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Cardiac Overexpression of Antioxidant Catalase Attenuates Aging-Induced Cardiomyocyte Relaxation Dysfunction

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Abstract

Catalase, an enzyme which detoxifies H₂O₂, may interfere with cardiac aging. To test this hypothesis, contractile and intracellular Ca²⁺ properties were evaluated in cardiomyocytes from young (3–4 mo) and old (26–28 mo) FVB and transgenic mice with cardiac overexpression of catalase. Contractile indices analyzed included peak shortening (PS), time-to-90% PS (TPS₉₀), time-to-90% relengthening (TR₉₀), half-width duration (HWD), maximal velocity of shortening/relengthening (\pm dL/dt) and intracellular Ca²⁺ levels or decay rate. Levels of advanced glycation endproduct (AGE), Na⁺/Ca²⁺ exchanger (NCX), sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA2a), phospholamban (PLB), myosin heavy chain (MHC), membrane Ca²⁺ and K⁺ channels were measured by western blot. Catalase transgene prolonged survival while did not alter myocyte function by itself. Aging depressed \pm dL/dt, prolonged HWD, TR₉₀ and intracellular Ca²⁺ decay without affecting other indices in FVB myocytes. Aged FVB myocytes exhibited a steeper decline in PS in response to elevated stimulus or a dampened rise in PS in response to elevated extracellular Ca²⁺ levels. Interestingly, aging-induced defects were nullified or significantly attenuated by catalase. AGE level was elevated by 5-fold in aged FVB compared with young FVB mice, which was reduced by catalase. Expression of SERCA2a, NCX and K_{v1.2} K⁺ channel was significantly reduced although levels of PLB, L-type Ca²⁺ channel dihydropyridine receptor and β -MHC isozyme remained unchanged in aged FVB hearts. Catalase restored NCX and K_{v1.2} K⁺ channel but not SERCA2a level in aged mice. In summary, our data suggested that catalase protects cardiomyocytes from aging-induced contractile defect possibly via improved intracellular Ca²⁺ handling.

Keywords

Catalase; cardiomyocytes; aging; shortening/relengthening; Ca²⁺ cycling protein

INTRODUCTION

Cardiac senescence, or cardiac aging, is referred to a dramatic decline in cardiac pump function and contractile reserve with advanced age. It is an irreversible biological process which contributes to high cardiovascular mortality (Lakatta et al. 1987; Lakatta 2000). Although occult diseases and sedentary life style may contribute to senescence-related changes in

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cardiovascular function, biological aging or advanced age itself has been demonstrated to be the single most independent risk factor for deteriorated cardiac morphology and function (Fleg et al. 1995; Lakatta et al. 1987; Lakatta 2000). Several rationales have been speculated for cardiac aging including prolonged action potential duration, myosin isozyme switch, impaired intracellular Ca^{2+} homeostasis, altered membrane structure and permeability and accumulation of reactive oxygen species (ROS), which may lead to abnormal cardiac contractile function (Kass et al. 2001; Lakatta 1999; Lakatta 2000). Although the precise link between advanced age and altered cardiac contractile function has not been fully elucidated, the “free radical theory of aging” seems to draw the most attention for development of cardiac aging (Yang et al. 2005). Data from our recent studies revealed that cardiac overexpression of heavy metal scavenging antioxidant metallothionein prolongs life span, alleviates aging-associated cardiac contractile dysfunction, insulin insensitivity and mitochondrial damage (Fang et al. 2005; Yang et al. 2006). To further study the role of enzymatic antioxidants on cardiac senescence, the present study was designed to examine the effect of catalase, an enzyme for H_2O_2 detoxification, on aging-elicited alteration in cardiomyocyte contractile function and intracellular Ca^{2+} cycling proteins. We hypothesized that catalase shifts redox (i.e., antioxidant/oxidant) balance to be pro-antioxidant and thus protects against oxidative stress-induced cardiac insult under senescence. To this end, a cardiac-specific catalase transgenic mouse line was employed in which catalase was constitutively over-expressed (~50 fold) in myocytes, distributing from sarcoplasmic reticulum (SR), nucleus to peroxisomes (Zhou and Kang 2000). Cardiomyocyte contractile and intracellular Ca^{2+} transient properties were examined at young (3–4 month) and old (26–28 month) ages. Protein abundance of major intracellular Ca^{2+} regulating proteins sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA2a), phospholamban (PLB), $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), membrane ion channels such as voltage-gated L-type Ca^{2+} channel dihydropyridine receptor (DHPR) and K^+ channels, myosin heavy chain isozyme and advanced glycation endproduct (AGE), a protein post-translational modification product highly correlated with aging, were evaluated in hearts from young and old catalase transgenic and wild-type FVB mice.

MATERIALS AND METHODS

Cardiac catalase over-expression transgenic mice and Kaplan-Meier’s survival curve

All animal procedures used here were approved by the Animal Care and Use Committee at the University of Wyoming (Laramie, WY, USA). Production of cardiac-specific catalase transgenic mice was described in detail previously (Kang et al. 1996; Zhang et al. 2003). In brief, an 8-kb catalase transgene driven by a myosin heavy chain (MHC) promoter containing the entire coding sequences of the catalase cDNA was purified on a matrix of diatomaceous earth and filtered through a 0.22- μm filter. Approximately 100 copies of purified transgene insert were microinjected into one pronucleus of each one-cell mouse embryo of inbred strain FVB. The transgene transcription of catalase was controlled by mouse α -MHC gene. To identify transgenic founder mice, genomic DNA was isolated from 1-cm tail clips from 4-week-old mice. DNA was subjected to Southern and dot blot analyses, which were probed with a 550-bp *SmaI/NotI* fragment derived from the rat insulin II portion of the catalase transgene. This probe was hybridized to an 8000-bp *EcoRI* fragment of the transgene, consistent with the presence of a unique *EcoRI* site in the catalase transgene (600 bp upstream of the MHC transcription initiation site). Founder mice were bred with mice of the same strain and transgenic offspring were identified by a polymerase chain reaction (PCR) using a primer pair derived from the MHC promoter and rat catalase cDNA. Reverse: aat atc gtg ggt gac ctc aa; Forward: cag atg aag cag tgg aag ga (Zhang et al. 2003). Transgenic positive mice (heterozygotes) and their transgenic negative littermates (FVB) were used for experiments. Adult male FVB and catalase transgenic mice at young (3–4 month-old) and old (26–28 month-old) ages were used in this study unless otherwise stated. To configure the Kaplan-Meier

survival curve, FVB and catalase mice of both genders were separated after weanling. All mice were housed (7–8 mixed mice per cage until they were genotyped at ~ 3 month of age) in a pathogen free environment in our animal facility within School of Pharmacy. Mice were maintained with a 12/12-light/dark cycle with free access to regular rodent chow and tap water. Mortality was recorded every three months (excluding unexpected death such as fighting).

Measurement of catalase enzyme activity

Frozen ventricular tissues were homogenized and sonicated on ice in a buffer containing 20 mM MOPS, 620 mM sucrose, 1 mM EDTA at pH 7.2. The homogenate was centrifuged at $13,000 \times g$ for 30 min (4°C) and protein concentration in the extracted supernatant fraction was determined by the Bradford assay (Bradford 1976). Ice-cold catalase buffer (50 mM potassium phosphate monobasic, 50 mM sodium phosphate dibasic, pH 7.0) was added to extracted samples and H₂O₂ was used to initiate the reaction. Catalase activity value was calculated from the spectrophotometric reading at 240 nm with respect to appropriate concurrent blanks (Brown-Borg et al. 1999).

