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Modulation of cardiac mitochondrial permeability transition and apoptotic signaling by endurance training and intermittent hypobaric hypoxia

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ABSTRACT

Background: Modulation of the mitochondrial permeability transition pore (MPTP) and inhibition of the apoptotic signaling are critically associated with the cardioprotective phenotypes afforded by both intermittent hypobaric-hypoxia (IHH) and endurance-training (ET). We recently proposed that IHH and ET improve cardiac function and basic mitochondrial capacity, although without showing addictive effects. Here we investigate whether a combination of IHH and ET alters cardiac mitochondrial vulnerability to MPTP and related apoptotic signaling.

Methods: Male Wistar rats were divided into normoxic-sedentary (NS), normoxic-exercised (NE, 1 h/day/5 week treadmill-running), hypoxic-sedentary (HS, 6000 m, 5 h/day/5 weeks) and hypoxic-exercised (HE) to study susceptibility to calcium-induced cardiac MPTP opening. Mitochondrial cyclophilin D (CypD), adenine nucleotide translocator (ANT), Bax and Bcl-2 protein contents were semi-quantified by Western blotting. Cardiac caspase 3-, 8- and 9-like activities were measured. Mitochondrial aconitase and superoxide dismutase (MnSOD) activity and malondialdehyde (MDA) and sulphydryl group (–SH) content were determined.

Results: Susceptibility to MPTP decreased in NE and HS vs. NS and even further in HE. The ANT content increased in HE vs. NS. Bcl-2/Bax ratio increased in NE and HS compared to NS. Decreased activities in tissue caspase 3-like (HE vs. NS) and caspase 9-like (HS and HE vs. NS) were observed. Mitochondrial aconitase increased in NE and HS vs. NS. No alterations between groups were observed for caspase 8-like activity, MnSOD, CypD, MDA and –SH.

Conclusions: Data confirm that IHH and ET modulate cardiac mitochondria to a protective phenotype characterized by decreased MPTP induction and apoptotic signaling, although without visible addictive effects as initially hypothesized.

1. Introduction

In addition to their key role for cellular energy supply, mitochondria are also determinant players in establishing cytosolic calcium homeostasis [1–3]. Mitochondria accumulate calcium in the matrix via a specific uniporter and possess different pathways for calcium extrusion. One classic example of the important physiological role of calcium as an intra-mitochondrial signal for ATP production is shown by studies demonstrating that physiological calcium uptake increases mitochondrial NADH production and activates ATP synthase and the adenine nucleotide translocator [2]. On the other hand, mitochondria are also deeply involved in cell death signaling [4–6]. If excessive mitochondrial calcium accumulation occurs, a common event in several cardiac pathological conditions including ischemia–reperfusion (I/R), a phenomenon known as permeability transition (PT) can be developed. The PT is characterized by the loss of the impermeability of the mitochondrial inner membranes, which is suggested to be mediated by the formation and opening of pores of protein nature in the inner mitochondrial membrane, termed the mitochondrial permeability transition pores (MPTP) [7–9]. Increased mitochondrial oxidative stress, and calcium overload in the presence of phosphate lead to enhanced MPTP induction [10,11]. These deleterious conditions lower the matrix calcium

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threshold needed for MPTP induction, which ultimately can lead to mitochondrial outer membrane disruption and release of pro-apoptotic factors contained in the inter-membrane space.

Endurance training (ET) and intermittent hypobaric hypoxia (IHH) are non-pharmacological strategies that have been recognized to counteract several forms of cardiac and mitochondrial stress and dysfunction [12,13]. Specifically, endurance training affords protection against distinct cardiac pathophysiological events causing mitochondrial dysfunction and particularly resulting in increased MPTP vulnerability [12,14,15]. In addition, cardiac signaling and mechanical remodeling induced by ET also lead to mitochondrial alterations which contribute to an overall cardioprotective phenotype, including decreased reactive oxygen species (ROS) generation [16], increased resistance to calcium-induced mitochondrial permeability transition pore (MPTP) opening [17,18], and decreased mitochondrial-dependent apoptotic signaling [18–20].

Some studies suggest that cardiac tolerance to I/R can also be augmented by previous long-term exposure to intermittent hypobaric-hypoxia (IHH) [13,21]. Although the biological and molecular mechanisms associated with the cardioprotective phenotype conferred by chronic IHH are not completely understood, increased tolerance of heart mitochondria to calcium-induced MPTP opening seems to contribute to the cardioprotection provided by IHH against I/R injury [22].

