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Keyword:	intermittent hypoxia, mitochondrial biomarkers, eccentric exercise, muscle damage

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**Modulation of mitochondrial biomarkers by intermittent hypobaric hypoxia
and aerobic exercise after eccentric exercise in trained rats**

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ABSTRACT

Unaccustomed eccentric contractions induce muscle damage, calcium homeostasis disruption and mitochondrial alterations. Since exercise and hypoxia are known to modulate mitochondrial function, we aimed to analyze the effects on eccentric exercise-induced muscle damage (EEIMD), in trained rats, of two recovery protocols based on: 1) intermittent hypobaric hypoxia (IHH) and 2) IHH followed by exercise. The expression of biomarkers related to mitochondrial biogenesis, dynamics, oxidative stress and bioenergetics was evaluated. Soleus muscles were excised before (CTRL) and 1, 3, 7 and 14 days after an EEIMD protocol. The following treatments were applied one day after the EEIMD: passive normobaric recovery (PNR), four-hour daily exposure to passive IHH at 4000m (PHR) or IHH exposure followed by aerobic exercise (AHR). Citrate synthase activity had reduced 7 and 14 days after application of the EEIMD protocol. However, this reduction was attenuated in AHR rats at day 14. PGC-1 α and Sirt3 and TOM20 levels had decreased after 1 and 3 days, but the AHR group exhibited increased expression of these proteins, as well as of Tfam, by the end of the protocol. Mfn2 greatly reduced during the first 72 h, but returned to basal levels passively. At day 14, AHR rats had higher levels of Mfn2, OPA1 and Drp1 than PNR animals. Both groups exposed to IHH showed a lower p66shc(ser³⁶)/p66shc ratio than PNR animals, as well as higher complex IV subunit I and ANT levels. These results suggest that IHH positively modulates key mitochondrial aspects after EEIMD, especially when combined with aerobic exercise.

Key words: intermittent hypoxia, mitochondrial biomarkers, mitochondrial biogenesis, mitochondrial dynamics, eccentric exercise, muscle damage

INTRODUCTION

Unaccustomed eccentric contractions induce muscle damage with subsequent swelling, delayed-onset muscle soreness, inflammation, leakage of intracellular proteins, increased production of reactive oxygen species (ROS), myophagocytosis of necrotic fibers and a decrease in maximal force generation (Schwane et al. 1983, Armstrong et al. 1983, Teague and Schwane 1995, MacIntyre et al. 1996, Close et al. 2004). Several mechanisms that explain the etiology of eccentric exercise-induced muscle damage (EEIMD) have been proposed. Although the precise order and sequence of the events are still a matter of scientific debate, it is clear that the early events that lead to EEIMD include: the overstretching and disruption of sarcomeres (Talbot and Morgan 1996), failure in the excitation-contraction coupling (Warren et al. 1993) and disruption of the cytoskeletal elements involved in force transmission (Fridén and Lieber 1998). After these initial events, inflammatory cells start accumulating in the damaged area, myofibrillar proteins degrade, ROS production increases and calcium homeostasis is disrupted (Clarkson and Sayers 1999, Sayers and Hubal 2007). Specifically, an increased concentration of intracellular calcium following eccentric contractions has been found in both human and rodent models (Duan et al. 1990, Fridén and Lieber 1996, Sonobe et al. 2008), probably as a consequence of the disruption of the muscle and sarcoplasmic reticulum membranes (Chen et al. 2007). This increase in intracellular calcium concentration activates calpain, a calcium-dependent non-lysosomal protease that cleaves cytoskeletal and mitochondrial proteins (Vissing et al. 2008). Despite some controversy (Molnar et al. 2006), eccentric exercise and calcium overload have been associated with impaired mitochondrial function, namely decreased respiratory control ratio (RCR), complex V activity, repolarization and maximal energization, mitochondrial swelling, and mitochondrial permeability transition pore (mPTP) opening

(Ratnay et al. 2011, Magalhães et al. 2013b), which is related to the activation of apoptosis cascade signaling. However, little is known about the effects of eccentric exercise on mitochondrial dynamics, such as fission and fusion, biogenesis and the expression of electron transporter chain complexes.

