

Metallothionein antagonizes aging-induced cardiac contractile dysfunction: role of PTP1B, insulin receptor tyrosine phosphorylation and Akt

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Summary

Aging is often accompanied by reduced insulin sensitivity and cardiac dysfunction. However, the causal relationship between the two remains poorly understood. This study was designed to determine the impact of cardiac-specific overexpression of antioxidant metallothionein (MT) on aging-associated cardiac dysfunction and impaired insulin signaling. Contractile and intracellular Ca^{2+} properties were evaluated in left ventricular myocytes including peak shortening (PS), maximal velocity of shortening/relengthening (\pm dL/dt), time-to-PS (TPS), time-to-90% relengthening (TR_{90}), fura-2 fluorescence intensity change (ΔFFI) and intracellular Ca^{2+} decay rate. Expression of insulin receptor, protein-tyrosine phosphatase 1B (PTP1B), phosphorylation of insulin receptor (Tyr1146) and Akt were evaluated by Western blot analysis. Aged wild-type FVB and MT transgenic mice (26–28 months old) displayed glucose intolerance and hyperinsulinemia. Cardiomyocytes from aged FVB mice exhibited prolonged TR_{90} and intracellular Ca^{2+} decay associated with normal PS, \pm dL/dt, TPS and ΔFFI compared with those from young (2–3 months old) mice. Western blot analysis revealed reduced Akt expression and insulin (5 mU g^{-1})-stimulated Akt phosphorylation, elevated PTP1B expression and diminished basal insulin receptor tyrosine phosphorylation associated with comparable insulin receptor expression in aged FVB mouse hearts. All of these aging-related defects in cardiac contractile function and insulin signaling (although not hyperinsulinemia and glucose intolerance) were significantly attenuated or ablated by MT transgene. These data indicate that enhanced antioxidant defense is beneficial for aging-induced cardiac contractile dysfunction and alteration in insulin signaling.

Key words: antioxidant; insulin resistance; intracellular Ca^{2+} transients; myocyte; shortening.

Introduction

Cardiac function declines with advanced age, which may account for a major cause of death in the elderly (Lakatta, 1999; Yang *et al.*, 2005). Although interactions among senescence, occult disease and physical inactivity may trigger senescence-related changes in cardiovascular function, the 'aging process' per se is a unique determinant of cardiac morphology and contractile performance and is thus considered as an independent risk factor for cardiovascular diseases (Lakatta, 1999). Several mechanisms have been speculated for the development of cardiac aging including action potential prolongation, myosin heavy chain isozyme switch and free radical accumulation, all of which lead to dysregulation of intracellular Ca^{2+} homeostasis and excitation-contraction (E-C) coupling in aged hearts (Lakatta, 1999; Kass *et al.*, 2001; Yang *et al.*, 2005). Furthermore, clinical and experimental evidence has shown a prevalence of insulin resistance and metabolic syndrome in the pathogenesis of cardiac aging (Kamide *et al.*, 1997; Desrois *et al.*, 2004). Compromised cardiac function is often associated with insulin resistance and is characterized by impaired cardiac efficiency, reduced ventricular function and coronary heart disease (Dutta *et al.*, 2001; Hintz & Ren, 2002; Mazumder *et al.*, 2004; Fang *et al.*, 2005). However, the link between insulin resistance and aging-associated cardiac contractile defects has not been precisely defined. The aim of the present study was to examine the impact of cardiac-specific overexpression of metallothionein, a low molecular weight heavy metal scavenger, on cardiomyocyte contractile function and insulin signaling in advanced age. Using state-of-the-art techniques, we evaluated single cardiomyocyte contractile function and expression of insulin receptor, tyrosine phosphorylation of insulin receptor, as well as postreceptor signal molecules including the serine/threonine protein kinase Akt and protein-tyrosine phosphatase 1B (PTP1B) in young and aged wild-type (FVB) and metallothionein transgenic mouse hearts.

Results

Experimental animals

There is no overt difference in body, heart, liver and kidney weights between age-matched FVB and metallothionein mice. The body and organ weights were significantly heavier in aged mice compared with young ones, as expected. Heart size

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Table 1 General features of young and old FVB and metallothionein (MT) mice

	FVB-young	FVB-old	MT-young	MT-old
Body weight (g)	15.8 ± 0.9	30.7 ± 0.5*	15.1 ± 0.8	28.9 ± 0.6*
Heart weight (mg)	82 ± 5	159 ± 4*	83 ± 5	150 ± 4*
HW/BW (mg g ⁻¹)	5.30 ± 0.12	5.16 ± 0.10	5.57 ± 0.12	5.21 ± 0.11
Liver weight (g)	0.76 ± 0.06	1.60 ± 0.05*	0.70 ± 0.05	1.46 ± 0.05*
LW/BW (mg g ⁻¹)	46.4 ± 1.1	52.1 ± 1.3*	45.5 ± 1.3	50.3 ± 0.9*
Kidney weight (g)	0.21 ± 0.02	0.49 ± 0.02*	0.20 ± 0.01	0.42 ± 0.02*
KW/BW (mg g ⁻¹)	12.9 ± 0.3	16.0 ± 0.3*	13.0 ± 0.4	14.6 ± 0.3*
Insulin (ng mL ⁻¹)	0.098 ± 0.015	0.274 ± 0.045*	0.106 ± 0.018	0.297 ± 0.072*

HW, heart weight; LW, liver weight; KW, kidney weight; Mean ± SEM, **P* < 0.05 vs. corresponding young mouse group, *n* = 40–54 mice per group.

