SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Changes of the excitation-contraction coupling of skeletal muscle in aged mice

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The Examination takes place at Life Science Building, University of Debrecen
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The PhD Defense (online format) takes place at 14:00 PM, on December 14th, 2020.

Publicity is guaranteed during the online Defense. If you are willing to participate, please indicate via e-mail to szentesi.peter@med.unideb.hu until 16:00 PM on December 12th, 2020. Due to technical reasons later sign-ups are not possible and you will not be able to join the online Defense.

Introduction

The skeletal muscle is one of the biggest organs which is 40 % of the body weight. It is extremely important during normal life as it plays an important role in movement, posture and energy metabolism of the body. Skeletal muscle mass can be increased with exercise, but decreases in the absence of exercise and in old age. Due to better living conditions, life expectancy at birth has now been extended, resulting in an increasing elderly population. As a result, sarcopenia in old age (a decrease in skeletal muscle mass and strength) has also grown into a public disease. Any therapy that could slow down muscle wasting in old age or accelerate regeneration after muscle injury would have a significant positive economic and social impact. Therefore, the background and treatment options for sarcopenia are being extensively studied. Muscle mass decreases with age. This process accelerates after the age of 70, however, the biological processes involved in these mechanisms are not yet fully explained. The ability and strength of muscles to regenerate also decreases with age. An important participant in these processes may be the slowed metabolic rate, which contributes to the decrease in muscle mass. These phenomena can lead to decreased physical activity, malnutrition, chronic inflammation, and decreased hormone secretion. The skeletal muscle has impressive plasticity that allows it to adapt to lifelong functioning. We hypothesize that greater muscle mass gained at a young age may be a promising starting point during aging to maintain adequate muscle and physical performance. However, increased muscle mass does not necessarily lead to increased performance. For example, such a phenotype is myostatin-deficient mouse, which shows decreased *in vivo* physical activity. On the other hand, there is a general opinion that constant moderate exercise keeps the muscles in good shape. Muscle formation contributes to meet the complex needs of increased activity through increased capillary density and enzymatic activity to enhance muscle metabolism. Furthermore, increased production of contractile proteins allows for greater contractile force. Sarcopenia is a very complex mechanism that involves many more phenomena than a decrease in physical activity with age.

The effects of diet and changes in hormone production over a lifetime contribute strongly to a decrease in muscle mass. The nature and extent of the contribution of these two factors to the healthy and functional maintenance of muscle is not yet known. During aging, for example, the loss of skeletal muscle function may be the result of oxidative stress resulting from the accumulation

of reactive oxygen or nitrogen species (ROS/RNS). Under physiological conditions, the oxidative balance between ROS/RNS production and their removal is regulated by the antioxidant system. Physiological concentrations of these reactive radicals play an important role in several signaling pathways, and both their too low and too high levels can be disadvantageous to cellular functions. The skeletal muscle uses a lot of oxygen, so it is able to produce large amounts of ROS/RNS. One of the most important sources of this is the mitochondria, which capacity reduced during aging. In aging muscles, increased oxidative stress can lead to changes in excitation contraction coupling (ECC) and Ca^{2+} homeostasis. Selenium is a trace element with antioxidant properties and plays an important role in several muscle functions. In cows, selenium deficiency causes white muscle disease (weakness and degeneration of the skeleton and heart muscle). Similar myopathy has been described in humans. Severe selenium deficiency is associated with insufficient skeletal muscle function. In our previous work in young mice, we have shown that a diet supplemented with selenium increases the release of calcium from the sarcoplasmic reticulum (SR) and thus improves skeletal muscle performance *in vivo* and *in vitro*. These effects were accompanied by increased level of selenoprotein N in the muscles, which may result in increased oxidative stress tolerance during long-term contractions. Selenoproteins are involved in chemical reactions such as oxidative reductive reactions and play an essential role in the defense against unstable oxygen-containing molecules. Although the exact function of selenoprotein N is not known, it probably protects cells from oxidative stress. It is active in many tissues before birth and is involved in the formation of muscle tissue (myogenesis). It plays an important role in normal muscle functions and also in calcium homeostasis. A decrease in muscle selenoprotein N content has been shown with advancing age.

Ankyrin belongs to the family of protein adapters and is involved in the organization of special membrane domains. All the three ankyrin genes (Ank1, Ank2, and Ank3) encode a large number of ankyrin isoforms that share a common general structure through multiple splicing events. In striated muscles, all the three ankyrin genes encode muscle-specific isoforms The ankyrin isoform found in most organs has a high molecular weight (220–130 kDa), but the internal promoter in the Ank1 gene expresses small muscle-specific isoforms that are 20–25 kDa, and collectively we call them sAnk1 (sAnk1.5, sAnk1.6, sAnk1.7, and sAnk1.9). These sAnk1 isoforms lack most canonical ankyrin domains, but contain a unique hydrophobic domain at their

