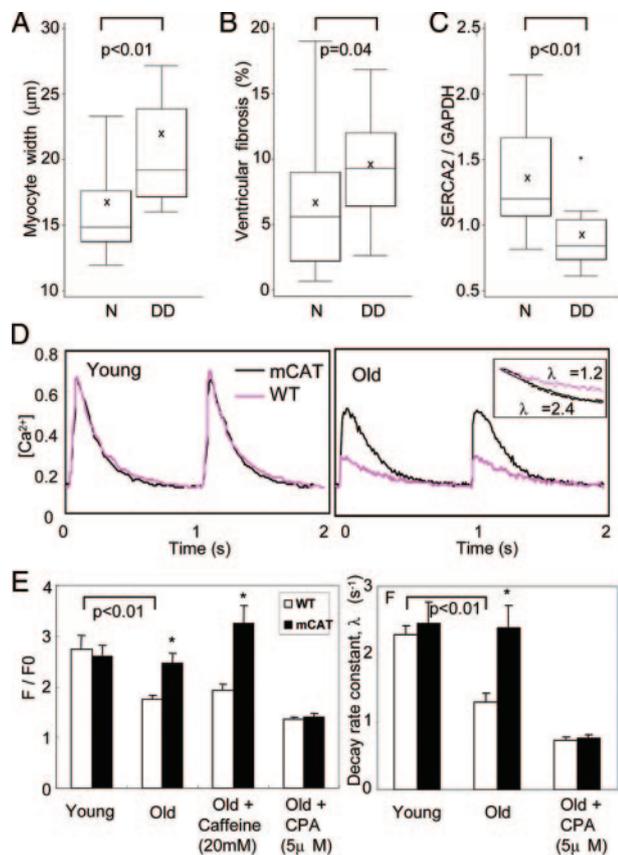
To further investigate the role of SERCA2 in aging, we examined  $[Ca^{2+}]_i$  transients in ventricular myocytes isolated from mouse hearts, loaded with the  $Ca^{2+}$  indicator dye Fluo-4, and paced at 1 Hz. Compared with young cardiomyocytes, old WT cardiomyocytes had significantly lower  $[Ca^{2+}]_i$  transient amplitude and slower rates of decay, whereas old mCAT cardiomyocytes had significantly preserved calcium transient amplitude and decay rates (Figure 6D and 6E). Stimulation with caffeine, which opens ryanodine receptors and unloads the sarcoplasmic reticulum calcium,<sup>5</sup> showed that old WT cardiomyocytes had significantly lower sarcoplasmic reticulum calcium loads. This is consistent with the lower  $[Ca^{2+}]_i$  transient amplitude observed in old WT cardiomyocytes. Inhibition of SERCA2 with cyclopiazonic acid (5  $\mu\text{mol/L}$ ) completely abolished the beneficial effects of mCAT on calcium transient amplitude and rate of decay (Figure 6E and 6F), suggesting that the protective effect of mCAT in aged cardiomyocytes is mediated by better preservation of SERCA2 activity. Overall, the decline of ventricular SERCA2 thus appeared to be the most important factor associated with age-dependent cardiac diastolic dysfunction, presumably by causing impaired myocardial relaxation.



**Figure 5.** Calcium handling proteins and cardiac angiotensin II. Western blots of calcium handling proteins were quantified relative to GAPDH. A, SERCA2 protein was significantly reduced in aged WT heart and better preserved in old mCAT (OmCAT) heart. B, NCX protein was significantly increased in aged heart and lower in OmCAT compared with old WT [OWT]. C, Linear regression demonstrates an inverse correlation between SERCA2 and NCX protein levels (all ages:  $\beta = -0.61$ ,  $R^2 = 0.49$ ,  $P < 0.01$ ; old mice only:  $\beta = -0.82$ ,  $R^2 = 0.52$ ,  $P = 0.001$ ). D, Cardiac Ang II concentration (fmol/mg total protein) measured by mass spectrometry was significantly increased with age in both WT and mCAT mice. E, Phosphorylated and total protein levels of phospholamban did not change significantly with age or mCAT expression. YWT indicates young WT; OmCAT, old mCAT.