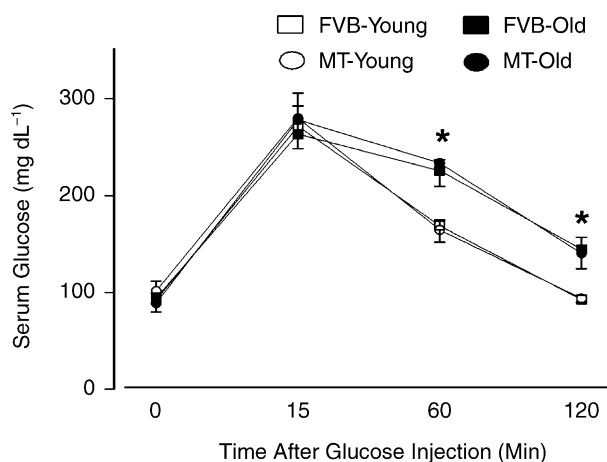


Fig. 1 Intraperitoneal glucose tolerance test (IPGTT) displaying serum glucose concentrations in response to intraperitoneal glucose challenge (2 g glucose per kg body weight) in young or old FVB and metallothionein (MT) mice. The mice were fasted for 12 h before tests were conducted. Mean ± SEM, *n* = 8–10 mice per group, **P* < 0.05 between young and old groups.

(normalized to body weight) was similar between young and aged mice whereas the size of liver and kidney was significantly enlarged in old mice. Plasma levels of insulin were significantly elevated in aged mice compared with those of young mice (Table 1). Following acute intraperitoneal glucose challenge (2 g kg⁻¹ body weight), the plasma glucose levels in young FVB or MT mice started to decline after peaking at 15 min. The plasma blood glucose levels in young mice returned to near baseline value after 120 min. However, the postchallenge glucose levels remained at much higher levels between 15 and 120 min in aged FVB or MT mice (Fig. 1), indicating glucose intolerance and insulin resistance in FVB or MT mice at old age. There was no difference in basal fasting glucose levels among all four mouse groups tested, excluding potential contribution of full-blown diabetes mellitus.

Mechanical and fluorescent properties of myocytes from young and old mice

The resting cell length was similar in myocytes from young and old FVB and metallothionein groups. Representative traces

shown in Fig. 2 depict contractile profiles of myocytes isolated from young and old FVB and metallothionein mice. Peak shortening amplitude (PS), maximal velocity of shortening and relengthening (\pm dL/dt) as well as duration of shortening (TPS) were comparable between myocytes from young and aged mice, regardless of FVB or metallothionein strain. Myocytes from aged FVB mice displayed significantly prolonged TR₉₀ compared with those from young FVB mice, suggesting aging-related diastolic dysfunction. Interestingly, metallothionein transgene effectively protected ventricular myocytes from aging-induced diastolic prolongation (Fig. 2). Further experiments using the intracellular fluorescence dye fura-2 revealed reduced intracellular Ca²⁺ clearing rate associated with normal baseline intracellular Ca²⁺ levels (resting FFI) and electrically stimulated fura-2 fluorescence intensity rise (Δ FFI) in aged FVB myocytes, consistent with the observation of prolonged TR₉₀ and normal PS in these myocytes. Similar to its effect on cell shortening, metallothionein abolished aging-induced prolongation in intracellular Ca²⁺ clearing without affecting resting FFI and Δ FFI (Fig. 3).

Effect of increasing stimulation frequency on myocyte shortening

Rodent hearts normally contract at high frequencies, whereas our mechanical recording was performed at 0.5 Hz. To evaluate the impact of aging on cardiac contractile function under higher frequencies, we increased the stimulus frequency to 5.0 Hz (300 beats min⁻¹) 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. Figure 4 shows a steeper negative staircase of PS with increased stimulus frequency in aged FVB myocytes compared with young FVB myocytes. The percentage decrease of PS from 0.1 Hz was significantly greater in aged FVB myocytes than young counterparts at higher frequencies (3.0 Hz and 5.0 Hz), suggesting reduced intracellular Ca²⁺ cycling ability or stress tolerance with aging. Consistent with its effect on mechanical function and intracellular Ca²⁺ transients, metallothionein transgene effectively prevented high stimulus frequency-induced reduction in myocyte shortening capacity at old age.

