High-Intensity Exercise Training Induces Mitonuclear Imbalance and Activates the Mitochondrial Unfolded Protein Response (UPR_{MT}) in the Skeletal Muscle of Aged Mice

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MATERIALS AND METHODS

Animals

Male C57BL/6J mice aged two months (young) and 24 months (old) were obtained from the University of Campinas Breeding Center. The animals were maintained in 12h:12h light/dark cycles and were housed five animals per cage, with water and food ad libitum. The experiments were approved by the ethics committee (CEUA 4573-1/2017) and followed the University guidelines for animals used in experimental studies and experiments according to the Guide for the Care and Use of Laboratory Animals, approved by the National Research Council and recently revised (Academies, 2011).

Single session of High-Intensity Interval Exercise (HIIE) protocol

Aged male C57BL/6J animals (25 months old) were submitted to a single session of high-intensity interval exercise (HIIE). Firstly, the animals performed 5-days acclimatization protocol (10 min of running per day at 8m/min). After 48h resting, the animals performed the Incremental Load Test (details below), to evaluate the maximal aerobic capacity (MAC). Later, after 48h resting, the animals were submitted to a single bout of HIIE, during 40 minutes. The protocol consisted in warm up (5 min, at 8 m/min), 10 bouts of 2 minutes (85% MAC), interspaced by an active recovery (1 min at 8m/min), and cool-down (5 min, at 8 m/min). After 4 hours of rest, the animals were euthanized to tissue extraction.

High-Intensity Interval Training Protocol

The animals were subjected to a treadmill high-intensity interval training protocol, based on previous protocols of Hafstad et al. (A D Hafstad et al., 2011; Anne D Hafstad et al., 2013) and Lund et al. (Lund et al., 2015). After five days of acclimatization (10 minutes per day of running at 8 m/min), the animals from the HIIT group were trained five days per week, for 30 minutes per day, on a 15º treadmill slope. Each training session consisted of 10 bouts, at a running speed of 85-90% VO^2_{max}, interspaced by resting periods running at 8 m/min, for 60 seconds. Before and after each training session, the animals ran for 5 minutes to warm up and 5 minutes to cool down. Additional information is given in Supplementary Table 1.

Grip Resistance Inverted Screen Test

To measure muscular strength resistance in all four limbs, the animals performed an inverted screen test protocol. The screen was made of fine stainless steel cables and had a diameter of 60 cm, with squares 1.5 mm in diameter. To ensure that the animals held on to the screen against the weight of gravity, we placed a pool below it, 30 cm in depth with water at a temperature of 17 $\pm$ 1 ºC. The test protocol consisted of two days of acclimatization, followed by one day of the test. On all days, each animal made three attempts. We only considered the highest value (time-to-exhaustion) reached on the day of the test.

Maximal Strength Grip Test

The animals also performed the grip force test to assess strength in their hind paws. The protocol consisted of three warm-up attempts, and five maximal grip strength measurement attempts. In each attempt, a researcher gently held each mouse by the tail and allowed it to grasp the horizontally positioned metal bar of the Grip Strength System (AVS Projetos®, São Carlos, São Paulo, Brazil) with its hind paws. The highest force value that was applied to the metal bar was recorded as the peak tension (N) and was used as the performance parameter. The highest and lowest attempts were excluded, and we calculated the average of the rest.

Incremental Load Test

After the acclimatization period, the animals performed an incremental load test to determine the maximal speed of exhaustion (MSE), using a protocol pre-established by Ferreira and co-workers (Ferreira et al., 2007). The animals initiated the test at 6 m/min, increasing 3 m/min every 3 minutes until exhaustion. Exhaustion was defined as the time that the rodents were unable to respond to gentle encouragement to run and stayed at the end of the treadmill for more than 15 seconds. The speed achieved, running distance, and time to exhaustion were monitored. This physical evaluation protocol allowed us to determine the aerobic capacity and the intensity of efforts during the HIIT.

Lactate Analysis

12.5 µL blood samples were collected with heparinized capillary microtubes from the tail tip of the animals, before and during the treadmill tests and the physical training sessions. The blood was

deposited into plastic tubes (Eppendorf®) containing 200 µL of trichloroacetic acid (4%) and stored at 2 to 8 Cº. The samples were homogenized with a reagent composed of hydrazine hydrate, EDTA, glycine, β-nicotinamide adenine dinucleotide (NAD), and LDH and incubated for 60 minutes. Absorbance (340 nm) was obtained using a microplate reader (ASYS EXPERT PLUS UV, BIOCHROM, UK) and blood lactate concentration was measured. The results are presented in Appendix A2.