NH2 terminus that binds them to SR. Of these smaller muscle isoforms, sAnk1.5 is most widely expressed, while the other isoforms are found at lower levels. The cytoplasmic end of sAnk1.5 contains a specific sequence that allows direct binding to obscurin. It is a sarcomeric protein required to maintain M-band stability in skeletal muscle. The interaction between sAnk1.5 and obscurin is essential to keep SR in close proximity to myofibrils. The evidence for this, was the lack of sAnk1 isoforms in primary cell cultures from rat *flexor digitorum brevis* (FDB) muscle resulted in anastomosis in the longitudinal tubules of the SR, while it had little effect on the junctional SR. In contrast, the expression levels and localizations of the channel and pump of SR did not differ for fast and slow muscle fibers from sAnk1 KO mice compared to control mice. Almost identical changes in the skeletal muscle fibers of obscurin KO mice were observed in the morphology of SR, confirming the importance of the interaction between sAnk1 and obscurin in preserving the structure of SR. In further studies, it was found that with age, structural damage and large-area contractures developed in the fibers from sAnk1 KO mice, as well as tubular aggregates containing SR proteins. All these structural changes were accompanied by a deterioration in the electrophysiological properties and strength of the sAnk1 KO muscles. These results are consistent with previous research that sAnk1-deficient cultured muscle fibers show reduced Ca^{2+} release from SR.

Aims

The decrease in muscle mass during aging, which may be a consequence of the popular disease sarcopenia, is associated with a significant loss of muscle strength and increased fatigue. This may also be due to the fact that as the age progresses, the production of reactive oxygen species also increases, which further destroys the cells. Furthermore, the presence of ROS also affects the proteins involved in ECC, leading to decreased function of Ca^{2+} homeostasis, which is key to normal skeletal muscle function. Our goal was to find out how to slow down or reverse this process. Our questions to be answered were:

- Does the antioxidant selenium dietary supplement reverse the decline in muscle function that occurs with aging?
- How does lifelong exercise affect the maintenance of muscle function in old age?
- How do these affect the function of proteins involved in ECC and muscle calcium homeostasis?
- How dose selenoprotein N level change as a result of selenium supplementation in old mice?

The sAnk1 proteins present in skeletal muscle play an important role in keeping the SR close to the myofibril, whose normal function is essential for Ca^{2+} homeostasis. Since it is not yet known whether the absence of this protein affects Ca^{2+} homeostasis of skeletal muscle fiber in young and old sAnk1 KO mice, we had the following questions:

- Does protein deficiency affect skeletal muscle fiber intracellular Ca^{2+} homeostasis in sAnk1 KO mice?
- How does aging affect these changes?

Materials and methods
Animal models

Our animal experiments were performed in accordance with the directives of the European Union (86/609 / EEC). The experimental protocol was performed with the permission of the Animal Welfare Committee of the University of Debrecen (31/2012 / DE MÁB). Mixed-sex animals were housed in plastic cages with metal lattice lids. Mice were fed normal rodent food and had *ad libitum* access to water. The lighting of the animals' rooms was automatically solved by alternating 12 hours of light and 12 hours of dark cycles, and the temperature was kept in the range of 22–25°C. Our experiments were performed on mixed-sex C57 BL/6, *Cmpt* and sANK1 KO mice.

In the series of experiments examine the effects of aging and the measurements were performed on 5 groups of animals. The first group, called the trained control, consisted of C57 BL/6 mice that were kept on a normal diet and placed in a cage equipped with a treadmill for training, which could be used voluntarily for 14 days every 2 months from 2 months of age until the end of their lives. The second and third, i.e., untrained young (4 months) and untrained old (20–22 months) control groups also included C57 BL/6 mice. They also were given a normal diet, but there was no treadmill in their cage. In the fourth, i.e., untrained *Cmpt* group, 20–22-month-old *Cmpt* mice were also kept on a normal diet and in a normal cage, i.e., without a treadmill. The fifth group, selenium fed untrained, consisted of 20–22-month-old C57 BL/6 mice maintained under similar conditions to the other untrained groups but given a diet with increased selenium content.

Volunteer running mill:

Mice were placed in a cage containing a mouse treadmill. The mills were connected to a computer, and then the data were recorded at 20-min intervals for 14 days. For each mouse, we calculated the average and maximum speed per day, the distance traveled, and the length of time spent running.

Grip test:

The foreleg grip test was performed so that when the animals reliably grasped the metal frame attached to the force gauge, they were gently pulled away from the force gauge by their tails. Mice were kept parallel to the frame connected to the force meter. The maximum force was recorded before the animal released the frame. The signals were digitized at 2 kHz and stored on an online connected computer. The test was repeated 10-15 times per mouse and then the data were averaged to obtain a single data point per animal. Measurements of the trained groups were always performed before the 14-day running period. For all other groups, the grip test was performed on the day the animals were sacrificed.

***In vitro* experiments:**

The animals were anesthetized and sacrificed according to the approved protocol of the Animal Welfare Committee of the University of Debrecen (31/2012 / DE MÁB), (22/2011 / DE MÁB, 2012). After pentobarbital anesthesia (27 mg/kg) and *cervical dislocation*, *m. flexor digitorum brevis* (FDB), *m. extensor digitorum longus* (EDL), *m. soleus* (SOL) and *m. quadriceps femoris* muscles were used.