Isolation of mouse cardiomyocytes

Hearts were rapidly removed from anesthetized mice and mounted onto a temperature-controlled (37°C) Langendorff system. After perfusing with a modified Tyrode's solution (Ca²⁺ free) for 2 min, the heart was digested with a Ca²⁺-free KHB buffer containing liberase blendzyme 4 (Hoffmann-La Roche Inc., Indianapolis, IN, USA) for 20 min. The modified Tyrode solution (pH 7.4) contained the following (in mM): NaCl 135, KCl 4.0, MgCl₂ 1.0, HEPES 10, NaH₂PO₄ 0.33, glucose 10, butanedione monoxime 10, and the solution was gassed with 5% CO₂-95% O₂. The digested heart was then removed from the cannula and left ventricle was cut into small pieces in the modified Tyrode's solution. Tissue pieces were gently agitated and pellet of cells was resuspended. Extracellular Ca²⁺ was added incrementally back to 1.20 mM over a period of 30 min. Isolated myocytes were used for experiments within 8 hours of isolation. Normally, a yield of at least 50–60% viable rod-shaped cardiomyocytes with clear sarcomere striations was achieved. Isolation with low yield of myocytes (< 40%, usually as a result of over-digestion) was not used. Myocytes with obvious sarcolemmal blebs or spontaneous contractions were not chosen for study. Only rod-shaped myocytes with clear edges were selected for mechanical and intracellular Ca²⁺ studies (Duan et al. 2002).

Cell shortening/relengthening and response to various stimulus frequencies or extracellular Ca²⁺

Mechanical properties of ventricular myocytes were assessed using a SoftEdge MyoCam® system (IonOptix Corporation, Milton, MA, USA) (Duan et al. 2002). In brief, cells were placed in a Warner chamber mounted on the stage of an inverted microscope (Olympus, IX-70) and superfused (~1 ml/min at 25°C) with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, at pH 7.4. The cells were field stimulated with supra-threshold voltage at a frequency of 0.5 Hz (unless otherwise stated), 3 msec duration, using a pair of platinum wires placed on opposite sides of the chamber connected to a FHC stimulator (Brunswick, NE, USA). The myocyte being studied was displayed on the computer monitor using an IonOptix MyoCam camera. An IonOptix SoftEdge software was used to capture changes in cell length during shortening and relengthening. Cell shortening and relengthening were assessed using the following indices: peak shortening (PS) - indicative of peak ventricular contractility, time-to- 90% PS (TPS₉₀) - indicative of contraction duration, and time-to-90% relengthening (TR₉₀) – represents cardiomyocyte relaxation duration, half-width duration (HWD) – duration between 50% shortening and 50% relengthening and an indicative of late contraction and early relaxation, maximal velocities of shortening (+ dL/dt) and relengthening (-dL/dt) - indicatives of maximal velocities of ventricular pressure rise/fall. In the case of

altering stimulus frequency from 0.1 Hz to 5.0 Hz, the steady state contraction of myocyte was achieved (usually after the first 5–6 beats) before PS was recorded. The contractile response of myocytes to increasing concentrations of extracellular Ca^{2+} (0.5 to 3.0 mM) was performed after switching extracellular Ca^{2+} concentration to a different perfusion level for 5 min.

Intracellular Ca^{2+} transient measurement

Myocytes were loaded with fura-2/AM (0.5 μM) for 10 min and fluorescence measurements were recorded with a dual-excitation fluorescence photomultiplier system (Ionoptix). Myocytes were placed on an Olympus IX-70 inverted microscope and imaged through a Fluor \times 40 oil objective. Cells were exposed to light emitted by a 75W lamp and passed through either a 360 or a 380 nm filter, while being stimulated to contract at 0.5 Hz. Fluorescence emissions were detected between 480–520 nm by a photomultiplier tube after first illuminating the cells at 360 nm for 0.5 sec then at 380 nm for the duration of the recording protocol (333 Hz sampling rate). The 360 nm excitation scan was repeated at the end of the protocol and qualitative changes in intracellular Ca^{2+} concentration were inferred from the ratio of fura-2 fluorescence intensity (FFI) at two wavelengths (360/380). Fluorescence decay time was measured as an indication of the intracellular Ca^{2+} clearing rate. Both single and bi-exponential curve fit programs were applied to calculate the intracellular Ca^{2+} decay constant (Duan et al. 2002).

Western blot analysis

Protein levels of SERCA2a, PLB, NCX, $\text{Kv}_{1.2}$ potassium channel, voltage-gated L-type Ca^{2+} channel dihydropyridine receptor α subunit (DHPR- α) and the post-translational modification product AGE were examined by standard Western blot. Left ventricular tissues were homogenized and centrifuged at $1,000 \times g$ for 10 min. The supernatants were then centrifuged at $70,000 \times g$ for 30 min at 4°C . The 100,000-g pellets were cellular membrane fractions and were used for immunoblotting of SERCA2a, PLB, NCX, $\text{Kv}_{1.2}$, DHPR- α and AGE. Membrane proteins (35 $\mu\text{g}/\text{lane}$) were separated on 10 – 15% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad, Hercules, CA, USA) and transferred to polyvinylidene difluoride membranes. The membrane was blocked with TBS-0.05% Tween 20 (TBS-T) with 5% nonfat dry milk for 60 min and was then incubated with monoclonal anti-SERCA2a (1:2,000, Affinity BioReagentTM, Golden, CO, USA), monoclonal anti-PLB (1:2,000, Abcam Inc, Cambridge, MA, USA), polyclonal anti-NCX (1:1,000, Swant, Bellinzona, Switzerland), monoclonal anti- $\text{Kv}_{1.2}$ (1:1,000, Upstate Biotechnology, Lake Placid, NY, USA), polyclonal anti-DHPR- α (1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and anti-AGE monoclonal (1:1,000) (Trans Genic Inc., Kumamoto, Japan) in TBS-T with 5% BSA overnight at 4°C . The antigens were detected by the luminescence method (ECL Western blotting detection kit, Amersham) with peroxidase-linked anti-mouse (SERCA2a, PLB, $\text{Kv}_{1.2}$), anti-rabbit (NCX) or anti-goat (DHPR- α) IgG (1:5,000 dilution). Quantification of band and AGE was determined using Quantity One density of SERCA2a, NCX, PLB, $\text{Kv}_{1.2}$, DHPR- α software (Bio-Rad, version 4.4.0, build 36) after conversion of radiographic film to TIFF files (8-bit grayscale) using a Scanjet 7400c (Hewlett-Packard, Palo Alto, CA, USA) and reported in optical density per square millimeter. Densitometry was performed by outlining the bands with the volume rectangle tool initially set on the control band of interest (Zhang et al. 2003).

MHC isoform analysis by gel electrophoresis

Samples were prepared for gel electrophoresis according to method described by Blough and colleagues (Blough et al. 1996). Each sample (20–30 mg heart tissue) was placed in a microcentrifuge tube and 30 μl of sample buffer was added per mg of tissue. The sample was homogenized for ~ 10 sec, heated for 2 min at 95°C , chilled on ice for 5 min before being

centrifuged. The supernatant was saved and diluted 1:10 with sample. Three μl of diluted samples was loaded for gel electrophoresis performed at 8°C in a PROTEAN II unit (Bio-Rad, Hercules, CA, USA). Stacking gels consisted of 4% acrylamide (acrylamide:bis = 50:1) and 5% glycerol (v/v, pH 8.8). Gels were run at a constant voltage of 200 V for 30 hr, fixed for a minimum of 2 hr in 5% glutaraldehyde before being silver-stained and scanned with a Bio-Rad GS-800 densitometer to determine the amount of MHC- β as a percentage of total MHC (Li et al. 2006).

