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Exercise modulates liver cellular and mitochondrial proteins related to quality control signaling

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ABSTRACT

Aims: The effects of exercise on cardiac and skeletal muscle, including the increase on mitochondrial function, dynamics, biogenesis and autophagy signaling are well described. However, these same effects on liver mitochondria, important in the context of hepatocyte ability to mitigate drug-induced injury and obesity-related disorders, are not fully understood. Therefore, the effects of two distinct chronic exercise models (endurance training—ET and voluntary physical activity—VPA) on liver cellular and mitochondrial quality control were analyzed.
Main methods: Eighteen male-adult Sprague–Dawley rats were divided into sedentary (SED), ET (12-week treadmill) and VPA (12-week voluntary free wheel). Liver mitochondrial alterations were evaluated by semi-quantification of proteins involved in oxidative stress (SIRT3, p66shc, p66(Ser36)), biogenesis (citrate synthase, PGC-1α and mtTFA), dynamics (MFN1, OPA1 and DRP1) and auto(mito)phagy (Beclin-1, Bcl-2, LC3II/LC3I, p62, Parkin and PINK) signaling. Liver ultrastructural alterations were also evaluated.
Key findings: Both exercise models induced beneficial alterations on liver mitochondrial morphology and increased mitochondrial biogenesis (PGC-1α and mtTFA), autophagy-related proteins (Beclin-1, LC3-II, LC3II/LC3I), and DRP1 and SIRT3 proteins. Increased citrate synthase activity and OPA1, p62 and Parkin content as well as decreased PINK protein levels were only observed after ET. VPA decreased OPA1, Beclin-1/Bcl-2, Parkin and p66(Ser36). Mitochondrial density and circularity increased in both exercised groups.
Significance: Both chronic exercise models increased proteins related with mitochondrial biogenesis and alteration proteins involved in mitochondrial dynamics and autophagy signaling, suggesting that exercise can induce liver mitochondrial adaptive remodeling and hepatocyte renewal.

1. Introduction

The mechanisms by which physical exercise modulates the liver mitochondrial phenotype, including alterations in the phosphorylative system and its components, substrate oxidation capacity, mitochondrial biogenesis, oxidative damage and antioxidants, as well as the induction of permeability transition and apoptotic signaling have been described [1,2]. Moreover, as it has recently been demonstrated by our group [3], exercise-induced adaptations result in improved liver mitochondrial function, increasing the resistance to deleterious conditions such as drug- and obesity-related disorders [2,4]. However, emerging importance has been attributed to the interplay between mitochondrial biogenesis, dynamics and auto(mito)phagy in exercise-induced regulation of cellular adaptation to stress, although this was never explored in the context of the hepatic tissue. Therefore, in complement to the previously referred and published paper [3], the present work aimed to analyze the effects of two distinct physical exercise modalities (ET and VPA), which respectively intend to mimic systematic training programs and voluntary daily physical activity, on markers of mitochondrial biogenesis, fusion and fission, and autophagy. As autophagy and mitochondrial dynamics deregulation have been implicated in the pathogenesis of common liver diseases, since inadequate activation of mitochondrial repair processes may contribute to accumulation of mitochondrial damage [5], it is likely that the manipulation of these interrelated processes through exercise may hold potential therapeutic value. Our current work hypothesis is that physical exercise increases protein markers related to the maintenance of mitochondrial quality control contributing, at least in part, to a more fitness and resistance phenotype.

2. Materials and methods

2.1. Animals

All experimental procedures were conducted in accordance with the Directive 2010/63/EU of the European Parliament and were approved

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by the local board. Eighteen male Sprague–Dawley rats (aged 21 days old in the beginning of the protocol) were housed in collective cages (two rats per cage) in 12 h light/dark cycles with free access to food and water and were randomly divided into three groups (n = 6 per group): sedentary (SED), endurance training (ET) and voluntary physical activity (VPA).

2.2. Endurance training

The animals from the ET group were exercised 5 days/week (Monday–Friday) in the morning (between 10:00 and 12:00 AM) for 12 weeks on a LE8700 motor driven treadmill (Panlab, Harvard, USA). The treadmill speed was gradually increased over the course of the 12-week training period. The protocol included 5 days of habituation to the treadmill with 10 min of running at 15 $m \cdot min^{-1}$, with daily increases of 5–10 min until 30 min was achieved. Habituation was followed by one consecutive week of continuous running (30 $min \cdot day^{-1}$) at 15 $m \cdot min^{-1}$ and was gradually increased until 60 $min \cdot day^{-1}$ on the second week [6].

2.3. Voluntary physical activity

The animals from the VPA group were housed in a polyethylene cage equipped with a running wheel [perimeter = 10.5 cm, Type 304 Stainless steel (2154F0106-1284L0106) Technicplast, Casale Litta, Italy]. The rats were allowed to exercise voluntarily with unlimited access to the running wheel 24 h/day. Running distance was recorded using a digital counter (ECO 701 Hengstler, Lancashire, UK).

2.4. Animal sacrifice and liver extraction

Forty-eight hours after the last exercise bout, non-fasted rats were euthanized by cervical dislocation between 9:00 and 10:00 AM to eliminate possible effects due to diurnal variation. After expedite opening of the abdominal cavity, rat livers and hearts were rapidly excised, rinsed, carefully dried and weighed.

