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Influence of cardiac-specific overexpression of insulin-like growth factor 1 on lifespan and aging-associated changes in cardiac intracellular Ca^{2+} homeostasis, protein damage and apoptotic protein expression

Qun Li and Jun Ren

Division of Pharmaceutical Sciences & Center for Cardiovascular Research and Alternative Medicine, University of Wyoming, Laramie, WY 82071, USA

Summary

A fall in circulating levels of cardiac survival factor insulin-like growth factor 1 (IGF-1) contributes to cardiac aging. To better understand the role of IGF-1 in cardiac aging, we examined the influence of cardiac IGF-1 overexpression on lifespan, cardiomyocyte intracellular Ca^{2+} homeostasis, protein damage, apoptosis and expression of pro- and anti-apoptotic proteins in young and old mice. Mouse survival rate was constructed by the Kaplan–Meier curve. Intracellular Ca^{2+} was evaluated by fura-2 fluorescence. Protein damage was determined by protein carbonyl formation. Apoptosis was assessed by caspase-8 expression, caspase-3 and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay. Pro- and anti-apoptotic proteins including Bax, p53, pp53, Bcl2, Omi/HtrA2, apoptosis repressor with caspase recruitment domain (ARC) and X-linked inhibitor of apoptosis protein (XIAP) were assessed by Western blot. Aging decreased plasma in IGF-1 levels, elevated myocyte resting intracellular Ca^{2+} levels, reduced electrically stimulated rise in intracellular Ca^{2+} and delayed intracellular Ca^{2+} decay associated with enhanced protein carbonyl formation, caspase-8 expression and caspase-3 activity in FVB mice, all of which with the exception of elevated resting intracellular Ca^{2+} were attenuated by IGF-1. Aging up-regulated expression of Bax, Bcl2 and ARC, down-regulated XIAP expression and did not affect p53, pp53 and Omi/HtrA2. The IGF-1 transgene attenuated or nullified aging-induced changes in Bax, Bcl2 and XIAP. Our data suggest a beneficial role for IGF-1 in aging-induced survival, cardiac intracellular Ca^{2+} homeostasis, protein damage and apoptosis possibly related to pro- and anti-apoptotic proteins.

Key words: aging; cardiomyocytes; IGF-1; intracellular Ca^{2+}; pro- and anti-apoptotic protein.

Introduction

Cardiac aging is an irreversible biological process mainly manifested as abnormal cardiac contractility especially diastolic function in the elderly (Lakatta, 1999, 2002). It is believed that cardiac aging may be the ultimate consequence of a synergistic action of impaired intracellular Ca^{2+} homeostasis, altered membrane permeability and accumulation of reactive oxygen species (Lakatta, 1999, 2000, 2002). Although the precise mechanisms behind these aging-induced changes have not been defined, deficiency in the growth hormone (GH) and insulin-like growth factor 1 (IGF-1) axis with increased age has attracted much attention over recent years (Ceda *et al*., 2002, 2005; Yang *et al*., 2005). GH and IGF-1 are essential for the maintenance of bodily structure and function, the deficiency of which is associated with altered body composition, cytokine and neuroendocrine activation, cardiac atrophy and impaired cardiac function (Rincon *et al*., 2005). Aging-associated declines in circulating IGF-1 levels have been associated with neuronal aging, symptoms of neurodegeneration and cardiovascular diseases including compromised heart pumping and cardiomyocyte function (Ren & Brown-Borg, 2002; Rincon *et al*., 2005; Tang, 2006). IGF-1, the mediator of many of the effects of GH in peripheral tissues, improves myocardial function in the setting of both healthy and failing hearts (Ren *et al*., 1999). The therapeutic value of IGF-1 has been implicated in cardiac disorders related to, but not limited to, heart failure, myocardial infarction and diabetic cardiomyopathy (Ren *et al*., 1999). Nonetheless, the impact of IGF-1 levels on cardiac aging and lifespan has been controversial. Defects in IGF-1 receptor-associated signaling have been shown to significantly extend lifespan in models ranging from invertebrates to mice (Bluher *et al*., 2003; Brown-Borg, 2003). Surplus of GH and consequently IGF-1, on the other hand, appears to shorten lifespan although conflicting reports have also been seen (Brown-Borg, 2003; Laron, 2005). Therefore, the aim of our present study was to examine the influence of cardiac-specific overexpression of IGF-1 on lifespan, cardiac intracellular Ca^{2+} homeostasis, protein damage, apoptosis and expression of key pro- and anti-apoptotic proteins including p53 (and its activation), Bax, Bcl2, the serine protease Omi/HtrA2,

Correspondence
Prof. Jun Ren, Division of Pharmaceutical Science & Center for Cardiovascular Research and Alternative Medicine, University of Wyoming, Laramie, WY 82071, USA. Tel.: (307) 766 6131; fax: (307) 766 2953; e-mail: jren@uwyo.edu

