Free Radical Biology and Medicine 225 (2024) 286–295

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Effects of intermittent exposure to hypobaric hypoxia and cold on skeletal muscle regeneration: Mitochondrial dynamics, protein oxidation and turnover

Sergio Sánchez-Nuño [a,1], Garoa Santocildes [b,*,1], Josep Rebull [b], Raquel G. Bardallo [b], Montserrat Girabent-Farrés [a], Ginés Viscor [b], Teresa Carbonell [b], Joan Ramon Torrella [b]

[a] Campus Docent Sant Joan de Déu, Universitat de Vic - Universitat Central de Catalunya (UVIC-UCC), C/ Sant Benito Menni, 18-20, 08830, Sant Boi de Llobregat, Spain

[b] Department of Cell Biology, Physiology and Immunology, Faculty of Biology, Universitat de Barcelona, Av. Diagonal 643, 08028, Barcelona, Spain

ARTICLE INFO

Keywords:
Intermittent hypobaric hypoxia
Intermittent cold exposure
Electron transport chain
Redox
Protein oxidation

ABSTRACT

Muscle injuries and the subsequent regeneration events compromise muscle homeostasis at morphological, functional and molecular levels. Among the molecular alterations, those derived from the mitochondrial function are especially relevant. We analysed the mitochondrial dynamics, the redox balance, the protein oxidation and the main protein repairing mechanisms after 9 days of injury in the rat gastrocnemius muscle. During the recovery rats were exposed to intermittent cold exposure (ICE), intermittent hypobaric hypoxia (IHH), and both simultaneous combined stimuli. Non-injured contralateral legs were also analysed to evaluate the specific effects of the three environmental exposures. Our results showed that ICE enhanced mitochondrial adaptation by improving the electron transport chain efficiency during muscle recovery, decreased the expression of regulatory subunit of proteasome and accumulated oxidized proteins. Exposure to IHH did not show mitochondrial compensation or increased protein turnover mechanisms; however, no accumulation of oxidized proteins was observed. Both ICE and IHH, when applied separately, elicited an increased expression of eNOS, which could have played an important role in accelerating muscle recovery. The combined effect of ICE and IHH led to a complex response that could potentially impede optimal mitochondrial function and enhanced the accumulation of protein oxidation. These findings underscore the nuanced role of environmental stressors in the muscle healing process and their implications for optimizing recovery strategies.

1. Introduction

Skeletal muscle regeneration is an important homeostatic process that allows restoring the architecture of damaged muscle fibres and maintaining muscle mass after injury [1]. During the recovery process that follows a skeletal muscle injury, the maturation of myofibers is accompanied by the fine remodelling of tissue architecture, with matrix rearrangement and angiogenesis [1]. Changes in the fibre structural organization during regeneration also imply mitochondrial network remodelling which is a critical process in maintaining cellular homeostasis and is intimately related to mitochondrial function [2]. At this point, mitochondrial homeostasis (equilibrium between mitophagy and mitogenesis) during skeletal muscle recovery seems to be crucial for tissue maturation [3]. In this way, inadequate mitophagy led by the suppression of peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC1α) induces delayed differentiation and maturation of myoblast [4].

The main function of mitochondria is related to ATP production through oxidative phosphorylation (OXPHOS) in the electron transport chain (ETC) complexes located on the inner mitochondrial membrane [5,6]. However, other mitochondrial roles have been described in skeletal muscle as reviewed by Alway et al. [4], reactive oxygen species (ROS) production being one of the most relevant on regulating skeletal muscle regeneration [7,8]. The nuclear factor erythroid 2-related factor 2 (NRF2) and its target genes, such as heme oxygenase-1 (HO-1), trigger the main protective mechanisms against the high ROS levels that occur

* Corresponding author.
E-mail address: gsantocildes@ub.edu (G. Santocildes).
[1] Equally contributed.

https://doi.org/10.1016/j.freeradbiomed.2024.09.032
Received 1 August 2024; Received in revised form 12 September 2024; Accepted 21 September 2024
Available online 21 September 2024

after pathological muscle injury [4]. An unbalance between ROS production and antioxidant activity could lead to oxidative stress, damaging cell biomolecules (lipids, proteins and DNA) during muscle myogenesis [8]. However, the role of ROS during the process of muscle injury recovery is controversial since recent evidence indicates that a physiological pro-oxidative environment is essential for myoblast differentiation [9].

Proteins are the most abundant biomolecules in skeletal muscle tissue [10]. Due to their structure, proteins are prime targets for being disrupted by ROS and can undergo various forms of oxidation, even forming adducts with 4-hydroxynonenal (4-HNE) (reviewed by Ref. [11]). Linked to the oxidative damage process, proteins have numerous quality control and repairing mechanisms that are crucial for protein turnover and cellular viability during skeletal muscle recovery. Heat shock proteins (HSPs) [12], the ubiquitin-proteasome system (UPS) and lysosomes are the main proteolytic systems [11,13–15].

The muscle regeneration process has been thoroughly studied, the regeneration phases and the cellular and molecular mechanisms involved are well known. However, a search for therapies to improve this process is still underway with the main objective of optimizing and reducing the time needed for complete molecular, cellular, and structural recovery. Intermittent hypobaric hypoxia (IHH) appears as an emerging therapy to be used in different pathological situations [16]. Considering that hypobaric hypoxia could lead to beneficial responses, both at systemic and muscular levels [17–21]. Recent studies have focused on the potential effect of intermittent hypoxia on muscle damage repair [22,71,23,70,24]. Given that hypobaric hypoxia and cold always coexist in the biosphere, and that these factors both separately and in combination do not produce the same physiological responses [25–27], we decided to merge these two stressors as a treatment for muscle injury. IHH and intermittent cold exposure (ICE), both combined and separately, produce an interesting modulation of the muscle recovery process, with IHH showing the most significant outcomes, as we have shown in a recent publication [24]. In this previous work, we found that 9 days of ICE and also combined ICE with IHH enhanced muscle regeneration at histological level (reducing centrally nucleated fibres and fibres positive for the developmental myosin heavy chain), while IHH exposure showed complete histological and functional recovery from the injury. IHH can be postulated as an anti-fibrotic treatment as it reduced collagen deposition. Moreover, an increase in the pSer473-Akt/total Akt ratio after 9 days in muscle gastrocnemius treated with ICE, IHH and their combination, together with an increase in the pThr172AMPKα/total AMPKα ratio after the IHH treatment, provide clues about the molecular mechanisms involved in enhanced muscle regeneration.

There is evidence suggesting that oxidative stress plays a key role in myogenesis and skeletal muscle physiology and pathology showing that moderate levels of ROS are critical for cell signalling and the regulation of gene expression, inducing cellular adaptations necessary for muscle growth [4,8]. For this reason, the present work aimed to evaluate the effect after 9 days of ICE, IHH and the simultaneous combination of both environmental factors on the protein expression of the electron transport chain (ETC), on mitochondrial dynamics, redox balance, protein oxidation and its turnover and degradation pathways in injured and contralateral non-injured rat gastrocnemius.

2. Materials and methods

2.1. Animals

A total of 20 adult male Sprague-Dawley rats (Envigo, Italy) with an average body mass of 254 ± 28 g were surgically injured in the right gastrocnemius muscle [28]. After the injury, rats were randomly distributed into four different groups: Control (CTRL), intermittent cold exposure (COLD), intermittent hypobaric hypoxia exposure (HYPO) and intermittent exposure to simultaneous cold and hypobaric hypoxia (COHY). Animals were submitted to the assigned intervention procedure for 9 days. The right non-injured gastrocnemius muscle of each animal was taken as the individual control condition [29].

All procedures adhered to the European Union guidelines for the care and management of laboratory animals and were conducted under the license granted by the Catalan authorities (reference no. 1899). Approval for these procedures was obtained from the University of Barcelona's Ethical Committee for Animal Experimentation (reference no. 8784) for the project titled "Synergistic effect of cold and hypoxia in the repair of induced muscle damage in laboratory rats," funded by the Ministry of Economy and Competitiveness (Spanish Government) under code DEP2013-48334-C2-1-P.

2.2. Intervention

No treatment was made for CTRL rats that rested at room temperature (25 °C) in their cages under normoxia conditions. COLD rats were kept in a cold room (4 °C) for 4 h per day and HYPO rats were exposed for 4 h per day at a simulated altitude of 4500 m in a 136-L polymethyl methacrylate plastic hypobaric chamber using a rotational vacuum pump (TRIVAC D5E; Germany). Finally, COHY animals combined both conditions with the hypobaric chamber placed into the cold room.

2.3. Tissue sampling

After 9 days all rats were euthanized, and both the injured (INJ) and the non-injured (NIN) gastrocnemius muscles were excised, frozen at −80 °C and subsequently homogenized with Precellys® Evolution tissue homogenizer (Bertin Technologies, France) in urea lysis buffer: 89.29 % of Urea 6M-1% SDS; 8.93 % of Phosphostop (Sigma Aldrich, USA); 1.78 % of Protease Inhibitor Cocktail (Sigma Aldrich, USA). Protein was extracted from the supernatant after 12 min of centrifugation (at 16 °C/25,000 G) and quantified with PierceTM BCA Protein Assay Kit (ThermoFisher Scientific, USA) in Tecan INFINITE 200 (Tecan, Austria).