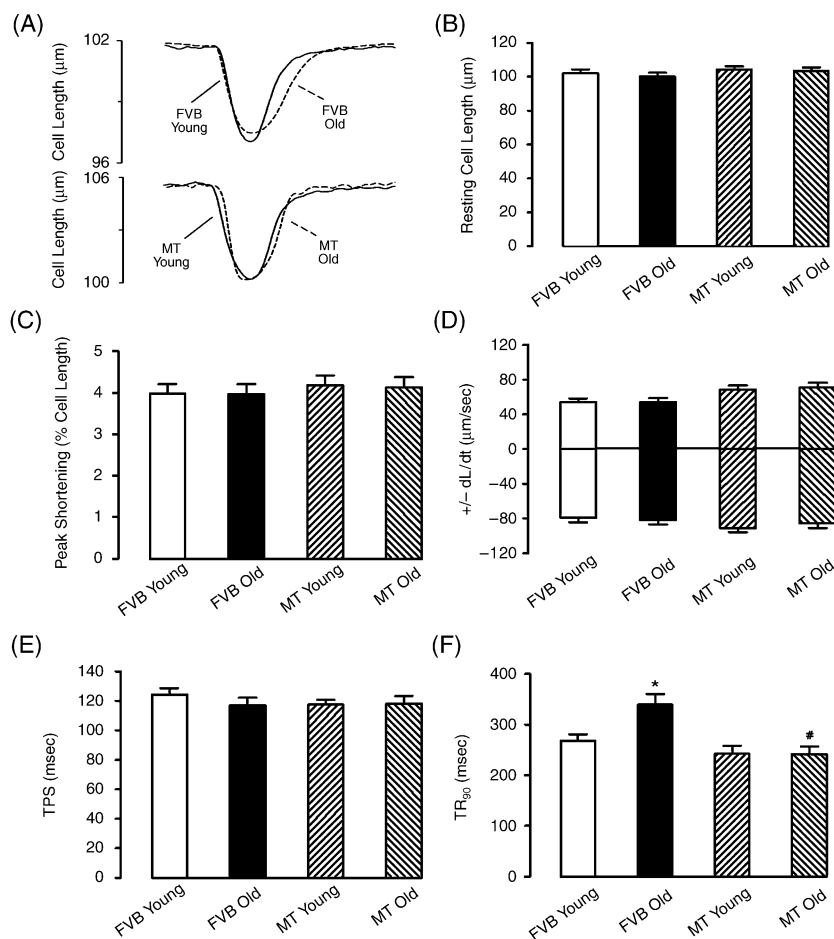


Fig. 2 Contractile properties of cardiomyocytes from young or old FVB and metallothionein (MT) mouse hearts. (A) Representative traces depicting cell-shortening profile in each of the four groups; (B) resting cell length; (C) peak shortening (PS, normalized to CL); (D) maximal velocity of shortening (+dL/dt) and relengthening (-dL/dt); (E) time-to-90% PS (TPS); and (F) time-to-90% relengthening (TR₉₀). Mean ± SEM, $n = 124$ cells per group, * $P < 0.05$ vs. FVB-young group, # $P < 0.05$ vs. FVB-old group.

Protein expression of Akt, pAkt, insulin receptor β , insulin receptor tyrosine phosphorylation and PTP1B

Aging is often associated with reduced insulin signaling (Iossa *et al.*, 2004; Couzin, 2005), which is supported by our observation of glucose intolerance (Fig. 1). To causally correlate cardiomyocyte mechanical function with insulin signaling, we examined the effect of aging and metallothionein transgene on cardiac expression of several essential insulin signaling molecules at both receptor and postreceptor levels including insulin receptor β , insulin receptor tyrosine phosphorylation, Akt, pAkt and PTP1B. Our results indicated that aging reduced total Akt protein expression in the hearts under either basal or insulin-stimulated (5 mU g^{-1} body weight for 10 min) conditions. While aging did not affect basal or 'tonic' phosphorylation of Akt in the absence of insulin, it significantly reduced insulin-stimulated Akt phosphorylation. Acute insulin stimulation significantly enhanced Akt phosphorylation (pAkt) in young FVB mice. However, the fold increase in pAkt was significantly reduced in the aged FVB group. Interestingly, metallothionein ablated aging-induced changes in total Akt expression and insulin-stimulated Akt phosphorylation without affecting those in young hearts (Fig. 5). Further study revealed that aging significantly up-

regulated expression of PTP1B and reduced basal or 'tonic' insulin receptor tyrosine phosphorylation without affecting insulin receptor β and insulin-stimulated tyrosine phosphorylation. Metallothionein transgene reconciled aging-induced up-regulation of PTP1B and down-regulation in basal or tonic insulin receptor tyrosine phosphorylation. Metallothionein transgene did not affect expression of PTP1B, insulin receptor β and insulin receptor tyrosine phosphorylation in young murine hearts (Fig. 6).

Discussion

The major findings of our present study are that advanced aging elicits glucose intolerance, hyperinsulinemia, cardiac contractile dysfunction and impaired intracellular Ca^{2+} handling. The aging-induced cardiac contractile and intracellular Ca^{2+} dysfunctions were causally associated with reduced total Akt level and insulin-stimulated Akt phosphorylation, diminished tonic insulin receptor tyrosine phosphorylation and increased levels of the negative regulator of insulin signaling PTP1B. Interestingly, these aging-associated alterations in contractile function (at both low and high stimulus frequencies) and insulin signaling were either significantly attenuated or abolished by cardiac overexpression of the antioxidant metallothionein. Since metallothionein transgene