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Table 1 General features of young and old FVB and IGF-1 mice

	FVB–young	FVB–old	IGF-1–young	IGF-1–old
BW (g)	20.1 ± 0.6	28.7 ± 0.8*	20.1 ± 1.2	29.3 ± 0.9*
HW (mg)	117 ± 5	161 ± 12*	121 ± 8	165 ± 8*
HW/BW (mg g^{-1})	5.89 ± 0.32	5.56 ± 0.36	6.08 ± 0.30	5.76 ± 0.39
LW (g)	0.95 ± 0.03	1.58 ± 0.07*	1.03 ± 0.04	1.50 ± 0.08*
LW/BW (mg g^{-1})	48.9 ± 2.1	54.8 ± 1.1	53.2 ± 2.3	51.0 ± 2.2
KW (g)	0.26 ± 0.01	0.44 ± 0.02*	0.24 ± 0.01	0.44 ± 0.02*
KW/BW (mg g^{-1})	12.8 ± 0.4	15.2 ± 0.5*	12.3 ± 0.3	15.2 ± 0.5*
Blood glucose (mg dL^{-1})	100.3 ± 5.3	101.1 ± 6.1	95.3 ± 7.2	103.6 ± 7.9
Plasma IGF-1 (ng mL^{-1})	97.9 ± 7.7	47.7 ± 4.9*	157.1 ± 5.2†	87.2 ± 6.8†

BW, body weight; HW, heart weight; LW, liver weight; KW, kidney weight; mean ± SEM, *$P < 0.05$ vs. corresponding young group, †$P < 0.05$ vs. corresponding FVB group, n = 14–16 mice per group.

apoptosis repressor with caspase recruitment domain (ARC) and X-linked inhibitor of apoptosis protein (XIAP). As an attempt is being made to delay cardiac aging and age-associated myocardial cell death via IGF-1 supplements, an understanding of the mechanistic basis of the apparent paradox between lifespan and cardiac health should be pertinent to the clinical application of IGF-1 to the elderly with compromised heart function.

Results

General feature of experimental animals

General features of young and aged male FVB and IGF-1 transgene mice are shown in Table 1. In young mice, transgenic IGF-1 overexpression did not elicit any notable effect on body, heart, liver and kidney weights compared with age-matched FVB mice. Elderly mice had heavier body and organ weights as well as enlarged size (organ weight normalized to body weight) of kidneys but not other organs compared with young counterparts. Aging significantly reduced plasma IGF-1 levels. The IGF-1 transgene elevated plasma IGF-1 levels in young mice and nullified aging-induced declines in plasma IGF-1 levels. Fasting blood glucose levels were not affected by either IGF-1 transgene or age. The Kaplan–Meier curve comparison depicts that IGF-1 transgenic mice display significantly better survival rates than FVB mice. The median lifespan was 724.0 and 890.5 days for FVB and IGF-1 mice, respectively ($P = 0.0262$). The survival curves of the two mouse lines begin to separate from each other after ~450 days of age with IGF-1 mice exhibiting a reduced mortality rate (Fig. 1). However, there was no significant difference between FVB and IGF-1 mice in the 'maximum lifespan' estimated by the proportion of mice alive at the 90th percentile for survival ($P = 0.15$, data not shown).

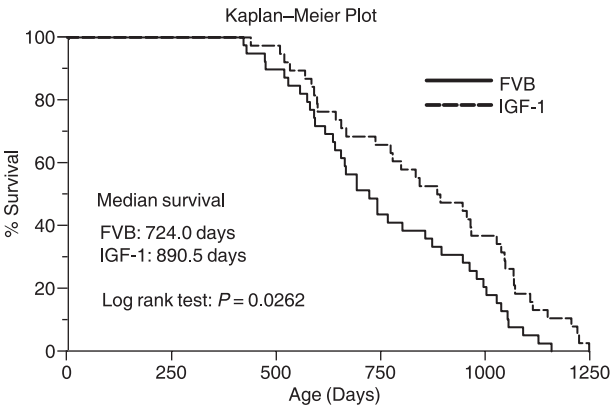

Fig. 1 Cumulative survival curve (Kaplan–Meier survival plot) of male FVB and IGF-1 mice. The cumulative survival rate was plotted against age in days. Log-rank test was performed to compare the FVB and IGF-1 lines ($P = 0.026$) (n = 39 and 38 mice for FVB and IGF-1 mice, respectively).