Because mitochondria are the main powerhouse of the cell and play an important role in oxidative stress, apoptosis signaling and calcium homeostasis, they have emerged as an interesting target for the prevention and treatment of muscle pathologies, including EEIMD. It has been demonstrated that non-pharmacological tools such as endurance training and hypoxia exposure, which are well-known mitochondrial modulators, are effective at improving mitochondrial function. For instance, Ratnay et al. (2013) found attenuated mitochondrial calcium overload and preserved RCR and mPTP Ca^{+2} sensitivity in the vastus lateralis of male rats after an acute bout of eccentric exercise using a downhill training protocol, while increased subsarcolemmal mitochondrial biogenesis has been found in the same muscle of humans submitted to hypoxic training (Desplanches et al. 2014). Furthermore, Magalhães et al. (2013a, 2014) reported the positive modulation of rat cardiac mitochondrial energetics and signaling by endurance training and intermittent hypobaric hypoxia (IHH). Similarly, exercise-induced mitochondrial cardioprotective effects have also been found in a rat model of doxorubicin-induced cardiotoxicity (Marques-Aleixo et al. 2015). Specifically, these studies showed that adequate doses of intermittent hypoxia and/or exercise can induce beneficial mitochondrial adaptive responses, such as increased efficiency of ATP production, reduced susceptibility to mitochondrial permeability transition pore (mPTP) opening, decreased caspase-3 activity and Bcl-2/Bax ratio and higher resistance against anoxia/reoxygenation stress. This positive modulation, produced by both endurance

exercise and intermittent hypoxia, could be, at least partially, due to an increase of mitochondrial biogenesis and dynamics, as well as reduced oxidative stress.

Thus, the main goal of the present study was therefore to analyze the effects of intermittent hypobaric hypoxia, alone or combined with aerobic exercise, in different markers of mitochondrial biogenesis, dynamics, oxidative stress and energetics after strenuous eccentric exercise. Because muscle damage is especially relevant in athletes, all the experiments were conducted in aerobic trained rats.

MATERIAL and METHODS

Animals

Fifty-two male Sprague-Dawley rats were used in this study. During the experimental protocol, animals were maintained at an average temperature of 23°C under a light-dark cycle of 12 h/12 h with food and water *ad libitum*. All animals underwent training on a treadmill for a month. Three days after the last training session, four rats were euthanized (CTRL group). The remaining rats were submitted to an EEIMD protocol and randomly divided into three experimental groups the next day: 1) PNR (passive and normoxic recovery, n=16), rats that passively recovered from EEIMD in normoxic conditions; 2) PHR (passive and hypoxic recovery, n=16), rats that passively recovered from EEIMD following a protocol of IHH exposure; and 3) AHR (active and hypoxic recovery, n=16), rats that performed a light running session on a treadmill immediately after IHH. Animals from these three groups were euthanized immediately after the last intervention at four different time points (n=4 each): 1, 3, 7 and 14 days after the EEIMD protocol (t01, t03, t07 and t14, respectively). Fig. 1 shows a schematization of the experimental design.

All procedures were performed in accordance with the internal protocols of our laboratory, which were authorized by the University of Barcelona's Ethical Committee for Animal Experimentation and ratified, in accordance with current Spanish legislation, by the Departament de Medi Ambient i Habitatge (Generalitat de Catalunya).

Aerobic training protocol

All rats underwent an aerobic training protocol. All training sessions were carried out at room temperature ($21 \pm 2^{\circ}\text{C}$) on a treadmill (LE 8710, Panlab, Barcelona, Spain). The aerobic training protocol consisted of an adaptation phase (5 days/week during two weeks), in which the duration and intensity of the running exercise were gradually increased in order to habituate the animals to the treadmill and the new activity, and then two weeks (5 days/week) in which the speed of the treadmill was set at $45 \text{ cm}\cdot\text{s}^{-1}$ and the duration of the exercise at 35 min. After the fourth day of the adaptation phase, and until the end of the training protocol, the animals trained twice daily (with a recovery interval of 6 h). This daily double session of running allowed rats to adapt physiologically to the endurance exercise and optimized the duration of the whole protocol.

Eccentric exercise-induced muscle damage protocol

Skeletal muscle damage was induced by eccentric exercise consisting of downhill running with a decline of 15° and at $50 \text{ cm}\cdot\text{s}^{-1}$ until exhaustion (Armstrong et al. 1983). This protocol began three days after the end of the training period and was applied twice on the same day: one session in the morning and one in the afternoon, with a resting period of 4 h between the end of the first session and the beginning of the second session.