Statistical analyses

Data were presented as Mean \pm SEM. Log rank test was used for Kaplan-Meier survival comparison. Statistical significance ($p < 0.05$) for each variable was determined by analysis of variance (ANOVA) or t-test, where appropriate.

RESULTS

General characteristics of mice and the Kaplan-Meier survival curve

General features of young and old FVB and catalase transgene mice are shown in Table 1, transgenic catalase over-expression did not elicit any notable effect on body, heart weights and heart size (heart-to-body weight ratio) compared with those of age-matched FVB wild-type littermates. Older mice display heavier body and heart weights (although not heart size, i.e., heart weight normalized to body weight) compared with young counterparts, as expected. The fasting blood glucose levels were similar among all four mouse groups studied, excluding potential contribution of diabetes to aging-induced effects, if any. The Kaplan-Meier curve comparison depicts that catalase transgenic mice display significantly better survival rates than FVB mice in both genders. The median lifespan was 3 and 6 month longer in male (30 months) and female (33 months) catalase mice, respectively, than that of FVB mice (27 months in both genders). The survival curves of FVB and catalase mice begin to separate from each other after 15 months of age with catalase mice exhibiting a reduced mortality rate in both genders (Fig. 1). To verify cardiac over-expression of catalase, catalase enzyme activity assay was performed. Result shown in Fig. 2 depicts a nearly 6-fold increase in cardiac catalase activity in young transgenic mice compared with the wild-type FVB mice. Aging itself triggered a significant decline in cardiac catalase activity, which can easily be masked by cardiac over-expression of catalase.

Mechanical and intracellular Ca^{2+} properties of cardiomyocytes

Mechanical properties obtained under extracellular Ca^{2+} of 1.0 mM and a stimulus frequency of 0.5 Hz indicated that resting cell length and peak shortening (PS) amplitude were all similar in cardiomyocytes among young and old male FVB and catalase mice. However, myocytes from old FVB mice displayed significantly reduced maximal velocity of shortening/relengthening ($\pm \text{dL}/\text{dt}$) compared with myocytes from young FVB mice. The reduced $\pm \text{dL}/\text{dt}$ was associated with normal shortening duration (TPS_{90}), prolonged relengthening duration (TR_{90}) and half-width duration (HWD) in old FVB myocytes. Interestingly, aging-induced mechanical dysfunctions were not observed in old catalase transgenic mice, suggesting a beneficial role of the antioxidant catalase against aging-associated cardiac dysfunction. Catalase transgene itself did not affect mechanical contractile properties in young mouse group (Fig. 3). Our further study using the intracellular fluorescence dye fura-2 revealed reduced intracellular Ca^{2+} clearing rate (both single and bi-exponential curve fit), enhanced baseline intracellular Ca^{2+} levels associated with unchanged electrically-stimulated rise in intracellular Ca^{2+} in aged FVB cardiomyocytes, consistent with the observation of prolonged TR_{90} and normal PS in these myocytes. Similar to its effect on cell shortening, catalase abolished aging-induced prolongation in intracellular Ca^{2+} clearing rate and elevation in resting intracellular

Ca²⁺ level in aged cardiomyocytes without affecting any indices tested in young cardiomyocytes (Fig. 4).

Effect of increasing stimulation frequency or extracellular Ca²⁺ concentration on myocyte shortening

Murine hearts normally contract at high frequencies, whereas our mechanical recording was conducted at 0.5 Hz. To evaluate the impact of aging on cardiac contractile function under higher frequencies, we raised stimulus frequency up to 5.0 Hz (300 beats/min) and recorded the steady-state peak shortening. Cells were initially stimulated to contract at 0.5 Hz for 5 min to ensure steady-state before commencing the frequency study. All the recordings were normalized to PS at 0.1 Hz of the same myocyte. Fig. 5A shows negative staircases in PS amplitude in cardiomyocytes from all four mouse groups when stimulus frequency was increased from 0.1 Hz to 0.5 Hz or higher. The percent decrease of PS from its value at 0.1 Hz was significantly greater in aged FVB myocytes than young myocytes at higher frequencies (0.5 – 5.0 Hz), suggesting reduced intracellular Ca²⁺ cycling or stress intolerance under advanced aging. Similar to its effect on myocyte contractile function, catalase transgene ablated high stimulating frequency-induced stepper reduction in PS among aged cardiomyocytes without affecting the response pattern in young cardiomyocytes. To examine the influence of extracellular Ca²⁺ levels on PS amplitude, as an estimate of the myofilament Ca²⁺ sensitivity, myocytes were first stimulated to contract at 0.5 Hz in a contractile buffer containing 0.5 mM CaCl₂. After the steady-state PS had been recorded, the extracellular Ca²⁺ level was increased to 1.0, 2.0 and 3.0 mM. Fig.5B shows that increases in extracellular Ca²⁺ concentration from 0.5 mM to 3 mM resulted in a positive staircase in PS in myocytes from young FVB mice. Interestingly, the increase in PS in response to elevated extracellular Ca²⁺ concentration was significantly dampened in myocytes from aged FVB mice, suggesting a reduced Ca²⁺ sensitivity in aged cardiomyocytes. Catalase restored reduced Ca²⁺ sensitivity in aged cardiomyocytes without affecting the response in young cardiomyocytes.

Expression of AGE and intracellular Ca²⁺ transport/regulatory proteins

Western blot analysis demonstrated a significant elevation in the levels of post-translational modification product AGE in aged FVB hearts which was nullified by catalase. Catalase itself significantly reduced the levels of AGE in young hearts (Fig. 6A). Cardiac contractile function is maintained by a cascade of intracellular Ca²⁺ regulatory/cycling proteins including SERCA, Na⁺-Ca²⁺ exchanger (NCX), phospholamban and membrane ion channels (Bers 2002). Aging has been demonstrated to affect the expression and function in some of these Ca²⁺ cycling proteins (Koban et al. 1998; Pavlovic et al. 2005; Schmidt et al. 2002; Xu and Narayanan 1998). Data obtained from our study indicated that aging down-regulated expression of SERCA2a, NCX and K_{v1.2} potassium channel without any significant effect on expression of phospholamban and voltage-gated L-type Ca²⁺ channel dihydropyridine receptor α -subunit (DHPR- α). Catalase significantly alleviated aging-induced reduction in NCX and K_{v1.2} potassium channel expression without affecting the level of SERCA2a. Protein expression of SERCA2a, NCX, phospholamban, K_{v1.2} and DHPR- α was unaffected by catalase transgene in young mice (Fig. 6 and Fig. 7). It was indicated that expression of MHC isoforms is also closely associated with cardiac function phenotype (Olsson et al. 2004). We thus examined the pattern of MHC isozyme distribution in aged mouse heart. Result shown in Fig. 7A and Fig. 7B revealed similar distribution of β -MHC isozyme in young and old mouse hearts, which was not altered by catalase transgene.

