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Synergistic impact of endurance training and intermittent hypobaric hypoxia on cardiac function and mitochondrial energetic and signaling

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ABSTRACT

Background: Intermittent hypobaric-hypoxia (IHH) and endurance-training (ET) are cardioprotective strategies against stress-stimuli. Mitochondrial modulation appears to be an important step of the process. This study aimed to analyze whether a combination of these approaches provides additive or synergistic effects improving heart-mitochondrial and cardiac-function.
Methods: Two-sets of rats were divided into normoxic-sedentary (NS), normoxic-exercised (NE, 1 h/day/5 weeks treadmill-running), hypoxic-sedentary (HS, 6000 m, 5 h/day/5 weeks) and hypoxic-exercised (HE) to study overall cardiac and mitochondrial function. *In vitro* cardiac mitochondrial oxygen consumption and transmembrane potential were evaluated. OXPHOS subunits and ANT protein content were semi-quantified by Western blotting. HIF-1α, VEGF, VEGF-R1 VEGF-R2, BNP, SERCA2a and PLB expressions were measured by qRT-PCR and cardiac function was characterized by echocardiography and hemodynamic parameters.
Results: Respiratory control ratio (RCR) increased in NE, HS and HE *vs.* NS. Susceptibility to anoxia/reoxygenation-induced dysfunction decreased in NE, HS and HE *vs.* NS. HS decreased mitochondrial complex-I and -II subunits; however HE completely reverted the decreased content in complex-II subunits. ANT increased in HE. HE presented normalized ventricular–arterial coupling (Ea) and BNP myocardial levels and significantly improved myocardial performance as evaluated by increased cardiac output and normalization of the Tei index *vs.* HS.
Conclusion: Data demonstrates that IHH and ET confer cardiac mitochondria with a more resistant phenotype although without visible addictive effects at least under basal conditions. It is suggested that the combination of both strategies, although not additive, results into improved cardiac function.

1. Introduction

Several non-pharmacological approaches, including endurance training (ET) and intermittent hypobaric hypoxia (IHH) have been considered effective preventive strategies against cardiac and mitochondrial dysfunction caused by different stressors [1,2]. Endurance training has been considered an effective trigger for protection against distinct cardiac pathophysiological events [3–5]. The activation of several signaling pathways and consequent metabolic and redox remodeling has been associated with a cardioprotective phenotype. ET-induced myocardial adaptations include induction of myocardial heat shock proteins, increased myocardial cyclooxygenase-2 activity, elevated endoplasmic reticulum stress proteins, nitric oxide production, improved function of sarcolemmal adenosine triphosphate (ATP)-sensitive potassium channels and increased myocardial antioxidant capacity [3]. In addition, the signaling and mechanical remodeling induced by ET also leads to alterations on mitochondrial physiology which contributes to the overall cardioprotective phenotype. Furthermore, despite some controversy [6], cardioprotection afforded by chronic exercise might also be mediated by activation of mitochondrial ATP-sensitive potassium channels (mitoKATP) [7].

On the other hand, published data suggest that cardiac tolerance to acute oxygen deprivation can also be prevented by previous long-term exposure to IHH associated with natural or simulated high altitude conditions. In fact, a significant amount of data [2,8] showed that previous IHH decreases myocardial infarction size, reduces the number of

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ventricular arrhythmias, and improves the recovery of cardiac contractile function against acute ischemia–reperfusion (I/R) injury. Although the molecular mechanisms related with the cardioprotective effect of chronic IHH are not completely understood, a selection of putative candidates has been proposed including an increased generation of reactive oxygen species (ROS) [9], nitric oxide [10], and the up-regulation of protein kinase C-delta [9], which would serve to prime the tissue to better handle later deleterious stimuli. In addition, both the activation of the mitoKATP channels [11,12] and the increased tolerance of heart mitochondria to calcium-induced MPTP opening [13] seem to contribute to the cardioprotection provided by IHH against I/R injury. Therefore, both the cardioprotective phenotype provided by ET and IHH may share, at least in part, some common signaling pathways. Consequently, an important question is whether hearts submitted to intermittent hypoxia can be further primed with ET so that the protective phenotype is increased.

Our hypothesis for this work is that IHH exposure plus an ET regimen in an animal model act in an additive or synergistic manner to increase the putative protective adaptations in the heart, especially at the mitochondrial level. To answer our relevant experimental question, male rats were submitted to IHH and ET alone and sequentially and hemodynamic measurements before and after volume overload, as well as echocardiography were used to assess cardiac function. Moreover, mitochondrial bioenergetics and resistance to *in vitro* anoxia and reoxygenation were also determined.

2. Methods

2.1. Reagents

ECL-Plus was purchased from GE Healthcare UK (RPN2132) and PVDF-membrane from BioRad-US (62-0182). OXPHOS cocktail antibodies were purchased from MitoSciences/Abcam-US (MS604), anti-SIRT3 antibody from Cell Signaling-US (2627S), 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). Rat VEGF EIA Kit was purchased from R&D systems Europe (RRV00). All other chemicals were purchased from Sigma Aldrich (Portugal).