Intermittent hypobaric hypoxia exposure

IHH sessions started the day after the EEIMD protocol and were performed using a hypobaric chamber with a volume of about 450 L. A relative vacuum was created with a rotational vacuum pump (TRIVAC D5E, Leybold, Cologne, Germany) whose air-flow rate was regulated at the inlet by a micrometric valve. Inner pressure was controlled by two differential sensors (ID 2000, Leybold, Cologne, Germany), which drove a diaphragm pressure regulator (MR16, Leybold, Cologne, Germany). The target pressure of 462 torr (equivalent to 4,000 m of altitude) was achieved steadily over a period of about 15 min. Once this pressure had been reached, the chamber pressure was maintained and regulated for 4 h. At the end of the session, normal barometric pressure was gradually restored over a period of 15 min. The total number of days of hypobaric hypoxia exposure varied according to the sampling schedule. Animals assigned to t01 were in the hypobaric chamber for a single session, whereas t14 animals were subjected to two weeks of daily exposure. Animals had *ad libitum* access to food and water kept in open-air reservoirs inside the hypobaric chamber during the hypoxia sessions.

Running exercise recovery protocol

AHR rats were enrolled in a recovery exercise program consisting of a daily session of aerobic exercise immediately after a hypobaric hypoxia session according to the sampling schedule. These rats were placed on a treadmill to run in accordance with a low-impact exercise program. The exercise session lasted 20 min with a gradual increase in speed until they reached $30 \text{ cm} \cdot \text{s}^{-1}$ and a gradual increase in inclination from 0° to 5° .

Plasma and muscle sampling

After the last intervention, rats were euthanized by heart excision under urethane anesthesia ($5 \text{ ml} \cdot \text{kg}^{-1}$ from a $30 \text{ g} \cdot \text{dL}^{-1}$ solution). Soleus muscles, which are widely used

in EEIMD studies due to its susceptibility to eccentric contractions and are recruited during aerobic exercise, were excised, rinsed in saline solution, immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Blood samples were obtained from the vena cava, placed in EDTA tubes and centrifuged at $1,300\text{ g}$ for 10 minutes. Plasma was collected and stored at -80°C until assayed.

Citrate synthase activity assay

Approximately 10 mg of soleus muscle were homogenized in 100 mL of ice-cold medium containing 75 mM of Tris-HCl, 2 mM of MgCl_2 and 1 mM of EDTA (pH 7.6). In accordance with Srere's method (1969), the homogenate was centrifuged at $11,000\text{ g}$ and the supernatant used to measure enzymatic activity spectrophotometrically.

Creatine kinase and myoglobin biomarkers

A commercially available ELISA (Life Diagnostics Inc., PA, USA) was used for the quantitative detection of creatine kinase isoenzyme MM (CK-MM) and myoglobin in plasma samples, in accordance with the manufacturer's instructions.

Western blotting

Thirty milligrams of soleus muscle were homogenized in ice-cold lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% DOC and 0.1% SDS) supplemented with protease and phosphate inhibitor cocktails (P8340 and P5726, Sigma) and centrifuged at $10,000\text{ g}$ for 10 min. The protein concentration of the collected supernatants was determined using the bicinchoninic acid assay (Thermo Scientific, IL, USA). Equivalent amounts of muscle protein were separated by SDS/PAGE (10%) and transferred onto PVDF membranes (Millipore, MA, USA). Membranes were blocked with nonfat dry milk and incubated with anti-peroxisome

proliferator-activated receptor gamma co-activator protein (PGC-1 α) (sc-5816), anti-mitochondrial transcription factor A (Tfam) (sc-23588), anti-translocase of the outer membrane 20 (TOM20) (sc-11415), anti-Mitofusin 2 (Mfn2) (sc-50331) and anti-adenine nucleotide translocator (ANT) (sc-9299) from Santa Cruz (CA, USA); with anti-Optic atrophy 1 (OPA-1) (ab119685) and anti-OXPHOS Western blotting cocktail (ab110413) (containing antibodies against CI, CII, CIII, CIV and CV subunits: anti-NDUFB8, anti-CII-30 kDa, anti-CIII-Core protein 2, anti-CIV subunit I and anti-CV alpha subunit, respectively) from Abcam (Cambridge, UK); with anti-dynamin-related protein 1 (Drp-1) (#8570), anti-Bax (#2772), anti-Bcl-2 (#2870), anti-sirtuin 3 (SIRT3) (#2627) and anti-p66sch (#2432) from Cell Signaling (Danvers, MA, USA); and with anti-p66shc(pSer36) (6E10) from Calbiochem Merck Millipore (Darmstadt, Germany). After incubation with the corresponding secondary antibodies (Santa Cruz, CA, USA), membranes were photographed using a ChemiDoc XRS+ imaging system and analyzed with Image Lab software (both from Bio-Rad Laboratories). Ponceau-S staining was used to normalize differences in protein loading or transfer, and the final data were expressed as the percentage variation of the control values (% CTRL) or PNR values (% PNR).