DISCUSSION

Our study presented for the first time that cardiac-specific overexpression of catalase attenuated aging-induced cardiac contractile and intracellular Ca²⁺ handling abnormalities. Catalase

transgene prolonged lifespan, ameliorates aging-induced decline in peak cardiac contractile capacity under increased stimulus frequency and elevated extracellular Ca^{2+} concentration. The catalase-induced cardiac protection against aging-induced cardiac defects may be underscored by alteration in AGE level and protein expression of certain Ca^{2+} regulatory proteins such as Na^+ - Ca^{2+} exchanger and potassium channel. These data supported the notion that ROS especially H_2O_2 , the main enzymatic target of catalase, may play a significant role in the pathogenesis of aging-associated cardiac contractile defects. Since catalase transgene itself did not overtly alter cardiac contractile and intracellular Ca^{2+} properties in young mice, indicating that catalase may not be innately harmful to cardiac function even at the levels of 50-fold overexpression. Therefore, it may be considered as a beneficial route to delay the cardiac aging process and to minimize senescence-associated high cardiac mortality (Cutler 2005).

Mounting evidence from human and experimental animals has indicated compromised cardiac function in senescence (Fleg et al. 1995; Lakatta 1999; Li et al. 2005). Studies from various murine strains including C57BL/6 and FVB mice depicted diminished cardiac contractile function and enhanced susceptibility to injury such as ischemia-reperfusion, due to either aging-associated decrease in coronary circulation and collateral flow or aging-related decline in intrinsic myocardial contractile capacity (Azhar et al. 1999; Fang et al. 2005; Li et al. 2005; Willems et al. 2003; Yang et al. 2006). Results from our present study revealed reduced maximal velocity of contraction and relaxation, prolongation of relaxation and half-width duration in aged FVB cardiomyocytes, similar to previously published report in aged cardiomyocytes (Fang et al. 2005; Li et al. 2005; Yang et al. 2006). Our data demonstrated that catalase transgene attenuated aging-induced cardiac contractile dysfunction, prolonged intracellular Ca^{2+} clearing, reduced Na^+ - Ca^{2+} exchanger and $\text{Kv}_{1,2}$ expression as well as AGE accumulation, indicating that facilitated intracellular Ca^{2+} cycling and reduced oxidative stress. Our observation of improved lifespan and cardiac contractile function in aged catalase mice is consistent with recent finding that median and maximum life spans were maximally increased by ~5 months and 5.5 months, respectively, in transgenic mice overexpressing human catalase localized to the peroxisome, the nucleus, or mitochondria (Schriner et al. 2005). The discrepancy in lifespan prolongation between our present study and the one done by Schriner and colleagues (Schriner et al. 2005) may be due to different sites of catalase overexpression. Nonetheless, results from these two studies are complimentary to each other. Transgenic mice with catalase overexpression localized to peroxisome, nucleus or mitochondria exhibit delayed cardiac pathology, reduced oxidative damage, H_2O_2 production and H_2O_2 -induced aconitase inactivation as well as diminished onset of mitochondrial deletion (Schriner et al. 2005). These data was in support of our observation of improved cardiac contractile function, intracellular Ca^{2+} handling, contractile capacity in response to higher stimulus or extracellular Ca^{2+} and Ca^{2+} cycling/transport proteins in hearts from cardiac specific catalase over-expression mice.

Data from our current study depicted impaired intracellular Ca^{2+} handling especially prolonged intracellular Ca^{2+} clearance and elevated resting intracellular Ca^{2+} levels in aged cardiomyocytes. Cardiac intracellular Ca^{2+} homeostasis is maintained by a number of membrane ion channels and Ca^{2+} transport/regulatory proteins within cardiomyocytes (Bers 2002). SERCA and Na^+ - Ca^{2+} exchanger are considered the main machineries to remove Ca^{2+} from cytosolic space for relaxation to occur. However the relative contribution for intracellular Ca^{2+} extrusion by SERCA and Na^+ - Ca^{2+} exchanger are still controversial, and may vary among different species (Bers 2002; Hove-Madsen and Bers 1993; Li et al. 1998). Cannell estimated that Na^+ - Ca^{2+} exchanger accounts for ~75% of intracellular Ca^{2+} extrusion in cardiomyocytes (Cannell 1991). However, Bers suggested that Na^+ - Ca^{2+} exchanger contributes to less than 10% of intracellular Ca^{2+} removal while SERCA is responsible for 90% Ca^{2+} removal in mouse hearts (Bers 2002). Our data revealed remarkable down-regulation of both Na^+ - Ca^{2+} exchanger and SERCA2a but not phospholamban, an endogenous inhibitor of

SERCA, in myocytes from aged wild-type FVB mice. The reduced SERCA2a and Na⁺-Ca²⁺ exchanger expression may account for, at least in part, impaired cardiomyocyte relaxation (prolonged relaxation and half-width duration), prolonged intracellular Ca²⁺ clearance and possibly intracellular Ca²⁺ overload in senescent myocytes. In addition, reduced K_{v1.2} protein expression in aged FVB hearts also suggest possible contribution of potassium channel in prolonged repolarization phase of action potential and thus elongated relaxation duration. Unchanged DHPR- α and β -MHC seem to rule out contribution from L-type Ca²⁺ channel and myosin isozyme switch in cardiomyocyte dysfunction under the present experimental setting. Somewhat conflicting data have been seen for expression or function of cardiac Ca²⁺, K⁺ channels and MHC isozyme under senescence including augmented (Dibb et al. 2004; Schuyler and Yarbrough 1990), unchanged (Josephson et al. 2002; Krishnamurthy et al. 2004; Long et al. 1999) or depressed (Liu et al. 2000; Long et al. 1999; Ranki et al. 2002) Ca²⁺ or K⁺ channel expression as well as β -MHC distribution. Animal species, strains, gender and experimental conditions may play significant roles in these discrepant findings. Nonetheless, our data revealed that aging-induced reduction in Na⁺-Ca²⁺ exchanger and K_{v1.2} channel but not SERCA2a was reconciled by the catalase transgene, suggesting possible roles of Na⁺-Ca²⁺ exchanger and K_{v1.2} channel rather than SERCA2a in catalase-elicited beneficial effect on cardiomyocyte aging. In addition, our observation that catalase ameliorates aging-induced decline in peak cardiac contractile capacity or cardiomyocyte Ca²⁺ sensitivity under increased stimulus frequency and elevated extracellular Ca²⁺ concentration also depicts a role of enhanced SR reserve and myofilament Ca²⁺ sensitivity in the beneficial effect of catalase. Although direct link between antioxidant capacity and cardiac intracellular Ca²⁺ homeostasis is still somewhat vague, an ample of evidence has suggested impaired intracellular Ca²⁺ handling following enhanced oxidative stress. H₂O₂ has been demonstrated to impair SERCA function and SR Ca²⁺ uptake as well as reduce SR intracellular Ca²⁺ release through reaction with S-H groups of RyR (Zaidi and Michaelis 1999). H₂O₂ has also been suggested to alter Ca²⁺ release from mitochondria and induce DNA damage (Ramasarma 1990). By catalyzing conversion of H₂O₂ to oxygen and water, catalase is expected to shift redox balance towards antioxidant end, leading to increased myocardial antioxidant capacity, which may offset the detrimental effect of H₂O₂.

