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PHYSICAL EXERCISE IMPROVES BRAIN CORTEX AND CEREBELLUM MITOCHONDRIAL BIOENERGETICS AND ALTERS APOPTOTIC, DYNAMIC AND AUTO(MITO)PHAGY MARKERS

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Abstract—We here investigate the effects of two exercise modalities (endurance treadmill training-TM and voluntary free-wheel activity-FW) on the brain cortex and cerebellum mitochondrial bioenergetics, permeability transition pore (mPTP), oxidative stress, as well as on proteins involved in mitochondrial biogenesis, apoptosis, and quality control. Eighteen male rats were assigned to sedentary-SED, TM and FW groups. Behavioral alterations and *ex vivo* brain mitochondrial function endpoints were assessed. Proteins involved in oxidative phosphorylation (OXPHOS, including the adenine nucleotide translocator), oxidative stress markers and regulatory proteins (SIRT3, p66shc, UCP2, carbonyls, MDA, -SH, aconitase, Mn-SOD), as well as proteins involved in mitochondrial biogenesis (PGC1α, TFAM) were evaluated. Apoptotic signaling was measured through quantifying caspase 3, 8 and 9-like activities, Bax, Bcl2, CypD, and cofilin expression. Mitochondrial dynamics (Mfn1/2, OPA1 and DRP1) and auto(mito)phagy (LC3II, Beclin1, Pink1, Parkin, p62)-related proteins were also measured by Western blotting. Only the TM exercise group showed increased spontaneous alternation and exploratory activity. Both exercise regimens improved mitochondrial respiratory activity, increased OXPHOS complexes I, III and V subunits in both brain subareas and decreased oxidative stress markers. Increased resistance to mPTP and decreased apoptotic signaling were observed in the brain cortex from TM and in the cerebellum from TM and FW groups. Also, exercise increased the expression of proteins involved in mitochondrial biogenesis, autophagy and fusion, simultaneous with decreased expression of mitochondrial fission-related protein DRP1. In conclusion, physical exercise improves brain cortex and cerebellum mitochondrial function, decreasing oxidative stress and apoptotic related markers. It is also possible that favorable alterations in mitochondrial biogenesis, dynamics and autophagy signaling induced by exercise contributed to increased mitochondrial plasticity leading to a more robust phenotype.

Key words: exercise, brain, mitochondrial metabolism, mitochondrial quality control.

INTRODUCTION

Regular physical exercise results in significant outcomes regarding cognitive function in both epidemiological and animal studies (Radak et al., 2001a; Cotman et al., 2007; Hillman et al., 2008; Hopkins et al., 2011; Gomes et al., 2012). Moreover, physical exercise protects neurons against aging-associated deleterious alterations (Dishman et al., 2006; Cotman et al., 2007; Boveris and Navarro, 2008; Radak et al., 2008) including the onset of neurodegenerative disorders (Adlard et al., 2005; Um et al., 2008, 2011; van Praag, 2008; Lau et al., 2011). However, the cellular and molecular mechanisms underlying this exercise-induced protective phenotype in the brain are still elusive.

In opposition to contractile tissues such as skeletal and cardiac muscles, in which physical exercise exerts multiple contractile-dependent effects, the metabolic remodeling in the brain tissue is likely related to other systemic alterations that occur during and after exercise. Augmented mitochondrial activity, including ATP synthesis, regulation of redox balance, maintenance of calcium homeostasis, and decreased cell death threshold may explain the observed protective phenotype (Marques-Aleixo et al., 2012). Generally, exercise-induced brain mitochondrial adaptations include increased content and/or activity of several enzymes involved in aerobic energy production (Ding et al., 2006; Dietrich et al., 2008; Kirchner et al., 2008), increased activity of mitochondrial complexes I, III and IV (Navarro and Boveris, 2004), decreased

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Abbreviations: ANT, adenine nucleotide translocator; BSA, bovine serum albumin; CS, citrate synthase; DCIP, 2,6-dichlorophenolindophenol; DNPH, 2,4-dinitrophenylhydrazine; EGTA, ethylene glycol tetraacetic acid; FW, free wheel voluntary physical activity; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mn-SOD, manganese-dependent superoxide dismutase; mPT, mitochondrial permeability transition; mPTP, mPT pore; OXPHOS, oxidative phosphorylation; RCR, respiratory control ratio; SED, sedentary; TM, treadmill endurance training; TPP^+, tetraphenylphosphonium.