Cardiomyocyte intracellular CA^{2+} properties

Our fura-2 fluorescence recording revealed elevated resting intracellular Ca^{2+} levels, depressed electrically stimulated fura-2 fluorescent intensity increase (ΔFFI) and reduced intracellular Ca^{2+} clearing rates (both mono- and bi-exponential curve fit) in cardiomyocytes from aged FVB mice. The IGF-1 transgene abolished the aging-induced decrease in ΔFFI and prolongation in intracellular Ca^{2+} clearing without affecting aging-elicited elevation in resting intracellular Ca^{2+} levels. In fact, IGF-1 itself enhanced resting intracellular Ca^{2+} levels but did not affect any other fluorescence indices measured (Fig. 2).

Effect of IGF-1 and age on protein carbonyl formation and apoptosis

Results shown in Fig. 3 indicate that protein carbonyl formation, caspase-8 expression and caspase-3 activity, indicators for protein oxidative damage and apoptosis, were significantly elevated in myocardium from aged FVB mice. However, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) staining failed to reveal any significant increase in the TUNEL-positive nuclei in aged FVB mouse hearts. There was essentially no difference in the TUNEL-positive apoptotic cells among all four mouse groups tested. The IGF-1 transgene itself reduced protein carbonyl formation without affecting apoptosis in young mice. Interestingly, aging-induced elevation in cardiac protein

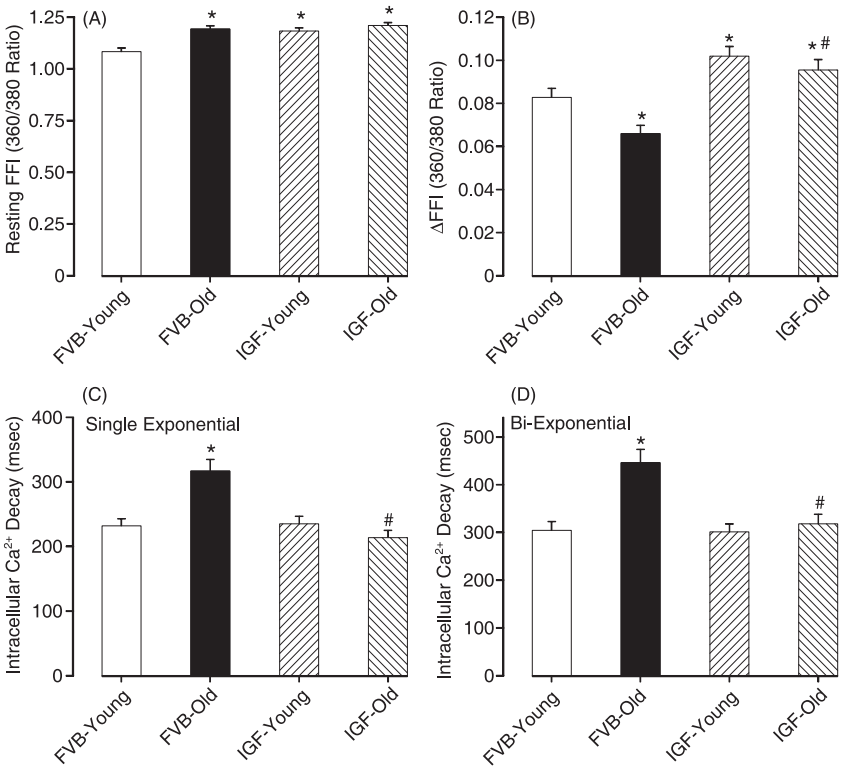

Fig. 2 Intracellular Ca^{2+} properties of cardiomyocytes from young or old FVB and IGF-1 mice. (A) Resting fura-2 fluorescence intensity (FFI); (B) electrically stimulated rise in FFI (ΔFFI); (C) mono-exponential intracellular Ca^{2+} transient decay rate; and (D) bi-exponential intracellular Ca^{2+} transient decay rate. Mean ± SEM, n = 61–68 cells per group, $*P < 0.05$ vs. FVB–young group, $\#P < 0.05$ vs. FVB–old group.