**Increased Cardiac Angiotensin II in Aging**

Angiotensin II (Ang II) is known to induce cardiomyocyte hypertrophy and apoptosis, increase cardiac fibrosis, and impair cardiomyocyte relaxation,<sup>16</sup> compatible with all of the above aging-related changes. We therefore measured the cardiac Ang II octapeptide in young and old LV tissue using mass spectrometry. Cardiac Ang II concentrations

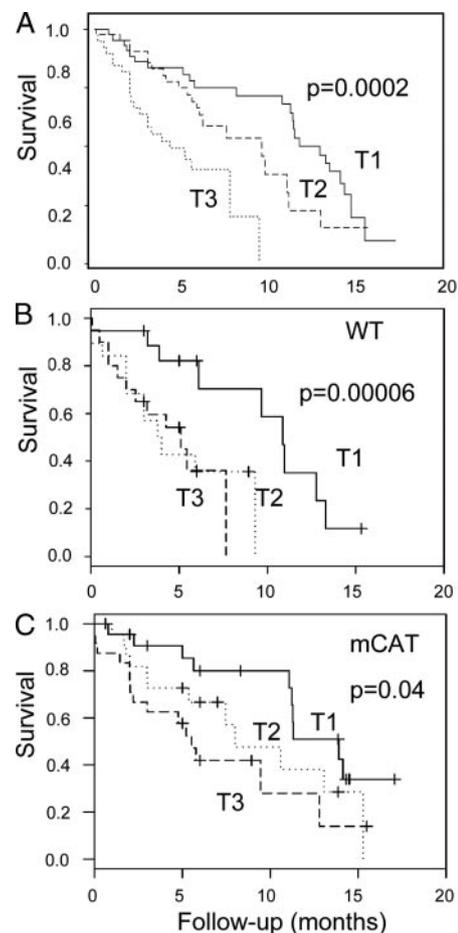


**Figure 6.** Plausible mechanisms of diastolic dysfunction in aged heart. A, Myocardial fiber width was significantly greater in old mice with diastolic dysfunction (DD) compared with those with normal diastolic function (N). B, Ventricular fibrosis (%) was higher in old mice with DD. C, SERCA2 protein was significantly decreased in mice with DD. D, Representative calcium transients of isolated cardiomyocytes from young and old mouse hearts loaded with Fluo-4 and paced at 1 Hz. Inset, old WT vs old mCAT decay rates. E, Quantitative analysis showed significant decreases in calcium transient amplitude (F/F<sub>0</sub>) and decay rate constant,  $\lambda$  (s<sup>-1</sup>), in old WT and protection in old mCAT cardiomyocytes. Sarcoplasmic reticulum Ca<sup>2+</sup> load was also significantly higher in old mCAT cardiomyocytes, as shown by mobilization of Ca<sup>2+</sup> by caffeine. The protective effect of mCAT was completely abolished by treating the old cardiomyocytes with cyclopiazonic acid (CPA), a SERCA2 inhibitor. \* $P < 0.025$ .

increased significantly with age (Figure 5D). mCAT did not appear to reduce the age-related increase in cardiac Ang II, suggesting that the mechanism of mCAT protection is downstream of Ang II.

### Cardiac Aging Phenotype as Predictors of Follow-Up Mortality

To investigate whether the cardiac aging phenotype determined by echocardiography is a predictor of follow-up mortality, we performed survival analysis of 130 old WT and mCAT mice that underwent echocardiography at midlife (age, 16 to 24 months) in the longevity cohort. Univariate Cox regression analyses verify that age, MPI, and LVMI were significant predictors of mortality (the Table). A multivariate Cox model was used to estimate the best linear combination of these variables ( $\beta_2$  MPI +  $\beta_3$  LVMI) to create



**Figure 7.** Kaplan-Meier analysis of follow-up mortality stratified by cardiac aging risk score tertiles. Cardiac aging risk score was calculated as a linear combination of MPI and LVMI obtained from the multivariate Cox model 1 ( $\beta_2$  MPI +  $\beta_3$  LVMI). Mice with the higher tertile of cardiac aging score (T3) had significantly shorter survival than those with the lower tertile (T1) when scores were analyzed for WT and mCAT mice together (A) or separately (B, WT; C, mCAT).

a cardiac aging risk score for each mouse based on these variables. Cox regression adjusted for age at the time of echocardiography (Cox model 2) verifies that this risk score provides a biologically meaningful measure of the risk of death; mice in the highest tertile (T3) had a significantly increased hazard of all-cause mortality, with a hazard ratio of 2.88 (95% confidence interval, 1.43, 5.82;  $P = 0.003$ ), compared with mice in the lowest tertile of cardiac aging score (T1) (the Table and Figure 7A). As evidence for our hypothesis, we found a marked difference in the distribution of cardiac aging risk scores between mCAT and WT mice: At midlife, the proportions of mCAT mice in T1, T2, and T3 were 91%, 48%, and 24% versus 9%, 52%, and 76% for WT ( $\chi^2 = 39.4$ ,  $P < 10^{-7}$ ). Kaplan-Meier analysis based on tertiles of cardiac risk scores calculated separately for WT and mCAT mice also stratified each of these genotypes into high- and low-mortality-risk groups (Figure 7B and 7C, respectively).

