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Effects of carotenoid dietary supplements on muscle performance and calcium homeostasis

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Introduction

Skeletal muscle

Muscle tissue is made up of different cell types that are specialized for contraction. Each cell type contains contractile proteins called actin and myosin, but they differ in their distribution, their amino acid sequence and the function of the proteins that regulate them. We distinguish three types of 'classical' contractile muscle cells that originate from the mesoderm: smooth muscle, cardiac muscle and skeletal muscle. While smooth muscle cells do not have regularly distributed sarcomers, cardiac and skeletal muscle cells have a well-organized striated structure that can be clearly observed by light microscopy.

Special membrane structures of skeletal muscle

T-tubule and sarcoplasmic reticulum

The skeletal muscle has a specially modified endoplasmic reticulum called the sarcoplasmic reticulum (SR). The tubules of the SR and the transverse tubules (T-tubules), which are formed by invaginations of the sarcolemma, are in very close physical proximity to each other in the triads. The triad plays a fundamental role in electro-mechanical coupling processes. The longitudinal tubule of the SR meshes around the myofibrils and plays an important role in muscle relaxation, as it is the site of the sarco-(endo)plasmic reticulum calcium ATPase (SERCA) pumps that are active transporters. The functional significance of T-tubules lies in their ability to allow simultaneous activation of the entire cross-section of skeletal muscle fibres. These processes occur through electro-mechanical coupling, whereby the action potential propagating through the T-tubules activates calcium channels in the SR, resulting in calcium release from the sarcoplasmic reticulum. This calcium plays a role in the process of muscle contraction.

The electro-mechanical coupling in skeletal muscle

Muscle contraction and relaxation are regulated by rapid changes in cytoplasmic Ca^{2+} levels. In a physiologically functioning skeletal muscle tissue, Ca^{2+} signaling is established by excitation contraction coupling (ECC), whereby the action potential (electrical signal) results in Ca^{2+} release (mechanical response). The main components in this signal conversion are the triads mentioned above, since in the triads a specific mechanical interaction between the voltage-dependent L-type Ca^{2+} channels (CaV1.1), also known

as dihydropyridine receptors (DHPRs), and type 1 ryanodine receptors (RyR1, a Ca^{2+} channel in the SR membrane) is established during ECC. After depolarization of the T-tubule membrane, DHPRs undergo a conformational change that leads to the opening of RyR1s Ca^{2+} channels due to mechanical coupling. The subsequent rapid and massive release of Ca^{2+} into the myoplasm triggers muscle contraction. The calcium ions released in the myoplasmic space bind to troponin-C molecule and thereby activate the contractile system. The interaction of actin and myosin, which is the sliding of thin and thick filaments side by side, produces muscle contraction and force generation. The SERCA pump actions and Ca^{2+} (re)uptake by the mitochondria allows muscle relaxation, preparing the muscle for the next cycle of ECC.

Mitochondria

Since they are main ATP sources, their influence on redox status, their ability to modulate pH levels and their role in calcium balance as a buffer system, mitochondria are key to the proper functioning of muscle. In the absence of ATP and calcium, muscles are unable to contract, so any alteration in mitochondria can lead to myopathy or other diseases linked to poor skeletal muscle function.

The skeletal muscle mitochondria actively contribute to the spatial and temporal distribution of Ca^{2+} through the mitochondrial Ca^{2+} uniporter (MCU), a 40-kDa, Ca^{2+} selective channel. Mitochondria shape myoplasmic Ca^{2+} concentrations and are energetically contribute to muscle metabolism through Ca^{2+}-mediated regulation, which influences mitochondrial tricarboxylic acid cycle (TCA) and electron transport chain activity.

In skeletal muscle mitochondria, an essential player in its Ca^{2+}-based regulation is the MICU1 (mitochondrial calcium uptake 1) protein, a modulating, gating component of the MCU (mitochondrial calcium uniporter) complex, whose loss-of-function mutations can lead to the development of severe clinical muscle phenotypes in patients (e.g. severe muscle weakness or chronic fatigue).

The dynamics of the mitochondrial network are governed by a delicate balance between fusion and fission processes. Fusion allows for content mixing within the mitochondrial population, thus preventing the permanent loss of essential components. Fission is necessary for the generation of new mitochondria, but also contributes to quality control by

allowing the removal of damaged mitochondria, thus promoting apoptosis in the presence of high cellular stress.

Mitofusins 1 and 2 (Mfn1/2) are involved in the fusion of the outer mitochondrial membrane (OMM), while optic atrophy 1 (OPA1) promotes the fusion of the inner mitochondrial membrane (IMM). Fission of mitochondria is controlled by the proteins mitochondrial fission 1 (Fis1), dinamin-related protein 1 (Drp1) and mitochondrial fission factor (Mff).

In addition to pathological conditions, ageing is also a factor that affects mitochondrial function. The mitochondrion is considered to be the main mediator in the development of ageing and age-related diseases, as the dysfunction of this cellular organelle contributes to the development of several human diseases. Since antioxidants are able to suppress the overproduction of ROS, it is hypothesized that they may also be able to slow down the ageing process.

Reactive oxygen species

The "Free-Radical Theory of Ageing" was established in 1950, which explains the mechanism of ageing at the cellular and molecular level. The theory states that reactive oxygen species (ROS), which are produced during cellular metabolism, damage cellular components and that the resulting damage is responsible for ageing.

The most abundant forms of intracellular ROS include superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($\bullet OH$), hydrogen peroxide (H_2O_2) and lipid peroxidases (LOOH). The majority of ROS molecules are produced in skeletal muscle during contraction as well as at rest. In mammalian cells, the main source of ATP is the mitochondrial electron transport chain (ETC), which can produce $O_2^{\bullet}$ molecules at at-least 11 different sites on both sides of the inner mitochondrial membrane.

Reactive oxygen radicals can adversely affect several participants of the ECC mechanism. Both DHPR and RyR1 are affected by ROS agents, and therefore the redox state of the cell may dominantly affect skeletal muscle calcium homeostasis and thus muscle contraction.

Ageing and sarcopenia

Ageing is a physiological process that causes a general decline in physical and mental ability. The musculoskeletal system is greatly affected, as muscle mass and function are progressively reduced, fatigue/exhaustion,

weakness and loss of mobility occur, which impair the quality of life of older people. Today, an increasing proportion of the world's population are ageing people, and this proportion is expected to triple by 2050.

