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NADPH oxidase-derived superoxide impairs calcium transients and contraction in aged murine ventricular myocytes

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ABSTRACT

Since aging increases oxidative stress, we analyzed the contribution of reactive oxygen species (ROS) to the contractile dysfunction of aged ventricular myocytes and investigated whether short-term interference with ROS formation could normalize contractile performance.

Isolated ventricular myocytes from young (2–4 months) and aged (24–26 months) male mice (C57BL/6) were used. We analyzed sarcomere shortening and calcium transients (Indo-1 fluorescence) of voltage clamped ventricular myocytes and myofilament ATPase activity (malachite green assay). Expression of calcium handling proteins (Western blots) and NADPH oxidase subunits (real-time PCR) was quantified, as well as NADPH oxidase activity (lucigenin chemiluminescence).

We found that aged myocytes showed decelerated shortening/relengthening without changes in fractional shortening. Calcium transient decay was similarly decelerated, but the amplitude of calcium transients was increased with aging. Calcium sensitivity of myofilaments of aged myocytes was reduced. These age-dependent changes occurred without altered calcium handling protein expression but were reversed by the superoxide scavenger tiron.

Aged myocytes showed increased NADPH oxidase expression and activity. Pharmacological inhibition of NADPH oxidase (diphenylene iodonium; apocynin) normalized age-dependent deceleration of shortening/relengthening.

In summary, we show that increased superoxide formation by upregulated NADPH oxidase contributes significantly to age-dependent alterations in calcium handling and contractility of murine ventricular myocytes.

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1. Introduction

The aging heart is characterized by changes in the calcium handling of cardiac myocytes. Particularly calcium removal from the cytosol by re-uptake into the sarcoplasmic reticulum (SR) is decelerated in aged cardiac myocytes favouring the development of diastolic dysfunction at higher frequencies (Lakatta, 1993). Slowed relengthening of isolated aged ventricular myocytes was also demonstrated in rodent models of cardiac aging (Capasso et al., 1992; Li et al., 2005; Yang et al., 2006).

In the human heart age-dependent diastolic dysfunction was attributed to a reduced expression of the SR calcium ATPase SERCA2 (Lakatta, 1993). Similarly, in aged rat hearts reduced SERCA2 expression and activity was demonstrated (Buttrick et al., 1991; Froehlich et al., 1978; Lompre et al., 1991). Li et al. (2007) recently reported reduced SERCA2 expression and activity in aged hearts of FVB mice, whereas we previously showed diastolic dysfunction in aged ventricular myocytes of C57BL/6 mice without changes in protein expression of SERCA2 (Isenberg et al., 2003). Therefore, depending on the strain used calcium handling may be additionally modulated by post-translational mechanisms in aged rodent ventricular myocytes.

It was suggested that reactive oxygen species (ROS) are involved in age-related dysfunction. Concerning the aged rat heart, increased ROS formation by mitochondrial electron transport chain or NADPH oxidase and reduced activity of the antioxidative enzyme superoxide dismutase were reported that promote a prooxidative shift in redox-balance (Adler et al., 2003; Sawada and Carlson, 1987; van der Loo et al., 2005). Calcium handling of cardiac myocytes is redox-sensitive. Perfusion of isolated rabbit hearts with hydrogen peroxide increased cytosolic calcium concentration (Corretti et al., 1991) and TNF-α or ischemia induce changes in calcium handling that are normalized by application of ROS scavengers (Cailleret et al., 2004; Dworschak et al., 2004). Recently, it was reported that lifelong constitutive over-expression of antioxidative enzymes prevents age-dependent diastolic dysfunction in murine ventricular myocytes providing further evidence for oxidative stress to be involved (Dai et al., 2009; Ren et al., 2007; Yang et al., 2006).

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Nevertheless, the role of ROS for contractile dysfunction of cardiac myocytes and its sources have not been directly proven in aged non-transgenic mice. Furthermore, it remains to be elucidated whether contractile dysfunction cannot only be prevented by lifelong interventions but once established also can be reversed i.e. by antioxidative treatment.

To address these questions, we determined expression of calcium handling proteins and NADPH oxidase subunits. We analyzed contraction, calcium transients and myofilament ATPase activity of isolated ventricular myocytes from young and aged mice. To identify the role of ROS and NADPH oxidase, pharmacological approaches were used.

We show that increased superoxide formation by upregulated NADPH oxidase significantly contributes to age-dependent alterations in calcium transients and myofilament function that result in contractile dysfunction of aged murine ventricular myocytes.

2. Material and methods

2.1. Isolation of murine ventricular myocytes

All procedures were approved by the local authorities and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). We used young (2–4 months) and aged (24–26 months) male mice of the C57BL/6 strain. Mice were killed by cervical dislocation and ventricular myocytes were isolated by standard collagenase dissociation technique as previously described (Kamkin et al., 2003).

2.2. Contraction

Myocytes were superfused with tyrode solution (1.8 mmol/L calcium, 37 °C) and voltage clamped to a holding potential of −80 mV. Electrode solution was composed of (in mmol/L) 140 CsCl, 5 NaCl, 4 Na_2ATP, 4.5 $MgCl_2$, 0.005 EGTA and 10 Hepes (pH 7.4). Trains of voltage steps were continuously applied at 0.5 Hz (10 ms pre-pulse to −45 mV followed by test-pulse to 0 mV for either 40 ms (short pulse) or 160 ms (long pulse)). After equilibration (3 min) frequency was sequentially increased: 0.5, 1, 2, 4 and 6 Hz (1 min each; 6 Hz for short pulses only).

Sarcomere length was continuously recorded using the IonOptix MyoCam system (scan rate 240 Hz). The last 10 contractions of every frequency train were averaged for fractional sarcomere shortening, time to peak shortening (TPS) and time to 70% relengthening (TR_{70}) using IonOptix software.