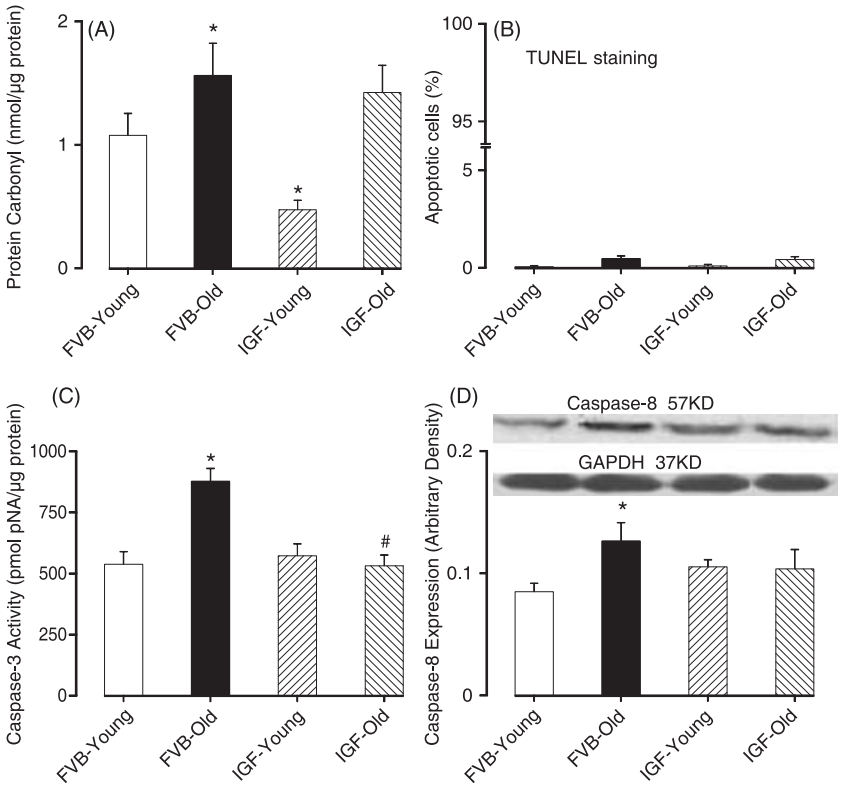

Fig. 3 Protein carbonyl formation (A), TUNEL staining (B), caspase-3 activity (C) and caspase-8 expression (D) in myocardium from young or old FVB and IGF-1 mice. Inset: representative gel blots depicting expression of caspase-8 and GAPDH (loading control) using specific antibodies. Mean ± SEM, n = 6–12 samples per group, $*P < 0.05$ vs. FVB young group, $\#P < 0.05$ vs. FVB–old group.