2.4. Protein extraction and western blotting

From each animal, muscle protein was extracted from INJ and NIN gastrocnemius and analysed with western blot. For the electrophoresis, 10 % and 12.50 % SDS–polyacrylamide gels with 15 wells were used and samples were solubilized in an electrophoresis loading buffer.

Western Blots were conducted as previously described [27] loading a control sample that consisted of a tibialis anterior muscle homogenate (by triplicates in each run) to control the intra- and inter-membrane variability. To confirm the experiment's viability, the control samples' variation coefficient has been set to be less than 20 % to accept the results of the gel. Equal protein content was loaded in each well of the same gel. However, the amount of loaded protein was adjusted by a linearity verification depending on the analysed protein (from 15 to 55 μg). Once protein was transferred to the membrane, a Ponceau S staining (Sigma-Aldrich, USA) was made to verify the loading variation of protein for each lane.

Membranes were blocked with 4 % bovine serum albumin (BSA) in TBS-T (Tris-buffered saline containing 0.1 % Tween 20) for 15 h and were incubated against its specific antibodies adjusting dilution for each one in TBS-T- BSA 4 %. The following antibodies were used: OXPHOS, PARKIN, PGC1α and SIRT-1 from Abcam (England); NRF2, HSP90, and 4-HNE from Novus Biological (USA); KEAP1, HO-1 from Santa Cruz Biotechnology (USA); eNOS (BD Biosciences, USA); 19S, 20S from Enzo Life Sciences (USA); and Ubiquitin (Sigma, USA). Subsequently, membranes were incubated with the corresponding secondary horseradish peroxidase (HRP)-conjugated antibody (Thermo Fisher Scientific) diluted 1:5000 in 5 % Blotto in TBS-T for 1 h at room temperature. The specific bands were visualized with Clarity TM Western ECL Substrate Kit (Bio-Rad Laboratories, USA), and the chemiluminescence signal was measured using the Odyssey Fc Imaging System (LI-COR Inc.

Biotechnology, USA) and quantified with the Image Studio Software (v. 5.2.5, LI-COR Inc. Biotechnology). Muscle signalling data were reported in arbitrary units as the sample band intensity relative to the Ponceau S stain, and the final data were normalized to the mean value (band densities) of the three control samples loaded on all the gels.

2.5. Statistical analysis

The normal distribution of the data was tested by the Shapiro–Wilk test. For the comparison of dependent variables between experimental groups (CTRL, COLD, HYPO, COHY) the non-parametric Kruskal-Wallis test was run using Dunn's test as *post hoc* for multiple comparisons. To analyse statistical differences of dependent variables between two specific recovery groups and for the comparison between non-injured and injured gastrocnemius, we applied a Mann-Whitney *U* test. All statistical analysis was undertaken using SPSS for Windows, v29.0 (IBM Corp, USA), and all differences were considered statistically significant at $p < 0.05$. Data are presented as box-and-whisker plots, the box represents the interquartile range and shows the first and the third quartiles separated by the median. Whisker endpoints represent the minimum and maximum values, and the mean is indicated with a black dot.

3. Results

Our study encompassed the protein expression of mitochondrial dynamics by analysing mitogenesis (SIRT1 and PGC1α) and mitophagy biomarkers (PARKIN), and the mitochondrial ATP production potential by scrutinizing the protein expression of complexes that constitute the oxidative phosphorylation pathway (OXPHOS) in the electron transport chain (ETC). To gauge the redox balance, the protein expression of the key redox biomarkers (NRF2, KEAP1 and HO-1) and eNOS were measured. Finally, to assess protein quality status and its turnover levels of protein oxidation, 4-HNE adducts, HSP90 and the UPS proteolytic system (20S, 19S and UBQ) were examined.

3.1. Mitochondrial dynamics and electron transport chain (ETC)

Results of SIRT1, PGC1α and PARKIN expression are shown in Fig. 1. No substantial differences in mitogenesis biomarkers were evidenced, neither between groups nor when comparing NIN versus INJ legs of any treatment (Fig. 1A and B). Regarding mitophagy, the INJ leg of all groups showed lower protein expression than CTRL in PARKIN (HYPO: $p = 0.142$, COHY: $p = 0.117$) with a statistically significant difference ($p < 0.05$) in COLD (Fig. 1C). The injury induced a higher significant expression of PARKIN in CTRL animals.

The expression of representative complexes of the ETC is presented in Fig. 2. Biomarkers from all complexes showed a statistically significant lower expression after COHY treatment in the INJ leg when compared to its contralateral NIN. Regarding the comparison between treatments, INJ muscle from COLD had elevated protein expression than CTRL in complexes I, II, III and V (Fig. 2A–C and Fig. 2E), whilst the combination of COLD with IHH (COHY) decreased the expression of complexes I and V (Fig. 2A and E). It is important to note that the expression of complex IV decreased in all treatments compared to CTRL in the injured leg (Fig. 2D).

3.2. Redox balance and protein oxidation

No statistical differences were found between INJ and NIN gastrocnemius in the protein expression of NRF2, HO-1 and KEAP1 (Fig. 3). Any treatment induced significant changes in the expression of the redox balance biomarkers (Fig. 3). Regarding eNOS (Fig. 4A), a statistically significant higher expression in the INJ muscle was observed in COLD and HYPO compared to CTRL, although the combined exposure (COHY) did not result in any significant change. COLD induced statistically higher oxidized protein accumulation (4-HNE) compared to CTRL and COHY (Fig. 4B).

3.3. Protein turnover

Fig. 5 shows the protein expression of HSP90 and the 19S and 20S proteasome subunits. Notably, no statistical differences between groups were observed between INJ and NIN muscles for any analysed marker. COLD exhibited a greater expression of the chaperone HSP90 (Fig. 5A). COLD and COHY showed a statistically significant reduction of the regulatory UPS subunit (19S) in the INJ muscle compared to CTRL (Fig. 5B). No statistical differences were observed between groups in 20S core subunit of the proteasome (Fig. 5C).

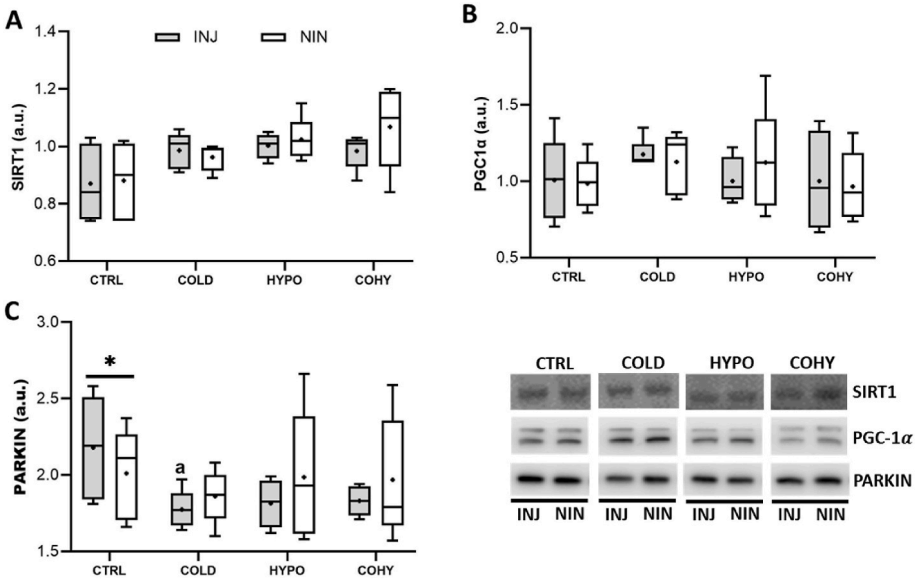

Fig. 1. Protein expression of mitochondrial dynamics markers in injured and non-injured gastrocnemius muscle. (A) SIRT1, (B) PGC1α, (C) PARKIN. Data are expressed in arbitrary units (a.u.) and the mean is indicated with a black dot. The sample size was n = 4–5 per group. Statistically significant differences are indicated as: *$p < 0.05$ vs NIN; [a] $p < 0.05$ vs CTRL. INJ, injured gastrocnemius; NIN, non-injured gastrocnemius; CTRL, control; COLD, intermittent cold exposure; HYPO, intermittent hypobaric hypoxia exposure; COHY, simultaneous intermittent cold and hypobaric hypoxia exposure.