Skeletal Muscle Tissue Sampling

Body mass was monitored during the 4 weeks of intervention. Twenty-four hours after the last session of HIIT, the animals were anesthetized with an intraperitoneal injection of chlorohydrate of ketamine (300 mg/kg, Parke-Davis, Ann Arbor, MI) and xylazine (30 mg/kg, Rompun, Bayer, Leverkusen). Corneal and pedal reflexes were verified and assured. Samples from the left paw of mixed gastrocnemius weighing 50mg were extracted and homogenized in a 400 uL tissue buffer, at 4 °C using a Bead Ruptor 12 Homogenizer (OMNI®) operated at 3000 rpm, for 120 seconds. The lysates were centrifuged (Eppendorf® 5804R) at 11000 rpm at 4 °C for 15 min to remove insoluble material, and the supernatants were used for the assay. The protein content was determined according to the bicinchoninic acid (BCA) method. Proteins were denatured by boiling in Laemmli sample buffer containing 100 mM DTT.

Western Blot Analysis

The samples were applied in polyacrylamide gel for separation by electrophoresis (SDS-PAGE) and transferred to 0.45 µm nitrocellulose membranes. Next, the membranes were blocked for 3 minutes in a Ponceau Red staining solution to guarantee that all samples contained similar total protein content. After that, the membranes were blocked with 5% of non-fat dry milk (NFDM) at room temperature for one hour and finally incubated with specific antibodies. Specific bands were labeled by chemiluminescence, and visualization was performed by exposing the membranes in specific software (GeneSys Software®). The Ponceau and protein band images were quantified using the UN-SCAN-IT® software. The GAPDH and α-Tubulin proteins were used as a qualitative endogenous control. The western blotting results were normalized by Ponceau quantification at the molecular weight of each revealed membrane, and the final values were presented as percentages relativized by the control group. The entire membranes, Ponceaus, endogenous values, and the statistical analyses of the western blots are shown in the Appendix A2.

Antibodies and Chemicals

The primary antibodies anti-MTCO1 (Bioss®, nº 3953R), anti-SDHA (Santa Cruz Biotechnology®, sc-166909), anti-Yme1L1 (Proteintech®, nº11510-1-ap), anti-Lonp1 (Bioss®, nº 4245r), anti-CLpP (Abcam, nº 124822), anti-UQCRC1 (Abcam®, nº 125882), anti-NRF1 (Abcam®, nº 55744), anti-TFAM (Abcam®, nº 131607), and anti-VDAC (Cell Signalling®, nº 4866s) were used according to the manufacturer's instructions. The antibodies anti-GAPDH (Cell Signalling®, nº 2118s) and anti-α-Tubulin (Cell Signalling®, nº 2144s) were used as an endogenous control. The Ponceau Red staining solution (nº P7170) was imported from Sigma Aldrich®. The secondary antibodies anti-mouse IgG (#7076) and anti-rabbit IgG (nº 7074) were imported from Cell Signalling®.

Real-Time Quantitative RT-PCR for Gene Expression Analysis

The total gastrocnemius RNA was isolated using TRIzol reagent (50 mg of muscle per 1µL of TRIzol) (Invitrogen, Grand Island, NY, USA). Two micrograms (µg) of total RNA was used as a template for cDNA synthesis, using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). The RT-qPCR was performed using 300 ng of cDNA, 7.5-60 µM primers, 2.0x Sybr Green Master Mix (Invitrogen®), and DEPC-treated water. The cycling parameters and relative content of mRNA were analyzed as previously described (Lenhare et al., 2017). The primers are described in Supplementary Table 2.

Real-Time Quantitative PCR of Mitochondrial-Encoded Genes

The protocol to evaluate mitochondrial DNA copy number was carried out according to previously described protocols (Stephenson et al., 2016). The primers were designed for three gene regions encoded only by mitochondrial genes. The extraction of the genomic DNA was performed according to the Invitrogen® PureLink™ Genomic DNA kit instructions. Then, the quantitative PCR was performed using the total DNA extracted from the tissues. To guarantee the protocol accuracy, each gene was relativized by Rpl13a, a housekeeping gene encoded only by the nuclear DNA. The primers utilized and individual values are also available in Supplementary Table 3.

Bioinformatic Analysis

All databases were obtained on the website genenetwork.org. The human data were extracted from the "GTEX v5 human muscle-skeletal GTEx RefSeq project (Sep15)" dataset, obtained through the GTEXv5 project for aged subjects (60-70 years old). Pearson's correlations were calculated among phenotypes using the data previously described (Andreux et al., 2012; Bagley, Szumlinski, & Kippin, 2019; Hook et al., 2018; Neuner, Heuer, Huentelman, O'Connell, & Kaczorowski, 2019; Philip et al., 2010; Pjetri et al., 2012; Porcu & Morrow, 2014; Williams et al., 2016). The *R* software was used to build the heatmap graphs. The *R* software can be accessed at *https://www.r-project.org/.* The Spearman's correlation between UPRmt genes and OXPHOS-complexes data were also obtained from the GTEXv5 project for aged subjects (60-70 years old).

Statistical Analysis

All results were expressed as mean ± standard error of the mean (SEM). The data were analyzed by Student's *t*-tests, multiple *t*-tests, or one-way analyses of variance (ANOVA). Post-hoc tests were performed utilizing the Holm-Sidak method for multiple comparisons and Bonferroni post-hoc test for ANOVA. The statistical significance used was fixed at a p-value <0.05. All statistical analyses and graphic images were performed and created in the GraphPad Prism 8.0 software.