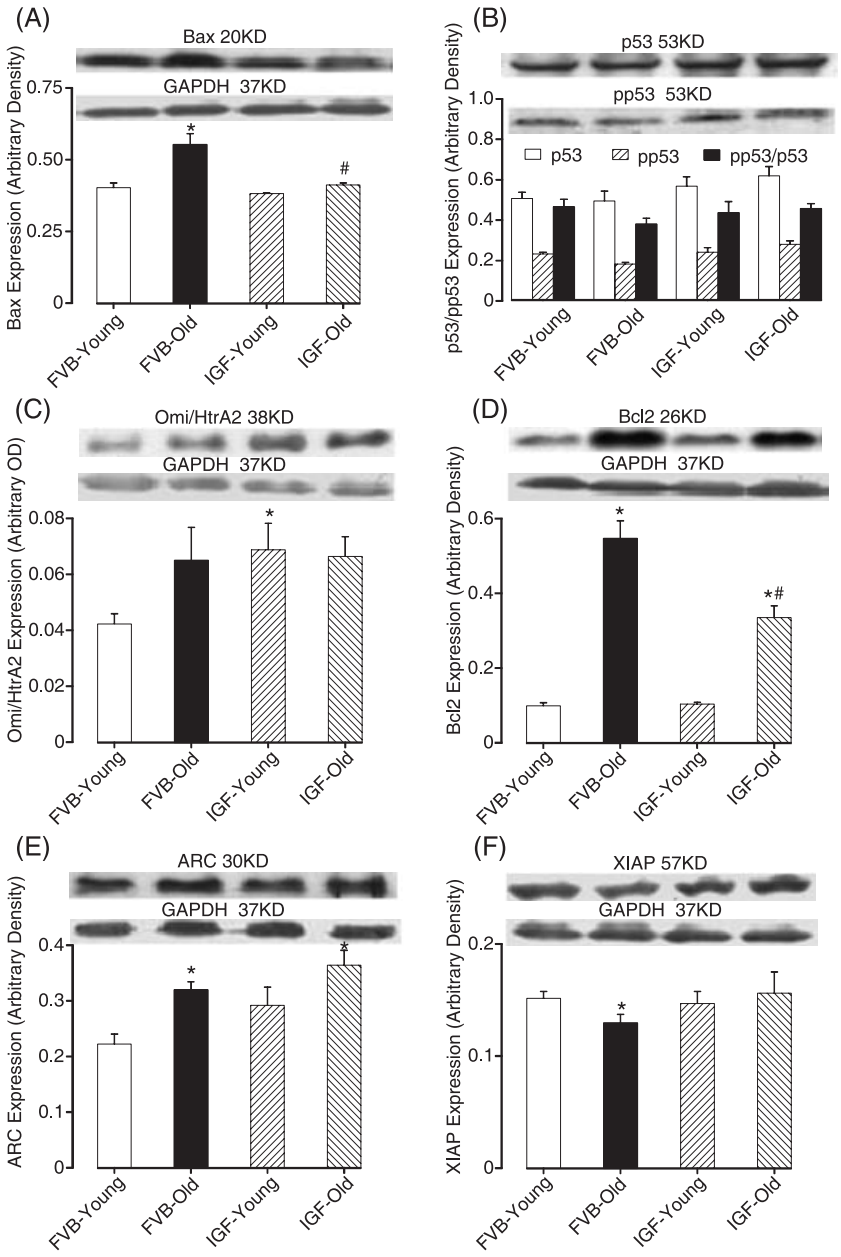

Fig. 4 Western blot analysis exhibiting expression of Bax (A), p53 and pp53 (B), Bcl2 (C), Omi/HtrA2 (D), ARC (E) and XIAP (F) in ventricles from young and old FVB and IGF-1 mice. Inset: gel blots depicting expression of above-mentioned proteins and GAPDH (loading control) using specific antibodies. Mean ± SEM, $n = 4–7$ samples per group, $*P < 0.05$ vs. FVB–young group, $\#P < 0.05$ vs. FVB–old group.

oxidative damage and apoptosis (caspase-8 and -3) were significantly attenuated by the IGF-1 transgene, suggesting the likelihood that IGF-1 offers cardiac protection by preventing protein oxidative damage and apoptosis.

Protein expression of pro- and anti-apoptotic proteins

Immunoblot data revealed significant elevations in the expression of Bax, Bcl2, ARC and a down-regulated XIAP expression in the aged FVB hearts. IGF-1 itself did not affect the expression of Bax, p53, pp53, Bcl2, ARC and XIAP, although it significantly up-regulated expression of the pro-apoptotic mitochondrial serine protease Omi/HtrA2 in young mice. The aging-induced up-regulation of ARC was not affected by IGF-1. Interestingly, IGF-1 alleviated aging-induced up-regulation of Bax and Bcl2, as well as down-regulation of XIAP (Fig. 4).

Discussion

Our present study revealed that cardiac-specific overexpression of IGF-1 significantly prolonged lifespan, rescued aging-induced intracellular Ca^{2+} homeostasis, protein damage and apoptosis in cardiomyocytes. The IGF-1-induced protection against cardiac aging may be associated with attenuation of aging-induced changes in Bax, Bcl2 and XIAP proteins. Furthermore, cardiac-specific IGF-1 overexpression attenuated aging-induced declines in plasma IGF-1 levels. As IGF-1 itself did not significantly affect intracellular Ca^{2+} homeostasis, tissue protein damage or apoptosis

in young mouse hearts, its beneficial role against aging-induced cardiac defect indicates clinical potential for this growth factor in delaying the cardiac aging process and minimizing senescence-associated high cardiovascular mortality.

Cardiac contractile dysfunction especially prolonged diastolic duration has been demonstrated in advanced age (Fleg *et al*., 1995; Lakatta, 1999; Li *et al*., 2005, 2007) in a manner similar to IGF-1 deficiency (Ren & Brown-Borg, 2002). Our current study revealed that aged cardiomyocytes displayed elevated resting intracellular Ca^{2+}, dampened intracellular Ca^{2+} release in response to electrical excitation and delayed intracellular Ca^{2+} clearance, indicating disrupted intracellular Ca^{2+} homeostasis especially Ca^{2+} resequestration. The IGF-1 transgene protected against aging-induced drops in intracellular Ca^{2+} release and intracellular Ca^{2+} clearance. The fact that IGF-1 itself did not affect these intracellular Ca^{2+} properties (with the exception of resting intracellular Ca^{2+} levels) in young mouse hearts indicates that this growth factor may not be innately harmful to cardiac intracellular Ca^{2+} handling. Several mechanisms may be proposed for the IGF-1-elicited beneficial effects against aging-induced intracellular Ca^{2+} dysregulation. First, our data revealed that IGF-1 is capable of alleviating aging-induced cardiac protein damage and apoptosis. Enhanced oxidative damage and apoptosis may directly interrupt cardiac intracellular Ca^{2+} homeostasis and excitation–contraction coupling (Chien, 1999; Goldhaber & Qayyum, 2000). Second, we found that IGF-1 significantly attenuated aging-induced changes in Bax, Bcl2 and XIAP, but not in p53, pp53 and Omi/HtrA2, indicating involvement of both pro- and anti-apoptotic proteins in IGF-1-induced protection. Generally speaking, few mouse deaths are attributable to heart failure. Thus, the preserved cardiac intracellular Ca^{2+} handling in aged IGF-1 hearts may not necessarily be the ultimate cause of improved lifespan in IGF-1 mice especially without any necropsy data. It is somewhat surprising that aging up-regulated the expression of the anti-apoptotic protein Bcl2. Although the effect of aging on cardiac Bcl2 regulation is largely unknown and neither an explanation for our puzzling observation is readily available, a number of reports have indicated that heart failure (which is often seen in aging) can lead to up-regulated Bcl2 levels, possibly as a compensatory mechanism against enhanced apoptosis (Latif *et al*., 2000; Moorjani *et al*., 2007). Furthermore, the balance between Bcl2 and Bax may be one of the ultimate deciding factors for cardiomyocyte apoptosis. With age, mitochondrial damage and dysfunction has been shown to trigger apoptosis with enhanced expression of the pro-apoptotic protein Bax (Pollack *et al*., 2002). It was demonstrated that IGF-1 protects against diabetes-induced cardiac pathology, contractile dysfunction and oxidative damage associated with antagonism of p53 activation (Kajstura *et al*., 2001). Our data support the notion of enhanced Bax with advanced age, but did not favor any role of p53, p53 activation and Omi/HtrA2 in IGF-1-elicited protection against cardiac aging. Although the mechanisms behind aging-induced elevation of anti-apoptotic proteins Bcl2 and ARC are not clear at this point, they may serve as compensatory mechanisms against elevated cardiomyocyte apoptosis. On the other hand, the inhibitors of the apoptosis protein (IAP) family including XIAP, which suppress apoptosis by inhibiting activation and activity of caspases, were shown to be down-regulated in advanced age (Gupta, 2004), which is consistent with our current experimental finding. The fact that IGF-1 may reconcile aging-induced changes in Bcl2 and XIAP suggests potential involvement of these anti-apoptotic proteins in aging-elicited cardiomyocyte apoptosis although further study is warranted. Last but not the least, our TUNEL-staining study revealed little apoptotic cell death despite an overt increase in capase-8/-3 expression/activity in aged hearts. This discrepancy demonstrates that aging may have initiated early stage of apoptosis (caspase activation) that has not yet resulted in global disruption of the normal nuclear chromatin structure of DNA.