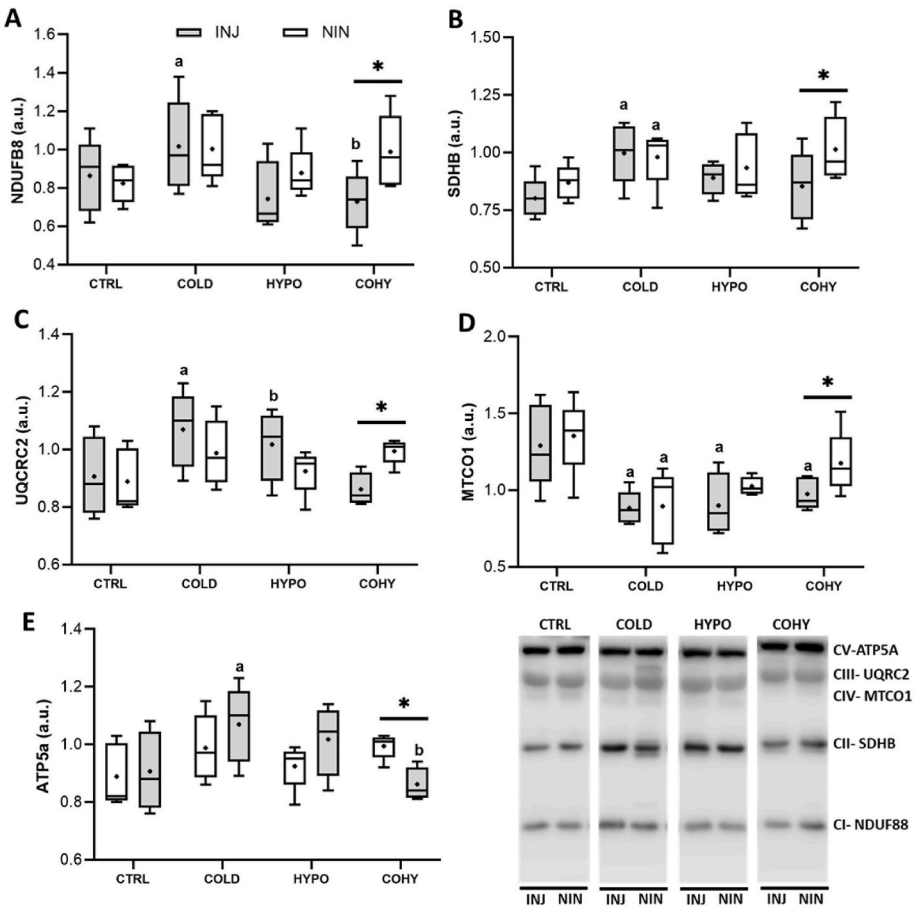

Fig. 2. Protein expression of OXPHOS in injured and non-injured gastrocnemius muscle. (A) Subunit NDUFB8 from the NADH dehydrogenase at complex I. (B) Subunit SDHB from the succinate dehydrogenase at complex II. (C) Subunit UQCRC2 from the CoQ-cytochrome *c* reductase at complex III. (D) Subunit MTCO1 from the cytochrome *c* oxidase at complex IV. (E) Subunit ATP5a from the ATP synthase enzyme at complex V. Data are expressed in arbitrary units (a.u.) and the mean is indicated with a black dot. The sample size was n = 4–5 per group. Statistically significant differences are indicated as: *$p < 0.05$ vs NIN; [a] $p < 0.05$ vs CTRL; [b] $p < 0.05$ vs COLD between muscles of the same condition. INJ, injured gastrocnemius; NIN, non-injured gastrocnemius; CTRL, control; COLD, intermittent cold exposure; HYPO, intermittent hypobaric hypoxia exposure; COHY, simultaneous intermittent cold and hypobaric hypoxia exposure.

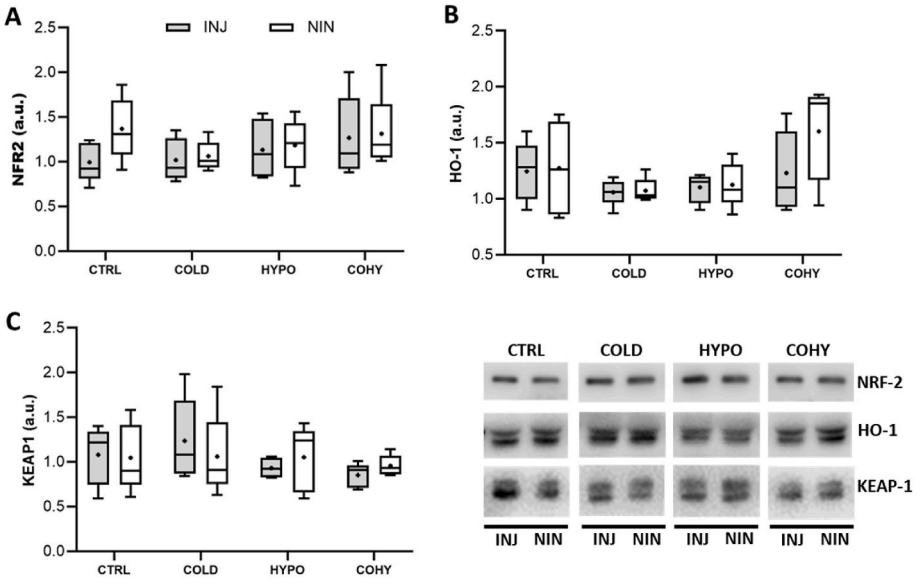

Fig. 3. Protein expression of redox balance markers in injured and non-injured gastrocnemius muscle. (A) NRF2, (B) KEAP1, (C) HO-1. Data are expressed in arbitrary units (a.u.) and the mean is indicated with a black dot. The sample size was n = 4–5 per group. INJ, injured gastrocnemius; NIN, non-injured gastrocnemius; CTRL, control; COLD, intermittent cold exposure; HYPO, intermittent hypobaric hypoxia exposure; COHY, simultaneous intermittent cold and hypobaric hypoxia exposure.

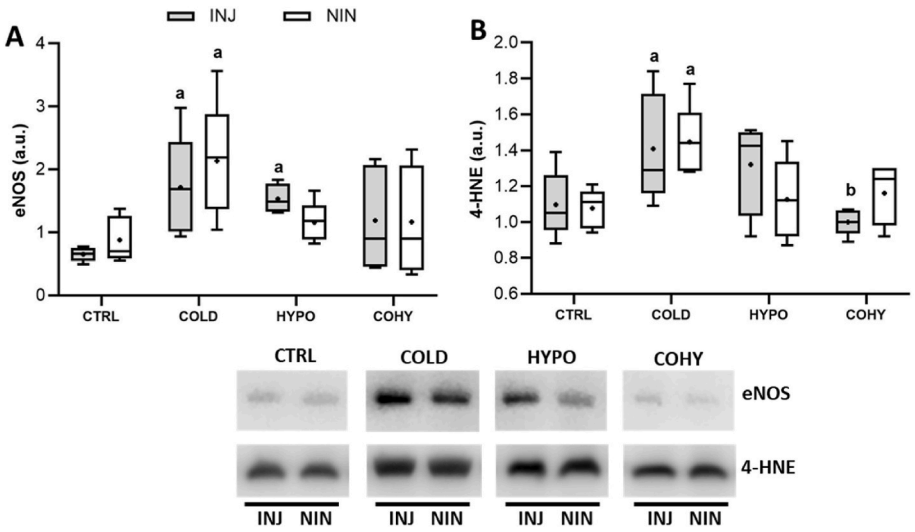

Fig. 4. Protein expression of eNOS and 4-HNE protein labelling in injured and non-injured gastrocnemius muscle. (A) eNOS, (B) total 4-HNE protein labelling. Data are expressed in arbitrary units (a.u.) and the mean is indicated with a black dot. The sample size was n = 4–5 per group. Statistically significant differences are indicated as: [a] $p < 0.05$ vs CTRL; [b] $p < 0.05$ vs COLD between muscles of the same condition. INJ, injured gastrocnemius; NIN, non-injured gastrocnemius; CTRL, control; COLD, intermittent cold exposure; HYPO, intermittent hypobaric hypoxia exposure; COHY, simultaneous intermittent cold and hypobaric hypoxia exposure.

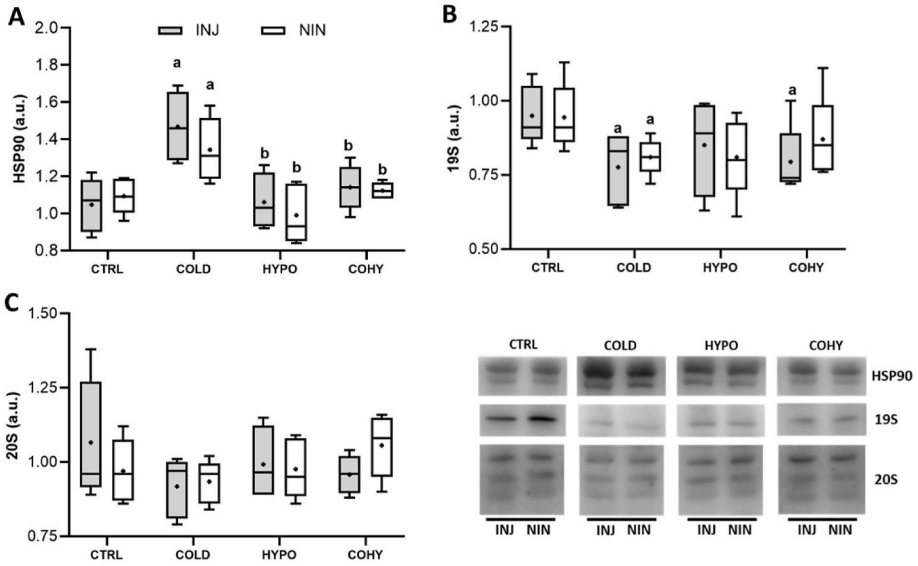

Fig. 5. Protein expression HSP90 and 26S proteasome in injured and non-injured gastrocnemius muscles. (A) HSP90, (B) rpt1 19S regulatory subunit of 26S proteasome, (C) 20S proteolytic subunit of 26S proteasome. Data are expressed in arbitrary units (a.u.) and the mean is indicated with a black dot. The sample size was n = 4–5 per group. Statistically significant differences are indicated as: [a] $p < 0.05$ vs CTRL; [b] $p < 0.05$ vs COLD between muscles of the same condition. INJ, injured gastrocnemius; NIN, non-injured gastrocnemius; CTRL, control; COLD, intermittent cold exposure; HYPO, intermittent hypobaric hypoxia exposure; COHY, simultaneous intermittent cold and hypobaric hypoxia exposure.