To date, a number of theories have been proposed to explain the physiology of ageing, such as genetic predisposition, programmed ageing, DNA degradation, endocrine dysfunction, increased free radicals and mitochondrial dysfunction. Cellular changes during ageing have a direct impact on skeletal muscle function, which may contribute to a decline in muscle strength and overall physical performance. During ageing, voltage sensitivity and a decrease in L-type calcium channel currents may be associated with inadequate voltage-dependent Ca^{2+} release and a specific decrease in muscle strength.

Another explanation for the decrease in muscle strength may be the altered Ca^{2+} release from the SR, which might be triggered by alteration of RyR1 by oxidative stress, most likely due to altered mitochondrial function.

Sarcopenia is the loss of skeletal muscle volume associated with ageing, which is a major health challenge today. Sarcopenia is characterized by a reduction in muscle mass and strength, increased susceptibility to fatigue and reduced muscle contraction velocity. The reduction in muscle mass is mainly due to a reduction in the number and size of fibres; however, there is also a degeneration of neuromuscular junctions as a consequence of stem cell exhaustion, leading to a loss of motor units.

Disturbances in the regulation of Ca^{2+} homeostasis and increased oxidative stress and ROS production are mechanisms that contribute to the age-related decline in muscle strength.

Oxidants and antioxidants

O_2^- produced by cellular organelles or cytoplasmic enzymes is rapidly and spontaneously converted to H_2O_2, a process catalyzed by superoxide dismutase (SOD) isoforms. H_2O_2 is the primary redox signaling molecule in the redox regulation of biological phenomena.

Antioxidants

An antioxidant is any substance that can bind reactive oxygen species or inhibit oxidation processes in the cell.

Vitamin A (retinol) is involved in redox processes and is considered a potential antioxidant in exercise-induced oxidative stress. However, in some studies, its effects were not beneficial as it increased oxidative stress in skeletal muscle, leading to tissue damage.

Antioxidants may be able to directly bind free radicals, inhibit ROS production and reduce peroxide concentrations and restore the oxidative state of the membrane. The other mechanism of action of antioxidants is to activate the body's antioxidant genes and prevent the oxidation of low-density lipoprotein (LDL) cholesterol, thus providing anti-apoptotic protection to the brain, heart and liver.

Carotenoids and their effects

Carotenoids are precursors of vitamin A, which are natural pigments in fruits and vegetables. They provide the yellow, orange or red color in nature thanks to their highly conjugated bonds.

They can be divided into two main groups according to their chemical structure:

1) carotenes, that contain only hydrogen and carbon;

2) xanthophylls, which have in their chemical structure, besides oxygen, several functional groups such as epoxy, methoxy, hydroxy, carbonyl and carboxy.

Carotenoids have received special attention in the last decades due to their strong antioxidant, regenerative, antiproliferative, anti-inflammatory and potentially anti-ageing effects, for which they are also called geroprotectors.

Vitamin A (retinol) is involved in the redox processes of the body and as a potential antioxidant may act against the production of ROS induced by physical stress. Vitamin A derivatives, collectively known as retinoids, play a key role in vertebrate development. They play a role in the differentiation of certain epithelial cells and are also important in vision, growth, reproduction and protection against infection.

Geroprotective carotenoids: astaxanthin (AX) and krill oil

The group of xanthophylls includes astaxanthin (AX), which has recently attracted much attention and was first isolated from lobster by Kuhn and Soerensen. This particular carotenoid is responsible for the bright red

color of most aquatic animals, such as salmon and crustaceans, as well as the pink color of flamingos. AX is not produced *de novo* in our body and must be obtained from an external source, mostly from food via an adequate diet.

The main source of AX is krill oil, which is extracted from the small crustacean *Euphausia superba*, a common species in the Pacific Ocean; krill oil contains between 0.1 and 1.5 mg/ml astaxanthin, depending on the processing method. In addition to AX, krill oil also contains vitamin A and E, minerals, ω-3 polyunsaturated fatty acids (ω-3 PUFAs), phospholipids (PL) and flavonoids, and is now being investigated as a potential health-promoting dietary supplement. The PUFAs: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are in the form of long-chain ω-3 fatty acids that can be used directly by the body, unlike fatty acids which are in the phospholipid form.

The effects of AX on skeletal muscle

During the physiological function of skeletal muscle, ROS and NOS (nitric oxide species) are generated from mitochondrial or non-mitochondrial sources such as NADPH, xanthine oxidase, phospholipase A2 and NO synthase. During exercise, our body maintains an appropriate oxidative balance, which is achieved by a sophisticated endogenous antioxidant system. All this requires antioxidant proteins such as glutathione peroxidase, superoxide dismutase, thioredoxins, peroxiredoxins and catalase, which are able to reduce ROS, and substrates of the internal antioxidant system such as glutathione, which can bind ROS.

With regular exercise, this endogenous defense system is also upgraded and in pathological conditions such as diabetes or cancer, our endogenous antioxidant system may be our most effective health protective mechanism. As a result, the use of antioxidants in medical treatments is widespread, and people who participate in sporting activities also use them and expect enhanced performance from their consumption. The most widely used antioxidants are the well-known vitamins C and E. In some cases, however, the use of these vitamins could increase ROS production and the chances of developing human diseases, hence the need to use antioxidants with more specific actions.

In humans, the positive effects of AX on skeletal muscle have been demonstrated in a number of studies; Sawaki and colleagues showed that 6 mg of AX consumed for a month reduced lactic acid levels in skeletal muscle after a 1200 m sprint run. Wolf and colleagues have shown that AX increased

the respiration rate of mitochondria by increasing the diffusion of oxygen across the mitochondrial membrane and thus increasing cellular energy production. Several data suggest that AX is able to protect mitochondria from oxidative stress, as AX is able to cross membranes, which is one its specific effects. AX can therefore play a prominent role for people with active lifestyles and athletes who are interested in higher performance and faster recovery.