Muscle strength measurement:

EDL (fast) and SOL (slow) muscles were manually dissected using tweezers and scissors. Then it was placed horizontally in an experimental chamber continuously perfused with carbogen (95% O_2 and 5% CO_2) and 10 ml/min Krebs solution (135 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM Hepes, 10 mM glucose, 10 mM $NaHCO_3$; pH 7.2; room temperature) so they were washed to maintain vitality. One end of the muscle was attached to a capacitive mechano-electric force transducer and the other end was punctured with an insect needle. To induce short contractions, two platinum electrodes placed under the muscle were used to deliver 2 ms long pulses of supramaximal amplitude at 0.5 Hz. Similar individual pulses were used to induce tetanus at 200 Hz for 200 ms (EDL) and at 100 Hz for 500 ms (SOL). Fatigue was induced by administering 150 tetanus, repeating the tetanus at a frequency of 0.5 Hz. To quantify fatigue, the amplitude of consecutive tetanus was normalized to the first tetanus. Muscles that were visibly damaged during manual preparation were excluded from the evaluation. After the measurements, the maximum diameter of the muscles was determined under

a light microscope. From this, the cross-sectional area (CSA) of the muscles was calculated assuming a circular cross-section. The force data were normalized to this cross-sectional area before averaging.

Isolation of individual skeletal muscle fibers:

Calcium concentration measurements were performed on individual FDB skeletal muscle fibers. For manual muscle dissection, a calcium-free Tyrode's solution containing the following in mM was used: 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 5 ethylene glycol bis (β-aminoethyl ether) -N, N, N ', N '-tetraacetic acid (EGTA), 11.8 HEPES; 1 gL^{-1} glucose; pH = 7.4. To isolate individual skeletal muscle fibers, FDB was enzymatically digested. For this, 0.2% type I collagenase (Sigma, St Louis, MO, USA) was added to the preparation solution, and the muscle was kept at 37°C for 35-65 minutes. Digestion was then stopped with enzyme-free normal Tyrode's solution (1.8 mM $CaCl_2$) and the muscles were stored at 4°C until further use. Prior to the measurements, individual skeletal muscle fibers were obtained from the FDB muscles by mild manual trituration.

Whole cell intracellular Ca^{2+} concentration measurement:

Changes in intracellular Ca^{2+} concentration were observed using Fura-2 AM dye. This fluorescent dye is ratiometric, so the absorption maximum (340 nm) of the Ca^{2+}-bound form differs from that of the non-Ca^{2+}-bound form (380 nm). After excitation at the wavelength corresponding to the two different absorption maximum on the individual muscle fiber, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) can be calculated from the ratio of the intensities of the fluorescent light emitted.

Isolated individual FDB fibers were used for fluorescence measurements and triturated on a laminin-coated cover slide. The solution was then replaced with a dye solution containing 5 µM Fura-2 AM and 150 nM neostigmine in 1 ml of normal Tyrode. The latter was due to inhibition of the extracellular acetylcholinesterase enzyme to prevent activation of the dye before it entered the fibers. The fibers were loaded for 60 minutes at 37°C in the dye containing solution. The fibers were then kept in dye-free Tyrode's solution at room temperature for 30 minutes before measurements. Alternating excitation (340 and 380 nm) was performed with a PTI instrument equipped with a dual wavelength monochromator (Deltascan, Photon Technology International, New

Brunswick, NJ, USA), while the emission was measured at 510 nm using photon electron multipliers and interference filters at a frequency of 10 Hz on a computer.

Intracellular calcium concentration was calculated in each case from the ratio of the measured fluorescence intensities using *in vivo* calibration. At the beginning of the measurements, the background was first recorded in a cell-free area on the given cover slide. Using an external perfusion system, the fibers were continuously perfused with normal Tyrode's solution. A locally positioned perfusion capillary needle (Perfusion Pencil®; AutoMate Scientific, Berkeley, CA, USA) was used to treat selected fibers. The solution delivery of the 0.35 ml/min needle was controlled by a local perfusion system (Valve Bank ™ 8 version 2.0, AutoMate Scientific, Berkeley, CA, USA). By directing the needle with the micro-manipulator onto the fiber to be measured, it was achieved that the different solutions reached the selected fiber almost immediately and only. To study calcium release, Ca^{2+} transients were induced with a depolarizing solution containing the same amount of KCl instead of 120 mM NaCl. This was added to the selected cell by local perfusion for 5 s. To determine Ca^{2+} released into the myoplasm, calcium binding to intracellular binding sites and removal from the intracellular space were modeled as follows: Ca^{2+} can bind intracellularly to SERCA pump, troponin C, parvalbumin ; while the remove Ca^{2+} pump activity was taken into account. The maximum delivery rate of the calcium pump was determined from the descending phase of Ca^{2+} following transient stimulation by fitting the most appropriate parameters. All other parameters of the model were kept constant. The Ca^{2+} release rate (flux) was calculated as the time derivative of Ca^{2+} transient. The amount of Ca^{2+} released during long depolarization (5 s) is a good estimate of the calcium content of SR. Another measurement protocol was used to directly measure the Ca^{2+} content of SR. First, normal Tyrode's solution was added to the fibers by local perfusion, and then it was replaced with calcium-free Tyrode's solution containing 5 mM EGTA. The intracellular Ca^{2+} depot was emptied using a cocktail (200 µM 4-chloro-M-cresol and 10 µM cyclopiazonic acid) in calcium-free Tyrode. As a result of the cocktail, large amounts of Ca^{2+} were released from the SR through the RyRs. All administered solutions contained 50 µM BTS to reduce fiber movement during calcium release. The resting $[Ca^{2+}]_i$ value was calculated by averaging the fluorescence ratios in

normal Tyrode. The Ca^{2+} content of SR was calculated as the difference between the resting $[Ca^{2+}]_i$ and the measured (when the cocktail was added).