Total ubiquitination and the most abundant bands (100, 75, 37, and 25 kDa) are shown in Fig. 6. The INJ muscle after COHY treatment had a statistically significant lower protein ubiquitination than the NIN, except for the 100 kDa band. COHY also increased the amount of ubiquitination compared to CTRL in the NIN. Notably, the 100 kDa band showed significant increases in all groups compared to CTRL both in INJ and NIN muscles (Fig. 6E).

4. Discussion

Muscle injuries at the distal myotendinous junction are common in sports practice. Despite the growing number of options for addressing muscle recovery from a clinical perspective, the primary treatment currently applied to muscle injuries is known as RICE (Rest, Ice, Compression, Elevation). Previous studies have also described that IHH improves muscle recovery. Additionally, we also know that muscle recovery involves mitochondrial restructuring, which can affect ROS signalling and oxidative damage to the structural proteins of myocytes. For all these reasons this study was focused on describing the effect of 9 days of exposure to intermittent cold or/and hypobaric hypoxia on the recovery of surgically injured rat gastrocnemius muscle. We aimed to understand the molecular events occurring during the muscle regeneration process, the mitochondrial status, the redox balance, the protein oxidation and its turnover.

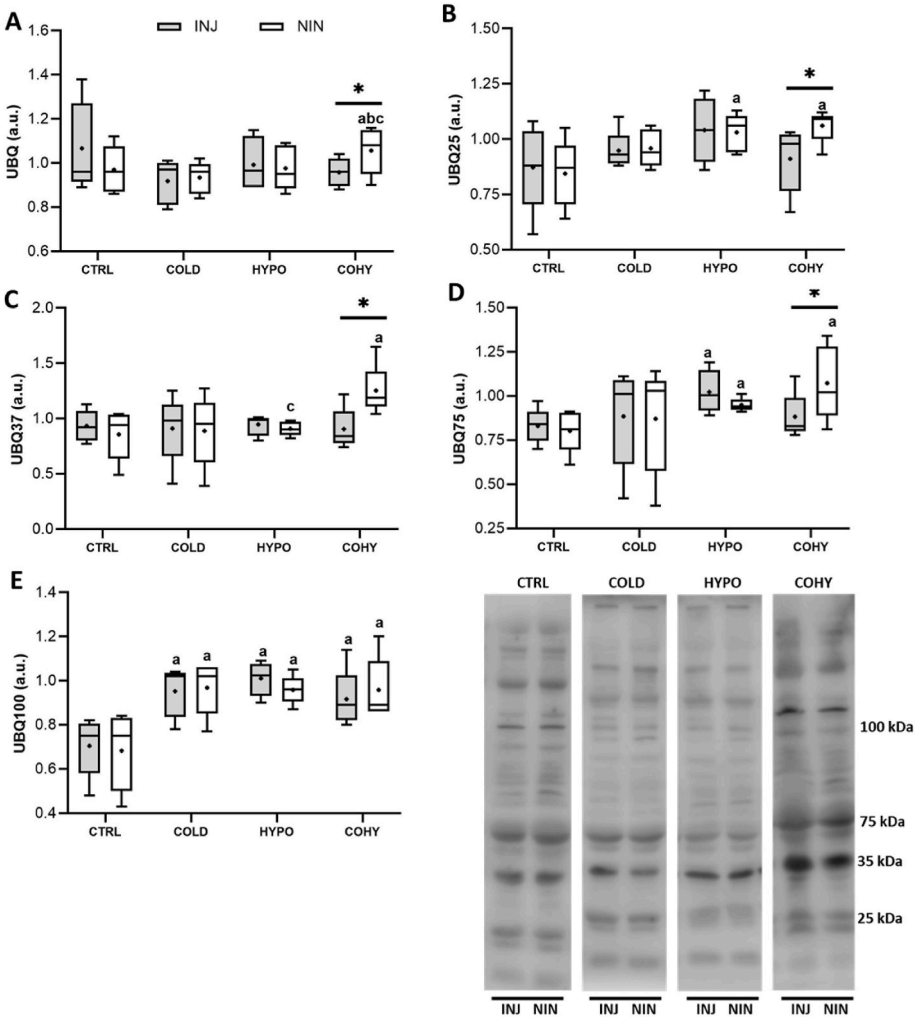

Fig. 6. Ubiquitinated proteins (UBQ) in injured and non-injured gastrocnemius muscle. (A) Total ubiquitination, (B) ubiquitinated 25 kDa band, (C) ubiquitinated 37 kDa band. (D) ubiquitinated 75 kDa band (E) ubiquitinated 100 kDa band. Data are expressed in arbitrary units (a.u.) and the mean is indicated with a black dot. The sample size was n = 4–5 per group. Statistically significant differences are indicated as: *p < 0.05 vs NIN; [a] p < 0.05 vs CTRL; [b] p < 0.05 vs COLD; [c] p < 0.05 HYPO between muscles of the same condition. INJ, injured gastrocnemius; NIN, non-injured gastrocnemius; CTRL, control; COLD, intermittent cold exposure; HYPO, intermittent hypobaric hypoxia exposure; COHY, simultaneous intermittent cold and hypobaric hypoxia exposure.

4.1. Mitochondrial dynamics in the recovery process

It is well described that skeletal muscle recovery after an injury follows a specific timing, being the mitochondrial reorganization a very relevant process [1]. Some mitogenic factors such as AMPK, SIRT1 and PGC1α, and mitophagy inductors such as PINK1 and PARKIN are described as key biomarkers of mitochondrial dynamics [30–33]. Research has been conducted to understand how exposure to cold and intermittent hypobaric hypoxia affects mitochondrial dynamics and metabolism [34]. However, to the best of our knowledge, the detailed mechanisms involved in these dynamics are not well described due to the complexity and variety of the experimental protocols and procedures applied. Previous studies from our group demonstrated an increase in PGC1α after a 7-day protocol of IHH (4000 m 4h/day) for recovering from muscle damage provoked after eccentric exercise [23]. Cao et al. [35] reported that, after a week of ICE (4 °C 3h/day), mitophagy was stimulated in apolipoprotein E deficient mice, inducing post-translational modifications of multiple proteins such as SIRT1. These findings did not match our results in rats, where no changes were observed in the expression of PGC1α and SIRT1 in any of the studied groups (Fig. 1A and B). PGC1α activation is mediated by SIRT1, both molecules being essential for the synthesis and assembly of the inner membrane proteins [3], mainly in muscles with a high proportion of slow fibres [36]. Gurd et al. [37] suggested a negative correlation between SIRT1 nuclear activity and the whole muscle SIRT1 protein content. Thus, an increased SIRT1 nuclear activity with a decreased whole SIRT1 muscle content could explain the lack of differences in the expression of SIRT1 that we have found between our groups and when comparing INJ versus NIN muscles. Additionally, AMPK plays a fundamental role in the activation of SIRT1, and consequently in the activation of mitogenesis pathways [38], which explains our recent findings on AMPK enhanced expression after 9 days of ICE [24]. Regarding mitophagy, since PARKIN has a pivotal role in remodelling dysfunctional or damaged segments of unhealthy mitochondria [39,40], it was anticipated that its activation would follow injury in the skeletal muscle tissue, as has been suggested by Esteca et al. [41] who attributed PARKIN-mediated mitophagy a key role in MuSCs differentiation. This is corroborated by the significative increase in the PARKIN expression that we have found in the INJ leg of our CTRL animals (Fig. 1C). Since our data revealed a downward trend in PARKIN expression, being significative in COLD, after 9 days from injury, mitophagy might not be actively ongoing at this time point of the recovery process when the removal of injury-damaged mitochondria could already be completed. We hypothesize that high AMPK levels, as described by Santocildes et al.

[24] after ICE, could influence a lower expression of PARKIN. Unfortunately, we have not found other studies addressing this specific aspect and the absence of day 0 data complicates the determination of the mitophagy activation timeline. However, we know that the ending of the mitophagy process is primarily supported by the decline in the percentage of dMyHC-positive fibres at day 9 [24], indicating that regenerating muscle fibres are transitioning from a phase of degeneration to maturation at this point of the recovery process.

Thus, our data suggest that at this point of the recovery process, muscle tissue could be acclimated to the exposure conditions, suggesting a particular homeostasis for the mitochondrial network dynamics irrespective of ICE and IHH. This does not mean that the different stressors do not play a crucial role in the mitochondria restructuration and distribution during the recovery process [2], nor that the choice of ambient conditions is irrelevant. Probably these effects have been decisive in the early stages of the regeneration process as it was reviewed by Forcina et al. [1].