Aims

Reactive oxygen radicals (ROS) produced by muscle work can play a role in damaging various components involved in the electro-mechanical coupling of muscles, including DHPRs and RyR1s. In addition, it is hypothesized that ROS may also affect mitochondrial calcium uptake. It remains to be clarified what effect do antioxidants, coming from external sources through diet, have on the electro-mechanical coupling of skeletal muscle and mitochondrial calcium homeostasis.

In the first part of my PhD work, I aimed to examine the changes (if any) in young adult healthy mice fed with AX supplemented rodent chow in terms of muscle strength, electro-mechanical coupling and mitochondrial calcium homeostasis. For this purpose, we formulated the following questions:

- To what extent does AX feeding affect the physical performance of mice, including muscle strength?
- How does AX feeding affect weight gain?
- How does AX affect skeletal muscle calcium homeostasis in mice, including electro-mechanical coupling and mitochondrial calcium homeostasis?

In the second part of my PhD research work, I aimed to investigate the potential molecular mechanisms underlying the nutritional supplementation of AX and krill oil in the skeletal muscle of ageing mice. We thus formulated the following goals for our investigations:

- What is the effect of AX and krill oil supplementation on the physical performance of ageing mice, including muscle strength?

• How do carotenoids affect skeletal muscle calcium homeostasis in ageing mice, including electro-mechanical coupling and mitochondrial calcium homeostasis?

• Does krill oil supplementation have an effect on the learning ability of ageing mice?

Materials and methods

Animal care

During the animal experiments, we followed the relevant guidelines of the European Community (86/609/EEC). The Institutional Animal Welfare Committee of the University of Debrecen approved the experimental protocol (3-1/2019/DEMAB). C57/Bl6 mixed-sex mice were housed in plastic cages with metallic roofs and kept at normal room temperature (22-25°C). Mice were provided with unlimited access to drinking water and food. The room was lit automatically in a 12-hour dark and 12-hour light cycle.

Astaxanthin and krill oil supplementation

In our experiments, we used AstaReal A1010 (AstaReal Co., Ltd., Nacka, Sweden), a natural biomass material containing astaxanthin, which is produced from the crushed and dried spores of the microalgae *Haematococcus pluvialis.*

To investigate the effect of AX on young, healthy animals, 18 4-6 month old, mixed-gender C57/Bl6 mice were randomly divided into two groups (AX and CTRL group). The AX diet (per os) lasted for 4 weeks, while littermate control animals received standard mouse chow. The special food was prepared by adding 4 g/kg of AstaReal A1010 (dissolved in 100% ethanol) to the standard pelleted mouse chow, so the final concentration would reach 0.02% AX. This concentration was selected based on the literature.

In some of our experiments, we used krill oil supplemented rodent chow (Rimfrost Inc. Alesund, Norway) at a concentration of 25 g/kg in order to reach the 0.02% AX concentration used in earlier experiments.

To investigate the effects of AX and krill oil on ageing animals, we used mixed-gender C57/Bl6 mice, aged between 13 and 17 months. In addition to the randomly formed groups fed with AX and CTRL, we also

created a third group (Krill), which received rodent chow supplemented with krill oil, at a final concentration of 25 g/kg, for 4 weeks.

In vivo experiments

Weight measurement

Before the start of the feeding protocol (day 1) and after the four-week feeding period (day 28), we measured the body weight of the mice belonging to the CTRL, AX and Krill groups. Changes in body weight were monitored together with food consumption, recorded and averaged per group.

Grip test

Mice were held horizontally to a bar connected to a capacitive force gauge, which was grasped by the mouse with its forepaws. Hind limbs were not allowed to touch any surface. When the mouse reliably grasped the rod with its forepaw, we gently pulled the animal away from the device by its tail until it released it. In this way, we measured the grasping ability, the grip strength. We recorded the maximum force measured before the animal released the bar. The data were stored on a computer connected to the measuring system. The test was repeated 10-15 times during the measurement, which was averaged to obtain the in vivo muscle strength value. The grip force thus obtained was normalized to the body weight of the animals. The measurements were performed before and after the feeding period. The changes were always compared to the control of the given group.

Barnes Maze test

The Barnes Maze protocol was used to evaluate the effect of dietary supplementation with krill oil on learning and memory in ageing mice. For the test, we used a 92 cm circular wooden platform with 20 holes (5 cm in diameter) evenly distributed along the edge. A plastic box filled with litter was installed under a particular hole. We positioned the platform so that it is surrounded by three distinct visual landmarks. The location of the platform was not changed during the experiment, and the platform was under continuous illumination. The movements of the mice on the platform were recorded with an overhead web camera (GeniusWideCam F100, Genius, New Taipei City, Taiwan). The protocol performed on each animal consisted of three parts:

- During habituation (day 1), the mouse was placed in the middle of the platform and covered with a closed cylinder (diameter 8 cm) for

1 minute. After the cylinder was removed, the mouse was allowed to familiarize itself with the environment for 5 min or until it found the escape box.

• The acquisition training took place over 10 days, twice a day at 1-hour intervals. The mouse was covered with the cylinder for 15 seconds, and after its removal, the mouse was given 3 minutes to find the location of the escape box. A bright light and 90 dB white background noise were used to induce the mice to escape. If the mice did not find the escape hole in time, we placed them in the escape box and kept them inside for 1 min for habituation purposes.

• The blind test was held 3 days after the acquisition training. All steps were identical except that the length of the test was only 1 minute and the escape box was removed.

Video analysis with the help of AnimalTracker

The AnimalTracker program was used to analyze the videos recording the movements of the animals during the Barnes Maze experiments. The Morris water maze module was adapted to the Barnes maze to calculate the time and distance traveled by the mice from the starting location to the target hole.

In vitro experiments

To sacrifice the experimental animals, we used cervical dislocation after CO_2 overdose. We dissected the *m. flexor digitorum brevis* (FDB) and *m extensor digitorum longus* (EDL) muscles for functional studies; for molecular biological studies we used *m. tibialis anterior* (TA) and *m. gluteal* muscles.