Measurement of elemental calcium release events (CREs) in permeabilized muscle fibers:

The isolated individual FDB muscle fibers were permeabilized with saponin and loaded with 0.1 mM fluo-3 dye. The fibers were hold in relaxing solution (components in mM: 6 $MgCl_2$, 5 Na_2-ATP, 125 K-glutamate, 10 HEPES, 1 EGTA, 0.13 $CaCl_2$, 10 glucose, 10 Na-phosphocreatine, pH 7.2) for 2-3 minutes in the presence of 0.002% saponin. Thus, by perforating the surface membrane, we were able not only to increase the number of elemental events on the fibers, but also to help calcium indicator dye enter the interior of the fiber. The permeabilization of the surface membrane was monitored under a microscope as an increase in the fluorescence intensity of fluo-3. This solution was then changed to a measuring solution (components in mM: 6 $MgCl_2$, 5 Na_2-ATP, 95 K_2SO_4, 10 HEPES, 1 EGTA, 0.13 $CaCl_2$, 10 glucose, 10 Na-phosphocreatine, pH 7.2). Images were recorded with a 40x oil immersion objective (NA = 1.3) on an ultra-fast Zeiss 5 LIVE laser scanning confocal microscope (Zeiss, Oberkochen, Germany). The fluo-3 dye was excited with an argon ion laser at 488 nm, and then the fluorescent light emitted by the fiber was collected above a wavelength of 505 nm. Fifteen minutes after the application of the measuring solution, a series of 120 images of 512×512 pixels (x, y) images were taken from each examined fiber at eight different locations at a speed of 67 ms/image. In the first image of each series, the background and the outline of the fiber were determined. The averaged background fluorescence was then subtracted from each pixel of all images in each series. To detect Z-lines in the fiber, the frequency spectra of all images were calculated using fast Fourier transform (FFT). The inverse FFT of the frequency components corresponding to the sarcomeres was then used to remove the Z-lines from the images. We used a stationary wavelet method with low-threshold screening to detect elemental Ca^{2+} release events (CRE or spark) on the fibers. Finally, the following parameters of the sparks were calculated: amplitude and two spatial widths at half of the maximum (FWHM), perpendicular (FWHM-x) and parallel (FWHM-y) to the Z-lines. Signal density (Signal Mass, SM) was calculated to estimate the amount of Ca^{2+} released during CRE.

Voltage Clamp measurements and Ca^{2+} transient analysis:

Images were recorded on isolated FDB fibers under voltage clamp conditions (Axoclamp 2B, Axon Instruments, Foster City, CA, USA) using the confocal microscope described above (Zeiss 5 Live) in an external solution of the following composition: (in mM) 140 TEA-CH$_3$SO$_3$, 1 CaCl$_2$, 3.5 MgCl$_2$, 10 HEPES, 1 4-aminopyridine, 0.5 CdCl$_2$, 0.3 LaCl$_3$, 0.001 TTX and 0.05 BTS, pH 7.2, osmolality 320 mOsm. The pipette was filled with an internal solution containing the rhod-2 fluorescent calcium indicator (components in mM: 110 N-methylglucamine, 110 L-glutamic acid, 10 EGTA, 10 Tris, 10 glucose, 5 Na ATP, 5 phosphocreatine Tris , 0.1 rhod-2, 3.56 CaCl$_2$, and 7.4 MgCl$_2$, pH 7.2, osmolality 320 mOsm). All experiments were performed at 20–22°C and the resting membrane potential was kept at −80 mV. The resistance of the pipette varied in the range of 1-2 MΩ. Analog compensation was used to correct for linear capacitive currents. Ca^{2+} transients were analyzed using a model that calculates the calcium release flux. The model takes into account changes in [Ca^{2+}]$_i$(t) in a single volume unit, including calcium removal processes such as the maximum transport rate (V$_{max}$) of the SERCA pump. The experiments were performed in the presence of calcium buffer (10 mM EGTA), so endogenous buffers were considered almost negligible in the calcium removal process. The voltage dependence of Ca^{2+} release activation is described by the Boltzmann function. Subtracting the removal flux of the pump from the calcium release flux (the flux through the calcium channels), the net flux exiting the SR can be determined. The integral of the net flux gives the SR calcium content that can be released by depolarization, also known as the amount of Ca^{2+} released (Δ[Ca^{2+}]$_{i\ total}$).