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expression/activation of several pro-apoptotic proteins (Cho et al., 2010; Um et al., 2011) and increased mitochondrial biogenesis (Steiner et al., 2011) and antioxidant capacity (Camiletti-Moiron et al., 2013). However, other still unknown mitochondrial-mediated mechanisms may also be involved, contributing to the improved functional phenotype associated with exercise. These mechanisms may involve altered susceptibility to the mitochondrial permeability transition (mPT), and improved mitochondrial dynamics and plasticity. In fact, as a result of calcium accumulation and oxidative stress, mitochondria may undergo mPT pore (mPTP) opening, leading to bioenergetic failure, release of pro-apoptotic proteins to the cytoplasm, and ultimately to mitochondrial-mediated apoptotic cell death (Toman and Fiskum, 2011). In addition, neurons are particularly susceptible to changes in mitochondrial dynamics. Also, impairments of mitochondrial physiology and quality control have been reported in several neuropathological conditions (Knott and Bossy-Wetzel, 2008; Twig and Shirihai, 2011). Balance of the mitochondrial network structure, resulting from a dynamic mitochondrial fusion, fission, auto(mito)phagy and mitochondrial biogenesis interaction are all necessary for a systematic response to the high-energy requirements of the nervous tissue (Chen and Chan, 2006; Gottlieb and Carreira, 2010). Therefore, the present study is a step forward into the understanding of the molecular mechanisms related to exercise-induced mitochondrial-mediated neuroprotection.

We thus aimed to analyze the effects of two long-term exercise modalities on brain cortex and cerebellum mitochondrial activity, susceptibility to mPTP opening and apoptotic signaling. Oxidative stress, oxidative phosphorylation (OXPHOS) subunits, molecular markers of mitochondrial biogenesis, mitochondrial fission and fusion as well as auto(mito)phagy signaling were also investigated. The further understanding of the mechanisms by which exercise exerts beneficial effects on brain function may improve the correct use of this preventive and therapeutic strategy against neurodegenerative diseases.

EXPERIMENTAL PROCEDURES

Reagents

Deionized water (18.7 MΩ) from an arium®611VF system (Sartorius, Göttingen, Deutschland) was used. Caspase 3, 8 and 9 substrates were purchased from Merck KGaA (Darmstadt, Germany), Commercial RANSOD kit from Randox Labs (Antrim, UK), chemiluminescent reagent ECL-Plus™ 104 (RPN2236) from GE Healthcare (Amersham BioSciences UK Ltd., Buckinghamshire, UK) and PVDF membranes (#IPVH00010) from Millipore (Billerica, MA, USA). Primary antibodies were purchased as follows: anti-OXPHOS (ab110413) and anti-PGC1-α (ab106814), anti-cyclophilin D (ab110324), anti-p62 (ab56416), anti-OPA1 (ab119685), anti-PINK1 (ab23707) and anti-Parkin (ab15954) from Abcam (Cambridge, UK); anti-Bax (#2772), anti-Bcl-2 (#2870), anti-DRP1 (#8570), anti-cofilin (#5175) anti-SIRT3 (#2627), anti-p66sch (#2432) and anti-beclin1 (#3495) from Cell Signaling Technology (Danvers, MA, USA); anti-LC3 (PD014) from MBL Medical & Biological Labs (Nagano, Japan); anti-DNP (D9656) and anti-UCP2 (SAB2501087) from Sigma Aldrich (Barcelona, Spain); anti-shc/p66(pSer^{36}) (6E10) from Calbiochem (Merck Millipore Darmstadt, Germany); anti-ANT (sc-9299), anti-Mfn1 (sc-50330), anti-Mfn2 (sc-50331), anti-TFAM (sc-23588), anti-TOM 20 (sc-11415) and anti-β-actin (sc-1616) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). All other chemicals were purchased from Sigma Aldrich (Barcelona, Spain).

Animals

All experimental procedures involving animals were performed in accordance with the European Convention for the Protection of Vertebrate Animal Used for Experimental and Other Scientific Purposes (CETS No. 123 of 18 March 1986 and 2005 revision) and the Commission Recommendation of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes [C (2007) 2525]. The authors are accredited by the Federation of Laboratory Animal Science Associations (FELASA) for animal experimentation (class c). The Ethics Committee of the Faculty of Sport, University of Porto approved the experimental protocol.

Eighteen male Sprague–Dawley rats (aged 21 days) were obtained from Charles River Laboratories (L'Arbresle, France) and randomly assigned to three groups ($n = 6$ *per* group): sedentary (SED), treadmill endurance training (TM), free wheel voluntary physical activity (FW). During the experimental protocol, animals were individually housed and maintained in a room under controlled environment (21–22 °C; 50–60% humidity), receiving food *ad libitum* (Scientific Animal Food and Engineering, A04) and water *ad libitum* in 12-h light/dark cycles.

Endurance treadmill training and Voluntary physical activity

The animals from the TM group 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 (Table 1). The protocol included 5 days of habituation to the treadmill with 10 min of running at 15 m/min, with daily increases of 5–10 min until 60 min of running was achieved (end of week 0). Habituation was followed by 12 weeks of continuous running (60 min/day) and the velocity increased gradually from 18 m/min to 30 m/min. The inclination of treadmill was maintained at 0° throughout the overall training protocol.

The animals from the FW group were individually housed in a polyethylene cage equipped with an activity wheel (circumference = 1.05 m, Type 304 Stainless steel (Cat. No. 2154F0106-1284L0106) Tecniplast, Casale Litta, Italy). The rats were allowed to exercise