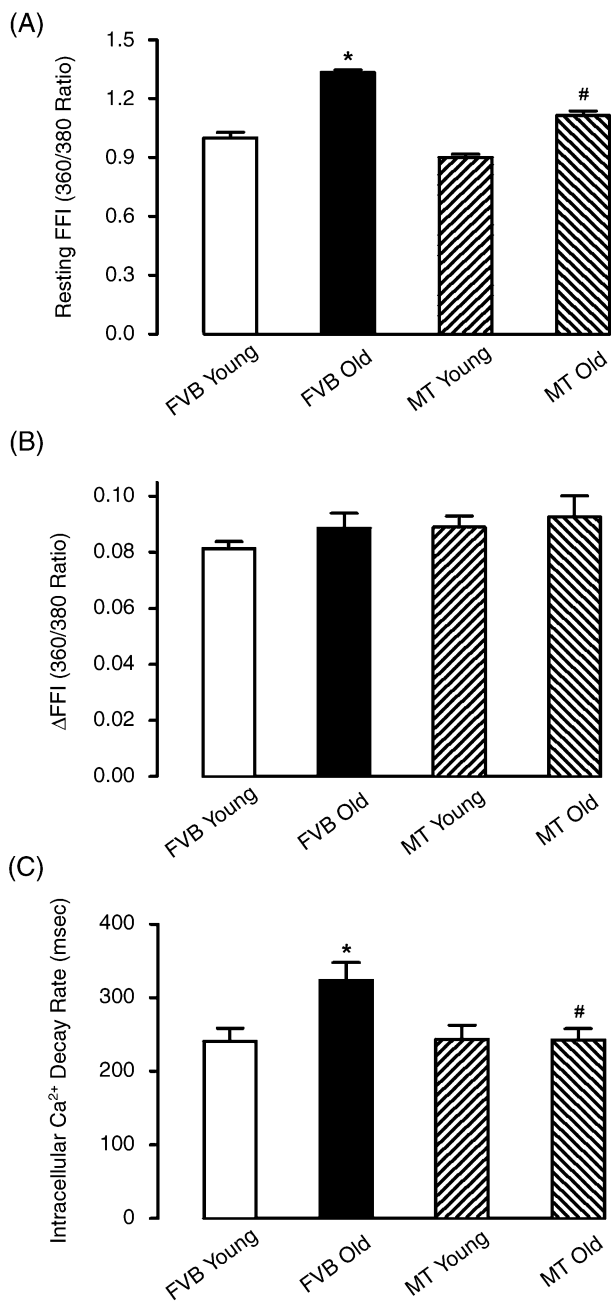


Fig. 3 Intracellular Ca^{2+} transient properties in cardiomyocytes from young or old FVB and metallothionein (MT) mice. (A) Resting fura-2 fluorescence intensity (FFI); (B) electrically stimulated increase in FFI (ΔFFI); and (C) intracellular Ca^{2+} transient decay rate. Mean \pm SEM, $n = 79$ cells per group, * $P < 0.05$ vs. FVB-young group, # $P < 0.05$ vs. FVB-old group.

itself did not affect whole body glucose intolerance and hyperinsulinemia, our results indicate that metallothionein overexpression or enhanced antioxidant capacity in the heart may be protective against aging-induced cardiac contractile dysfunction and impaired insulin signaling.

Compromised insulin signaling or its sensitivity often develops in advanced age (Iossa *et al.*, 2004; Couzin, 2005). Insulin insen-

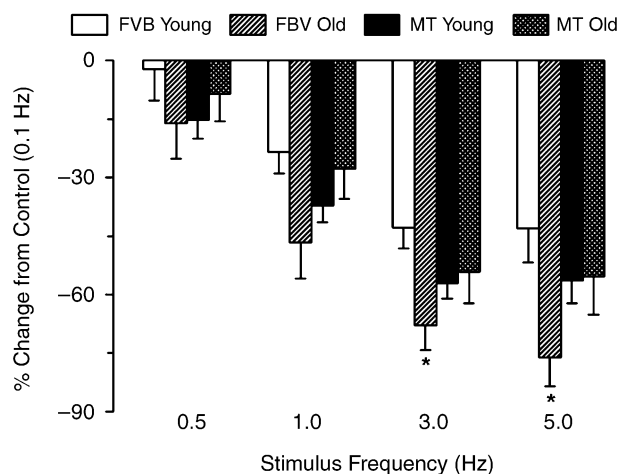


Fig. 4 Peak shortening (PS) amplitude of ventricular myocytes isolated from young or old FVB and metallothionein (MT) mice at different stimulus frequencies (0.1–5.0 Hz). PS was normalized to that of 0.1 Hz obtained from the same cell. Mean \pm SEM, $n = 28$ –30 cells per group, * $P < 0.05$ vs. FVB-young group.

sitivity or resistance may harm normal ventricular function through increased blood pressure as a result of sympathetic activation, renin-angiotensin system stimulation and direct myogenic effect on heart muscles (Reaven, 1995; Dutta *et al.*, 2001; Hintz & Ren, 2002). Improving insulin sensitivity by pharmacological intervention, lifestyle modification including diet, weight loss and physical exercise has been shown to benefit cardiac function and reduce blood pressure (Matthaei *et al.*, 2000; Wang *et al.*, 2004). This insulin sensitizing property of pharmacological and nonpharmacological manipulations may help to preserve ventricular function in the elderly. In our current study, aging elicited hyperinsulinemia and glucose intolerance but not fasting blood glucose levels, excluding potential contribution of diabetes to our aging murine model. Data from our study revealed that ventricular myocytes from aged FVB mice displayed prolonged relaxation duration (TR_{90}) while all other mechanical indices (PS, $\pm \text{dL}/\text{dt}$ and TPS) remained normal. Moreover, aged cardiomyocytes exhibited reduced cardiac contractile reserve or tolerance to stress manifested as significantly diminished PS at higher stimulus frequencies. These data are consistent with our previous findings using murine models at similar age (Li *et al.*, 2005). The impaired intracellular Ca^{2+} handling manifested as reduced intracellular Ca^{2+} clearance rate is likely responsible for prolonged relaxation duration (TR_{90}) and reduced intracellular Ca^{2+} cycling ability (reduced PS at high stimulus frequencies) in cardiomyocytes from aged FVB mice. It is noteworthy that the relaxation duration (TR_{90}) was prolonged whereas the maximal velocity of relaxation ($-\text{dL}/\text{dt}$) was normal in aged FVB cardiomyocytes. This discrepancy in relaxation parameters indicates that certain mechanisms responsible for rapid myocardial relaxation such as myosin heavy chain isozyme may be normal whereas factors responsible for reduced phrase ventricular filling or delayed cytosolic Ca^{2+} extrusion (e.g. Na^+ - Ca^{2+} exchanger, mitochondrial