ROS measurements:

Individual FDB fibers were loaded with 3 nM dihydroethidium in nTyr for 20 min at room temperature. The fluorescence intensity of the resting fibers was detected above 550 nm by excitation at 532 nm. The measurement was repeated on the same fiber after 100 tetanus evoked by field stimulation (2 ms long square pulses at 50 Hz for 100 ms). Background-corrected and fluorescence values were averaged over a selected area parallel to the longitudinal axis of the fibers. The calculated curves showed a periodic increase and decrease in fluorescence, reflecting the sarcomere pattern of the dye

distribution. We assumed that the mean values of the curves were proportional to the ROS production of the fibers.

Western blot analysis:

Tissue samples from the back limb, *musculus quadriceps femoris* were homogenized in lysis buffer (20 mM Tris-Cl, 5 mM EGTA, protease inhibitor coctail (1: 100) from Sigma) and digested on ice with a sonicator. The protein content of the samples was measured by a modified bicinchinic acid protein assay (Pierce, Rockford, IL, USA) using bovine serum albumin. For Western blot analysis, 1/5 volume of 5-fold electrophoresis sample buffer (310 mM Trise-HCl pH 6.8, 10% SDS, 50% glycerol, 100 mM dithiothreitol (DTT), 0.01% bromine phenol blue) was added to the whole muscle homogenate and then cooked at 80°C for 5 minutes.

10 µg of protein was separated by 5% and 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel for immunological detection of the tested proteins. Proteins were electrophoretically transferred to nitrocellulose membranes (BioRad, Vienna, Austria).

After blocking the nonspecific binding sites with 5% slim milk powder dissolved in PBS, the membranes were incubated overnight at 4°C with the appropriate primary antibodies.

After washing three times for 10 min with PBST (PBS, supplemented with 0.1% Tween 20), the membranes were incubated with the secondary antibody for 1 h. The latter was horseradish peroxidase (HRP) -conjugated goat immunoglobulin (Bio-Rad) raised against the primary antibody-producing species (rabbit or mouse) diluted 1: 1000 in PBS containing 5% slim milk powder. Signals were detected by enhanced chemiluminescence (ECL) reaction (Thermo Scientific, Rockford, IL, USA). The intensity of each band and the background of the same image were determined using ImageJ software (NIH, Bethesda, MD, USA). Background-corrected normalized values were given based on the intensity of household genes in the same samples. Finally, these data were expressed relative to the control.

Determination of selenium content in muscles:

Hydride Generation Atomic Fluorescence Spectrometry (HG-AFS) was used to estimate total selenium content in EDL muscle. The samples were wet digested by adding 5 ml of 65% HNO_3 to 1 g of the sample, digesting at 60°C for 60 minutes and then at 120°C for 240 minutes after adding 3 ml of 30% H_2O_2. The resulting samples were diluted to 15 mL with 3 M hydrochloric acid and filtered. HCl was used for dilution to form the hydride. Measurements were performed using an Atomic Fluorescence Spectrometer (Millennium Merlin, P.S. Analytical, Orpington, England) with the following settings: 15 l/min argon gas addition, 40 s measurement, 40 s wash time, 100 amplification. For the hydride generation reaction, 3 M hydrochloric acid was used as the acid solution and 1.4 w/v% $NaBH_4$ in 0.1 M NaOH was used as the reducing agent.

Data analysis and statistics:

Average data was expressed as mean ± mean error (SEM). Mean and SEM were calculated from weighted averages and weighted standard errors by appropriate weighting of the number of muscles from the same animal, while the number of samples was the number of animals in that group. Differences between groups of animals were assessed by one-way and two-way analysis of variance (ANOVA) and pairwise multiple comparison procedures (Bonferroni multiple comparison test; Student-Newman-Keuls method) using the Prism statistical program (GraphPad Software, San Diego, CA, USA). A p-value less than 0.05 was considered statistically significant. A casual mediation model (CMM) was developed to investigate whether the relationship between selenoprotein expression level and maximal tetanus force is altered by intracellular Ca^{2+} concentration as a mediator.

Results

In vivo experiments:

A grip test was used to check the *in vivo* muscle performance of the animals. Grip strength normalized to body weight decreased with age as the old control mice' body weight increased, but they produced a maximum force similar to that of young animals. Old myostatin-deficient mice carrying the Compact mutation (*Cmpt*) produced significantly higher maximal strength than old control animals, but after normalization to body weight, their performance was significantly lower than that of control mice. Selenium treatment did not change the maximal grip force in old age, but the grip force normalized to body weight became significantly higher than that of control mice. One group of control animals (8 mice) participated in 14-day voluntary running experiments (trained group) between 9 and 90 weeks of age (corresponding to 2 and 22 months). As expected, lifelong training of mice significantly increased the maximum grip strength compared to untrained old control animals. The results of the voluntary running experiments varied with age. After 2-3 days in the special cage, the average running speed stabilized at a given level. All other measured parameters (maximum daily speed, running time, and distance) followed a similar trend over the two weeks of running. All parameters of volunteering showed a decrease after 40 weeks of age in control mice. In addition, we performed a voluntary running experiment on 21-month-old animals that had not previously run in their lives (untrained group). Mean running speed and daily distance were significantly lower in the untrained control group at 85 weeks of age. Interestingly, untrained old animals spent almost as much time on running as old trained mice. Taken together, these experiments clearly indicated that aging of mice had a negative effect on their muscle performance. In order to elucidate the mechanisms underlying this phenomenon, we further examined *in vitro* force and ECC steps in detail.