Muscle force measurement

In order to conduct *in vitro* muscle strength measurement, the EDL muscles were placed horizontally in an experimental chamber, where they were continuously perfused with Krebs solution (10 ml/min) bubbled with carbogen (95% O2 and 5% CO2), at room temperature. One end of the muscle was attached to a capacitive mechano-electric force meter (Experimetria, Budapest, Hungary), while the other end was restrained with an insect needle. The muscles were then stretched to a length at which they could exert maximum force. After waiting for 5 minutes, two platinum electrodes touching the muscles from below were used to deliver 2 ms supramaximal (5

V) pulses. These pulses with a frequency of 0.5 Hz were used to induce at least 10 individual twitches. To induce tetanus, individual pulses (5 V, 2 ms) were applied with a frequency of 200 Hz for 200 ms. The resulting force was digitized at 2 kHz using a TL-1 DMA interface and stored on an online connected computer using Axotape software (Axon Instruments, Foster City, CA, USA). Finally, the force was normalized to the cross-sectional area of the given EDL muscle.

Isolation of FDB muscle cells

Calcium measurements were performed on individual skeletal muscle fibers from the FDB muscles. Manual preparation of FDB muscles in normal Tyrode's solution was followed by enzymatic digestion in calcium-free Tyrode's solution containing 0.2% collagenase type I (Sigma-Aldrich, St. Louis, MO, USA, catalog number: SCR103) at 37 °C, for 50 minutes. After the enzyme treatment, the FDB muscles were placed in normal Tyrode's solution and stored in a refrigerator at 4 °C until use (within 36 hours maximum). After gentle trituration of the muscle with a pipette, individual FDB fibers were obtained.

Voltage-clamp measurement and confocal microscopy

The patch clamp technique was used for calcium measurements on FDB fibers. At the beginning of the measurement, the isolated FDB fibers were placed in an external solution and their resting potential was recorded using the voltage-clamp technique (Axoclamp 200B, Axon Instruments, Foster City, CA, USA). To examine changes in cytosolic calcium, we used different depolarizing voltages through the pClamp 11.0 program (Molecular Devices, San Jose, CA, USA) and simultaneously performed line-scan recordings with a confocal microscope (Zeiss 5 Live, Oberkochen, Germany). The fibers were dialyzed through the patch pipette with an internal solution containing 50 μM rhod-2 and 10 mM EGTA; after 15-20 minutes of incubation, depolarizing pulses were applied. Rhod-2 was excited at 543 nm, the emission above 550 nm was recorded using a suitable filter. The experiments were performed at room temperature (20-22 °C). The resting potential was set to -80 mV, the pipette resistance varied between 2 and 4 MΩ.

The line-scan images were analyzed with a custom-made program, in which the model described by Royer was taken into account with the following parameters: K_d (rhod-2) = 1.58 μM and k_{ON} = 0.07 μM^{-1} ms^{-1}.

A Boltzmann function was used to describe the activation voltage (V_m) dependence:

$$Ca\ (V) = Ca_{max}/(1 + \exp(-(V_m - V_{50})/k) \quad \text{Eq. (1)}$$

In Eq.(1), V_{50} is the half-activating voltage, Ca_{max} is the maximum measured calcium value, while 1/k is the logarithmic slope of the function. The individual data points were normalized to the Ca_{max} value and plotted as a function of the membrane potential.

In some experiments, isolated FDB fibers were pretreated with 10 μM retinol for 3 h in normal Tyrode's solution at 4 °C.

Mitochondrial calcium uptake measurement

Mitochondrial calcium level changes in individual FDB fibers were investigated using rhod-2 AM calcium-sensitive dye following repeated electrical stimulation following Ainbinder et al. (2015). FDB fibers were incubated with 5 μM rhod-2-AM for 15 min at room temperature and then washed with normal Tyrode solution. The fibers were electrically stimulated (S88 Stimulator, Grass Technologies, Warwick, RI, USA) through a pair of platinum electrodes, which were placed near the fiber considered to be healthy. Five consecutive tetanus stimulation series (500 ms duration, 100 Hz) were applied to the fibers at a supramaximal activation voltage. x-y images (512,512 pixels, 0.5 ms/pixel) were taken at rest, after the 1st and 5th tetanus. The rhod-2 fluorescence values were calculated using the following equation:

$$F_{mito}=(F_{I\text{-}band} - F_{A\text{-}band})/F_{A\text{-}band} \quad \text{Eq. (2)}$$

Molecular Biology

RNA preparation, Reverse Transcription (RT) and quantitative Polimerase Chain Reaction (qPCR)

The total RNA fraction was isolated from homogenized TA skeletal muscle samples from CTRL, AX and krill oil-fed mice using TRI reagent (MRC, Cincinnati, OH, USA, cat. no. TR118). The isolated RNA was resuspended in nuclease-free water and stored at -80°C before starting the experiments. RNA concentration and quality were determined using a spectrophotometer at a wavelength of 260 nm (NanoDrop ND1000; Promega Biosciences, Madison, WI, USA). Isolated RNA was treated with DNase and RNase inhibitor (Ambion, Austin, TX, USA). Using the Omniscript RT kit

(Qiagen, Germantown, MD, USA; catalog number: 205113), 1000 ng of the isolated total RNA was converted into complementary DNA (cDNA) by reverse transcription. cDNA synthesis was performed using random hexamers in a reaction volume of 25 µl. For quantitative RT-PCR, we used Taqman Gene Expression Assays with Taqman™ Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA). Amplification was performed using a Light Cycler 480 Master instrument (Roche, Basel, Switzerland) (for plates cat. no. Roche: 04729692001; for sealing foils cat. no. Roche: 04729757001). Taqman probes purchased from Thermo Fisher Scientific (Waltham, MA, USA) were used for Taqman gene expression assays. The amplification program was 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Relative expression values of transcripts were calculated using the comparative Ct method and AP3D1 (Mm00475961_m1) was used for normalization. All qPCR reactions were performed in triplicate. Cp values were determined using Light Cycler 480 SW 1.5.0 software (Roche), and relative copy numbers were calculated using the DCp method. Finally, in the case of the studied samples (MCU, Mfn2, CB1, Drp1) and normalization gene (AP3D1), the ratios of the measured values gave the relative expression levels.