Experimental limitations: Although our current study has provided evidence that catalase may rescue aging-associated cardiomyocyte contractile dysfunction, caution should be taken to extrapolate data from isolated cardiomyocytes to whole heart. It is possible that advanced age affects the sensitivity of cardiomyocytes to the enzymatic insults during the isolation procedure whereas catalase protects the aged cardiomyocytes against the enzymatic insults. In addition, since the healthiest cardiomyocytes are usually chosen for mechanical recordings, therefore a small sub-population of catalase-expressing myocytes which exhibit functional improvement may not truly reflect the contractile state of the whole heart.

In conclusion, our study revealed that cardiac specific overexpression of catalase prolongs lifespan and rescues aging-induced contractile and intracellular Ca²⁺ dysfunction as well as reduced contractile capacity under high stimulus frequency or increased extracellular Ca²⁺ levels in cardiomyocytes possibly through improvement in intracellular Ca²⁺ regulatory protein or membrane ion channel, in addition to its known anti-apoptotic/anti-oxidant capacity. These data, in conjunction with prolonged life span in catalase over-expression mice, have shed some light on the clinical potential of catalase enzyme in aging-associated cardiac dysfunction.

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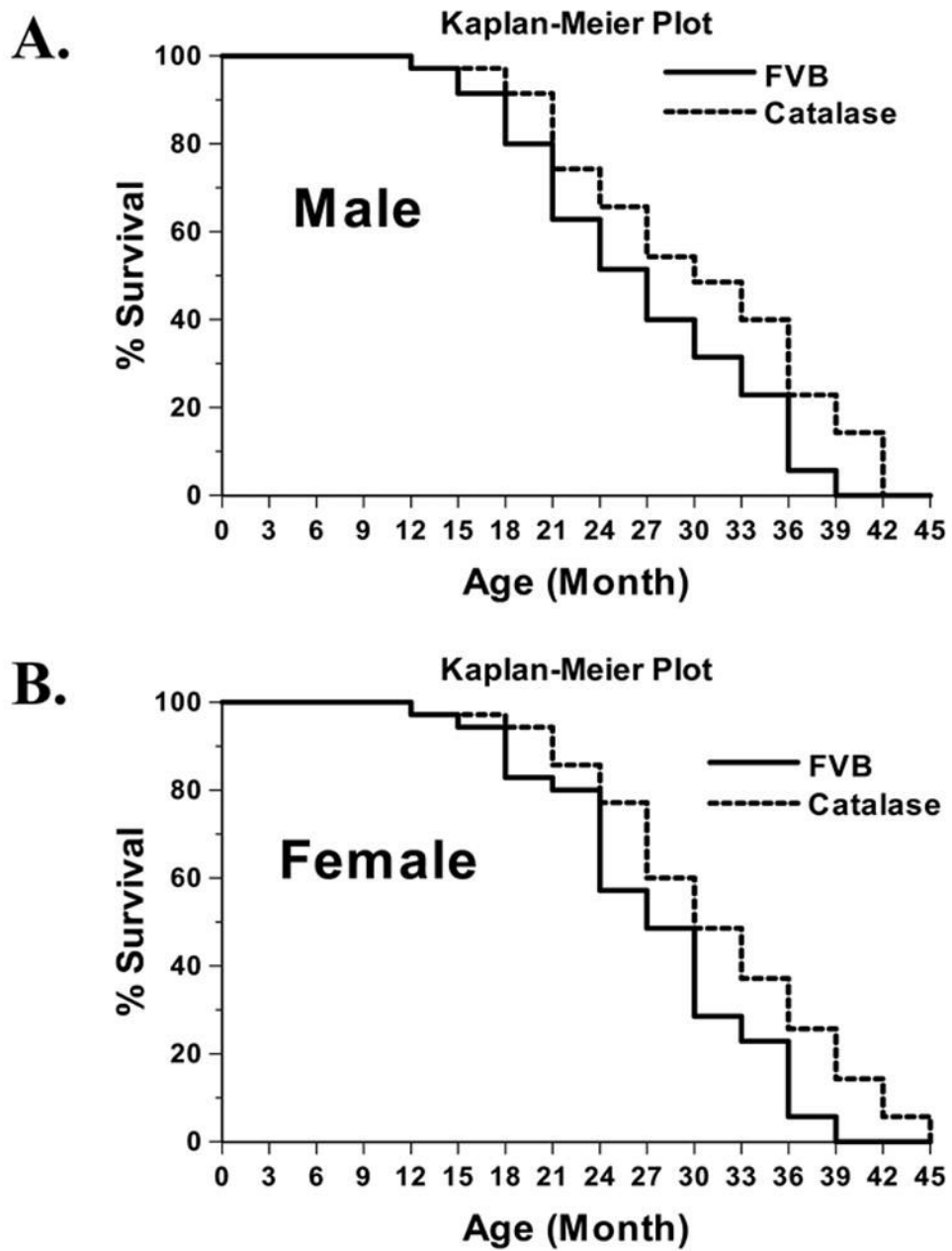


Fig 1. Cumulative survival curve (Kaplan-Meier survival plot) of male and female FVB and catalase mice. Survival rate of mice was checked every three months. The cumulative survival rate was plotted against the time course ($n = 35$ mice per group). Log rank test was performed to compare the FVB and catalase lines with a p value of 0.0117 and 0.0202 in male (panel A) and female (panel B) mice, respectively.