IGF-1 participates in the regulation of tissue remodeling, glucose metabolism, insulin sensitivity, lipid profile, myocardial growth and myocardial function in both physiological and pathophysiological conditions (Ren *et al*., 1999; Delafontaine & Brink, 2000; Lombardi *et al*., 2000). A fall in the serum IGF-1 level, which often accompanies the biological aging process, leads to abnormal body composition and metabolism (Paolisso *et al*., 1997). The severely IGF-1-deficient Ames dwarf mice with a prolonged lifespan displayed compromised cardiac excitation–contraction coupling in cardiomyocytes (Ren & Brown-Borg, 2002). Similarly, patients with IGF-1 deficiency exhibit cardiac dysfunctions reminiscent of aging, which is mainly manifested as reduced left ventricular mass, ejection fraction and diastolic filling. Data from our present study suggest that the beneficial effect of IGF-1 on cardiac aging may be mediated through restoration of plasma IGF-1 levels in aged mice. Data from the Jackson Laboratory revealed median lifespan of 760 days for FVB females and 598 days for FVB males. Our study observed a median lifespan of 724 days in FVB males. The discrepancy between our data and those from the Jackson Laboratory may be due to sample size and altitude (our university is 7200 feet above sea level). IGF-1 transgenic mice displayed a prolonged median but not maximum lifespan. This observation appears to be contradicted with the notion that IGF-1 deficiency or interruption of IGF-1 receptor signaling prolongs lifespan (Bluher *et al*., 2003; Brown-Borg, 2003). Nonetheless, our finding is supported by some recent observations that untreated patients with GH deficiency due to GH gene deletion or isolated IGF-I deficiency due to deletions or mutations of the GH receptor gene (Laron syndrome) displayed early signs of aging (wrinkled skin, obesity, insulin resistance and osteopenia), despite a long lifespan reaching ages of 80–90 years. This is supported by longer lifespan in animal models with genetic GH deficiencies such as Snell mice (Pit-1 gene mutations), the Ames mice (PROP-1 gene mutation) and the Laron mice (GH receptor gene knock-out). To the contrary, GH overexpressing transgenic mice and acromegaly patients secreting high amounts of GH show premature death (Laron, 2005). In addition to the effect of GH/IGF-1 on lifespan, data from our laboratory as well as others have depicted compromised cardiac function in IGF-1 deficiency and hypercontractile state of the heart with GH surplus

(Lombardi *et al*., 2000; Colligan *et al*., 2002; Ren & Brown-Borg, 2002). These observations led to trials with GH or IGF-1 treatment in the elderly and the establishment of the so-called 'rejuvenation clinics' (Rudman *et al*., 1990). The cardiac-specific IGF-1 transgenic mice have higher circulating levels of IGF-1 (GH levels are not available at this time), although we do not know if the elevated IGF-1 levels are solely from cardiomyocytes. Whether any IGF-1 transgenic modification-induced nonspecific alteration contributes to lifespan independent of serum IGF-1 levels is unknown and may not be excluded. Given the controversial findings between heart function and longevity at various GH/IGF-1 levels, the jury is still out on whether the seemingly compromised cardiac function under IGF-1 deficiency is indeed 'detrimental' to cardiac health and the ultimate longevity (Anversa, 2005).