Western Blot

For Western blot experiments, the whole cell lysate from *m. glutealis* was isolated using a mechanical force extraction method. Cell lysates were prepared from the tissue samples by applying shear force to the cells with stainless steel balls. Protein content was measured using a modified BCA protein assay (Pierce, Rockford, IL, USA). The samples were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 10% gels were loaded with an equal (40 µg) amount of protein per lane. The samples were then transferred to a nitrocellulose membrane (Bio-Rad). The protein-binding nitrocellulose membranes were blocked with a 5% milk-PBS solution. Proteins were detected with rabbit anti-MICU1 primary antibodies (Thermo Fisher Scientific, Waltham, MA, USA, PA5-77364, polyclonal, 1:500), rabbit anti-Mitofusin-2 (Cell signaling Technology, Danvers, MA, USA 9482 and 8570, 1:500) and labeled with mouse anti-Drp1 (Santa Cruz, Dallas, TX, USA, sc-271583, monoclonal, 1:500) diluted in milk containing 5% PBS. Horseradish peroxidase-conjugated goat anti-rabbit IgG Fc antibodies (Bio-Rad; 1:1000 in milk containing 5% PBS) were used as secondary antibodies, and immunoreactive bands were visualized with SuperSignal® West Pico or Femto Chemiluminescent Substrate enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) were visualized using a KODAK Gel Logic 1500

Imaging System (Eastman Kodak Company, Kodak, Tokyo, Japan). To determine equal loading, membranes were reprobed with mouse anti-α-actinin antibody (1:1000 dilution in milk containing 5% PBS, Santa Cruz, Dallas, TX, USA, sc-166524) and visualized as described above.

Statistical analysis

In this work, the data were given as the standard error of the mean (SEM). For *in vivo* grip test measurements, paired t-tests were used to compare data before and after carotenoid dietary supplementation for each group. For *in vitro* force measurement, the mean and SEM were calculated as the average of the values of muscles from the same animal, while the number of samples was the number of animals within a given group. One-way analysis of variance (ANOVA) and pairwise Bonferroni's multiple comparison method were used to compare CTRL, AX and krill oil data. In all other cases, statistical significance was assessed using the Prism statistical program (GraphPad Software, San Diego, CA, USA).

Results

AX and krill oil supplementation increases *in vivo* muscle force

Initially, we examined the chronic effect of AX feeding on body weight. On the 1st and 28th day of the experiment, we measured the body weight of the mice fed with standard rodent chow (CTRL group) and the AX supplemented group. In young adult mice, mean body weight gain was significantly lower in AX mice than in CTRL animals. Mean food intake was the same for the two groups (0.21 ± 0.01 (CTRL) and 0.22 ± 0.02 (AX) g/day/gram of body weight). The average body weights for the two groups were as follows: 27.7 ± 1.0 g (day 1) and 28.8 ± 0.8 g (day 28) in the CTRL group, while for the AX group: 28.1 ± 0.7 g (day 1) and 28.2 ± 0.7 g (day 28).

To determine the *in vivo* muscle performance of young adult animals, we used the grip test, both at the beginning and at the end of the AX supplementation. After 4 weeks, the AX-fed mice were able to generate more force than their control counterparts.

Similar results were obtained when ageing mice were fed with AX and krill oil. While AX significantly reduced body weight gain by the end of

the fourth week on the specific diet, the group of animals consuming the krill oil-supplemented diet showed similar weight gain trends as the CTRL group. Here, the change in body weight was neither attributable to reduced food intake nor to reduced muscle mass (average food consumption: 0.21 ± 0.01, 0.22 ± 0.02 and 0.21 ± 0.03 g/day/body weight for the CTRL, AX and Krill groups). As for ageing mice, we also found that compared to the CTRL group, both AX and krill oil significantly increased the grip force normalized to body weight after four weeks.

AX and krill oil supplementation increases *in vitro* muscle force

To determine whether the AX diet can directly alter muscle function, *in vitro* muscle strength was tested in young adult mice. There was no difference in the mean amplitude of the normalized single twitch of the EDL muscles between the AX-fed and CTRL groups (1.69±0.13 mN/mm^2 for CTRL and 1.76±0.08 mN/mm^2 for AX). Cross-sectional area of EDL muscles did not differ between groups (1.30 ± 0.07 mm^2 for CTRL and 1.27 ± 0.05 mm^2 for AX). In contrast, AX feeding significantly increased the force exerted during tetanus. In case of EDL muscles, no significant differences were found in the time to peak (TTP) and half relaxation time (HRT) in both groups.

In ageing mice, we found that the amplitude of normalized twitches (1.65 ± 0.12 mN/mm^2 for CTRL, 3.06 ± 0.63 mN/mm^2 for AX and 2.33 ± 0.38 mN/mm^2 for krill) and tetanus was significantly increased in animals treated with AX and Krill (8.13 ± 0.40 mN/mm^2 for CTRL, 14.52 ± 2.94 mN/mm^2 for AX and 10.24 ± 1.11 mN/mm^2 for krill). Based on our results, we can say that in ageing mice, nutritional supplementation with AX proved to be more effective than krill oil, as it caused a marked increase in muscle strength both in case of individual twitches and tetani.

At the same time, as a result of AX treatment, no significant differences in parameters such as TTP or HRT were found. In contrast, krill oil administration significantly prolonged the TTP value in case of single twitches compared to the control.

Voltage dependence of calcium transients remains unaltered after carotenoid supplementation

In order to assess the effects of AX and the krill oil-containing diet on the mechanical connection between DHPR and RyR1, one of the key steps

of the ECC mechanism, calcium transients were investigated using the whole-cell voltage-clamp technique. On enzymatically isolated FDB fibers, starting from a holding potential of -80 mV, 100 ms long, increasing depolarizations were applied ranging between -60 and +30 mV the every 10 mV. The delay between the two pulses was 1100 ms. In parallel, calcium transients were recorded on a Zeiss Live confocal microscope.

Using Eq.(1) introduced in the Materials and Methods, we fitted a Boltzmann function to the maximum fluorescence values recorded during the given measurement for each analyzed cell. Neither the half-activating voltage (V_{50}) nor the slopes of the fitted curves (1/k) were different when comparing the data of CTRL and AX fibers (V_{50} = -13.03 and -16.57 mV, 1/k = 7.91 and 10.04 for CTRL and AX mice). Based on our results we conclude that the voltage dependence of calcium transients did not change in the two groups, that is, the AX treatment probably has no effect on the activation of calcium release, i.e. on the mechanical interaction between DHPR and RyR1.