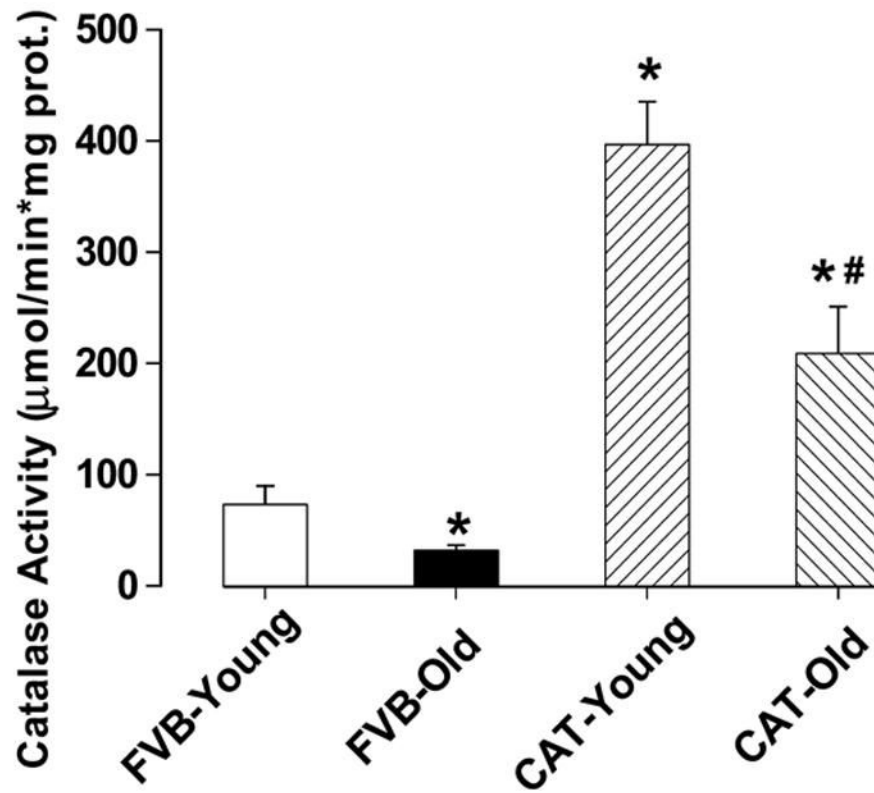
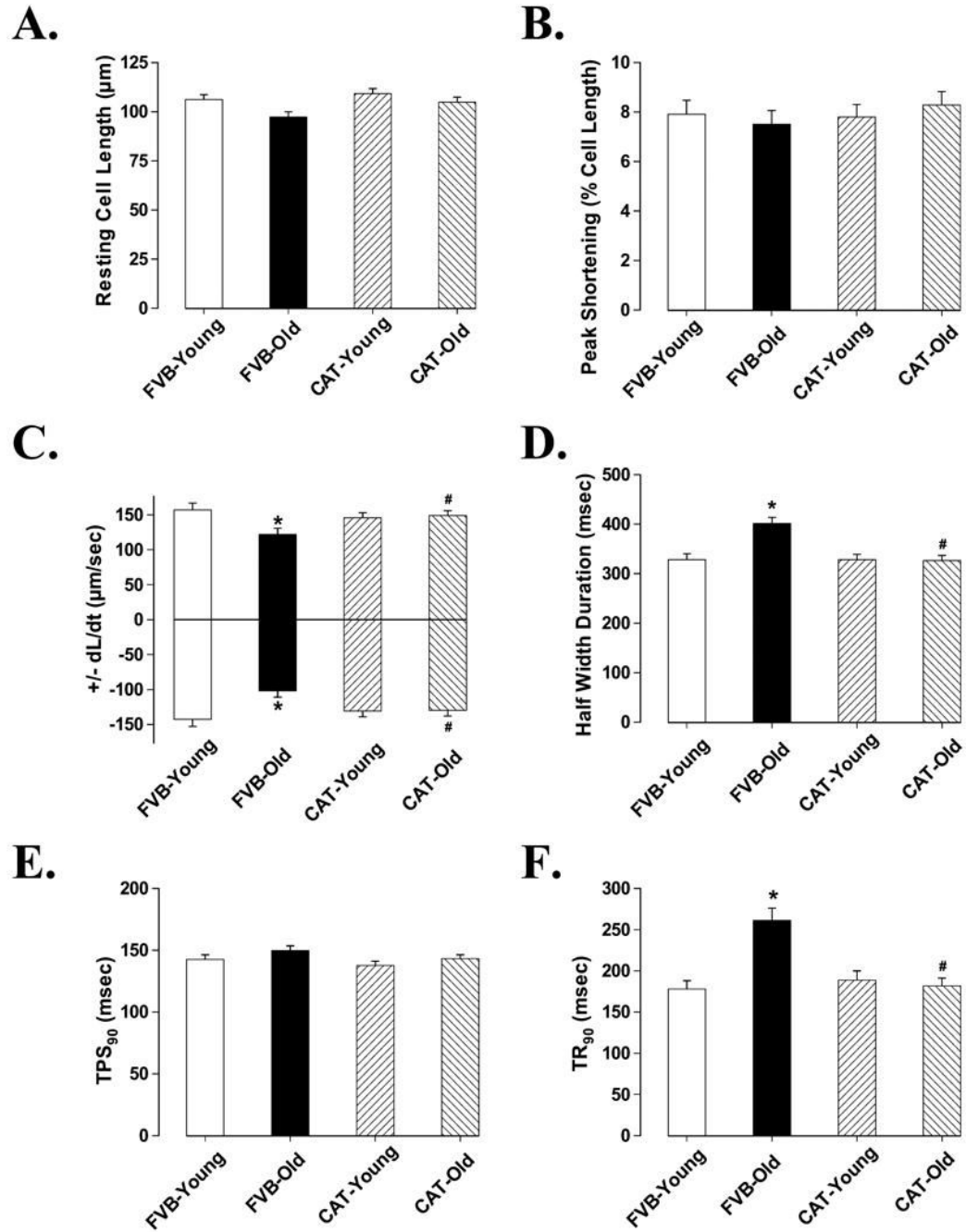


Fig. 2. Cardiac catalase activity in male young and old FVB and catalase (CAT) mice. Mean \pm SEM, $n = 8-9$ hearts per group, * $p < 0.05$ vs. FVB-young group, # $p < 0.05$ vs. FVB-old group.

**Fig 3.**

Contractile properties of cardiomyocytes from male young and old FVB and catalase (CAT) mice. A: Resting cell length; B: Peak shortening (PS, normalized to cell length); C: Maximal velocity of shortening ($+dL/dt$) and relengthening ($-dL/dt$); D: Half width duration; E: Time-to-90% PS (TPS_{90}); F: Time-to-90% relengthening (TR_{90}). Mean \pm SEM, $n = 68 - 69$ cells from 8 mice per group, * $p < 0.05$ vs. FVB-young group, # $p < 0.05$ vs. FVB-old group.

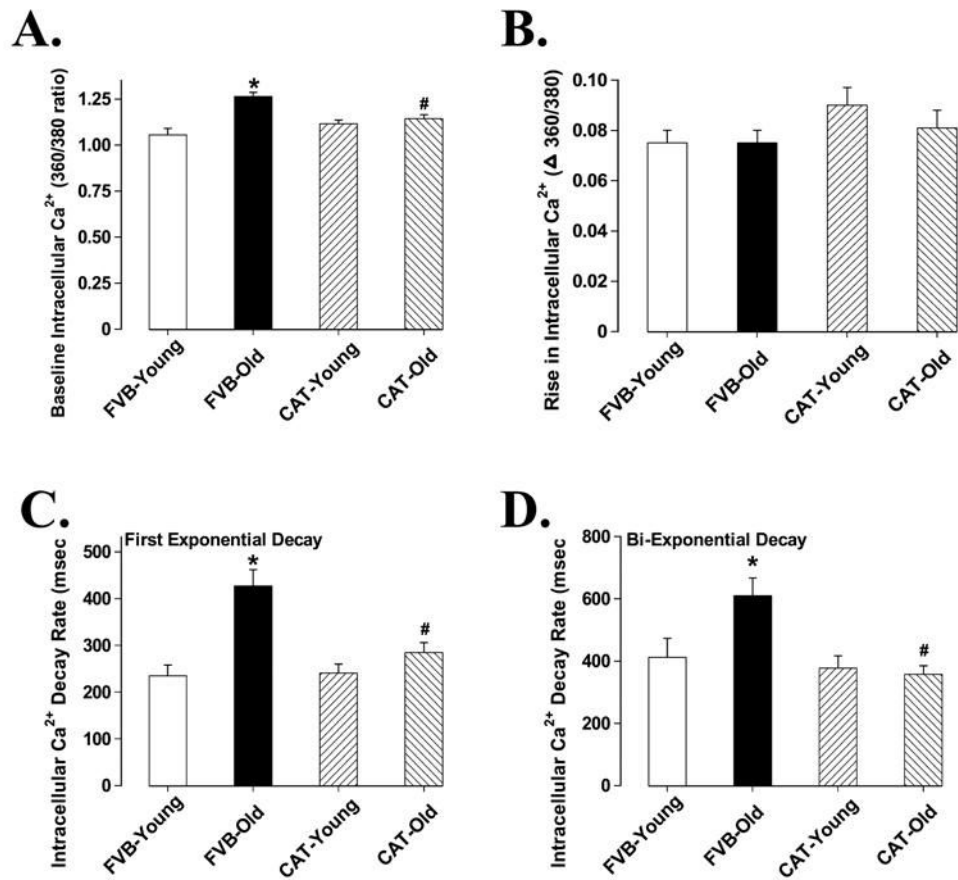


Fig 4. Intracellular Ca²⁺ properties of cardiomyocytes from male young and old FVB and catalase (CAT) mice. A: Baseline intracellular Ca²⁺ levels (360/380 ratio); B: Rise in intracellular Ca²⁺ levels in response to electrical stimulus (360/380 ratio); C: First exponential decay constant of intracellular Ca²⁺; and D: Bi-exponential decay constant of intracellular Ca²⁺. Mean ± SEM, n = 43 cells from 5 mice per group, * p < 0.05 vs. FVB-young group, # p < 0.05 vs. FVB-old group.