In vitro experiments:

Force measurement:

As expected, *in vitro* muscle strength decreases with age in control animals. To determine whether hypermuscular phenotype, lifelong exercise, or selenium dietary supplementation directly modifies muscle function, *in vitro* muscle strength measurements were performed in one young and four old animal groups. We found a significant difference in the mean amplitude of individual contractions of EDL muscles, from *Cmpt*, selenium-fed, trained, and control

animals. While selenium feeding and lifelong exercise increased, the *Cmpt* phenotype decreased the maximum of twitches. A similar significant difference was found in the tetanic force. Interestingly, these differences were not observed in the soleus muscle. There was no difference in individual twitch/tetanus ratio between animal groups. This parameter indicates normal functioning of the neuromuscular junction. Furthermore, selenium and exercise reduced muscle fatigue during the protocol containing 150 tetanus in EDL, but not in the SOL muscles of old animals. In contrast, both fast and slow muscles from old *Cmpt* mice were significantly more fatiguable than the same type of muscle from old control mice. No significant differences were found in fatigue of EDL muscle between young and old control mice.

Changes in intracellular calcium concentration ($\Delta[Ca^{2+}]_i$) in old animals:

We examined the effects of muscle hypertrophy, exercise, and selenium treatment on calcium homeostasis in individual FDB muscle fibers from old mice. Fibers were loaded with calcium-sensitive Fura-2 acetoxymethyl (AM) dye for measurements. Resting $[Ca^{2+}]_i$ was significantly higher in *Cmpt* fibers than in control mice. Selenium treatment and training significantly reduced the resting $[Ca^{2+}]_i$ to the level of young animals and all three were significantly lower than resting $[Ca^{2+}]_i$ in muscle fibers from old mice.

Depolarization with 120 mM KCl for 5 seconds was used to determine the calcium content of the SR, i.e. to measure the amount of calcium that could be released from the SR. This type of depolarization induced calcium transients in the presence of normal (1.8 mM) extracellular calcium concentration. Selenium feeding and training increased the amplitude of calcium transients, whereas in *Cmpt* animals it was smaller than in control mice. The pooled data confirm that selenium supplementation and exercise caused a statistically significant increase in the change of $[Ca^{2+}]_i$ compared to muscle fibers in control animals, indicating that under these conditions, calcium release from SR is higher. Selenium almost doubled, and training almost tripled the amplitude of calcium transients. In contrast, depolarization-induced calcium transients were significantly smaller in *Cmpt* animals than in control fibers.

Further study of the transients demonstrated that the amount of calcium released from SR after training was significantly higher and significantly lower in *Cmpt* animals than in untrained control fibers, whereas it was unchanged after selenium treatment. It should be noted that while the amount of calcium released from SR did not change in selenium-fed mice, the amplitude of

calcium increased significantly increased. This may suggest a modified kinetics of SR calcium release, which was confirmed by calculating the rate (flux) of SR Ca^{2+} release. It was found to be significantly higher in both selenium-fed and trained mice, while it was significantly lower in *Cmpt* animals compared to untrained control mice.

Composition of proteins that regulate [Ca^{2+}]$_i$ in the old muscle:

We examined the expression pattern of key ECC linked proteins in skeletal muscle. Interestingly, RyR1 showed a strong age-dependent decrease in all groups of untrained animals but not in trained mice. It is noticeable that the lifelong run retained the full-length RyR1 content at the level of young animals. On the other hand, a significantly increased amount of the lower molecular weight degradation product of RyR1 was observed in old animals. In contrast, lifelong training resulted in significantly less degradation of the normal 550 kDa protein. The expression pattern of RyR1 was confirmed by two different antibodies. Interestingly, in contrast to RyR1, there was no significant difference in normalized levels of SERCA and DHPR between different groups of old animals. In addition, we did not find a significant difference in the amount of DHPR and SERCA between young and old control animals. Since selenium feeding effectively compensate the age-related decrease in muscle performance (as discussed above), and in a previous study we showed that it increases the selenoprotein N (Sepn) content of muscles, it was worth investigating that whether selenoprotein N expression changes with age and, if so, whether selenium supplementation can compensate this change. The expression of 70 kDa Sepn showed a gradual decrease with age, in 22-month-old mice it was decreased to 31% of the value measured in neonatal animals. After a diet supplemented with selenium, a remarkable increase in selenoprotein N levels was observed. In parallel, the mean selenium content of EDL muscles in old mice was determined and was found to be significantly higher in selenium-fed mice (197.1±6.6 ng/g, n=4, p<0.05) than in control animals (140.5±1.8 ng/g, n=4).