In conclusion, our study revealed that cardiac-specific overexpression of IGF-1 rescues aging-induced intracellular Ca^{2+} homeostasis possibly through protected protein damage and apoptosis. Our data also revealed potential involvement of anti-apoptotic proteins in IGF-1-elicited cardioprotective effects against cardiac aging. These data should shed some light on the clinical application of IGF-1 in the treatment of aging-associated cardiac dysfunction.

Experimental procedures

Experimental animals

All animal procedures used in this study were approved by the Animal Care and Use Committees at the University of Wyoming (Laramie, WY, USA). Male FVB and IGF-1 heterozygous transgenic mice at young (3–4 months old) and old (26–28 months old) ages were used. The pigmentation of fur color was used as a marker for heterozygous IGF-1 (light brown) or wild-type FVB (white) mouse identification as described by Reiss *et al*. (1996). All animals were kept in our institutional animal facility with free access to standard laboratory chow and tap water. Serological test verified that the research mice housed in our animal facility to be specific pathogen free (SPF) including parvo virus, hepatitis and *Helicobacter*. All mice used for the lifespan analysis (the Kaplan–Meier survival curve and log-rank test) were assigned to a longevity cohort at birth and were not used for any biochemical, immunoblotting or intracellular Ca^{2+} transient tests. Maximum lifespan was estimated by the proportion of mice alive at the 90th percentile for survival as reported previously by Wang *et al*. (2004). Only male mice were used for this study. At the time of sacrifice, fasting blood glucose and plasma IGF-1 levels were measured using a glucose monitor (Accu-ChekII, model 792; Boehringer Mannheim Diagnostics, Indianapolis, IN, USA) and an enzyme-linked immunosorbent assay (ELISA) commercial kit from Diagnostic System Laboratory (Webster, TX, USA), respectively.

Isolation of mouse cardiomyocytes

Hearts were rapidly removed from anesthetized mice (ketamine : xylazine 5 : 2, 1.32 mg kg^{-1}, i.p.) and mounted onto a temperature-controlled (37 °C) Langendorff system. After perfusing with a modified Tyrode solution (Ca^{2+} free) for 2 min, the heart was digested for about 10 min with 0.9 mg mL^{-1} collagenase D in the modified Tyrode solution. The modified Tyrode solution (pH 7.4) contained the following (in mM): NaCl 135, KCl 4.0, $MgCl_2$ 1.0, HEPES 10, NaH_2PO_4 0.33, glucose 10, butanedione monoxime 10, and the solution was gassed with 5% CO_2–95% O_2. The digested heart was then removed from the cannula and the left ventricle was cut into small pieces in the modified Tyrode solution. Tissue pieces were gently agitated and the pellet of cells was resuspended. Extracellular Ca^{2+} was added incrementally back to 1.20 mM over a period of 30 min. Isolated myocytes were used for experiments within 8 h of isolation. Only rod-shaped myocytes with clear edges were selected for mechanical and intracellular Ca^{2+} studies (Li *et al*., 2007).

Intracellular CA^{2+} transient measurement

Murine myocytes were loaded with fura-2/AM (0.5 μM) for 10 min and intracellular Ca^{2+} fluorescence measurements were recorded with a dual-excitation fluorescence photomultiplier system (IonOptix Corporation, Milton, MA, USA). Myocytes were placed on an Olympus IX-70 inverted microscope and imaged through a Fluor ×40 oil objective. Cells were exposed to light emitted by a 75-W lamp and passed through either a 360- or 380-nm filter (bandwidths were ± 15 nm), 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 s 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 the fura-2 fluorescence intensity (FFI) at two wavelengths. Fluorescence decay time (both mono- and bi-exponential decay rates) was also measured as an indication of intracellular Ca^{2+} clearing rate (Norby *et al*., 2004).

Protein carbonyl assay

The carbonyl content of protein was determined as described by Dong *et al*. (2006). Briefly, proteins were extracted and minced to prevent proteolytic degradation. Nucleic acids were eliminated by treating the samples with 1% streptomycin sulfate for 15 min, followed by a 10-min centrifugation (11 000 ***g***). Protein was precipitated by adding an equal volume of 20% trichloroacetic acid (TCA) to protein (0.5 mg) and centrifuged for 1 min. The TCA solution was removed and the sample resuspended in 10 mM 2,4-dinitrophenylhydrazine (2,4-DNPH) solution. Samples were incubated at room temperature for 15–30 min. Following a 500 μL of 20% TCA addition, samples were centrifuged for 3 min. The supernatant was discarded, the pellet was washed in ethanol : ethyl acetate and allowed to incubate at room temperature for 10 min. The samples were centrifuged again for 3 min and the ethanol : ethyl acetate steps repeated two more times. The precipitate was resuspended in 6 M guanidine

solution, centrifuged for 3 min and insoluble debris was removed. The maximum absorbance (360–390 nm) of the supernatant was read against appropriate blanks (water, 2 M HCl) and the carbonyl content was calculated using the molar absorption coefficient of 22 000 M^{-1} cm^{-1}.