To study the relative contribution of cardiac aging to mortality, we estimated the incidence of all-cause mortality in mice that reached their natural end of life. The

incidence of mortality in the highest tertile of cardiac aging score (T3) versus those in the lower 2 tertiles of cardiac aging score was 16.2% and 7.3%, respectively. The attributable risk fraction indicated that 55% of mortality risk was attributable to the cardiac aging risk score. This is the first such analysis of cardiac functional parameters with aging that has been reported. Thus, cardiac aging is a significant predictor of mortality in mice despite the absence of hypertension, diabetes, or hypercholesterolemia, and the cardiac aging phenotype appears to contribute to approximately half of the mortality risk in the mouse longevity cohort.

### Discussion

Our results demonstrate that overexpression of mCAT protects mice from cardiac aging, providing direct evidence for the role of mitochondrial ROS in the aging of this vital organ. Several lines of evidence support this conclusion. In WT mice from the longevity cohort, we found age-dependent LV hypertrophy and a decline in cardiac performance (especially diastolic function), concomitant with the accumulation of oxidized mitochondrial proteins, mtDNA mutations, increased ventricular fibrosis, cardiomyocyte hypertrophy, a decline in SERCA2 protein, and activation of the calcineurin-NFAT pathway in the aged heart. These age-related alterations took place in the absence of significant cardiovascular risks such as diabetes, hypertension, or hypercholesterolemia, suggesting that these findings are primary changes of cardiac aging rather than secondary to other diseases. mCAT littermates were partially protected from all of the above age-related cardiac alterations, suggesting that these aging changes are related in substantial part to chronic ROS exposure and emphasizing the role of mitochondrial ROS in cardiac aging. Interestingly, we did not observe any additional benefit in protection against cardiac aging in mice with higher versus lower mCAT cardiac expression (online-only Data Supplement Table II), which is consistent with our previous observation that lifespan is not associated with the level of mCAT expression.<sup>7</sup> We have suggested that the location of the catalase, ie, targeting to mitochondria, is more important than the magnitude of catalase expression. Here, we show that mtDNA point mutations and deletions in the heart increased significantly with age but that the antioxidant function of mCAT protects cardiac mtDNA from these age-related mutations. These age-associated mtDNA point mutations had a spectrum similar to that observed in oxidative DNA damage.<sup>4</sup> Furthermore, because oxidative stress has been shown to induce mitochondrial biogenesis,<sup>12</sup> we also found that mtDNA copies increased in aged heart, with upregulation of transcription factors involved in mitochondrial biogenesis. These phenomena were reduced in old mCAT mice, suggesting that mitochondrial biogenesis in the aging heart is mediated by oxidative damage to mitochondria.

To investigate the signaling in age-associated cardiac hypertrophy, we examined 2 major pathways of cardiac hypertrophy, MEK1/2-ERK1/2 and calcineurin pathways, which are central regulators of compensated and pathological hypertrophy, respectively.<sup>13</sup> Although we did not find in-

creased expression or phosphorylation of ERK1/2 in aging, we found that the calcineurin-NFAT pathway was significantly activated in the aged heart. Calcineurin activity was increased by  $\approx$ 4-fold in the aged heart (Figure 4A). This phosphatase activates the transcription factor NFAT. Activated NFAT translocates into nucleus, where it interacts with the transcription factor (GATA4) to initiate transcription of hypertrophic fetal genes such as atrial and brain natriuretic peptides. Electrophoretic mobility shift assay showed that NFAT DNA binding activity is increased in aged heart (Figure 4B), consistent with increased mRNA levels of atrial and brain natriuretic peptides and modulatory calcineurin interacting protein-1 (Figure 4C). Furthermore, we found an age-dependent increase in GATA4 phosphorylation at Ser105 (Figure 4D), which has been reported to enhance its activity of DNA binding and transcription activation.<sup>14</sup> The activation of the calcineurin-NFAT pathway in age-associated cardiac hypertrophy was partially protected by mCAT, suggesting an interaction between ROS and hypertrophic signaling mediators. Although myocardial ROS has been implicated in the generation of cardiac hypertrophy and dysfunction, as shown by the antihypertrophic effect of several antioxidants,<sup>17,18</sup> the *in vivo* evidence for direct links between hypertrophic mediators and ROS has been scant.<sup>19</sup> ROS might cause oxidative and nitrative modification of signaling proteins and may thereby modulate signal transduction and transcriptional and translational regulation.<sup>20</sup> However, further studies are needed to elucidate the exact mechanism of these interactions.