Increased ROS production in old muscle:

An earlier study described that RyR1 is affected by oxidative stress, and therefore we hypothesized that selenoproteins may protect against the negative effects of ROS on the ryanodine receptor. The above results suggest that the muscles of old animals may be exposed to increased oxidative stress than those from young mice. To confirm this hypothesis, we examined the

oxidative state of muscle fibers by measuring ROS production. For this purpose, fibers isolated from FDB muscles of young (4 months old) and old (22 months old) mice were loaded with Dihydroethidium, and dye fluorescence was recorded before (F_b) and after (F_f) a fatigue protocol. The protocol consisted of 150 tetanus elicited by 2 ms long square pulses given at 50 Hz. ROS production was quantified by calculating the ratio of background-corrected fluorescences (F_f/F_b). This value was significantly higher after the fatigue protocol in older mice (1.54±0.14, n=4, p<0.05) compared to young muscle fibers (1.11±0.07, n=6). This confirmed our hypothesis that old muscles are exposed to increased oxidative stress.

Skeletal muscle fibers from sAnk1 KO mice decrease SR calcium content:

In the second part of our work, we also examined the effect of aging on skeletal muscle function in a special mouse model. In these animals, the sAnk1 protein, which plays a key role in SR localization, was missing. Previous studies have shown that with age, severe structural damage develops in muscle fibers from sAnk1 KO mice. Therefore, we examined how skeletal muscle calcium homeostasis changes in this mouse model with age.

The effect of sAnk1 deficiency on Ca^{2+} homeostasis was examined in individual FDB fibers from 4-month-old (young) and 10-month-old (old) mice. We first measured resting $[Ca^{2+}]_i$ on unstimulated FDB fibers from control and sAnk1 KO mice. Based on the measurements, resting $[Ca^{2+}]_i$ did not differ significantly in FDB fibers from either 4 or 10-month-old sAnk1 KO mice compared to the control. Ca^{2+} transients were evoked by the treatment with a calcium-releasing solution (calcium-releasing cocktail) in a calcium-free extracellular solution. Addition of the releasing cocktail to fibers from control mice resulted in large Ca^{2+} transients, which began to decrease as a result of calcium depletion while the cocktail was still present on the fiber. Measurements in genetically modified mice showed that the mean change in amplitude of Ca^{2+} transients ($\Delta[Ca^{2+}]_i$) was significantly smaller in fibers from 4 and 10-month-old KO mice compared to age-matched control mice. This may suggest that the SR volume, and thus the total Ca^{2+} content, is lower in sAnk1 KO mice than in control mice. In the following, $\Delta[Ca^{2+}]_i$ were examined using whole cell voltage clamp technique by gradually increasing membrane depolarization between −60 mV and +30 mV. The mean changes in the amplitude of Ca^{2+} transients were significantly smaller in 4-month-old KO mice compared to the control. In similar experiments, a difference was also observed between sAnk1 KO and control

10-month-old animals, but this did not reach a statistically significant level. When we calculated the total amount of Ca^{2+} released during the maximum depolarization pulse (+30 mV), we found a significant difference between sAnk1 KO and control mice only in 4-month-old mice, but not in 10-month-old mice. The parameters of the Boltzmann function describing the voltage dependence of transients were only slightly affected by the absence of sAnk1 in young and old animals, although the latter was less steep. Interestingly, the midpoint voltage of the Boltzmann curves shifted to the right by almost 20 mV, i.e., toward a more positive voltage for both groups of old (10-month-old) animals. To determine whether the decreased Ca^{2+} transients observed after treatment with the releasing cocktail or induced by membrane depolarization resulted from altered calcium transport by the SERCA pump, we calculated the maximum Ca^{2+} transport rate (V_{max}) of the pump in the fibers of control and sAnk1 KO mice. The analysis showed that V_{max} was significantly reduced in the old animals compared to the young in both the control (p=0.0057) and sAnk1 KO groups (p<0.0001). This suggests that a remarkable but not statistically significant decrease in Ca^{2+} transients with age can be partly explained by lower SERCA pump activity. However, the difference in Ca^{2+} transients observed between control and sAnk1 KO muscle fibers at 4 months of age was not attributable to the SERCA pump, as there was no difference in V_{max} between the two genotypes at this age (4.72±0.08 and 4.79±0.07 mM s^{-1}, p= 0.54, n=9 in both groups).

Spontaneous Ca^{2+} release events (CRE) are smaller in sAnk1 KO mice:

Measurement of spontaneous Ca^{2+} release events in saponin-permeabilized muscle fibers provides an opportunity to study the functional properties of RyR1 in a native environment independently of DHPR control. Using this method, we tested whether the absence of sAnk1 affects spontaneous Ca^{2+} release from SR. Spontaneous CRE measurements were performed by confocal microscopy with two-dimensional (XY) imaging on fluo-3 loaded FDB muscle fibers from 4 and 10 month old control and sAnk1 KO mice.

A significant decrease in the frequency of CREs was observed in the fibers of 4-month-old sAnk1 KO mice compared with age-matched control mice. The frequency of CREs also decreased in the fibers of 10-month-old control mice compared to young control animals. However, no further difference in the frequency of CREs was observed between KO and control mice in the old age.