Statistics

The results were reported as mean $\pm$ SE for each experimental group. Data were analyzed using a one-way ANOVA test followed by the Holm-Sidak *post hoc* test or Student's *t*-test when appropriate. A *p*-value < 0.05 was considered statistically significant. Regarding the Western blot results, statistical comparisons were only tested when samples were within the same membrane. Samples belonging to each temporal point (t01, t03, t07 or t14) were always loaded into the same gel, thereby allowing statistical comparisons. Additional gels containing CTRL and PNR t01-t14 samples

were run in order to statistically evaluate the time course of the analyzed proteins. All statistical analyzes were carried out with the statistical package SigmaPlot (Systat Software Inc.).

RESULTS

Biomarkers of muscle damage

Plasma levels of CK-MM and myoglobin were assessed in order to confirm the validity of the EEIMD protocol. As shown in Fig. 2, both CK-MM and myoglobin significantly increased in plasma after a double session of strenuous eccentric exercise ($p < 0.001$).

Citrate synthase activity

As shown in Fig. 3, no significant differences were observed during the first 72 h either among treatments or *versus* the CTRL group. At t07 and t14, however, all groups showed a significant decrease in CS activity when compared with their counterparts at t01 and t03 and when compared to CTRL animals. Furthermore, AHR rats exhibited higher CS activity than PNR and PHR animals at t14.

Protein expression of mitochondrial biogenesis markers

The content of two key promoters of mitochondrial biogenesis was assessed. As seen in Fig. 4A, PGC-1 α content significantly decreased one day after the EEIMD protocol (CTRL vs. PNR t01). AHR animals showed increased levels of PGC-1 α compared to PNR rats at all time points, and these were statistically significant at t07 and t14. PHR rats showed a similar trend to AHR animals, with a significant increase at t07. A non-significant decrease in Tfam content (Fig. 4B) was found at t03 (CTRL vs. PNR, $p = 0.06$). AHR animals showed a significant 1.6-fold increase over PNR rats at both t07 and t14, while PHR rats showed a significant decrease at t07 and an increase at t14.

Since mitochondrial biogenesis is accompanied by an increase in the protein import machinery, we also analyzed the content of TOM20 (Fig. 4C). The protein content was significantly reduced at t03 and t14 (CTRL vs. PNR). Both AHR and PHR groups exhibited higher levels of TOM20 than PNR at t14.

Protein expression of mitochondrial dynamics markers

Since mitochondrial dynamics play a key role in mitochondrial function, we semi-quantified the content of fusion (Mfn2, OPA-1) and fission (Drp-1) proteins. Strenuous eccentric exercise produced a significant 2-fold and 3-fold reduction in Mfn2 expression at t01 and t03 (CTRL vs. PNR), which partially reversed on the following days, t07 and t14 (Fig. 5A). Although the PNR group recovered its Mfn2 levels faster than the AHR group at t07, at t14 AHR rats showed significantly higher levels of Mfn2 than PNR animals. As shown in Fig. 5B, OPA-1 did not show significant alterations over time, although a decreasing trend was observed ($p = 0.08$ at t14, CTRL vs. PNR). Both PHR and AHR groups had significantly higher levels of OPA-1 than PNR at t07 and t14. The EEIMD protocol significantly increased fission protein Drp-1 levels, by up to 100% of the control at all temporal points (CTRL vs. PNR) (Fig. 5C). At t14, moreover, AHR rats showed expression levels 1.6-fold higher than PNR and PHR groups.

Apoptotic signaling

As observed in Fig. 6A, the content of the pro-apoptotic protein Bax significantly increased seven days after the EEIMD protocol (CTRL vs. PNR). The same trend was observed before (t03) and after (t14), although without statistical significance ($p = 0.09$ and $p = 0.06$, respectively). The combination of exercise and hypoxia showed a non-significant trend towards attenuation of this increase at t07. Furthermore, a significant reduction in the levels of anti-apoptotic protein Bcl-2 was observed at t03 (CTRL vs.

PNR). AHR animals showed higher levels of Bcl-2 than PNR ($p < 0.05$) and PHR ($p < 0.01$) groups at t14 (Fig. 6B). The Bax/Bcl-2 ratio significantly increased in PNR t03 rats when compared to CTRL and PNR t01 ($p < 0.01$) (Fig. 6C). AHR animals showed a non-significant reduction at t07 when compared to PNR ($p = 0.06$).