4.2. Mitochondrial oxidative phosphorylation

The elevated metabolic requirements for ATP during myogenesis and the recovery of skeletal muscle following injury rely on mitochondrial oxidative phosphorylation to support biosynthesis, cell differentiation and tissue reorganization [4], implying that muscle early regeneration requires high energy requirements [1,4]. Focusing on the INJ muscles, our data suggest a different recovery time course for OXPHOS expression depending on the treatment followed. COLD showed an increase in the expression of complexes I, II, III and V, which could be associated with physiological compensations leading to a higher density of these complexes on the inner mitochondrial membrane, as these increased expressions do not depend on mitochondrial dynamics [42]. Moreover, Sepa-Kishi et al. [43] suggested that cold exposure induces an endocrine response that contributes to enhancing carbohydrate metabolism, which was supported by our previous results where increased GLUT1 protein expression was found in COLD [27]. Moreover, we described an increased protein expression of the vascular endothelial growth factor (VEGF) and higher fibre capillarization after the COLD protocol [24,27], which could explain the better histologic recovery. Supporting these previous findings, this research showed an increase in the expression of eNOS in COLD (Fig. 4A) which relates to the required vasodilation for improving the tissue delivery of energy substrates and oxygen required for the energy production and for minimizing peripheral heat loss [44]. Increased expression of eNOS was also found in HYPO but the combination of cold and hypoxia (COHY) did not evidence the same response after 9 days of recovery. Exposure to both cold and hypoxia is known to be stressful for organisms, increasing energy requirement while decreasing oxygen availability, thus leading to a reduction in body weight by hypoxia [27,45] and an increase in metabolic rate to enhance thermogenesis by the cold [46,47]. Although our experimental design contemplates intermittent exposure, we can hypothesize that the stressful stimuli generated by these simultaneous environments likely induced an excessive catabolic condition, impairing COHY mitochondrial protein resynthesis and slowing the regeneration process, thereby limiting the benefits shown by the exposure to a unique environmental stressor.

This study also shows that exposure to hypoxia and cold independently and in combination decreases the expression of complex IV of the ETC. Specifically identified as cytochrome *c* oxidase, complex IV plays a central role in the final steps of electron transfer, orchestrating the reduction of molecular oxygen to water [48]. Our results agree with previous work on mice, where a decrease in the maximal activities of citrate synthase or cytochrome *c* oxidase in the gastrocnemius muscle after exposure to cold and hypoxia, either individually or in tandem, was observed [49]. Even though our study does not show higher levels of expression in ATP synthase (complex V), some authors suggest that during muscle recovery [1] and during exposure to cold and hypoxic environmental conditions [34], an increase in cellular ATP levels could be necessary. In this regard, Ramzan et al. [50] described in isolated rat cardiac muscle that cytochrome *c* oxidase could be inactivated by an increased ratio of ATP/ADP.

It is surprising that despite the activation of injury-repairing mechanisms, there is very little variability in the responses observed between INJ and NIN. Significant differences are only evidenced in the combined exposure (COHY). Specifically, there is a visible decrease in the expression of all complexes in INJ. We did not find any previous report in support of these findings. However, the similarity between INJ and NIN reinforces our hypothesis that, after 9 days of recovery, the muscle tissue has acclimated to these new conditions. This noteworthy finding underscores the complexity of mitochondrial responses to environmental stressors and provides valuable insights into the distinct adaptability of these key mitochondrial enzymes to hypoxic conditions.

4.3. Redox balance regulation

Nuclear factor erythroid 2-related factor 2 (NRF2) and Kelch-like ECH-associated protein 1 (KEAP1) are key players in the cellular defence against oxidative stress and inflammation [51]. While there is not an extensive body of literature directly linking NRF2 and KEAP1 to muscle regeneration, their role in cellular protection and maintenance could indirectly influence the regenerative processes in muscle tissue [52]. Moreover, the activation of NRF2 increases the antioxidant response element (ARE) gene expression, including HO-1, contributing to the cellular defence and repair mechanisms required during the regenerative process, mainly neutralizing reactive oxygen species (ROS). Some authors have described that NRF2 regulation could be affected by hypoxia [53] or by cold [54]. However, our results evidenced a lack of significant differences between INJ and NIN in NRF2, HO-1 and KEAP1 redox biomarkers in all experimental groups suggesting that, at least at this time point of the recovery process, they are not playing a protagonist role. Studies on cardiac muscle in mice where ICE was more severe (−20 °C) showed increased expression of NRF2 and antioxidant enzymes like superoxide dismutase (SOD) after 7 days [54]. Also, in human skeletal muscle, the NRF2/KEAP1 ratio was increased after acute hypoxia training [55]. As the NRF2 pathway is a key regulator of antioxidant expression, our findings indicate that after ICE and IHH exposure (COHY), the tissue assumed a redox balance. Probably the 9-day recovery period has masked the imbalances in redox homeostasis that occurred during the initial recovery days following the injury [1,51,52], or probably the quality/success of the recovery after an injury process may be due to other specific markers at this point [56].

In the context of physiological homeostasis, the genesis of ROS is typically concomitant with the acute release of oxidative phosphorylation within the mitochondrial framework. The orchestration of redox balance hinges on the delicate interplay between antioxidant levels and ROS generation. Existing literature shows that the preponderance of ROS emanates from complexes I and III of the electron transport chain (ETC) [56]. Thus, it seems evident that the increase in mitochondrial activity leads to an increase in ROS production. This has been demonstrated after acute exercise [57]. Several authors have described ROS production as a regulatory element in mitochondrial remodelling and cellular differentiation processes through signalling mechanisms. For instance, ROS production promotes the activation of PGC1α, which in turn regulates the activation of NRF2 [56]. Our results align with this evidence, as the increase in the expression of all mitochondrial complexes in COLD did not show changes in the expression of NRF2 or PGC1α. Moreover, the release of nitric oxide (NO) by eNOS contributes to a better irrigation of the muscle tissue increasing the levels of ROS generated at the mitochondrial level [58]. As commented previously, the eNOS protein expression increase in COLD and HYPO (Fig. 4A) might contribute to tissue recovery [24,27].

4.4. Protein oxidation and turnover/repairing pathways

Despite physiological protein oxidation occurs mainly due to ROS outbreak, an imbalance in antioxidant defences can lead to large-scale oxidative damage, resulting in significant damage to biomolecules and potentially causing cell death [11]. This would be evident through the accumulation of damaged oxidized metabolites, such as lipids and proteins as well as DNA [1,4]. Muscle damage recovery after injury [59], ICE [60], and IHH [61] induces an increase in ROS production, but most of its consequences on cell responses remain controversial. In skeletal muscle, protein is the most abundant component and during the recovery from injury protein quality control is crucial for restructuring damaged fibres, as well as the formation of new fibres [1]. Given the intricate nature of proteins, the spectrum of potential protein damage is notably diverse. In contrast to lipid oxidation, muscle tissue exhibits distinct repair mechanisms for reversible protein damage and degradation mechanisms for irreversible protein damage [11]. In our study, the evaluation of protein oxidation was done by semi-quantifying 4-hydroxynonenal (4-HNE) protein-adducts, which is considered the most toxic product of lipid peroxidation documented in mammals [62,63], inflicting damage to proteins through the formation of covalent adducts and expediting protein aggregation in tissues [11,64,65]. Our results did not show a greater accumulation of oxidized proteins on INJ vs NIN gastrocnemius after the recovery process, probably due to the increased protein turnover [1] and the proper functioning of repair mechanisms [11], suggesting again tissue acclimation at this recovery point. However, COLD evidenced a significant increase in 4-HNE accumulation in both INJ and NIN muscles. This increase in oxidized proteins could be a consequence of the higher ROS production by the increased expression of ETC complexes and lower expression of PARKIN and the increased production of NO by eNOS [8].

Due to the susceptibility of proteins to oxidation, numerous cellular pathways play crucial roles in repairing and eliminating damaged proteins, thereby preventing their accumulation and subsequent aggregation [66,67]. Protein oxidation and aggregation are closely linked to significant pathologies that may culminate in cell death. An initial line of defence against protein damage involves their association with heat shock proteins (HSPs) [12] and with the ubiquitin-proteasome system (UPS) [14]. The 26S proteasome is a substantial protein complex that consists of a central catalytic core (20S) flanked by two regulatory subunits (19S) [68]; [14]. Consistent with the literature, our results revealed a notable overexpression of HSP90 protein correlated with higher expression of eNOS following ICE exposure in COLD [69]. This data could suggest higher chaperone activity and increased protein-repairing pathways in ICE. Again, it is surprising that cold conditioning on HSP90 expression disappeared when combined with IHH (COHY). Moreover, the aggregation of proteins, stemming from the accrual of cross-linked 4-HNE proteins, present a challenge for degradation through the ubiquitin-proteasome system (UPS) [11]. The intricate nature of these aggregated structures renders them resistant to efficient degradation via the UPS machinery. Regarding proteasome 26S, COLD and COHY down expressed the 19S regulatory subunit. These data could suggest the negative influence that 4-HNE accumulation exerts on the regulation of the proteasome [11]. Although the combination of ICE and IHH also decreased the expression of 19S, the COHY group did not show high levels of 4-HNE, which could be related to lower mitochondrial activity or maybe to lower NO production by eNOS [7]. Before proteasome degradation, proteins must be ubiquitinated [14]. This study comprehensively assesses the overall ubiquitination status of proteins, additionally scrutinizing the ubiquitination of bands exhibiting pronounced protein accumulation in the blots. The classification of these bands was based on the molecular weight. Although the ubiquitination process is highly regulated, it can be challenging to obtain specific data by analysing ubiquitination patterns. However, these patterns can still provide valuable insights into the rate of protein turnover, whether due to physiological processes or oxidative stress. Surprisingly, NIN gastrocnemius showed higher levels of ubiquitination than INJ. We have not found bibliographical data that could help to interpret this finding. We can hypothesize that the pro-inflammatory pathways activated during muscle damage recovery led to more efficient ubiquitin tagging. Moreover, regarding the ubiquitin labelling protein patterns, particularly those with a molecular weight approximating 100 kDa, a notable increase was found in all experimental groups. Our data suggest that, even in the establishment of a renewed homeostasis, the tissue remains remarkably dynamic in terms of protein recycling. Considering the ongoing histological reorganization of muscle tissue, it is pertinent to note that the ICE and IHH exposure appear to amplify the repair processes. Fig. 7 shows a diagram linking all variables studied and showing that both IHH and ICE appear protective by increasing survival of mitochondria (decreased PARKIN expression indicative of mitophagy) and their function (increased ETC expression), decreasing ubiquitination of higher molecular weight proteins and balancing redox status. However, their combination does not provide much benefit on these variables as some of variables have opposing effects.