A liver homogenate was obtained in a RIPA buffer (#20-188) with protease and phosphate inhibitor cocktail, in a ratio of 100 $mg \cdot mL^{-1}$ using a Teflon pestle on a motor driven Potter-Elvehjem glass homogenizer at 0–4 °C three to five times for 5 s at low speed setting. Homogenates were centrifuged at 12 000 $\times g$ for 10 min at 4 °C and resulting supernatants were prepared and stored at −80 °C for later semi-quantification of protein expression by Western blotting. Protein content was spectrophotometrically determined by the Bradford method using bovine serum albumin (BSA) as standard [7].

2.5. Isolation of liver mitochondria

Liver mitochondria were daily isolated using conventional methods of differential centrifugation as described previously [8]. The final concentration of mitochondrial protein was spectrophotometrically determined using the Biuret method with BSA as standard [9]. Mitochondrial fractions were separated and prepared for later semi-quantification of proteins by Western blotting as detailed below [8].

2.6. Liver mitochondrial citrate synthase activity

Liver mitochondrial citrate synthase activity was measured using the method proposed by Coore et al. [10]. The colorimetric assay was performed against a blank test. Total citrate synthase activity was expressed in nanomoles per milligrams of mitochondrial protein ($\varepsilon_{412} = 13.6\ mM^{-1} \cdot cm^{-1}$).

2.7. Immunoblotting detection of liver tissue and mitochondrial proteins

Equivalent amounts of liver tissue or mitochondria (1 $mg \cdot mL^{-1}$) were denatured in sample loading buffer and separated by dodecyl sulfate–polyacrylamide gel electrophoresis SDS/PAGE (12% gels) as described by Laemmli [11], followed by blotting on PVDF membranes according to the method of Locke et al. [12]. The mitochondrial content of the outer mitochondrial translocator TOM20 or β-actin was used as a protein loading control for mitochondrial and liver tissue-quantified proteins, respectively. In addition, membranes were stained with Ponceau-S to verify the efficiency of transfer and equal protein loading. After blotting, membranes were blocked for 2 h with 5% (*w*/*v*) non-fat dry milk powder in T-TBS (Tris-buffered saline with 0.1% Tween 20) to decrease non-specific binding.

Membranes were incubated with anti-PGC-1α (3 $\mu g \cdot mL^{-1}$; ab106814 goat monoclonal IgG, Cambridge, UK), anti-p62 (2 $\mu g \cdot mL^{-1}$; ab56416 mouse monoclonal IgG, Cambridge, UK), anti-OPA1 (1 $\mu g \cdot mL^{-1}$; ab119685 mouse monoclonal IgG, Cambridge, UK), anti-PINK (1:1000; ab23707 rabbit polyclonal IgG, Cambridge, UK), anti-Parkin (1:500; #4211 mouse monoclonal IgG, Danvers, MA, USA), anti-DRP1 (1:1000; #8570 rabbit monoclonal IgG, Danvers, MA, USA), anti-Bcl-2 (1:1000; #2870 rabbit monoclonal IgG, Danvers, MA, USA) anti-SIRT3 (1:1000; #2627 rabbit monoclonal IgG, Danvers, MA, USA), anti-shc [p66] (1:500; #2432 rabbit polyclonal IgG, Danvers, MA, USA), anti-shc [p66 (pSer36)] (1:1000; 6E10 mouse monoclonal IgG, Merck Millipore, Darmstadt, Germany), anti-mtTFA (1:1000; sc-23588; goat polyclonal IgG, Dallas, TX, USA), anti-MFN1 (1:1000; sc-50330 rabbit polyclonal IgG, Dallas, TX, USA), anti-β-actin (sc-1616; goat polyclonal, Dallas, TX, USA) and anti-TOM20 (1:1000; sc-11415 rabbit polyclonal IgG, Dallas, TX, USA). Primary antibodies were diluted in TBS-T containing 2% of non-fat dried milk or BSA for 8 h at 4 °C. Following primary antibody incubation, membranes were washed and incubated with secondary horseradish-peroxidase-conjugated anti-mouse (1:10 000; sc-2005, Dallas, TX, USA), anti-goat (1:10 000; sc-2354, Dallas, TX, USA) or anti-rabbit (1:10 000; sc-4004, Dallas, TX, USA) antibodies for 2 h, at room temperature, containing 2% of non-fat dried milk or BSA. Protein bands were visualized by treating the immunoblots with ECL® Plus™, visualized with the ChemiDoc XRS + system (Bio-Rad Laboratories, Amadora, Portugal) and analyzed with the Image Lab software (Bio-Rad Laboratories, Amadora, Portugal). The densitometry analysis was carried out immediately before saturation of the immunosignal. Data were observed as band intensity of immunostaining values (arbitrary units) and the results were expressed as percentage variation of the SED control group.

2.8. Transmission electron microscopy (TEM)

The collected liver tissues were first fixed in 2.5% of glutaraldehyde solution, subsequently washed in phosphate buffer and post-fixed in 1% osmium tetroxide. The samples were then dehydrated in graded ethanol (95–100%) with 100% of propylene oxide as transitional solvent, and embedded in Epon resin blocks. Ultrathin sections (50–60 nm) were collected on copper grids, stained with uranyl acetate and lead citrate and finally examined under a transmission electron microscope (JEM1400, USA). Alterations in mitochondrial morphology were evaluated using the following parameters as described [13]: area of mitochondria (A_m), perimeter (P_m, length of the mitochondrial outline), circularity (a value of 1 indicates a perfect circle and values approaching 0 indicate an increasingly elongated shape; $4 \cdot \pi \cdot \text{area} / \sqrt{P_m}$), aspect ratio (AR, ratio between the major and minor axes of the ellipse equivalent to the mitochondria; max diameter / min diameter) and the percentage of the picture area occupied by mitochondria. Images were analyzed with ImageJ (Version 1.49b).