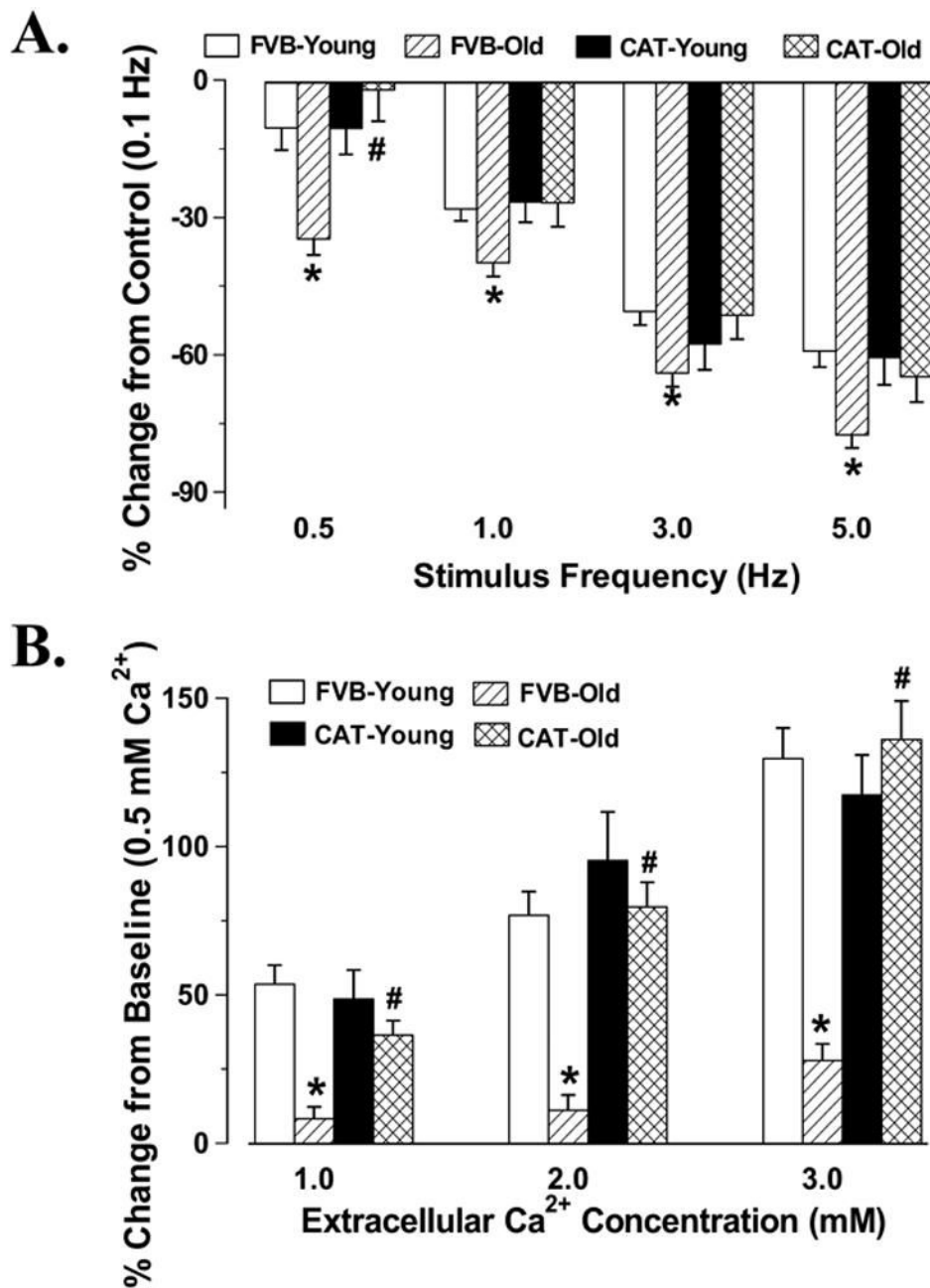


Fig. 5. Peak shortening (PS) amplitude of cardiomyocytes from male young or old FVB and catalase (CAT) mice at different stimulus frequencies (Panel A with an extracellular Ca²⁺ concentration of 1.0 mM) or different extracellular Ca²⁺ concentrations (Panel B with a stimulus frequency of 0.5 Hz). PS was shown as % change from PS value at respective baseline (0.1 Hz for panel A and 0.5 mM Ca²⁺ for panel B) of the same cell. Mean \pm SEM, n = 26 – 29 cells from 8 mice per group, * p < 0.05 vs. respective young group, # p < 0.05 vs. FVB-old group.