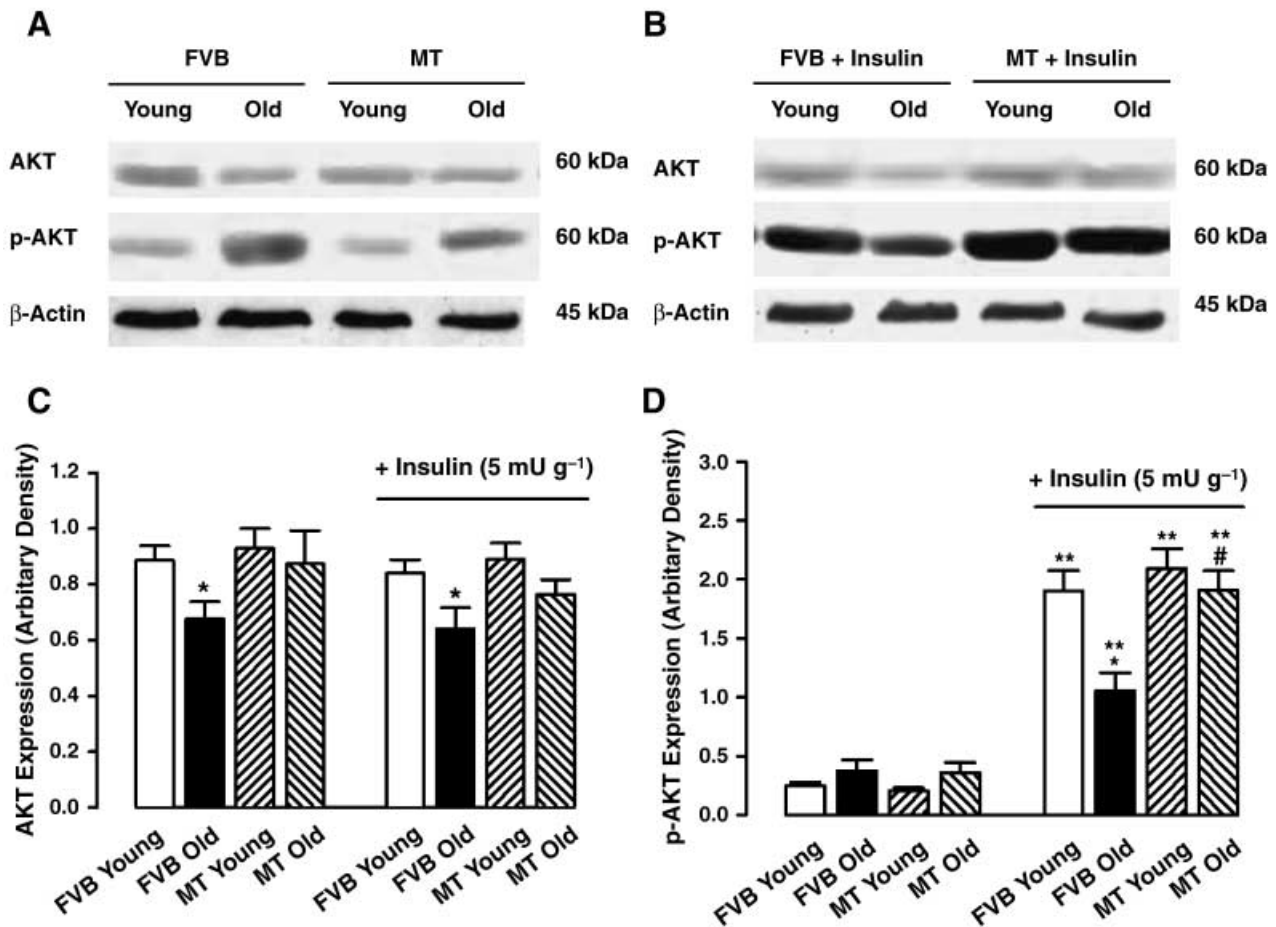


Fig. 5 Western blot analysis exhibiting total and phosphorylated Akt level in ventricles from young or old FVB and metallothionein (MT) mice challenged with or without insulin (5 mU g⁻¹, i.p. for 10 min). (A and B) Represent blots depicting total and phosphorylated Akt without and with insulin challenge using anti-Akt and anti-pAkt antibodies. (C) Total Akt expression; and (D) pAkt expression. β -actin was used as a loading control. Mean \pm SEM, $n = 7$ –11 samples per group, * $P < 0.05$ vs. FVB-young group, ** $P < 0.05$ vs. corresponding non-insulin treated group, # $P < 0.05$ vs. FVB-old group.

or sarcolemmal Ca²⁺ pumps) may be at fault. Further study is warranted to understand the precise role of these myocardial contractile or Ca²⁺ regulatory proteins under senescence.