Protein expression of mitochondrial oxidative stress markers

Protein levels of Sirt3, a pivotal regulator of oxidative stress, were assessed (Fig. 7A). A significant reduction one and three days after the EEIMD protocol ($p < 0.01$) was observed (CTRL vs. PNR). Sirt3 returned to its basal levels in PNR rats at t07, while AHR animals showed significantly higher levels than the other groups at t07 ($p < 0.01$) and t14 ($p < 0.05$).

The protein content of p66shc, in addition to its phosphorylated form (p66shc-pSer36) and their ratio, was used as the protocol endpoint measurement of mitochondrial oxidative stress (Fig. 7B-D). Both groups exposed to IHH exhibited a significant lower p66shc(pSer³⁶)/p66shc ratio than PNR rats, due to either a reduction in its phosphorylated form, as in the case of PHR rats ($p < 0.05$ vs. PNR), or an increase in its total form (AHR, $p < 0.05$ vs. PNR).

Protocol endpoint measurement of electron transport chain proteins and ANT

The content of electron transport chain (ETC) complexes I-IV and ATP synthase subunits and ANT was semi-quantified (Fig. 8) two weeks after the application of the EEIMD protocol. IHH induced a significant increase in the levels of complex I and IV synthase subunits, as well as those of ANT (PHR vs. PNR, $p < 0.05$). Despite the fact that AHR animals showed lower levels of complex I subunit than PHR rats, the former exhibited higher levels of complex IV subunit and ANT than PNR animals ($p < 0.05$).

DISCUSSION

Previous studies demonstrated that unaccustomed eccentric exercise induces an immediate and transient impairment of the mitochondrial function in skeletal muscle, such as decreased RCR, impaired calcium handling and mPTP opening (Ratnay et al. 2011, Magalhães et al. 2013b). However, the effects of EEIMD on the protein expression of markers of mitochondrial biogenesis, dynamics and bioenergetics, or of apoptosis and oxidative stress, have not been addressed before. Here we present the changes that EEIMD induced in the expression of different related proteins and how IHH, alone or combined with aerobic exercise, modulated these alterations.

Muscle damage markers and citrate synthase activity

As expected, increased plasma levels of CK-MM and myoglobin were found after a double session of exhaustive downhill running, thus confirming the presence of muscle damage. Soleus muscle CS activity was found to be unaltered during the first 72 h. Immediate alterations in mitochondrial function after eccentric exercise have been reported in rats (Ratnay et al. 2011, Magalhães et al. 2013b); as a consequence, a decrease in CS would be expected. In fact, CS activity has been found decreased, unaltered or even increased in both human and rats after acute exercise (Ji et al. 1988, Leek et al. 2001, Molnar et al. 2006). Furthermore, the application of a training protocol before the EEIMD could have conferred a protective phenotype, delaying the expected CS activity decrease. Indeed, CS activity decreased abruptly between days 3 and 7, thus suggesting that EEIMD could trigger a delayed response in the activity of certain enzymes, in addition to the immediate impairment found by other authors. At the end of the protocol, AHR rats showed a partial reversal of this reduction. Other studies had already demonstrated that IHH, alone and combined with aerobic exercise, can upregulate CS activity in both humans (Melissa et al. 1997) and rats (Esteva et al.

2009). As detailed below, this upregulation could be explained by the findings obtained for mitochondrial biomarkers of biogenesis, dynamics and oxidative stress.

Mitochondrial biogenesis markers

Our results showed that acute eccentric exercise induced an immediate decrease in PGC-1 α protein expression. This reduction in PGC-1 α could lead to impaired mitochondrial biogenesis, which would explain the decrease observed in the mitochondrial content marker CS over the subsequent days. Interestingly, the groups exposed to IHH (PHR and AHR) showed increased levels of PGC-1 α and its downstream transcription factor Tfam between seven and/or fourteen days after EEIMD, which would suggest mitochondrial biogenesis. The higher levels of TOM20 found at t14 in these groups reinforce this hypothesis, as this protein mediates in the mitochondrial protein import destined for the matrix, which is essential during mitochondrial biogenesis (Grey et al. 2000). Although exposure to chronic hypobaric hypoxia has usually been associated with decreased mitochondrial content and biogenesis (Hoppeler et al. 1990, Zhang et al. 2007), IHH has already been demonstrated to induce the opposite response, at least in rat lung, although the mechanism remains unknown (Chitra and Boopathy 2014). This apparent discordance is not surprising, as it is becoming increasingly evident that the effects of hypobaric hypoxia are strongly dependent on the dose, intensity and frequency of the exposure (Navarrete-Opazo and Mitchell 2014).