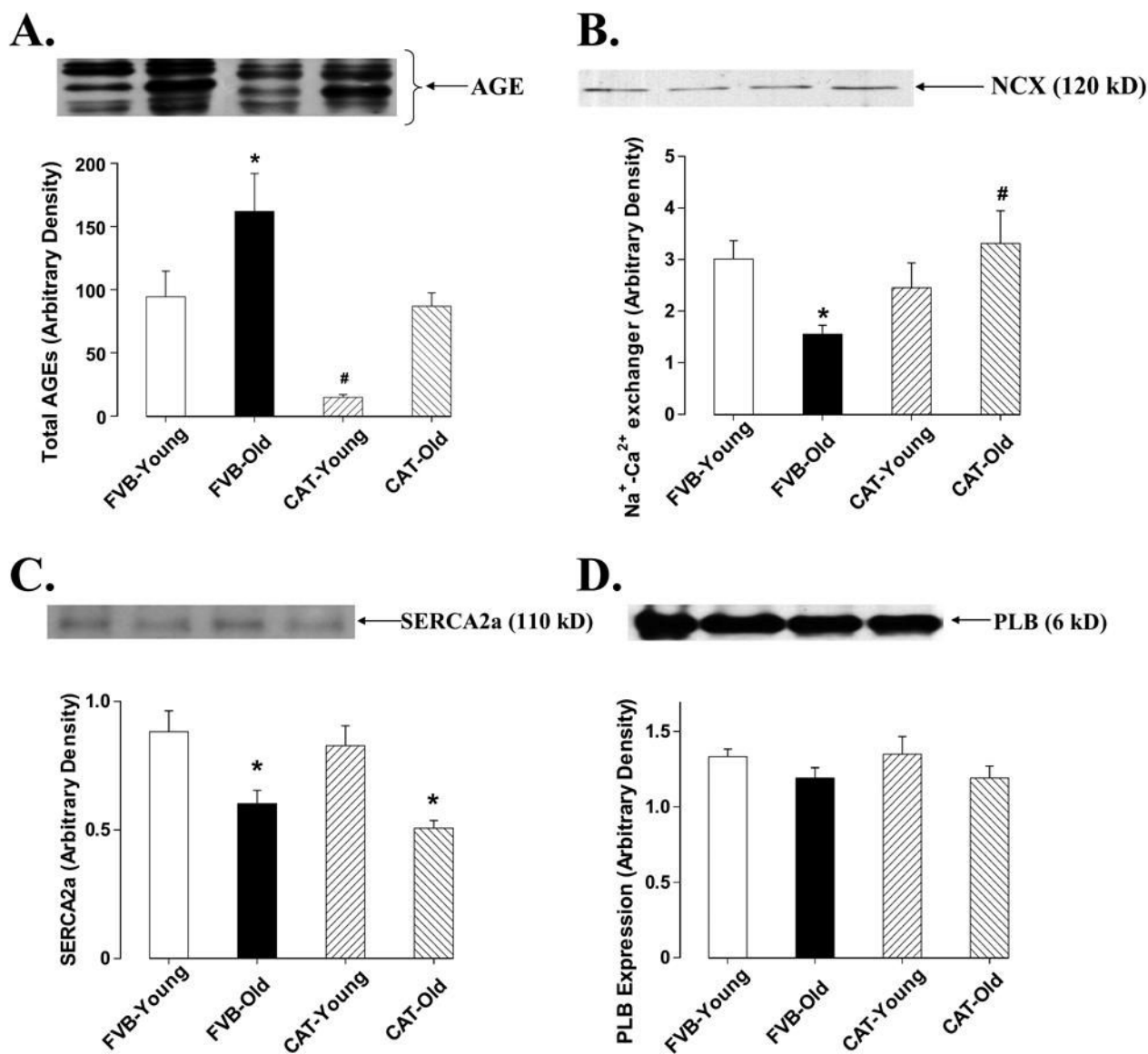


Fig 6. Western blot analysis of AGE, NCX, SERCA2a and PLB in male young or old FVB and catalase (CAT) transgenic mouse hearts. Insets: actual gel blots using specific anti-AGE, anti-NCX, anti-SERCA2a and anti-PLB antibodies. Mean \pm SEM, n = 9 – 13 samples per group, * p < 0.05 vs. FVB-young group, # p < 0.05 vs. FVB-old group.

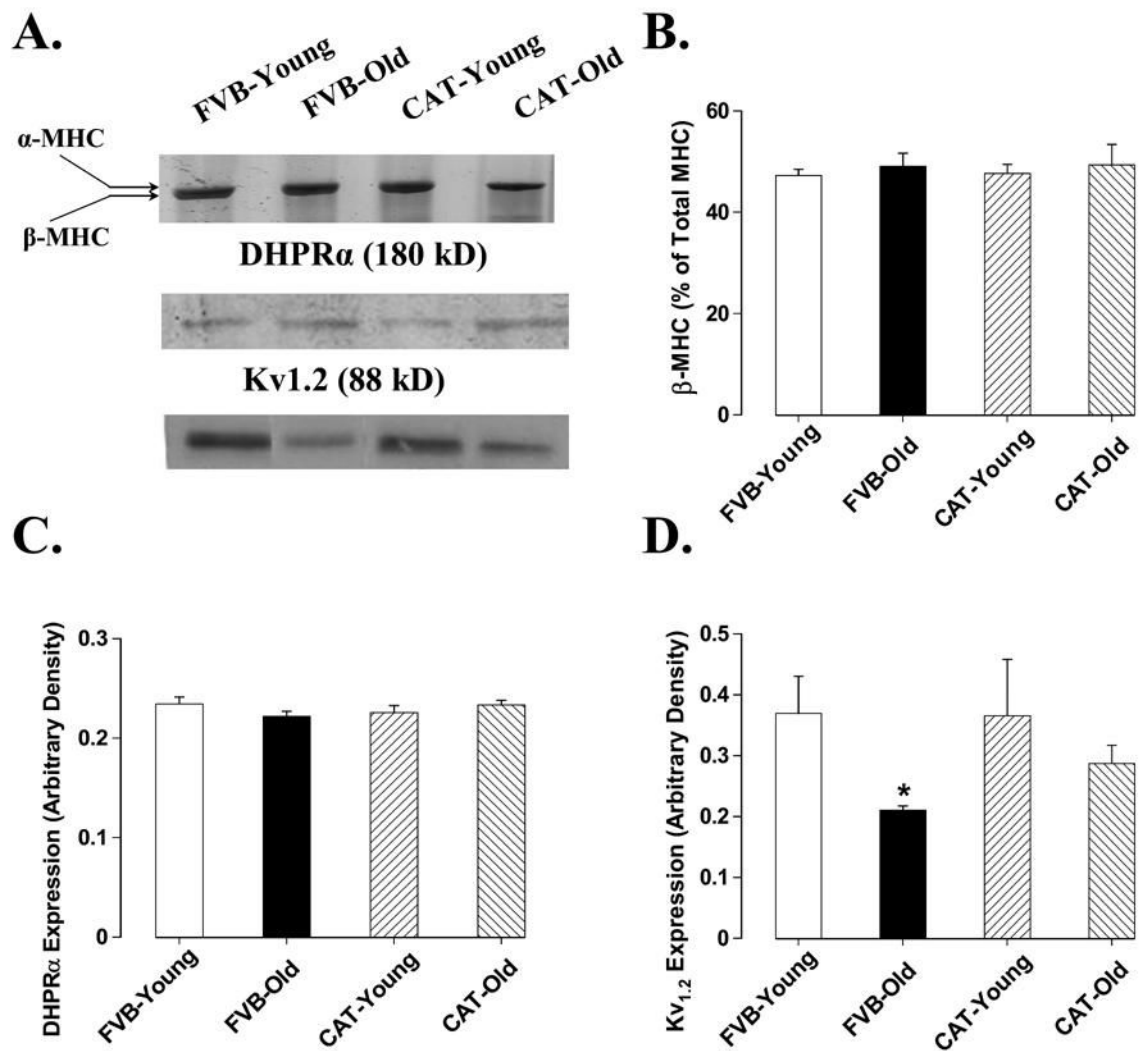


Fig 7. Western blot analysis of DHPR- α voltage-dependent Ca^{2+} channel, Kv1.2 potassium channel and myosin heavy chain (MHC) β -isozyme distribution in male young or old FVB and catalase (CAT) transgenic mouse hearts. Panel A: actual gel blots depicting protein expression using specific antibodies; Panel B-D: expression of DHPR- α , Kv1.2 and β -MHC. Mean \pm SEM, n = 4 – 5 samples per group, * p < 0.05 vs. FVB-young group, # p < 0.05 vs. FVB-old group.

Table 1

General features of young (3–4 month-old) and old (26–28 month-old) wild-type FVB and catalase (CAT) transgenic mice.

Mice group	FVB-Young	FVB-Old	CAT-Young	CAT-Old
Body weight (g)	20.4 ± 0.5	30.5 ± 0.9*	20.1 ± 0.5	31.1 ± 0.6*
Heart weight (mg)	106 ± 4	169 ± 8*	106 ± 3	160 ± 6*
Heart/Body weight (mg/g)	5.19 ± 0.11	5.55 ± 0.23	5.26 ± 0.07	5.14 ± 0.14
Fasting blood glucose (mM)	5.54 ± 0.31	5.71 ± 0.35	5.62 ± 0.37	5.43 ± 0.35

Mean ± SEM, n = 19 – 24 mice per group,

* p < 0.05 vs. FVB-young group.