The maximum of calcium transients calculated from the peak value of the fluorescence intensity (+10 mV depolarization) was not statistically different between the two groups (3.46 ± 0.22 for the CTRL and 3.68 ± 0.35 for the AX group). Since the depolarizing pulse duration was always constant (100 ms), the area under the F/F_0 transients was calculated from the beginning to the end of the pulse, and no statistical differences were found (172.1 ± 17.9 ms for CTRL and 176.4 ± 19.2 ms for AX; $p > 0.8$) These results suggest that the higher tetanic force observed in AX mice is not due to increased calcium release.

When we examined the effects of carotenoid antioxidant supplementation on the voltage dependence of calcium transients in ageing mice, we found that the diet containing krill oil pushed the V_{50} value slightly to the left, in other words towards more negative voltage values (V_{50} = 1.07, -5.80 and -8.77 mV and 1/k = 11.35, 12.78 and 8.65 for CTRL, AX and krill oil groups).

Unaltered calcium transients and calcium fluxes after carotenoid antioxidant supplementation

No significant changes were observed in the average F/F_0 peak fluorescence values between the studied groups, namely these values were 3.40 ± 0.39; 2.93 ± 0.31 and 3.53 ± 1.06 for CTRL, AX and krill oil, respectively. Overall, it can be said that the fluorescence, the calcium

concentrations calculated from it, and the calcium flux showed a very similar time course in the CTRL and carotenoid supplemented animals, meaning that there is no significant change in these respects after the use of special carotenoid-containing diet.

Retinol alters calcium release in skeletal muscle

In the next series of experiments, the acute effect of 10 μM retinol on the electro-mechanical coupling of skeletal muscle was investigated. Retinol is structurally similar to AX, but its intracellular metabolism is different. Our results show that the acute application of retinol shifted the half activating voltage of calcium transients and changed their slope (1/k = 7.91 and 14.76 mV, and V_{50} = -13.03 and -8.52 mV for CTRL and retinol groups, respectively). This effect was accompanied by a significant decrease in the peak value of calcium released from the SR in case of retinol pretreated fibers.

Since the sequestration of Ca^{2+} back into the intracellular store is the main regulator of the resting SR calcium content, we were curious whether the immediate - from activation to activation - Ca^{2+} uptake is also modified by the two investigated compounds. To test the effects of the drugs, we used a protocol to mimic muscle fatigue by applying a series of tetanic stimulation on CTRL fibers and treated with AX and retinol. Calcium transients were elicited by a series of depolarizing pulses to +30 mV with a delay of 1.5 s, each lasting 200 ms. In this experimental setup, calcium transients induced by continuous and repeated depolarization can be compared to tetanus twitches occurring during physiological muscle contraction. As a result of the serial stimulation, the F/F_0 value continuously decreased, as the calcium content of the store is depleted, and the calcium transients become smaller and smaller. We were surprised to find that this reduction was less dramatic in AX-treated fibers. In contrast, retinol treatment significantly reduced it. In all three cases, the fluorescence decrease was fitted with an exponential function. In the case of retinol application, the fitted exponential curve remained well below the CTRL and AX fitting, which indicates that after acute retinol treatment, muscle fibers are more prone to fatigue and were exhausted sooner.

Variable effects of carotenoid supplementation on activity dependent mitochondrial calcium uptake

For both animals groups (CTRL and AX), we experienced an increase in mitochondrial rhod-2 fluorescence intensity as a result of the stimulation, but in young animals, the AX diet seemed to have been more

effective, as it protected the mitochondria against calcium overload. Fluorescence averaged parallel to the axis of the fiber increased after tetanus stimulation, but the average fluorescence increase was smaller for the fibers of the AX-fed mouse group. In the ageing group, a diet supplemented with krill oil had a similar effect.

Variable effects of carotenoid supplementation on mitochondrial dynamics

In the continuation of our experiments, we were interested in elucidating the reasons behind the increased *in vivo* and *in vitro* force generation in connection with the applied nutritional supplement. Therefore, we investigated whether certain proteins involved in mitochondrial dynamics (fusion and fission) and Ca^{2+} regulation were affected by the special nutritional diet containing AX and krill oil. In order to characterize mitochondrial fusion, we examined Mfn2, in relation to mitochondrial fission, Drp1, and in relation to mitochondrial calcium uptake, we analyzed the expression level of MCU. In addition, we also studied the CB1 protein, as it was previously described that the expression level of the protein changes during ageing. In case of the CTRL, AX and krill oil-fed groups of ageing mice, quantitative PCR analysis was performed on the TA muscles and we found significant differences in MCU, Mfn2 and CB1 transcript levels after nutritional supplementation. AX significantly increased MCU mRNA transcript levels, while krill oil did not affect it. On the other hand, Mfn2 mRNA levels were significantly changed by the latter compared to CTRL, but only a slight increase was observed after AX supplementation.

CB1 transcripts remained unchanged after four weeks of the special diet. These results suggest that increased levels of Mfn2 and MCU transcripts in ageing muscle may be a consequence of changes in protein turnover. In order to get a more accurate picture, we examined the related protein levels using a semi-qualitative Western blot method, but we did not find any significant changes after dietary supplementation. Based on our results, we concluded that the four-week special diet apparently does not cause significant changes in the mitochondrial fusion of skeletal muscles.

Mitochondrial fission is a multistep process that mainly depends on the cytosolic GTPase dynamin-related protein 1 (Drp1). After AX feeding, our qPCR measurements showed a significantly increased Drp1 transcript level, while a surprisingly significant decrease was observed at the protein level compared to the CTRL group. In the group fed with krill oil, we did not

experience this kind of change at the mRNA level, but we experienced a similarly significant decrease at the protein level.

Krill oil and memory

To investigate the effect of ω-3 PUFAs present in krill oil (but not in AX) on learning ability and memory, we used the Barnes Maze experiment in ageing mice. Krill oil-supplemented diet lasted 4 weeks. Differences observed during repeated experiments (if any) were expected in the latency (duration) and distance (route) traveled to the escape hole, which can be interpreted as an indicator of hippocampus-dependent memory function. 8 control and 8 krill oil-fed mice were trained during the acquisition period for 10 consecutive days, and then a blind test was performed after a three-day rest period. On the acquisition days, the time elapsed until entering the target hole (latency) and the distance traveled until then were measured in centimeters. During the acquisition period, the mice gradually became familiar with the location of the target hole and spent less time and traveled a shorter distance each day until finding it.