The findings presented herein, indicative of the enhancement of reparative mechanisms following the ICE and IHH exposure, agree with prior outcomes from our laboratory. Notably, previous results demonstrated that IHH recovers the muscular contractile capacities within 9 days of exposure, notwithstanding the presence of fibres exhibiting reduced fibre cross-sectional area (FCSA). In contrast, ICE and the combined treatment needed a 21-day exposure period to elicit a complete restoration of their contractile capacities [24].

5. Conclusions

In conclusion, 9 days of ICE and IHH exposure conditioned mitochondria recovery, protein oxidation and proteasome expression. ICE reduced mitophagy and increased the expression of ETC complexes. This increased mitochondrial activity, along with the disruption of the regulatory 19S expression of proteasome, likely resulted in a greater accumulation of 4-HNE, despite the rise in HSP90 expression. IHH reduced the expression of complex III of ETC, related to ROS production. This interaction resulted on lower accumulation of 4-HNE, probably due to proteasome activity. Both ICE and IHH, when applied separately, elicited an increased expression of eNOS, which could have provoked vasodilation in the blood vessels supplying the muscle tissue, ensuring higher oxygen, metabolite and growth factors arrival to the injured muscles, and accelerating muscle recovery. ICE in combination with IHH decreased the expression of ETC complexes on INJ muscle compared with NIN, which in combination with lower eNOS expression led to less 4-HNE accumulation despite lower expression of 19S.

Our data suggest that mitochondrial dynamics and its implication in ROS production may not play a determining role after 9 days of muscle recovery, potentially being much more relevant during the initial days following the injury. At this stage of recovery, protein damage repair seems to be the most crucial factor for the tissue to regain its functional capacity.

Funding

This work was supported by the Spanish Ministry of Economy, Industry and Competitiveness (DEP2013-48334-C2-1-P)

CRediT authorship contribution statement

Sergio Sánchez-Nuño: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Garoa Santocildes:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Josep Rebull:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Raquel G. Bardallo:** Writing – review &

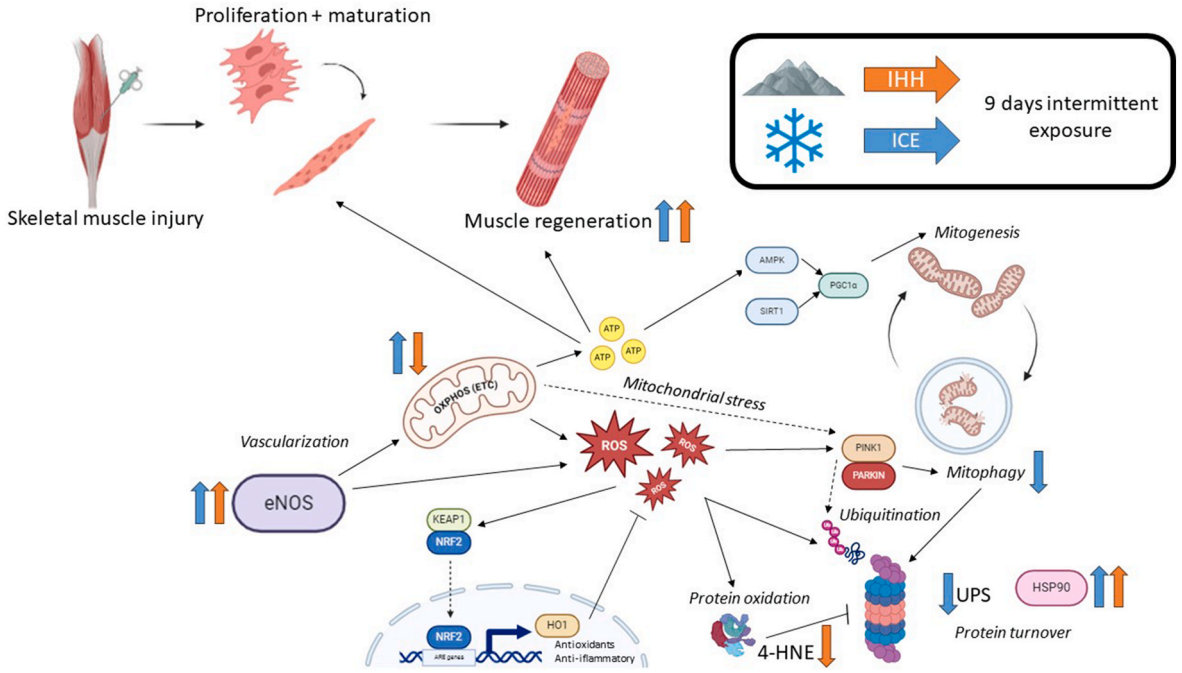

Fig. 7. Redox-related pathways in muscle regeneration. Muscle damage is characterized by elevated inflammation and the generation of reactive oxygen species (ROS). During skeletal muscle regeneration, each step has been shown to be regulated by redox activity [7,8]. Exposure to 9 days of ICE decreased PARKIN expression (mitophagy) and increased the expression of ETC complexes, while exposure to IHH decreased ROS production in the ETC. Both ICE and IHH increased eNOS expression and the ubiquitination of higher molecular weight proteins. ICE down expressed 19S regulatory subunit of 26S proteasome.

editing, Data curation, Conceptualization. **Montserrat Girabent-Farrés:** Writing – review & editing, Methodology, Formal analysis. **Ginés Viscor:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Teresa Carbonell:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Joan Ramon Torrella:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] L. Forcina, M. Cosentino, A. Musarò, Mechanisms regulating muscle regeneration: insights into the interrelated and time-dependent phases of tissue healing, Cells 9 (5) (2020), https://doi.org/10.3390/cells9051297. Article 5.

[2] F.A. Rahman, J. Quadrilatero, Mitochondrial network remodeling: an important feature of myogenesis and skeletal muscle regeneration, Cell. Mol. Life Sci. 78 (10) (2021) 4653–4675, https://doi.org/10.1007/s00018-021-03807-9.

[3] L.D. Popov, Mitochondrial biogenesis: an update, J. Cell Mol. Med. 24 (9) (2020) 4892–4899, https://doi.org/10.1111/jcmm.15194.

[4] S.E. Alway, H.G. Paez, C.R. Pitzer, The role of mitochondria in mediation of skeletal muscle repair, Muscles 2 (2) (2023), https://doi.org/10.3390/muscles2020011. Article 2.

[5] D. Nolfi-Donegan, A. Braganza, S. Shiva, Mitochondrial electron transport chain: oxidative phosphorylation, oxidant production, and methods of measurement, Redox Biol. 37 (2020) 101674, https://doi.org/10.1016/j.redox.2020.101674.

[6] R.-Z. Zhao, S. Jiang, L. Zhang, Z.-B. Yu, Mitochondrial electron transport chain, ROS generation and uncoupling, Int. J. Mol. Med. 44 (1) (2019) 3–15, https://doi.org/10.3892/ijmm.2019.4188, submitted for publication.

[7] E. Le Moal, V. Pialoux, G. Juban, C. Groussard, H. Zouhal, B. Chazaud, R. Mounier, Redox control of skeletal muscle regeneration, Antioxidants Redox Signal. 27 (5) (2017) 276–310, https://doi.org/10.1089/ars.2016.6782.

[8] D. Lian, M.-M. Chen, H. Wu, S. Deng, X. Hu, The role of oxidative stress in skeletal muscle myogenesis and muscle disease, Antioxidants 11 (4) (2022) 4, https://doi.org/10.3390/antiox11040755.

[9] N.S. Rajasekaran, S.B. Shelar, D.P. Jones, J.R. Hoidal, Reductive stress impairs myogenic differentiation, Redox Biol. 34 (2020) 101492, https://doi.org/10.1016/j.redox.2020.101492.

[10] A.J. Galpin, U. Raue, B. Jemiolo, T.A. Trappe, M.P. Harber, K. Minchev, S. Trappe, Human skeletal muscle fiber type specific protein content, Anal. Biochem. 425 (2) (2012) 175–182, https://doi.org/10.1016/j.ab.2012.03.018.