In a recent study, we analyzed the isolated and combined effects of ET and IHH on cardiac function and specifically on endpoints of mitochondrial capacity [23]. In that study, both cardioprotective strategies positively modulated cardiac function, as measured from hemodynamic and echocardiographic parameters as well as phosphorylation capacity indexes. Considering the clinical relevance of targeting MPTP and associated mitochondrial-driven apoptosis in the context of cardiac protection [10,11], our present specific aim is to evaluate the modulation of the MPTP and apoptotic signaling in the context of cardioprotective phenotypes afforded by ET and IHH. Our hypothesis is that combined ET and IHH addictively or synergistically decrease MPTP and apoptotic signaling in the heart.

2. Methods

2.1. Reagents

Caspase 3, 8 and 9 substrates were purchased from Calbiochem-UK (235400, 368057), ECL-Plus from GE Healthcare UK (RPN2132) and PVDF-membrane from BIORAD-US (62-0182). Anti-cyclophilin D antibody was purchased from MitoSciences/Abcam-US (MS604, MSA04), BAX and Bcl-2 antibodies from Cell Signaling-US (2627S, 2772, 2876), anti-ANT antibody from Santa Cruz-US (Sc-9299), and secondary antibodies were purchased from GE Healthcare-UK (RPN2124) and from Jackson ImmunoResearch-US (705-035, 111-035-003). All other chemicals were purchased from Sigma Aldrich (Portugal).

2.2. Animal care and treatment

Forty Wistar male rats (aged 5 weeks, weighting around 190 g at the beginning of the experiments) were randomly divided into four groups (n = 10 per group): normoxic-sedentary (NS), normoxic-exercised (NE), hypoxic-sedentary (HS) and hypoxic-exercised (HE). During the experimental protocol, all animals were housed in collective cages (two animals per cage) and maintained in a room at normal atmosphere (21–22 °C; 50–60% humidity) in 12 h light/12 h dark cycles, receiving standard food chow (A04-SAFE, Scientific Animal Food and Engineering, Augy, France) and water ad libitum. The study was approved by the local Institutional Review Board and follows the Guidelines for Care and Use of Laboratory Animals in research advised by the Federation of European Laboratory Animal Science Associations (FELASA). Several authors are accredited by FELASA to perform animal experimentation.

2.3. Intermittent hypobaric hypoxia (IHH) and endurance training (ET) regimens

Animals in the hypoxic groups (HS, HE) were submitted to an acclimatization period of 5 h per day during 7 days in a hypobaric chamber (following the first day of hypoxia exposure at 2500 m, altitude was incremented by 500 m/day until a simulated altitude of 6000 m i.e., 49.3 kPa was reached). After the hypoxic acclimatization period, the animals were exposed to intermittent hypobaric hypoxia during 5 weeks (5 h per day i.e., from 7 to 12 a.m., 5 days per week) at a simulated atmospheric pressure equivalent to an altitude of 6000 m [22]. The lag phase to reach the established simulated altitude and to return to sea level conditions corresponded to 12 min. After the hypoxic period, the HE group rested for 4 h before initiating the endurance treadmill training (from 4 to 5 p.m.). Animals of the normoxic groups (NS, NE) were maintained at an atmospheric pressure of 101.3 kPa (760 mm Hg) equivalent to sea level throughout the protocol.

In coordination with the hypoxic regimen schedule, animals from the exercised groups (NE, HE) were adapted to the treadmill running during 7 days (following the first two days of exercise at 15 $m \cdot min^{-1}$, speed was increased by 5 $m \cdot min^{-1}$ until 25 $m \cdot min^{-1}$ was reached). After the adaptation period, the endurance-trained animals ran 1 h per day during 5 weeks at a speed of 25 $m \cdot min^{-1}$ (0% gradient), whereas the non-exercised animals (NS, HS) were placed on a non-moving treadmill to minimize handling and environmental stress. All the animals completed the entire training program, hypobaric hypoxia exposure or both interventions.