The mechanical and intracellular Ca²⁺ defects triggered by advanced age were alleviated by metallothionein, in a manner similar to its effect on insulin resistance-induced cardiac contractile dysfunctions (Fang *et al.*, 2005). Metallothionein is believed to protect cardiomyocyte function in type 1 diabetes and insulin resistance through its antioxidant property (Ye *et al.*, 2003; Fang *et al.*, 2005). It is likely that reduction of oxidative stress is responsible for metallothionein-elicited protection against aging-associated cardiac contractile dysfunction, which is supported by the observation that metallothionein transgene is capable of antagonizing aging-induced elevation in NADPH oxidase subunit, superoxide generation, cytochrome c release and apoptosis (Yang *et al.*, 2006). Hyperinsulinemia has been shown to contribute to accumulation of reactive oxygen species, reactive nitrogen species and oxidative stress (Cai & Kang, 2001; Cai *et al.*, 2005) and may contribute to aging-induced cardiac dysfunction. Although involvement of oxidative stress and stress

signaling is beyond the scope of our current study, their important role in cardiac aging should not be ignored.

Alteration of several insulin signaling molecules has been identified in our current study, both at the receptor and post-receptor levels. Perhaps the most interesting finding was that alterations of these insulin signal cascades (insulin receptor tyrosine phosphorylation, Akt phosphorylation and PTP1B) correlate with aging-induced contractile dysfunction and protection from metallothionein. Our observation of improved function with the antioxidant metallothionein is consistent with earlier reports that abrogation of oxidative stress improves insulin sensitivity in sucrose diet-induced murine models of insulin resistance and the Ren2 rat model of tissue angiotensin II overexpression (Blendea *et al.*, 2005; Fang *et al.*, 2005). Interruption of insulin signaling usually starts with reduced expression and/or function of insulin receptor, insulin receptor substrate-1 (IRS-1), followed by diminished tyrosine phosphorylation of IRS-1 and activation of the postreceptor phosphatidylinositol-3 (PI-3) kinase–Akt cascade (Goodyear *et al.*, 1995). We observed normal expression of insulin receptor β , reduced tonic insulin receptor tyrosine phosphorylation,