Pre-incubations with tiron (1 mmol/L; Fluka), diphenylene iodonium (DPI, 10 µmol/L; Sigma) or apocynin (50 µmol/L; Sigma) were done for at least 2–3 h.

2.3. Calcium transients

Myocytes were loaded with Indo-1 AM (5 µmol/L; Invitrogen) for 30 min. Myocytes were superfused with tyrode solution (1.8 mmol/L calcium, 37 °C) and voltage clamped to a holding potential of −80 mV. Electrode solution was composed of (in mmol/L) 140 KCl, 5 Na_2ATP, 6 $MgCl_2$, 0.005 EGTA and 10 Hepes (pH 7.4). Myocytes were continuously stimulated with voltage steps at 1 Hz (10 ms pre-pulse to −45 mV followed by 160 ms test-pulse to 0 mV). The cells were excited at 340 nm. Emission was collected and amplified in bands of 395–425 nm and 450–490 nm. Background fluorescence at 410 nm and 470 nm was digitally subtracted before fluorescence ratio 410 nm/470 nm of Indo-1 loaded cells was calculated. For analysis, 10 consecutive traces of stimulation were averaged offline for transient amplitude, time to peak systolic calcium concentration (TPC) and time to 70% calcium decay (TD_{70}).

2.4. Myofilament ATPase activity

Myofilaments were prepared as described by Pi et al. (2003). Isolated cardiac myocytes were homogenized in relaxing buffer composed of (in mmol/L) 100 KCl, 1 $MgCl_2$, 2 EDTA, 1 DTT, 20 Pipes (pH 7.0), BSA (1 g/L), protease inhibitor cocktail (1:100; Sigma) and Ser/Thr phosphatase inhibitor cocktail (1:200; Sigma). It was supplemented with 5 mmol/L Na_2ATP and 0.33% Triton X-100. The homogenate was left on ice for 20 min. The myofilaments were pelleted, washed twice with and resuspended in relaxing buffer (without supplements).

Myofilament ATPase activity was determined according to Swartz et al. (1999) with modifications. The activation buffer was composed of (in mmol/L) 130 KCl, 4 $MgCl_2$, 1 NaN_3, 1 DTT, 4 Na_2ATP, 2 EDTA, 2 EGTA, 2 HEDTA, 20 Pipes (pH 7.0) BSA (1 g/L), protease inhibitor cocktail (1:100; Sigma) and Ser/Thr phosphatase inhibitor cocktail (1:200; Sigma). The ATPase reaction was carried out at 30 °C using 10 µg of myofilaments in a total volume of 100 µL activation buffer supplemented with $CaCl_2$ to yield different free Ca^{2+} concentrations (pCa 6.5 to 4.0; calculated using the WEBMAX C program at http://www.stanford.edu/~cpatton/webmaxc). After 10 min the reaction was stopped by addition of 50 µL ice-cold 25% TCA. Within this time, the reaction was linear using the highest calcium concentration (pCa 4.0).

The generated inorganic phosphate (P_i) was quantified by the malachite green assay (Carter and Karl, 1982). 20 µL of the supernatants from TCA precipitations was diluted to 100 µL. Subsequently, 70 µL of solution A (42 mmol/L Na_2MoO_4 dissolved in 1 mol/L HCl), 30 µL of solution B (0.042% malachite green in 1% polyvinyl alcohol) and 30 µL of solution C (30% H_2SO_4) were added. After colour development for at least 60 min, absorption was read at 630 nm.

Blebbistatin is a specific inhibitor of myosin II ATPase activity (Limouze et al., 2004). For every sample, ATPase reactions were performed using untreated myofilaments and blebbistatin pre-incubated myofilaments (100 µmol/L, 20 min). The difference was ascribed to phosphate generation by myofilament ATPase. Activity was determined as nmol P_i (mg protein)$^{-1}$ min^{-1} by comparison with a phosphate standard curve, which was linear to at least 500 µmol/L P_i.

2.5. Western blots

Western blot analyses were performed using hearts that were perfused with normal tyrode solution (1 min). Thereafter, hearts were immediately shock-frozen in liquid nitrogen. Hearts were homogenized in lysis buffer composed of (in mmol/L) 20 Tris, 250 sucrose, 3 EGTA, 20 EDTA, plus 0.5% Triton X-100, 0.5% SDS (pH 8.0), protease inhibitor cocktail (1:50; Sigma) and Ser/Thr phosphatase inhibitor cocktail (1:100; Sigma). After SDS-PAGE of total proteins and transfer onto polyvinylidene difluoride (PVDF) membranes, blots were incubated with antibodies specific for total phospholamban (Santa Cruz), DHP receptor α subunit (Dianova), RyR2 (Sigma) and calsequestrin (Upstate). Blots were visualised using the ECL Western blotting analysis system (Amersham Pharmacia Biotech). To correct for putative differences in gel loading and protein transfer efficiency, immunological signals were normalized to a subsequent Ponceau S staining of the blot. Values are given in arbitrary units (a.u.).

2.6. NADPH oxidase activity

NADPH oxidase activity was quantified by the lucigenin chemiluminescence assay using Krebs–Henseleit buffer composed of (in mmol/L): 146 NaCl, 5 KCl, 1 $CaCl_2$, 0.35 NaH_2PO_4, 1 $MgSO_4$, 10 glucose and 10 Hepes (pH 7.4). Isolated myocytes were homogenized in Krebs–Henseleit buffer supplemented with protease inhibitor cocktail (1:100; Sigma). Homogenate (100 µg), NADPH (100 µmol/L; Sigma) and dark

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