Caspase-3 assay

Caspase-3 is an enzyme activated during induction of apoptosis. The caspase-3 activity was determined according to our previously published method (Relling *et al*., 2006). Briefly, 1 mL of phosphate-buffered saline (PBS) was added to ventricular or brain tissues. Tissues were homogenized and centrifuged at 10 000 ***g*** at 4 °C for 10 min. The supernatant was discarded, and pellets were lysed in 100 μL of ice-cold lysis buffer [50 mM HEPES, pH 7.4, 0.1% CHAPS, 1 mM dithiothreitol (DTT), 0.1 mM EDTA and 0.1% NP40]. The assay for caspase-3 activity was carried out in a 96-well plate. Each well contained 30 μL of lysate, 70 μL of assay buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 100 mM NaCl, 10 mM DTT and 1 mM EDTA) and 20 μL of caspase-3 colorimetric substrate Ac-DEVD-pNA (Sigma Chemicals, St. Louis, MO, USA). The 96-well plate was incubated at 37 °C for 2 h, during which caspase in the sample was allowed to cleave the chromophore p-NA from the substrate molecule. Absorbance readings were obtained at 405 nm with the caspase-3 activity being directly proportional to the colorimetric reaction. Protein content was determined using the Bradford method (1976).

Tunel staining

TUNEL assessment of myonuclei positive for DNA strand breaks was determined using a fluorescence detection kit (Roche Applied Science, Indianapolis, IN, USA) and fluorescence microscopy. Cross-sections (5 μM) were placed in a cryostat (–23 °C) and then fixed in 4% paraformaldehyde for 20 min at room temperature. Sections were then permeabilized with 0.1% Triton-X-100 in 0.1% sodium citrate for 2 min on ice. TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT), fluorescein-dUTP was added to the sections in 50 μL drops and incubated for 60 min at 37 °C in a humidified chamber in the dark. The sections were rinsed three times in PBS for 5 min each. Following embedding, sections were visualized with an Olympus BX-51 microscope equipped with an Olympus MaguaFire SP digital camera. DNase I and label solution were used as positive and negative controls. To determine the percentage of apoptotic cells, the TUNEL-positive nuclei and TUNEL-negative cells were counted using the ImagePro image analysis software (Media Cybernetics, Bethesda, MD, USA) (Sgonc *et al*., 1994).

Western blot analysis

Protein levels of ARC, Bax, Bcl2, caspase 8, Omi/HtrA2, p53 and HILP (XIAP) were examined by Western blot. Left ventricular tissues were homogenized and centrifuged at 70 000 ***g*** for 20 min at 4 °C. The supernatants were used for immunoblotting of ARC, Bax, Bcl2, Omi/HtrA2, p53, phospho-p53 and HILP (XIAP). The extracted proteins in supernatants were separated on 10–15% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose blotting membranes. After blocking the membrane was incubated with rabbit anti-ARC polyclonal (1 : 1000; Santa Cruz, Santa Cruz, CA, USA), rabbit anti-Bax monoclonal (1 : 1000; Cell Signaling, Beverly, MA, USA), mouse anti-Bcl2 monoclonal (1 : 1000; Santa Cruz), rabbit anti-caspase-8 polyclonal (1 : 1000; Cell Signaling), rabbit anti-HtrA2/Omi monoclonal (1 : 1000, Abcam Inc., Cambridge, MA, USA), mouse anti-p53 monoclonal (1 : 1000; Oncogene, Cambridge, MA, USA), rabbit anti-phospho-p53 polyclonal (Ser392, 1 : 1000; Cell Signaling, Danvers, MA, USA) and mouse anti-HILP (XIAP) antibodies (1 : 1000; BD Biosciences, San Jose, CA, USA) overnight at 4 °C followed by incubation with secondary antibodies. The antigens were detected by the luminescence method. Quantification of band density was determined using Quantity One software (version 4.4.0, build 36; Bio-Rad, Hercules, CA, USA) and reported in optical density per square millimeter (Li *et al.*, 2007). For all Western blot analysis, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1 : 1000, Cell Signaling) was used as the loading control.

Statistical analyses

Data were presented as mean ± SEM. The 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.

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