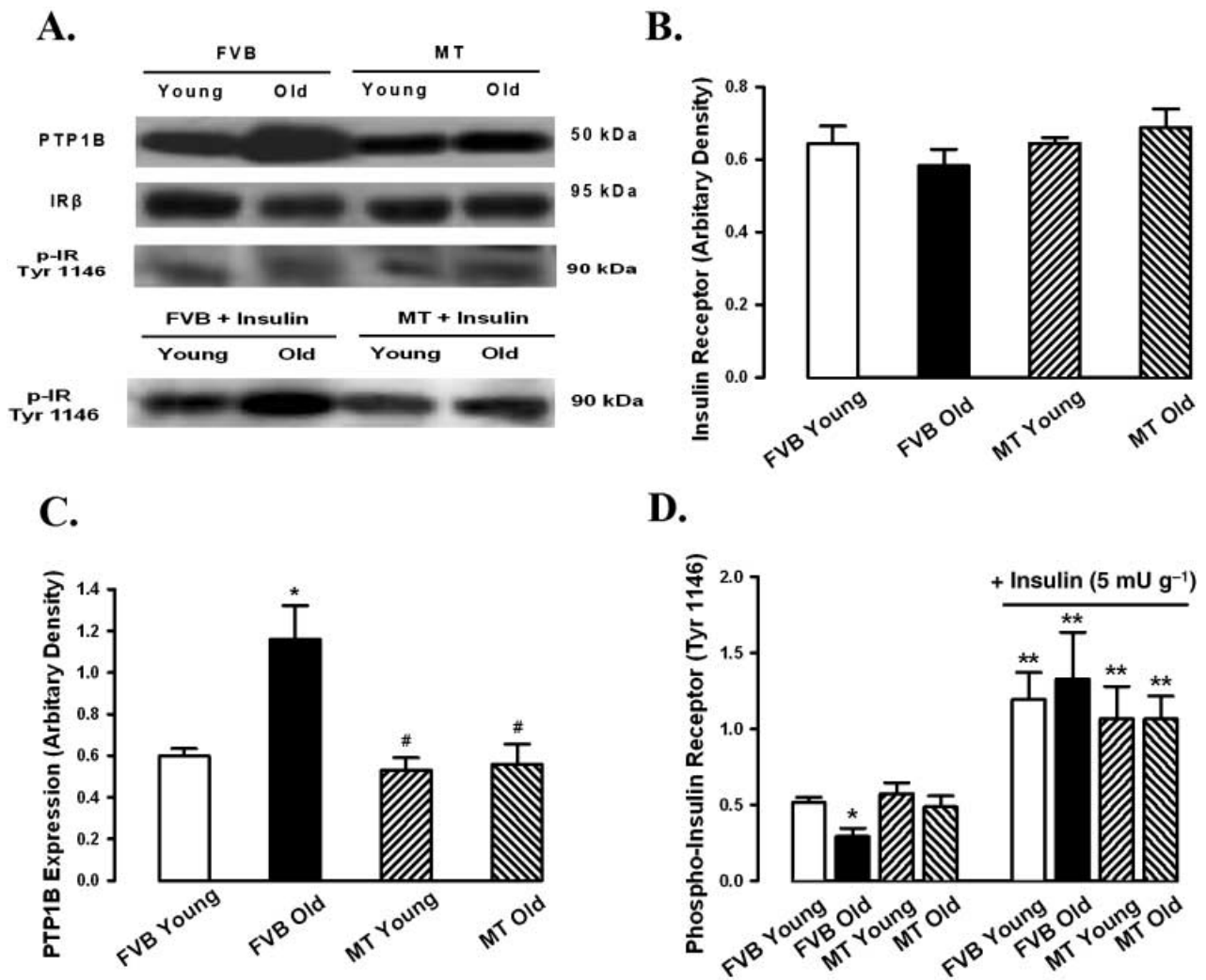


Fig. 6 Western blot analysis exhibiting insulin receptor, PTP1B and insulin receptor tyrosine phosphorylation (Tyr1146) in ventricles from young or old FVB and metallothionein (MT) mice challenged with or without insulin (5 mU g⁻¹, i.p. for 10 min). (A) Represent blots depicting insulin receptor, PTP1B and insulin receptor tyrosine phosphorylation (Tyr1146) (with or without insulin treatment) using respective antibodies; (B) insulin receptor expression; (C) PTP1B expression; and (D) phosphorylation of insulin receptor (Tyr1146) under basal and insulin-stimulated condition. Mean \pm SEM, $n = 6-11$, * $P < 0.05$ vs. FVB young-group, ** $P < 0.05$ vs. corresponding non-insulin treated group, # $P < 0.05$ vs. FVB-old group.

reduced total Akt levels and diminished fold increase of Akt phosphorylation in response to insulin in aged FVB hearts. Such loss in insulin signaling components under advanced aging is consistent with the enhanced expression of PTP1B, a negative modulator of insulin sensitivity (Zabolotny *et al.*, 2004). It is not surprising that aged hearts trigger up-regulation of PTP1B, which is extremely sensitive to reactive oxygen species via reversible oxidation (Meng *et al.*, 2004). Although oxidative stress aids the tyrosine phosphorylation-dependent response of insulin by transiently inactivating PTPs which normally suppress insulin signaling (Meng *et al.*, 2004), excessive reactive oxygen species under insulin resistant aging conditions is believed to up-regulate the redox sensitive proteins PTP1B. Although insulin-stimulated insulin receptor tyrosine phosphorylation appears to be normal under advanced aging conditions (Fig. 6D), it is likely that basal or tonic insulin receptor tyrosine phosphorylation contributes

predominantly to the aging-induced reduction in insulin sensitivity under our current experimental setting. It can be speculated that the metallothionein transgene may restore aging-induced loss in insulin signaling at several levels, insulin receptor tyrosine phosphorylation, total Akt, Akt phosphorylation as well as the negative insulin regulator PTP1B, which helps to preserve cardiac contractile function during aging. Nevertheless, additional study is required to validate the precise role of these signal molecules in insulin signaling-associated cardiac aging pathology using either confirmatory pharmacological interventions or specific gene manipulation approaches.

Myocardial aging is an inevitable and irreversible biological process characterized by a progressive decline of cardiac function (Lakatta, 1999, 2000; Lakatta *et al.*, 2001; Yang *et al.*, 2005). Accumulation of free radicals, oxidative stress and insulin resistance are perhaps the main players leading to aging-related

cardiovascular diseases including metabolic syndrome especially hypertension and diabetes, atherosclerosis and congestive heart failure (Lakatta, 1999; Yang *et al.*, 2005). Our findings suggest that antioxidants and insulin sensitizers such as vitamin and peroxisome proliferator-activated receptor- γ (PPAR- γ) agonist may have significant clinical application in the preservation of ventricular function in the elderly. Although it is not practical to overexpress metallothionein clinically in the elderly, our data suggest that the artificial 'jack-up' of the antioxidant defense using metallothionein may help to compensate for reduced antioxidant defense overall. In fact, evidence from our laboratory did not support an aging-associated decline in cardiac abundance of metallothionein (Yang *et al.*, 2006). Furthermore, our present study indicated that compromised insulin signaling may contribute to cardiac contractile dysfunction and impair intracellular Ca^{2+} homeostasis under advanced aging, which is alleviated by antioxidant metallothionein overexpression in hearts. These data suggest that improvement of insulin signaling itself may represent a potential therapeutic intervention for both myocardial aging and antioxidant treatment against cardiac aging. Although our study sheds some light on the interaction of oxidative stress, insulin signaling and cardiac aging-associated mechanical defects, the pathogenesis of cardiac contractile dysfunction under senescence still deserves further in-depth investigation. Future work should focus on both the genetic and environmental manipulation of endogenous antioxidant generation in the aging heart in an effort to alleviate aging-associated cardiovascular diseases. It is also pertinent to delineate the insulin signaling mechanism in the antioxidant defense against cardiac senescence and understand the transition from insulin sensitive to insulin resistant state in aging hearts.