2.2. Animal care and treatment

Two sets of 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/group): normoxic-sedentary (NS), normoxic-exercised (NE), hypoxic-sedentary (HS) and hypoxic-exercised (HE). The first set of animals was used to study *in vitro* mitochondrial function and related molecular biology markers and the second to perform echocardiography and *in vivo* cardiac hemodynamic evaluation. 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/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/day *i.e.*, from 7 to 12 a.m., 5 days/week) at a simulated atmospheric pressure equivalent to an altitude of 6000 m [adapted from 14]. 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, the 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/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, the set of animals used to study *in vitro* mitochondrial function were anesthetised 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.

2.5. Isolation of heart mitochondria

Mitochondria were prepared using conventional methods of differential centrifugation as described [15]. Mitochondrial and homogenate protein contents were determined by the Biuret method calibrated with BSA [16]. 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 oxygen consumption assays

Mitochondrial oxygen consumption was measured polarographically, at 25 °C, using a Biological Oxygen Monitor system (Hansatech Instruments) and a Clark-type oxygen electrode (Hansatech DW 1, Norfolk, UK). Reactions were performed in 0.75 mL closed, temperature-controlled and magnetically stirred glass chamber containing 0.5 mg of mitochondrial protein in a respiration buffer containing 65 mM KCl, 125 mM sucrose, 10 mM Tris, 20 mM EGTA, 2.5 mM KH_2PO_4, pH 7.4. After 1-min equilibration period, mitochondrial respiration was initiated by adding glutamate (10 mM) plus malate (5 mM) or succinate (10 mM) plus rotenone (4 mM). State 3 respiration was measured after adding 333 nmol ADP; state 4 was considered as the rate of oxygen after full ADP phosphorylation. The respiratory control ratio (RCR, state3/state 4) and the ADP/O ratio (nmol ADP phosphorylated by natom O consumed), were calculated according to Estabrook [17], using 474 natom $O \cdot mL^{-1}$ as the value for oxygen solubility at 25 °C in double distilled water.

To evaluate the mitochondrial response to *in vitro* anoxia/reoxygenation (A/R), anoxia was performed by exhausting oxygen in the reaction chamber after stimulating mitochondrial respiration with the addition of two ADP pulses in glutamate (10 mM) and malate (5 mM) energized mitochondria. Energized cardiac mitochondria were stimulated with an initial 333 nmol ADP pulse to obtain pre-anoxia respiratory rates. The anaerobic conditions were reached in state 4 through the addition of a second ADP pulse (1 mM), with the total period of anoxia set to 1 min. Anoxia was followed by 4 min of *in vitro* reoxygenation by exposing the reaction medium containing the mitochondrial suspension to open air while stirring the suspension. The polarographic oxygraph chamber was then closed and respiratory activities were measured again after the addition of another 333 nmol ADP pulse.

2.7. Mitochondrial membrane potential measurements

Mitochondrial transmembrane potential ($\Delta\psi$) was monitored indirectly based on the activity of the lipophilic cation tetraphenylphosphonium (TPP^+), followed by a TPP^+ selective electrode prepared as previously described [18]. Reactions were carried out in 1 mL of reaction buffer containing 65 mM KCl, 125 mM sucrose, 10 mM Tris, 20 mM EGTA, 2.5 mM KH_2PO_4, pH 7.4, supplemented with 3 μM TPP^+ and 0.5 mg/mL of protein with the temperature maintained at 25 °C. For measurements of $\Delta\psi$ with complex I substrates, energization was carried out with 10 mM of glutamate and 5 mM of malate and ADP-induced phosphorylation was achieved by adding 444 μM ADP. For measurements of $\Delta\psi$ with complex II substrates, 10 mM succinate supplemented with 4 μM rotenone were added to the medium containing 3 mM TPP^+ and mitochondria. The lag phase, which reflects the time needed to phosphorylate the added ADP, was also measured for both substrates.

2.8. Western blotting assays

Protein expression was determined by Western-blot, for that equal amounts of protein (50 μg) were denatured in sample loading buffer and separated by dodecyl sulphate–polyacrylamide gel electrophoresis (SDS/PAGE), followed by a transference to a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences). After blotting, membranes were blocked with 5% (w/v) non-fat dried milk or BSA in a Tris-buffered saline with Tween 20 (TBST-T). The membranes were then incubated with anti-ANT (1:1000 dilution; goat polyclonal, Santa Cruz Biotechnology sc-9299) and anti-Sirt3 (1:1000 dilution; rabbit monoclonal, Cell Signaling 2627). Primary antibodies were diluted in TBS-T containing 1% of non-fat dried milk or BSA for 2 h at room temperature (22–25 °C). After the incubation period, membranes were washed three times and incubated for 2 h with horseradish peroxidase-conjugated anti-goat for ANT (1:10,000; Jackson ImmunoResearch 705-035-003) or anti-rabbit for SIRT3 (1:10,000; Jackson ImmunoResearch 111-035-003). Immunoreactive bands were visualized using ECL® chemiluminescence reagents (Amersham Biosciences), according to the manufacturer's instructions, followed by exposure to X-ray films (Sigma Z370398). Films were then analyzed with Quantity One Software (BioRad Laboratories), and resulting absorbance values were expressed as the percentage variation of the NS group values.