Upstream of the above signaling pathways, Ang II induces cardiomyocyte hypertrophy and apoptosis, increases cardiac fibrosis, and impairs cardiomyocyte relaxation,<sup>16</sup> all consistent with our observed age-related changes. Inserra et al<sup>21</sup> proposed that Ang II is a crucial mediator of cardiovascular aging. They recently showed that long-term inhibition of Ang II reduces cardiac pathology and prolongs rat survival.<sup>22</sup> Consistent with this, we found that cardiac Ang II concentrations increased significantly with age. Cardiac Ang II levels in old mCAT mice were similar to those in old WT mice despite mCAT protection against cardiac aging, suggesting that mCAT acts downstream of Ang II. Ang II is known to promote superoxide generation through NADPH oxidase, to uncouple endothelial nitric oxide synthase, and to stimulate mitochondrial H<sub>2</sub>O<sub>2</sub> production in vascular endothelial cells *in vitro*.<sup>23</sup> Although blocking Ang II signaling provides survival benefit in patients with heart disease, the efficacy of such drugs for retarding intrinsic cardiac aging remains unknown.

Diastolic dysfunction is well documented in human cardiac aging. Impaired myocardial relaxation caused by functional decline in calcium handling proteins and increased myocardial stiffness related to cardiac hypertrophy and fibrosis are common causes of diastolic dysfunction. Among these, the decline in SERCA2 protein is the strongest predictor of diastolic dysfunction in aged mice (online-only Data Supplement Table III). We and others have found that old WT cardiomyocytes have a lower amplitude and prolonged decay rate of calcium transients,<sup>24</sup> and we have shown that old mCAT cardiomyocytes have calcium transient profiles

more comparable to young cardiomyocytes (Figure 6E). Significantly lower sarcoplasmic reticulum calcium loads in old WT cardiomyocytes (shown by caffeine stimulation; Figure 6D) are best explained by a chronic reduction in SERCA2 protein levels in old hearts because dephosphorylated phospholamban did not change significantly with age (Figure 5E) and the beneficial effect of mCAT was completely abolished when SERCA2 was inhibited by cyclopyrazonic acid (Figure 6E). Our data also show that NCX is upregulated in aged heart when SERCA2 is decreased. Such declines in SERCA2 and increases in NCX expression have previously been reported in cardiac aging in response to Ang II and in heart failure.<sup>25–27</sup> Ren et al<sup>28</sup> reported that overexpression of catalase in cardiomyocytes attenuated aging-induced cardiomyocyte relaxation dysfunction by increasing NCX rather than preserving SERCA2. In contrast, mCAT preserves SERCA2 in old mice, and upregulation of NCX is less apparent.

Although mice are the best-studied experimental model of mammalian aging, little evidence has directly linked the senescence of vital organs with survival. Echocardiographic data of our mouse longevity cohort suggested that both cardiac hypertrophy (increased LVMI) and functional decline (increased MPI) are significant predictors of mortality. We generated the cardiac aging risk score for each mouse in the cohort, estimated by the linear regression model of LVMI and MPI (Cox model 1, the Table), and showed that the cardiac aging risk score is an independent predictor of mortality. Estimation by an epidemiological approach suggested that approximately half of the natural deaths in the cohort were attributable to cardiac aging. Study of the end-of-life pathology of this longevity cohort has indicated that cardiac pathology was a contributing cause of death in about half of the C57BL6 mice,<sup>29</sup> consistent with the estimated attributable risk fraction for cardiac aging by echocardiography, suggesting that cardioprotection likely contributes to the lifespan extension previously observed in mCAT mice.<sup>5</sup> Such demonstration of improved organ function during aging may be even more relevant to human health than observations of extended longevity,<sup>30</sup> and this report shows that the murine model of cardiac aging is well suited for this approach.