Experimental procedures

Experimental animals

The experimental procedure was approved by our Institutional Animal Use and Care Committee at the University of North Dakota (Grand Forks, ND, USA) and University of Wyoming (Laramie, WY, USA). All animal procedures were in accordance with National Institutes of Health animal care standards. In brief, male FVB and metallothionein (MT) transgenic mice were used at either 2–3 months of age (denoted as 'young') or 26–28 months of age (denoted as 'old'). Mice were maintained with a 12-h light/12-h dark cycle with free access to tap water. The fur color was used as a marker for metallothionein (dark brown) or wild-type FVB (white) mouse identification as described (Ye *et al.*, 2003). For the insulin stimulation study, mice were injected intraperitoneally with insulin (5 mU g^{-1} body weight) for 10 min before sacrifice and cardiac tissue collection.

Intraperitoneal glucose tolerance test and plasma insulin

The intraperitoneal glucose tolerance test was performed as described (Hintz & Ren, 2002). Mice fasted for 12 h were given

an intraperitoneal (i.p.) injection of glucose (2 g kg^{-1}). Blood samples were drawn from the tail immediately before the glucose challenge, as well as 15, 60 and 120 min thereafter. Serum glucose levels were determined using an Accu-Chek III glucose analyzer (Accu-ChekII, model 792, Boehringer Mannheim Diagnostics, Indianapolis, IN, USA). Plasma insulin levels were measured by radioimmunoassay (Linco Research, St. Charles, MO, USA).

Isolation of mouse ventricular myocytes

Hearts were rapidly removed from anesthetized mice and immediately mounted on a temperature-controlled (37 °C) Langendorff perfusion system. After perfusion with modified Tyrode solution (Ca^{2+} free) for 2 min, the heart was digested for 10 min with 0.9 mg mL^{-1} collagenase D (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) in 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 and agitated into cell suspension. Extracellular Ca^{2+} was added incrementally back to 1.2 mM. Isolated myocytes were used for experiments between 1 and 8 h following isolation. Only rod-shaped viable myocytes were selected for mechanical and intracellular Ca^{2+} recording (Duan *et al.*, 2003).

Cell shortening/relengthening measurements

Mechanical properties of ventricular myocytes were assessed using a SoftEdge MyoCam® system (IonOptix Corporation, Milton, MA, USA) (Duan *et al.*, 2003). In brief, cells were placed in a Warner chamber mounted on the stage of an inverted microscope (Olympus IX-70, Olympus Optical Corporation, Tokyo, Japan) and superfused (~ 1 mL min^{-1} at 25 °C) with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 CaCl_2 , 1 MgCl_2 , 10 glucose, 10 HEPES, at pH 7.4. The cells were field stimulated with suprathreshold voltage at a frequency of 0.5 Hz, 3 ms duration, using a pair of platinum wires placed on opposite sides of the chamber connected to an FHC stimulator (FHC, Inc., Bowdoinham, ME, USA). The myocyte being studied was displayed on the computer monitor using an IonOptix MyoCam camera. A SoftEdge software (IonOptix Corp.) 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-PS (TPS) – indicative of systolic duration; time-to-90% relengthening (TR_{90}) – indicative of diastolic duration; and 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 (0.1–5.0 Hz), the steady-state contraction of myocyte was achieved (usually after the first five to six beats) before PS amplitude was recorded.

Intracellular Ca²⁺ 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 ×40 oil objective. Cells were exposed to light emitted by a 75 W 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 and 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 Ca²⁺ concentration were inferred from the ratio of fura-2 fluorescence intensity (FFI) at two wavelengths. Fluorescence decay time was measured as an indication of the intracellular Ca²⁺ clearing rate (Duan *et al.*, 2003).

Western blot analysis of Akt, pAkt, insulin receptor β, insulin receptor tyrosine phosphorylation and PTP1B

The total protein was prepared as described previously (Duan *et al.*, 2003). In brief, tissue samples from left ventricles were removed and homogenized in a lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% SDS and 1% protease inhibitor cocktail. Samples were then sonicated for 15 s and centrifuged at 12 000 *g* for 20 min at 4 °C. The protein concentration of the supernatant was evaluated using Protein Assay Reagent (Bio-Rad, Hercules, CA, USA). Equal amounts (50 μg protein per lane) of protein or prestained molecular weight markers were separated on 10% or 15% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad), then transferred electrophoretically to Nitrocellulose membranes (0.2 μm pore size, Bio-Rad). Membranes were incubated for 1 h in a blocking solution containing 5% milk in Tris-buffered saline (TBS), then washed briefly in TBS and incubated overnight at 4 °C with anti-Akt (1 : 1000), anti-pAkt (1 : 1000), anti-PTP1B (1 : 2500), anti-insulin receptor β (1 : 2500), anti-phospho-insulin receptor (tyr1146) (1 : 1000) and anti-β-actin (1 : 5000) antibodies. Anti-Akt, anti-pAkt (Ser473), anti-phospho-insulin receptor (Tyr1146) and anti-β-actin antibodies were obtained from Cell Signaling (Beverly, MA, USA). Anti-PTP1B antibody was from BD Biosciences Pharmingen (San Diego, CA, USA). Anti-insulin receptor β antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Following rinsing to remove excessive primary antibody, blots were incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody (1 : 5000). Antibody binding was detected using enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ, USA). The intensity of immunoblot bands was detected with a Bio-Rad Calibrated Densitometer (Model: GS-800). For all Western blot analysis experiments, β-actin was used as an internal loading control.

Data analysis

Data were expressed as mean ± SEM. Statistical comparisons were performed by analysis of variance (ANOVA) followed by Newman-Keuls *post hoc* test. Significance was defined as *P* < 0.05.

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