Mitochondrial dynamics markers

An imbalance between fusion and fission proteins was induced by eccentric exercise. During the first 72 h, the fission protein marker Drp-1 drastically increased, while the expression of Mfn2 halved. This imbalance towards mitochondrial fission could be due

to the presence of damaged mitochondria (Twig et al. 2008) and, together with the decrease in mitochondrial biogenesis markers, suggests that mitochondrial turnover was impaired by EEIMD. Two weeks afterwards, the Drp-1 levels were still significantly higher than before EEIMD. AHR rats exhibited even higher levels of Drp-1, but also of both fusion proteins Mfn2 and OPA-1, which would suggest that the higher rates of mitochondrial fission in this group were also compensated by higher fusion frequency. These high rates of both fusion and fission could indicate a more dynamic, adaptable mitochondrial network. Furthermore, the maintenance of an interconnected mitochondrial network is crucially important for keeping mitochondria energetically active (Tondera et al. 2009).

Apoptotic signaling

In line with the literature (Sudo and Kano 2009), increased apoptotic signaling was found three days after EEIMD, as reflected by the Bax/Bcl-2 ratio. This increase in apoptotic signaling at t03 coincided with the peak expression of Drp-1, which is involved in cytochrome *c* release (Frank et al. 2001) and, therefore, in apoptotic signaling. IHH with aerobic exercise seemed to confer a protective phenotype against apoptotic signaling, as AHR rats showed a lower Bax/Bcl-2 ratio at t07 and t14 than PNR animals. Similar results were found in cardiac mitochondria in a study carried out by Magalhães et al. (2014), in which rats exposed to IHH and aerobic exercise showed decreased apoptotic signaling.

Mitochondrial oxidative stress markers

Sirtuins are deacetylases that play an important role in aging and life span regulation. Specifically, Sirt3 is mainly located in mitochondria, and its function is to activate oxidative and antioxidant enzymes (Yu et al. 2012). At the same time, downregulation

of Sirt3 can be induced by oxidative stress (Wu et al. 2013). Thus, it is not surprising that after strenuous eccentric exercise, which is known to induce oxidative stress (Stagos et al. 2015), Sirt3 expression was found to be downregulated, although it returned to CTRL levels after seven days.

As reported by other studies (Ascensão et al. 2012), rats exposed to IHH and aerobic exercise (AHR) showed higher levels of Sirt3, whose upregulation has been associated with the activation of antioxidant defenses (Yu et al. 2012). Furthermore, Sirt3 is a target gene of PGC-1 α , which was also found to be upregulated, and contributes to the mitochondrial biogenesis mediated by PGC-1 α (Kong et al. 2010). In addition to its role in oxidative metabolism, oxidative stress and mitochondrial biogenesis, it is also responsible for the deacetylation and activation of OPA-1 (Samant et al. 2014), which suggests that IHH with aerobic exercise not only upregulated the content of this protein, but also increased its activity.

In addition to the increased levels of Sirt3 in AHR animals, rats exposed to IHH, alone or with aerobic exercise, exhibited a reduction in the p66shc(pSer³⁶)/p66shc ratio. Since p66shc plays an important role in oxidative response and is serine-phosphorylated by oxidative damage (Migliaccio et al. 1999), the fact that AHR rats showed increased levels of total p66shc, but the same amount of serine³⁶-phosphorylated p66shc, could indicate that these animals triggered an adaptive response to oxidative stress, thereby showing good correlation with the increase in Sirt3. On the other hand, PHR rats showed lower levels of p66shc(pSer³⁶), which would suggest that their protective phenotype against oxidative stress was mediated by another mechanism.

Electron transport chain proteins and ANT

The content of complex I-V subunits and ANT were measured at the end of the protocol in order to assess whether the changes observed in mitochondrial markers of biogenesis, dynamics and oxidative stress affected ECT-related proteins. Surprisingly, the increase found in PGC-1 α , Tfam and TOM20 did not align well with the results obtained from the analysis of the ETC complexes content. However, this apparent inconsistency could be a consequence of the sequence of the events involved in mitochondrial biogenesis, as there is a temporal delay between the gene and protein expression and the assembly of multi-subunit respiratory complexes (Hood 2001). Nevertheless, although no changes were observed in the protein content of the ATP synthase, IHH induced an increase in ANT, which is responsible for the exchange of ADP/ATP across the inner mitochondrial membrane. Thus, even with similar levels of ATP synthase, rats exposed to IHH could benefit from a higher flux of substrate. Moreover, ANT is a component of the MPTP, which mediates in apoptosis signaling (Baines et al. 2005). Decreased ANT content has been linked to increased susceptibility to Ca²⁺-induced MPTP opening (Oliveira and Wallace 2006). Consequently, an increase in ANT levels could be interpreted as a more protective phenotype against apoptosis.