### Conclusions

Our data emphasize the roles of mitochondrial ROS in cardiac aging, which is an independent predictor of mortality in mouse longevity cohort. This study also suggests potential clinical applications of mitochondria-targeted antioxidant drugs in cardiovascular diseases, despite the disappointing results from nontargeted antioxidants.

### Source of Funding

This work was supported by National Institutes of Health grants P30 AG013280 and P01 AG001751.

### Disclosures

None.

### References

- Lakatta EG, Levy D. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises, part II: the aging heart in health: links to heart disease. *Circulation*. 2003;107:346–354.
- Murphy SW. Diastolic dysfunction. *Curr Treat Options Cardiovasc Med*. 2004;6:61–68.
- Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. *Cell*. 2005;120:483–495.
- Vermulst M, Bielas JH, Kujoth GC, Ladiges WC, Rabinovitch PS, Prolla TA, Loeb LA. Mitochondrial point mutations do not limit the natural lifespan of mice. *Nat Genet*. 2007;39:540–543.
- Schriner SE, Linford NJ, Martin GM, Treuting P, Ogburn CE, Emond M, Coskun PE, Ladiges W, Wolf N, Van Remmen H, Wallace DC, Rabinovitch PS. Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science*. 2005;308:1909–1911.
- Dilly KW, Rossow CF, Votaw VS, Meabon JS, Cabarrus JL, Santana LF. Mechanisms underlying variations in excitation-contraction coupling across the mouse left ventricular free wall. *J Physiol*. 2006;572:227–241.
- Etches V, Frank J, Di Ruggiero E, Manuel D. Measuring population health: a review of indicators. *Annu Rev Public Health*. 2006;27:29–55.
- Zheng F, Plati AR, Potier M, Schulman Y, Berho M, Banerjee A, Leclercq B, Zisman A, Striker LJ, Striker GE. Resistance to glomerulosclerosis in B6 mice disappears after menopause. *Am J Pathol*. 2003;162:1339–1348.
- Khouri SJ, Maly GT, Suh DD, Walsh TE. A practical approach to the echocardiographic evaluation of diastolic function. *J Am Soc Echocardiogr*. 2004;17:290–297.
- Vermulst M, Wanagat J, Kujoth GC, Bielas JH, Rabinovitch PS, Prolla TA, Loeb LA. DNA deletions and clonal mutations drive premature aging in mitochondrial mutator mice. *Nat Genet*. 2008;40:392–394.
- Bielas JH, Loeb LA. Quantification of random genomic mutations. *Nat Methods*. 2005;2:285–290.
- St-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jager S, Handschin C, Zheng K, Lin J, Yang W, Simon DK, Bachoo R, Spiegelman BM. Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell*. 2006;127:397–408.
- Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol*. 2006;7:589–600.
- Liang Q, Wiese RJ, Bueno OF, Dai YS, Markham BE, Molkentin JD. The transcription factor GATA4 is activated by extracellular signal-regulated kinase 1- and 2-mediated phosphorylation of serine 105 in cardiomyocytes. *Mol Cell Biol*. 2001;21:7460–7469.
- Zile MR, Brutsaert DL. New concepts in diastolic dysfunction and diastolic heart failure: Part II: causal mechanisms and treatment. *Circulation*. 2002;105:1503–1508.
- Rosenkranz S. TGF-beta1 and angiotensin networking in cardiac remodeling. *Cardiovasc Res*. 2004;63:423–432.
- Date MO, Morita T, Yamashita N, Nishida K, Yamaguchi O, Higuchi Y, Hirotsani S, Matsumura Y, Hori M, Tada M, Otsu K. The antioxidant N-2-mercaptopyrionyl glycine attenuates left ventricular hypertrophy in in vivo murine pressure-overload model. *J Am Coll Cardiol*. 2002;39:907–912.
- Matsushima S, Ide T, Yamato M, Matsusaka H, Hattori F, Ikeuchi M, Kubota T, Sunagawa K, Hasegawa Y, Kurihara T, Oikawa S, Kinugawa S, Tsutsui H. Overexpression of mitochondrial peroxiredoxin-3 prevents left ventricular remodeling and failure after myocardial infarction in mice. *Circulation*. 2006;113:1779–1786.
- McKinsey TA, Kass DA. Small-molecule therapies for cardiac hypertrophy: moving beneath the cell surface. *Nat Rev Drug Discov*. 2007;6:617–635.
- Liu H, Colavitti R, Rovira II, Finkel T. Redox-dependent transcriptional regulation. *Circ Res*. 2005;97:967–974.
- Inserra F, Romano L, Ercole L, de Cavanagh EM, Ferder L. Cardiovascular changes by long-term inhibition of the renin-angiotensin system in aging. *Hypertension*. 1995;25:437–442.
- Basso N, Cini R, Pietrelli A, Ferder L, Terragno NA, Inserra F. Protective effect of long-term angiotensin II inhibition. *Am J Physiol Heart Circ Physiol*. 2007;293:H1351–H1358.
- Doughan AK, Harrison DG, Dikalov SI. Molecular mechanisms of angiotensin II mediated mitochondrial dysfunction: linking mitochondrial oxidative damage and vascular endothelial dysfunction. *Circ Res*. 2008;102:488–496.
- Lim CC, Apstein CS, Colucci WS, Liao R. Impaired cell shortening and relengthening with increased pacing frequency are intrinsic to the