Subsequently, the mean CRE amplitude (F/F_0) was calculated and a significant decrease was found in 4- and 10-month-old KO mice compared to the same age control. Analyzing the distribution of amplitudes of CREs, we showed that smaller amplitude CREs were present at a higher frequency in KO animals compared to age-matched controls. Analysis of the spatial dimension of the CREs showed that the width of the events measured at half of the maximum (FWHM) decreased significantly both parallel to the Z-lines (FWHM-Y) and perpendicular to it (FWHM-X) in the 4- and 10-month old KO mice. This was associated with an increased number of low-amplitude CREs in the fibers of KO mice. These changes were clearly reflected in the calculated amount of Ca^{2+} released, which is estimated by signal mass (SM) during CREs. The absence of sAnk1 was accompanied by a significant decrease in SM, regardless of the age of the mice. These data indicate that the amount of Ca^{2+} available for immediate release in the SR is decreasing or that the opening of Ca^{2+} channels is damaged, possibly at the same time in sAnk1 KO mice.

Summary

Sarcopenia, aging-related loss of muscle mass and muscle strength, is a disease that negatively affects the quality of life of an aging society and severely limits physical performance and daily activity. The chances of muscle damage may increase in the absence of the essential trace element selenium, which can also cause muscle pain, fatigue and weakness. Lack of muscle-specific proteins can have similar negative consequences in young and old age.

In our studies, we showed that the rate of voluntary running, maximal twitch and tetanic force, and the amount of Ca^{2+} released from the internal stores of muscles decreased, while the production of reactive oxygen radicals increased in old mice compared to young animals. These changes were associated with decreased calcium release channel RyR1 and Selenoprotein N content and increased RyR1 degradation. Both lifelong exercise and short-term selenium dietary supplementation were able to compensate for the decrease in muscle strength and Ca^{2+} release from SR in old mice. However, the increase in muscle mass at a young age was not able to do so, as demonstrated by our experiments in myostatin-deficient mice. Selenium supplementation significantly increased Selenoprotein N levels in old mice and we have shown that its beneficial effect on muscles is mediated through the RyR1 independent pathway.

We examined the calcium content of SR in sAnk1-deficient mice where the location of SR was not normal, which was significantly decreased in young and old sAnk1 KO animals, whereas additional structural changes appeared only during aging. We have shown that the absence of sAnk1 also affects intracellular Ca^{2+} homeostasis. While resting cytosolic Ca^{2+} levels did not change in fibers from sAnk1 KO mice, the amplitude of Ca^{2+} transients induced by depolarizing pulses decreased without changing their voltage dependence. Furthermore, analysis of spontaneous Ca^{2+} release events (sparks) on saponin-permeabilized fibers showed that the frequency of sparks was also significantly lower in young and old KO mice compared with the control. In addition, the amplitude and spatial dimension of the sparks, and thus the amount of calcium released, were also significantly lower in KO animals.

In summary, our results suggest that moderate exercise and selenium dietary supplementation have a beneficial effect on calcium release and muscle strength from SR in old age, while increased muscle mass does not necessary improve physical performance during aging. Furthermore, the lack of sAnk1 not

only has a negative effect on the structure of SR, but also causes its decreased Ca^{2+} storage capacity, which ultimately results in lower Ca^{2+} release and consequently weaker muscle strength. Our results may pave the way for new therapeutic options for sarcopenia and genetic muscle diseases in old age.

Appendix

Registry number: DEENK/221/2020.PL
Subject: PhD Publikációs Lista

Candidate: Dana Al-Gaadi
Neptun ID: EB7LHF
Doctoral School: Doctoral School of Molecular Medicine
MTMT ID: 10058622

List of publications related to the dissertation

1. Fodor, J., **Al-Gaadi, D.**, Czirják, T., Oláh, T., Dienes, B., Csernoch, L., Szentesi, P.: Improved Calcium Homeostasis and Force by Selenium Treatment and Training in Aged Mouse Skeletal Muscle.
Sci. Rep. 10, 1707, 2020.
DOI: http://dx.doi.org/10.1038/s41598-020-58500-x
IF: 3.998 (2019)

2. Pierantozzi, E., Szentesi, P., **Al-Gaadi, D.**, Oláh, T., Dienes, B., Sztretye, M., Rossi, D., Sorrentino, V., Csernoch, L.: Calcium Homeostasis Is Modified in Skeletal Muscle Fibers of Small Ankyrin1 Knockout Mice.
Int. J. Mol. Sci. 20 (13), 3361, 2019.
DOI: http://dx.doi.org/10.3390/ijms20133361
IF: 4.556

List of other publications

3. Sztretye, M., Geyer, N., Vincze, J., **Al-Gaadi, D.**, Oláh, T., Szentesi, P., Kis, G., Antal, M.,
   Balatoni, I., Csernoch, L., Dienes, B.: SOCE Is Important for Maintaining Sarcoplasmic
   Calcium Content and Release in Skeletal Muscle Fibers.
   Biophys. J. 113, 2496-2507, 2017.
   IF: 3.495

Total IF of journals (all publications): 12,049
Total IF of journals (publications related to the dissertation): 8,554

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on
the basis of the Journal Citation Report (Impact Factor) database.

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