2.4. Blood collection and heart harvesting for mitochondrial studies

Twenty-four hours after the last training session, animals were anesthetized with ketamine (75 $mg \cdot kg^{-1}$) and xylazine (5 $mg \cdot kg^{-1}$) and the abdominal cavity was opened to expose the inferior cava vein. A blood sample of 2 mL was collected in an EDTA-containing tube for the determination of hemoglobin concentration and hematocrit. After fast chest opening, rat hearts were rapidly excised, rinsed, carefully dried and weighed. Thereafter, a portion of 20–25 mg of left ventricle was separated, homogenized in homogenization buffer (20 mM Tris–HCl, pH 7.4) using a Teflon pestle on a motor-driven Potter-Elvehjem glass homogenizer at 0–4 °C (3–5 times for 5 s at a low speed setting with a final burst at a higher speed setting). Homogenates were centrifuged at 3000 ×*g* for 10 min at 4 °C and the resulting supernatant was stored at −80 °C for later determination of caspase 3-, 8- and 9-like activities.

2.5. Isolation of rat heart mitochondria

Mitochondria were prepared using conventional methods of differential centrifugation as described in Ref. [24]. Mitochondrial and homogenate protein contents were determined by the Biuret method calibrated with BSA [25]. All isolation procedures were performed at 0–4 °C. Considering the relatively greater abundance of intermyofibrillar (IMF) (~80%) compared with subsarcolemmal (SS) (~20%) mitochondria within the cells, a potentially dominant role for the IMF subfraction vs. the SS subfraction when studying mitochondrial alterations is expected.

2.6. Mitochondrial swelling measurement

Mitochondrial osmotic volume changes were followed by monitoring the decrease of absorbance at 540 nm with a Jasco V-630 spectrophotometer. The reaction was continuously stirred and the temperature was maintained at 25 °C. The assays were performed in 1 mL of reaction medium, supplemented with 4 μM rotenone, 10 mM succinate and 240 nmol calcium with 0.5 $mg \cdot mL^{-1}$ protein. Control trials were performed using 1 μM of cyclosporin-A, the classical in vitro MPTP inhibitor [26].

2.7. Caspase-like activity determination

To measure caspase 3-, 8- and 9-like activities, aliquots of heart ventricle homogenate were incubated in a reaction buffer containing 25 mM Hepes (pH 7.5), 10% (w/v) sucrose; 10 mM dithiothreitol (DTT), 0.1% CHAPS and 100 μM caspase substrate Ac (N-acetyl)-LEHD-pNA (Calbiochem, UK) for 2 h at 37 °C. Caspase 3-, 8- and 9-like activities were determined by following the detection of the chromophore p-nitroanilide after cleavage from the labeled substrate Ac-LEHD-p-nitroanilide at 405 nm. The method was calibrated with known concentrations of p-nitroanilide (Calbiochem, UK). Caspase-like activity was calculated by the p-Na released for equal protein loaded.

2.8. Mitochondrial aconitase and superoxide dismutase activity

To measure aconitase activity, cardiac mitochondria were resuspended, immediately before measurement in 0.5 mL of buffer containing 50 mM Tris–HCl (pH 7.4) and 0.6 mM $MnCl_2$, and submitted to 4 cycles of freezing and thawing. After that, aconitase activity was measured spectrophotometrically by monitoring the formation of cis-aconitase from isocitrate at 240 nm in 50 mM Tris–HCl (pH 7.4) and 0.6 mM $MnCl_2$ and 20 mM isocitrate at 25 °C according to Krebs and Holzach [27]. One unit of aconitase was defined as the amount of enzyme necessary to produce 1 μmol of cis-aconitase per min (molar extinction coefficient at 240 nm = 3.6 $mM^{-1} \cdot cm^{-1}$).

Mitochondrial superoxide (SOD) activity was estimated using the commercial Ransod kit from Randox (SD 125). Initially, heart mitochondria were diluted in 0.01 M of phosphate buffer (pH 7.0). SOD activity was spectrophotometrically measured at 550 nm, and calculated from the degree of inhibition of the generation of superoxide radicals produced by xanthine and xanthine oxidase, which react with 2-(4-iodophenyl)-3-(4-nitrophenol-5-phenyltetrazodium chloride) (INT) to form a red formazan dye. One unit of SOD was calculated as the amount that caused 50% inhibition of the rate of reduction of INT under the conditions of the assay.

2.9. Lipid peroxidation and sulfhydryl protein group measurement

The extent of lipid peroxidation was measured by the assay for thiobarbituric acid reactive substances (TBARS). A volume of the supernatant previously obtained was mixed with 2 volumes of 10% trichloroacetic acid and centrifuged for 2 min at 3000 *g*. Aliquots