- senescent mouse cardiomyocyte. *J Mol Cell Cardiol.* 2000;32:2075–2082.
25. Lakatta EG. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises, part III: cellular and molecular clues to heart and arterial aging. *Circulation.* 2003;107:490–497.
  26. Houser SR, Piacentino V 3rd, Weisser J. Abnormalities of calcium cycling in the hypertrophied and failing heart. *J Mol Cell Cardiol.* 2000;32:1595–1607.
  27. Domenighetti AA, Wang Q, Egger M, Richards SM, Pedrazzini T, Delbridge LM. Angiotensin II-mediated phenotypic cardiomyocyte remodeling leads to age-dependent cardiac dysfunction and failure. *Hypertension.* 2005;46:426–432.
  28. Ren J, Li Q, Wu S, Li SY, Babcock SA. Cardiac overexpression of antioxidant catalase attenuates aging-induced cardiomyocyte relaxation dysfunction. *Mech Ageing Dev.* 2007;128:276–285.
  29. Treuting PM, Linford NJ, Knoblaugh SE, Emond MJ, Morton JF, Martin GM, Rabinovitch PS, Ladiges WC. Reduction of age-associated pathology in old mice by overexpression of catalase in mitochondria. *J Gerontol A Biol Sci Med Sci.* 2008;63:813–822.
  30. Butler RN, Miller RA, Perry D, Carnes BA, Williams TF, Cassel C, Brody J, Bernard MA, Partridge L, Kirkwood T, Martin GM, Olshansky SJ. New model of health promotion and disease prevention for the 21st century. *BMJ.* 2008;337:a399.

### CLINICAL PERSPECTIVE

Old age is a major risk factor for cardiovascular disease because it prolongs exposure to hypertension, diabetes, hypercholesterolemia, and other cardiovascular risks. However, intrinsic cardiac aging, the slowly progressive structural changes and functional declines with age, also makes the heart more susceptible to stress and contributes to increased risk of cardiovascular mortality and morbidity in the elderly. Using echocardiography, we showed that cardiac aging in mice recapitulates human cardiac aging as documented by prior epidemiological studies: age-dependent increases in left ventricular mass index and diastolic dysfunction and worsening myocardial performance index. Cardiac aging in the mouse is accompanied by increases in mitochondrial protein oxidation, DNA deletions, mitochondrial biogenesis, and ventricular fibrosis, as well as decreased cardiac SERCA2 and activation of the calcineurin–nuclear factor of activated T-cell pathway. Although mitochondrial reactive oxygen species have been proposed as a cause of aging, their role in cardiac aging is unclear. We have previously shown that overexpression of catalase targeted to mitochondria (mCAT) prolongs the murine median lifespan by 17% to 21%. In this study, we demonstrated that all of the age-related changes were significantly attenuated in mCAT mice. Survival analysis showed that echocardiographic parameters are a significant predictor of mortality in mice, even in the absence of hypertension, diabetes, or hypercholesterolemia. This study emphasizes the critical role of mitochondrial reactive oxygen species in cardiac aging and the impact of cardiac aging on survival. These findings also suggest that mitochondria-targeted antioxidants may have a role in treating or preventing reactive oxygen species–related cardiovascular diseases, despite the disappointing results of clinical trials using nontargeted antioxidants.