[11] N. Chondrogianni, I. Petropoulos, S. Grimm, K. Georgila, B. Catalgol, B. Friguet, T. Grune, E.S. Gonos, Protein damage, repair and proteolysis, Mol. Aspect. Med. 35 (2014) 1–71, https://doi.org/10.1016/j.mam.2012.09.001.

[12] S. Pomella, M. Cassandri, F. Antoniani, S. Crotti, L. Mediani, B. Silvestri, M. Medici, R. Rota, A. Rosa, S. Carra, Heat shock proteins: important helpers for the development, maintenance and regeneration of skeletal muscles, Muscles 2 (2) (2023), https://doi.org/10.3390/muscles2020014. Article 2.

[13] A. Ciechanover, Intracellular protein degradation: from a vague idea thru the lysosome and the ubiquitin–proteasome system and onto human diseases and drug targeting, Biochim. Biophys. Acta Protein Proteonomics 1824 (1) (2012) 3–13, https://doi.org/10.1016/j.bbapap.2011.03.007.

[14] T. Jung, B. Catalgol, T. Grune, The proteasomal system, Mol. Aspect. Med. 30 (4) (2009) 191–296, https://doi.org/10.1016/j.mam.2009.04.001.

[15] F. Shang, A. Taylor, Ubiquitin–proteasome pathway and cellular responses to oxidative stress, Free Radic. Biol. Med. 51 (1) (2011) 5–16, https://doi.org/10.1016/j.freeradbiomed.2011.03.031.

[16] G. Viscor, J.R. Torrella, L. Corral, A. Ricart, C. Javierre, T. Pages, J.L. Ventura, Physiological and biological responses to short-term intermittent hypobaric hypoxia exposure: from sports and mountain medicine to new biomedical applications, Front. Physiol. 9 (2018), https://doi.org/10.3389/fphys.2018.00814.

[17] K. Heinicke, N. Prommer, J. Cajigal, T. Viola, C. Behn, W. Schmidt, Long-term exposure to intermittent hypoxia results in increased hemoglobin mass, reduced plasma volume, and elevated erythropoietin plasma levels in man, Eur. J. Appl. Physiol. 88 (6) (2003) 535–543, https://doi.org/10.1007/s00421-002-0732-z.

[18] H. Hoppeler, M. Vogt, Muscle tissue adaptations to hypoxia, J. Exp. Biol. 204 (18) (2001) 3133–3139, https://doi.org/10.1242/jeb.204.18.3133.

[19] C. Lundby, J.A.L. Calbet, P. Robach, The response of human skeletal muscle tissue to hypoxia, Cell. Mol. Life Sci. 66 (22) (2009) 3615–3623, https://doi.org/10.1007/s00018-009-0146-8.

[20] O. Mathieu-Costello, Muscle adaptation to altitude: tissue capillarity and capacity for aerobic metabolism, High Alt. Med. Biol. 2 (3) (2001) 413–425, https://doi.org/10.1089/15270290152608598.

[21] J.A. Neubauer, Invited Review: physiological and pathophysiological responses to intermittent hypoxia, J. Appl. Physiol. 90 (4) (2001) 1593–1599, https://doi.org/10.1152/jappl.2001.90.4.1593.

[22] T. Chaillou, N. Koulmann, A. Meunier, R. Chapot, B. Serrurier, M. Beaudry, X. Bigard, Effect of hypoxia exposure on the recovery of skeletal muscle phenotype during regeneration, Mol. Cell. Biochem. 390 (1) (2014) 31–40, https://doi.org/10.1007/s11010-013-1952-8.

[23] D. Rizo-Roca, J.G. Ríos-Kristjánsson, C. Núñez-Espinosa, E. Santos-Alves, I. O. Gonçalves, J. Magalhães, A. Ascensão, T. Pagès, G. Viscor, J.R. Torrella,