As a result of the experiment, it can be said that during the experiment, the mice fed with krill oil learned the location of the target hole faster and were more purposeful than the control animals, because we found a significant difference ($*p < 0.05$) between the two groups of animals in the latency (4–6. day) and in the distance traveled (day 6). By the end of the experiment, i.e. on the 14^{th} day, the average values of the previous two parameters were the same in both groups of animals, meaning that the effect of krill oil could only be detected and measured only in a part of the experiment.

Discussion

Nutraceuticals and skeletal muscle performance

Our study supports the fact that AX improves *in vivo* muscle performance and *in vitro* tetanus muscle strength without significant effects on skeletal muscle calcium homeostasis and electro-mechanical coupling.

Up to date, little was known about the effects of retinol on the skeletal muscle-type ECC mechanism. In our hand acute treatment of FDB muscle fibers with 10 µM retinol clearly resulted in a shift in the V_{50} value of

the voltage-activated calcium transients and significantly reduced the F/F_0 peak of the calcium transients.

A possible explanation is that AX may accelerate lipid utilization, which increases metabolic activity through retinoid signaling and antioxidant action. This idea is confirmed by our results, which show that mice fed AX diet gained less weight during the 4-week feeding period, while consuming the same amount of chow as mice in the control group (~0.2 g/day/bw). This is consistent with the work of Ikeuchi et al., who found that AX does not affect food intake but reduces body weight gain in obesity-prone experimental mice, possibly through increased energy expenditure. Similar results were reported by Ruiz et al., who first described the role of retinoid metabolism in skeletal muscle.

AX and mitochondrial Ca^{2+} signalling

Following Ca^{2+} release from the intracellular store about 10-18% of calcium is taken up by mitochondria using the mitochondrial uniporter (MCU) and other alternative channels. We observed a persistent increase in mitochondrial fluorescence after electrical stimulation, which probably plays a role in the activation of ATP generation by calcium, as previously suggested by Rossi et al. We found that in the AX-fed mice group, mitochondrial calcium uptake decreased after repeated tetanic stimulation compared to the CTRL group. Our results are consistent with the observation that AX supplementation positively affects mitochondrial calcium homeostasis and reduces activity-dependent mitochondrial calcium imbalance.

We hypothesized that AX treatment may slightly modify the expression level of the MCU calcium-sensitive regulator (MICU1) in skeletal muscle, but it had no significant effect on calcium homeostasis. During the initial depolarization activation, we did not see any differences between the mitochondrial calcium levels measured in the FDB fibers of the control and AX mouse groups; these differences were only detectable after the 5^{th} tetanic stimulation.

Another possibility is that AX affects the calcium sensitivity of MICU1 as Reane et al reported earlier that a splice variant of MICU1 has altered calcium binding properties. Whatever the case may be, mitochondrial calcium uptake during intense training may be one of our endogenous defense mechanisms against calcium overload, otherwise known as overload. On the other hand, in skeletal muscle, MICU1 affects ATP production by directly increasing the activity of essential metabolic enzymes in the mitochondria via

calcium. Others have shown that MICU1 deficiency does not alter mitochondrial function in resting muscle fibers but reduces the increase in oxygen consumption during calcium stimulation. It is conceivable that AX treatment reduces the direct phosphorylation of ATP through MICU1 and favors skeletal muscle energy utilization by shifting it towards fatty acid oxidation, resulting in lower body weight gain, as we found in the present study. Similar results were described in a skeletal muscle-specific MCU-deficient mouse model, where the deletion did not affect intracellular Ca^{2+} handling of myofibrils, but inhibited acute mitochondrial Ca^{2+} influx and Ca^{2+}-stimulated mitochondrial respiration, resulting in a reduction in acute exercise performance in mice. In addition, a recent study showed that muscle-specific MICU1 deficiency induced a catabolic response in both liver and adipose tissue, as the expression of enzymes involved in catabolism increased in the tested samples. According to our hypothesis, AX improves ATP phosphorylation, thus increasing muscle strength and fat metabolism will increase to compensate for this.

AX and glucose metabolism

Since AX has been associated with metabolic changes, we hypothesized that, like retinoic acid, AX improves muscle performance by increasing glucose uptake and/or glycogen stores. Ruiz et al. found that in mice retinoic acid activates mTORC2 and insulin signaling, and overexpression of SRP35 - a low molecular weight retinol dehydrogenase protein - improves muscle performance by increasing glucose uptake and glycogen stores.

Nutraceuticals and skeletal muscle performance in ageing

The intake of antioxidants, such as vitamins C, E, A and perhaps carotenoids such as the xanthophyll astaxanthin which we studied here, can reduce oxidative damage to the muscle and increase muscle performance. Krill oil has been associated with improvements in exercise and antioxidant/anti-inflammatory markers, and several clinical trials have been conducted in this area. One possible mechanism of action is that krill oil activates mTOR signaling and the combination of EPA and DHA increases the rate of muscle protein synthesis by increasing the activity of the mTOR-p70s6k signaling pathway in young and middle-aged men and women.

In the present research, we found that consumption of AX/krill oil for four weeks significantly improved muscle strength *in vivo* and *in vitro* in

both young and ageing mice. These changes occurred in addition to a slight decrease in body weight; the food intake of each animal group was practically the same, and no significant changes were found in the muscle mass of the mice, which suggests that the decrease in body weight cannot be explained by the loss of muscle mass. It is possible that AX supplementation accelerates glucose uptake and enhances lipid utilization, which increases retinoic acid metabolic activity in skeletal muscle tissue. In addition, AX consumption may inhibit ATP phosphorylation via MICU1 and shift energy expenditure toward more fatty acid oxidation, resulting in less body weight gain.

Nutraceuticals and calcium homeostasis during ageing

Another possible explanation for the increased muscle strength during nutritional supplementation could be the change in the activity of the RyR1 channel, in addition to the change in the calcium sensitivity of the contractile filaments (which was not investigated in this work).