Interestingly, IHH also increased the content of complex IV, which may have an indirect antioxidant effect by reducing ROS generation in complexes I and III and reducing electron leakage (Chen et al. 2003, Parise et al. 2005). This explains, at least partially, the reduced p66shc(pSer³⁶)/p66shc ratio in PHR and AHR rats, as p66shc is mainly phosphorylated at serine-36 by H₂O₂ (Migliaccio et al. 1999), whose production is derived from the activity of complexes I and III.

It is important to remember, however, that mitochondrial protein content alone is not enough to determine mitochondrial health, does not provide information about energy production efficiency and does not relate to the activity of the complexes.

Aerobic exercise relevance and dose-dependent effect of IHH

Overall, most of these alterations were found to be attenuated or reversed in rats exposed to IHH (PHR and AHR). This counteraction of the deleterious effects induced by EEIMD was substantially more robust in AHR rats, which would suggest that aerobic exercise enhances the IHH response. In fact, the rationale behind the use of a light session of aerobic exercise was to further challenge the oxygen homeostasis in order to effectively trigger a hypoxic response in the soleus muscle, as in a resting state the oxygen demand could be easily handled even in a hypoxic environment. Thus, the recovery exercise protocol was more a *hypoxic enhancer* than an independent treatment itself, especially when applied to trained subjects. It is important to note that all rats were trained with two daily sessions of 35 min at $45 \text{ cm}\cdot\text{s}^{-1}$ ($1.89 \text{ Km}\cdot\text{day}^{-1}$) before the EEIMD protocol, while the exercise applied during the recovery consisted of a single daily session of 20 min at $30 \text{ cm}\cdot\text{s}^{-1}$ ($0.36 \text{ Km}\cdot\text{day}^{-1}$). That was a reduction of a fifth of the training volume, while the intensity was decreased by 66%. Taking into account the importance of the training intensity in the maintaining or development of exercise-induced physiological adaptations (Hickson et al. 1982), it seems unlikely that the solely application of this exercise protocol could have induced the observed alterations in trained subjects.

Moreover, significant differences between rats exposed to IHH and normobaric rats were only found after seven and fourteen days, in agreement with the notion that the response to IHH is dose-dependent (Navarrete-Opazo and Mitchell 2014).

Conclusion

Our data showed that EEIMD downregulated the expression of mitochondrial biogenesis and fusion-related proteins (PGC-1 α , Mfn2), upregulated mitochondrial

fission markers (Drp-1) and induced an apoptotic response after three days of application. Although CS activity was not altered during the first three days, a significant decrease was found after the first and second weeks. IHH followed by aerobic exercise upregulated the protein content of mitochondrial biogenesis and dynamics markers and decreased the p66shc(pSer³⁶)/p66shc ratio, thus suggesting a reduction in oxidative stress. Concordantly, Sirt3, which modulates all these processes directly or indirectly, was found to be upregulated. All these findings seemed to eventually result in increased CS activity, which would suggest that the changes found at protein expression level effectively lead to functional adaptation.

These findings open the door for further studies in athletes in the field of the treatment of the muscle damage. Thus, the application of IHH combined with exercise emerges as a new potential therapeutic tool. In fact, altitude training (being the hypoxia natural or simulated in hypobaric chambers) is already used in humans in order to induce cardioprotective adaptations, muscular adaptations and improve exercise performance, so its application in muscle damage seems like a logical further step in the field of the hypoxic training.

CONFLICT OF INTEREST STATEMENT

The authors report no conflicts of interest associated with this manuscript.

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FIGURE CAPTIONS

Figure 1. Schematic representation of the experimental design. *EEIMD*, eccentric exercise-induced muscle damage; *PNR*, passive normoxic recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic recovery.

Figure 2. Validation of the *EEIMD* protocol in trained rats. A-B: Plasma concentration of the muscle damage biomarkers CK-MM and myoglobin. Asterisks (***) indicate $p < 0.001$ vs. CTRL. *PNR*, passive normoxic recovery.