Morphological and ultrastructural changes occur in skeletal muscles with ageing. Tubular aggregates of SR membranes accumulate, causing abnormal SR Ca^{2+} storage and Ca^{2+} release. Furthermore, molecular changes can occur that cause quantitative (reduced expression) and functional modifications in L-type voltage-dependent Ca^{2+} channels. In skeletal muscle fibers from ageing mice, functional uncoupling of DHPR and RyR1 reduces calcium release from the SR. RyR1s are highly sensitive to oxidative changes and their function is impaired in ageing muscle due to oxidation and nitrosylation of their cysteine residues. This results in Ca^{2+} leakage from the SR and causes muscle weakness.

After four-week long antioxidant diet, we did not experience any significant changes in the calcium signals induced by depolarizing pulses, nor in the peak values of calcium transients, the flux of Ca^{2+} release, and the amount of calcium released.

Nutraceuticals and mitochondrial function

While AX supplementation favorably reduced activity-dependent mitochondrial calcium uptake in young adult mice, similar effects were not observed in ageing mice. This may mean that due to the antioxidant effect of both AX and krill oil, ROS production in FDB muscles may decrease. It is conceivable that the four-week special diet was sufficient for the young adult mice for the drug to exert its beneficial effect. In ageing mice, where oxidative

stress and ROS production are more prominent, feeding for a similar period of time was probably not sufficient.

Several abnormalities have been described in mitochondria isolated from skeletal muscle of old mice, such as increased ROS production, reduced electron transport mechanisms in complexes I and V, and damage to mitochondrial DNA. Ainbinder et al described a gradual decline in Mfn2 protein expression in skeletal muscle, and this is not due to reduced gene expression or translation of Mfn2 mRNA. Similarly, Filadi et al found that cells lacking Mfn2 show reduced MCU expression, which may explain the reduction in mitochondrial Ca^{2+} signals. These results prompted us to investigate the effects of AX and krill oil treatment on mitochondrial dynamics and expression levels of key proteins involved in skeletal muscle calcium homeostasis. To this end, we first examined the relative mRNA transcript levels of Mfn2, MCU, Drp1 and CB1 using a quantitative RT-PCR reaction. In the AX-fed group, we observed a significant increase in MCU and DRP1 mRNA expression, while Mfn2 mRNA expression in the Krill group mice increased significantly. It is important to note that when quantifying the related protein levels of MICU1 and Mfn2, these showed no difference between the groups. Therefore, we concluded that the unchanged Mfn2 and MICU1 protein levels detected by the Western blot method may explain the unchanged parameters of calcium transients and Ca^{2+} signals observed during patch clamp experiments and repetitive stimulation-induced mitochondrial Ca^{2+} uptake experiments.

Krill oil and cognition

Age is a determining factor in terms of learning ability and has a significant impact on the morphology of brain structures involved in learning processes. AX is considered a potential neuroprotective compound that may be able to preserve brain health. Four weeks of krill oil supplementation had a positive effect on spatial learning and memory in ageing mice. Our data clearly show that cognitive ability improved in the ageing mice during the experiment, as the mice learned the exit location in the maze more quickly than their control counterparts.

Fish oil and krill oil contain significant amounts of PUFA (EPA and DHA) bound in different structural forms. In fish oil, EPA and DHA are found as triglycerides, so they are less bioactive than in krill oil, where they are mainly found in the form of phospholipids (in a ratio of 2:1), so they are more bioavailable, especially for the brain, which consists of up to 60% lipids (most

of it is PUFA, predominantly DHA). Our results indicate that the lipid composition and AX content of krill oil play a decisive role in improving cognitive functions in ageing mice. However, further studies are needed to explore whether AX alone has any direct effects on the central nervous system.

Summary

Ageing and frailty are associated with a loss of muscle strength, which is a direct consequence of a reduction in the quantity and quality of muscle fibres. The generation of reactive oxygen radicals, by-products of terminal oxidation, plays a leading role in ageing. Their negative effects can be reduced by antioxidant supplementation.

Astaxanthin (AX), a marine carotenoid, is a powerful natural antioxidant that protects against oxidative stress. AX and its main source (krill oil) are dietary supplements with numerous health-promoting, geroprotective, anti-inflammatory, and anti-fatigue effects. Retinol and its derivatives affect lipid and energy metabolism. To date, the effects of AX, krill oil and retinol on excitation-contraction coupling (ECC) of skeletal muscles are poorly understood.

In this work, we investigated the functional effects of AX and krill oil in young and aging mice. We investigated in vivo and in vitro skeletal muscle function, aspects of intracellular and mitochondrial calcium homeostasis, cognition and spatial memory in aging mice supplemented with krill oil.

In the first part of our work, we divided young (4-6 months old) C57Bl6 mice into two groups: the control group consumed normal mouse chow, while the other consumed AX for 4 weeks. In vivo and in vitro force generation and intracellular calcium homeostasis were investigated. In some experiments, acute retinol treatment was used. In the second part of our work, we used the same feeding protocol in aging mice, where we established a third group that consumed krill oil supplemented diet.

In vivo and in vitro force measurements revealed that antioxidant supplementation significantly improved muscle strength in both age groups. When we investigated the voltage dependence of calcium transients in

individual m. flexor digitorum brevis enzymatically isolated fibers using patch clamp technique, we found no significant changes following antioxidant supplementation in either age group. In young adult mice, retinol reduced peak calcium transients. The activity-dependent mitochondrial calcium uptake was lower in the AX group in young individuals, whereas no such difference was found in aging mice. We also showed that krill oil supplementation improved spatial memory and learning ability in aging mice.

Overall, our data support the general use of antioxidant supplements as geroprotectors because they improve learning ability and physical performance.

List of publications

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Candidate: Zoltán Singlár
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List of publications related to the dissertation

1. **Singlár, Z.**, Szentesi, P., Fodor, J., Angyal, Á., Csernoch, L., Sztretye, M.: Assessing the Potential of Nutraceuticals as Geroprotectors on Muscle Performance and Cognition in Aging Mice. *Antioxidants. 10* (9), 1415, 2021.
   DOI: http://dx.doi.org/10.3390/antiox10091415
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3. Sztretye, M., **Singlár, Z.**, Ganbat, N., Al-Gaadi, D., Szabó, K., Köhler, Z. M., Dux, L., Keller-Pintér, A., Csernoch, L., Szentesi, P.: Unravelling the Effects of Syndecan-4 Knockdown on Skeletal Muscle Functions.
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IF: 4.566

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