Figure 3. Effect of IHH and exercise after the *EEIMD* protocol on the citrate synthase activity normalized to muscle protein content. Statistically significant differences are indicated according to the following codes: * vs. CTRL; *a* vs. t01, *b* vs. t03 (for comparisons between different time points within the same treatment); ■ for comparisons between different treatments at the same time point. One, two and three repeated symbols correspond to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. *PNR*, passive normoxic recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic recovery.

Figure 4. Effect of *EEIMD* and the recovery protocols on the protein expression of different markers of mitochondrial biogenesis in the soleus muscle. *A*: relative content of PGC-1 α . *B*: relative content of Tfam. *C*: relative content of TOM20. Bands were normalized to Ponceau-S staining (the corresponding well is shown). Normalized bands were expressed as a percentage of the CTRL group. The bands shown are not necessarily arranged in the same order as in the gel (in that case, a white space separates the bands and wells). Statistically significant differences are indicated according to the following codes: * vs. CTRL; ■ for comparisons between different treatments at the same time point. One, two and three repeated symbols correspond to $p < 0.05$, $p < 0.01$

and $p < 0.001$, respectively. *PNR*, passive normoxic recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic recovery.

Figure 5. Effect of EEIMD and the recovery protocols on the protein expression of different markers of mitochondrial dynamics in the soleus muscle. *A*: relative content of Mfn2. *B*: relative content of OPA-1. *C*: relative content of Drp-1. Bands were normalized to Ponceau-S staining (the corresponding well is shown). Normalized bands were expressed as a percentage of the CTRL group. The bands shown are not necessarily arranged in the same order as in the gel (in that case, a white space separates the bands and wells). Statistically significant differences are indicated according to the following codes: * vs. CTRL; *a* vs. t01, *b* vs. t03 (for comparisons between different time points within the same treatment); ■ for comparisons between different treatments at the same time point. One, two and three repeated symbols correspond to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. *PNR*, passive normoxic recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic recovery.

Figure 6. Effect of EEIMD and the recovery protocols on the protein expression of different markers of apoptosis signaling in the soleus muscle. *A*: relative content of Bax. *B*: relative content of Bcl-2. *C*: Bax/Bcl-2 ratio. Bands were normalized to Ponceau-S staining (the corresponding well is shown). Normalized bands were expressed as a percentage of the CTRL group. The bands shown are not necessarily arranged in the same order as in the gel (in that case, a white space separates the bands and wells). Statistically significant differences are indicated according to the following codes: * vs. CTRL; *a* vs. t01 (for comparisons between different time points within the same treatment); ■ for comparisons between different treatments at the same time point. One and two repeated symbols correspond to $p < 0.05$ and $p < 0.01$, respectively. *PNR*,

passive normoxic recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic recovery.

Figure 7. Effect of EEIMD and the recovery protocols on the protein expression of different markers of mitochondrial oxidative stress in the soleus muscle. *A*: relative content of Sirt3. *B*: relative content of p66shc(pser³⁶) at the end of the recovery protocol. *C*: relative content of p66shc at the end of the recovery protocol. *D*: p66shc(pser³⁶)/p66shc ratio at the end of the recovery protocol. Bands were normalized to Ponceau-S staining (the corresponding well is shown). Normalized bands were expressed as a percentage of the CTRL group (in *A*) or as a percentage of the PNR group (in *B-D*). The bands shown are not necessarily arranged in the same order as in the gel (in that case, a white space separates the bands and wells). Statistically significant differences are indicated according to the following codes: * vs. CTRL; *a* vs. t01 (for comparisons between different time points within the same treatment); ■ for comparisons between different treatments at the same time point. One, two and three repeated symbols correspond to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. *PNR*, passive normoxic recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic recovery.

Figure 8. Effect of the recovery protocols on the expression of electron transport chain complex subunits and ANT in the soleus muscle at the end of the recovery protocol. *A*: relative content complex I NDUF8 subunit. *B*: relative content of complex II 30 kDa subunit. *C*: relative content of complex III core 2 subunit. *D*: relative content of complex IV subunit I. *E*: relative content of ATP synthase subunit alpha. *F*: relative content of ANT. Bands were normalized to Ponceau-S staining (the corresponding well is shown). Normalized bands were expressed as a percentage of the PNR. The bands shown are not necessarily arranged in the same order as in the gel (in that case, a white

space separates the bands and wells). Symbol ■ indicates statistical differences between different treatments ($p < 0.05$). *PNR*, passive normoxic recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic recovery.

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