Intermittent hypobaric hypoxia combined with aerobic exercise improves muscle morphofunctional recovery after eccentric exercise to exhaustion in trained rats, J. Appl. Physiol. 122 (3) (2017) 580–592, https://doi.org/10.1152/japplphysiol.00501.2016.
[24] G. Santocildes, G. Viscor, T. Pagès, J.R. Torrella, Simulated altitude is medicine: intermittent exposure to hypobaric hypoxia and cold accelerates injured skeletal muscle recovery, J. Physiol. (2023), https://doi.org/10.1113/JP285398 *n/a*(n/a).
[25] N. Banchero, S.R. Kayar, A.J. Lechner, Increased capillarity in skeletal muscle of growing Guinea pigs acclimated to cold and hypoxia, Respir. Physiol. 62 (2) (1985) 245–255, https://doi.org/10.1016/0034-5687(85)90118-5.
[26] S. Ramos-Romero, G. Santocildes, D. Piñol-Piñol, C. Rosés, T. Pagés, M. Hereu, S. Amézqueta, J.R. Torrella, J.L. Torres, G. Viscor, Implication of gut microbiota in the physiology of rats intermittently exposed to cold and hypobaric hypoxia, PLoS One 15 (11) (2020) e0240686, https://doi.org/10.1371/journal.pone.0240686.
[27] G. Santocildes, G. Viscor, T. Pagès, S. Ramos-Romero, J.L. Torres, J.R. Torrella, Physiological effects of intermittent passive exposure to hypobaric hypoxia and cold in rats, Front. Physiol. 12 (2021), https://doi.org/10.3389/fphys.2021.673095.
[28] P. Contreras-Muñoz, A. Fernández-Martín, R. Torrella, X. Serres, M. D. la Varga, G. Viscor, T.a.H. Järvinen, V. Martínez-Ibáñez, J.L. Peiró, G. Rodas, M. Marotta, A new surgical model of skeletal muscle injuries in rats reproduces human sports lesions, Int. J. Sports Med. 37 (2015) 183–190, https://doi.org/10.1055/s-0035-1555933.
[29] G. Santocildes, M. Merino, F. Fabiani, T. Pagès, M. Marotta, G. Viscor, J.R. Torrella, Histomorphological and functional contralateral symmetry in the gastrocnemius muscles of the laboratory rat, J. Anat. 241 (3) (2022) 692–701, https://doi.org/10.1111/joa.13674.
[30] G.W. Dorn, Evolving concepts of mitochondrial dynamics, Annu. Rev. Physiol. 81 (81) (2019) 1–17, https://doi.org/10.1146/annurev-physiol-020518-114358, 2019.
[31] L. Liu, Y. Li, G. Chen, Q. Chen, Crosstalk between mitochondrial biogenesis and mitophagy to maintain mitochondrial homeostasis, J. Biomed. Sci. 30 (1) (2023) 86, https://doi.org/10.1186/s12929-023-00975-7.
[32] H.-M. Ni, J.A. Williams, W.-X. Ding, Mitochondrial dynamics and mitochondrial quality control, Redox Biol. 4 (2015) 6–13, https://doi.org/10.1016/j.redox.2014.11.006.
[33] L. Tilokani, S. Nagashima, V. Paupe, J. Prudent, Mitochondrial dynamics: overview of molecular mechanisms, Essays Biochem. 62 (3) (2018) 341–360, https://doi.org/10.1042/EBC20170104.
[34] P. Mishra, D.C. Chan, Metabolic regulation of mitochondrial dynamics, JCB (J. Cell Biol.) 212 (4) (2016) 379–387, https://doi.org/10.1083/jcb.201511036.
[35] Y. Cao, M. Zhang, Y. Li, J. Lu, W. Zhou, X. Li, H. Shi, B. Xu, S. Li, O-GlcNAcylation of SIRT1 protects against cold stress-induced skeletal muscle damage via amelioration of mitochondrial homeostasis, Int. J. Mol. Sci. 23 (23) (2022), https://doi.org/10.3390/ijms232314520. Article 23.
[36] J. Tonkin, F. Villarroya, P.L. Puri, M. Vinciguerra, SIRT1 signaling as potential modulator of skeletal muscle diseases, Curr. Opin. Pharmacol. 12 (3) (2012) 372–376, https://doi.org/10.1016/j.coph.2012.02.010.
[37] B.J. Gurd, Y. Yoshida, J.T. McFarlan, G.P. Holloway, C.D. Moyes, G.J.F. Heigenhauser, L. Spriet, A. Bonen, Nuclear SIRT1 activity, but not protein content, regulates mitochondrial biogenesis in rat and human skeletal muscle, Am. J. Physiol. Regul. Integr. Comp. Physiol. 301 (1) (2011) R67–R75, https://doi.org/10.1152/ajpregu.00417.2010.
[38] B. Luen Tang, Sirt1 and the mitochondria, Mol. Cell. 39 (2) (2016) 87–95, https://doi.org/10.14348/molcells.2016.2318.
[39] D.A. Hood, J.M. Memme, A.N. Oliveira, M. Triolo, Maintenance of skeletal muscle mitochondria in health, exercise, and aging, Annu. Rev. Physiol. 81 (81) (2019) 19–41, https://doi.org/10.1146/annurev-physiol-020518-114310, 2019.
[40] J.-P. Leduc-Gaudet, S.N.A. Hussain, E. Barreiro, G. Gouspillou, Mitochondrial dynamics and mitophagy in skeletal muscle health and aging, Int. J. Mol. Sci. 22 (15) (2021), https://doi.org/10.3390/ijms22158179. Article 15.
[41] M.V. Esteca, M.B. Severino, J.G. Silvestre, G. Palmeira Dos Santos, L. Tamborlin, A. D. Luchessi, A.S. Moriscot, Å.B. Gustafsson, I.L. Baptista, Loss of parkin results in altered muscle stem cell differentiation during regeneration, Int. J. Mol. Sci. 21 (21) (2020) 8007, https://doi.org/10.3390/ijms21218007.
[42] H. Chen, M. Lu, Q. Lyu, L. Shi, C. Zhou, M. Li, S. Feng, X. Liang, X. Zhou, L. Ren, Mitochondrial dynamics dysfunction: unraveling the hidden link to depression, Biomed. Pharmacother. 175 (2024) 116656, https://doi.org/10.1016/j.biopha.2024.116656.
[43] D.M. Sepa-Kishi, Y. Sotoudeh-Nia, A. Iqbal, G. Bikopoulos, R.B. Ceddia, Cold acclimation causes fiber type-specific responses in glucose and fat metabolism in rat skeletal muscles, Sci. Rep. 7 (1) (2017) 15430, https://doi.org/10.1038/s41598-017-15842-3.
[44] R.D. Hyldahl, J.M. Peake, Combining cooling or heating applications with exercise training to enhance performance and muscle adaptations, J. Appl. Physiol. 129 (2) (2020) 353–365, https://doi.org/10.1152/japplphysiol.00322.2020.
[45] T. Dünnwald, H. Gatterer, M. Faulhaber, M. Arvandi, W. Schobersberger, Body composition and body weight changes at different altitude levels: a systematic review and meta-analysis, Front. Physiol. 10 (2019), https://doi.org/10.3389/fphys.2019.00430.
[46] R.J. Brychta, K.Y. Chen, Cold-induced thermogenesis in humans, Eur. J. Clin. Nutr. 71 (3) (2017) 345–352, https://doi.org/10.1038/ejcn.2016.223.
[47] S. Yu Tsibul'nikov, L.N. Maslov, N.V. Naryzhnaya, V.V. Ivanov, Yu B. Lishmanov, Specific features of adaptation of rats to chronic cold treatment, Dokl. Biol. Sci. 470 (1) (2016) 214–216, https://doi.org/10.1134/S0012496616050033.
[48] T. Yamashita, G.A. Voth, Insights into the mechanism of proton transport in cytochrome c oxidase, J. Am. Chem. Soc. 134 (2) (2012) 1147–1152, https://doi.org/10.1021/ja209176e.
[49] J.L. Beaudry, G.B. McClelland, Thermogenesis in CD-1 mice after combined chronic hypoxia and cold acclimation, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 157 (3) (2010) 301–309, https://doi.org/10.1016/j.cbpb.2010.07.004.
[50] R. Ramzan, A.M. Dolga, S. Michels, P. Weber, C. Culmsee, A.J. Rastan, S. Vogt, Cytochrome c oxidase inhibition by ATP decreases mitochondrial ROS production, Cells 11 (6) (2022), https://doi.org/10.3390/cells11060992. Article 6.
[51] J.W. Kupiec-Weglinski, NRF2: new mechanistic insights and therapeutic perspectives, Antioxidants Redox Signal. 40 (10–12) (2024) 632–635, https://doi.org/10.1089/ars.2023.0398.
[52] I. Bellezza, I. Giambanco, A. Minelli, R. Donato, Nrf2-Keap1 signaling in oxidative and reductive stress, Biochim. Biophys. Acta Mol. Cell Res. 1865 (5) (2018) 721–733, https://doi.org/10.1016/j.bbamcr.2018.02.010.
[53] T.L. Duarte, N.P. Talbot, H. Drakesmith, NRF2 and hypoxia-inducible factors: key players in the redox control of systemic iron homeostasis, Antioxidants Redox Signal. 35 (6) (2021) 433–452, https://doi.org/10.1089/ars.2020.8148.
[54] P. Cong, Y. Liu, N. Liu, Y. Zhang, C. Tong, L. Shi, X. Liu, X. Shi, Y. Liu, Z. Tong, M. Hou, Cold exposure induced oxidative stress and apoptosis in the myocardium by inhibiting the Nrf2-Keap1 signaling pathway, BMC Cardiovasc. Disord. 18 (1) (2018) 36, https://doi.org/10.1186/s12872-018-0748-x.
[55] A. Gallego-Selles, M. Martin-Rincon, M. Martinez-Canton, M. Perez-Valera, S. Martín-Rodríguez, M. Gelabert-Rebato, A. Santana, D. Morales-Alamo, C. Dorado, J.A.L. Calbet, Regulation of Nrf2/Keap1 signalling in human skeletal muscle during exercise to exhaustion in normoxia, severe acute hypoxia and post-exercise ischaemia: influence of metabolite accumulation and oxygenation, Redox Biol. 36 (2020) 101627, https://doi.org/10.1016/j.redox.2020.101627.
[56] E. Barbieri, P. Sestili, Reactive oxygen species in skeletal muscle signaling, J. Signal Trans. 2012 (1) (2012) 982794, https://doi.org/10.1155/2012/982794.
[57] C. Kang, K.M. O'Moore, J.R. Dickman, L.L. Ji, Exercise activation of muscle peroxisome proliferator-activated receptor-γ coactivator-1α signaling is redox sensitive, Free Radic. Biol. Med. 47 (10) (2009) 1394–1400, https://doi.org/10.1016/j.freeradbiomed.2009.08.007.
[58] E. Supruniuk, J. Górski, A. Chabowski, Endogenous and exogenous antioxidants in skeletal muscle fatigue development during exercise, Antioxidants 12 (2) (2023), https://doi.org/10.3390/antiox12020501. Article 2.
[59] S. Di Meo, G. Napolitano, P. Venditti, Mediators of physical activity protection against ROS-linked skeletal muscle damage, Int. J. Mol. Sci. 20 (12) (2019), https://doi.org/10.3390/ijms20123024. Article 12.
[60] X. Wang, H. Che, W. Zhang, J. Wang, T. Ke, R. Cao, S. Meng, D. Li, O. Weiming, J. Chen, W. Luo, Effects of mild chronic intermittent cold exposure on rat organs, Int. J. Biol. Sci. 11 (10) (2015) 1171–1180, https://doi.org/10.7150/ijbs.12161.
[61] T.L. Clanton, Hypoxia-induced reactive oxygen species formation in skeletal muscle, J. Appl. Physiol. 102 (6) (2007) 2379–2388, https://doi.org/10.1152/japplphysiol.01298.2006.
[62] H. Esterbauer, K.H. Cheeseman, Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. En *Methods in Enzymology*, Vol. 186, pp. 407–421, Academic Press, 1990, https://doi.org/10.1016/0076-6879(90)86134-H.
[63] W.A. Pryor, N.A. Porter, Suggested mechanisms for the production of 4-hydroxy-2-nonenal from the autoxidation of polyunsaturated fatty acids, Free Radic. Biol. Med. 8 (6) (1990) 541–543, https://doi.org/10.1016/0891-5849(90)90153-A.
[64] T. Carbonell, N. Alva, S. Sanchez-Nuño, S. Dewey, A.V. Gomes, Subnormothermic perfusion in the isolated rat liver preserves the antioxidant glutathione and enhances the function of the ubiquitin proteasome system, Oxidative Medicine and Cellular Longevity, 2016 (1) (2016) 9324692, https://doi.org/10.1155/2016/9324692.
[65] M. Perluigi, R. Coccia, D.A. Butterfield, 4-Hydroxy-2-Nonenal, a reactive product of lipid peroxidation, and neurodegenerative diseases: a toxic combination illuminated by redox proteomics studies, Antioxidants Redox Signal. 17 (11) (2012) 1590–1609, https://doi.org/10.1089/ars.2011.4406.
[66] B.S. Berlett, E.R. Stadtman, Protein oxidation in aging, disease, and oxidative stress, J. Biol. Chem. 272 (33) (1997) 20313–20316, https://doi.org/10.1074/jbc.272.33.20313.
[67] E.R. Stadtman, B.S. Berlett, Reactive oxygen-mediated protein oxidation in aging and disease, Chem. Res. Toxicol. 10 (5) (1997) 485–494, https://doi.org/10.1021/tx960133r.
[68] M.H. Glickman, A. Ciechanover, The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction, Physiol. Rev. 82 (2) (2002) 373–428, https://doi.org/10.1152/physrev.00027.2001.
[69] M.B. Harris, B.M. Mitchell, S.G. Sood, R.C. Webb, R.C. Venema, Increased nitric oxide synthase activity and Hsp90 association in skeletal muscle following chronic exercise, Eur. J. Appl. Physiol. 104 (5) (2008) 795–802, https://doi.org/10.1007/s00421-008-0833-4.
[70] T. Chaillou, N. Koulmann, A. Meunier, P. Pugnière, J.J. McCarthy, M. Beaudry, X. Bigard, Ambient hypoxia enhances the loss of muscle mass after extensive injury, Pflueg. Arch. Eur. J. Physiol. 466 (3) (2014) 587–598, https://doi.org/10.1007/s00424-013-1336-7.
[71] D. Rizo-Roca, J.G. Ríos-Kristjánsson, C. Núñez-Espinosa, E. Santos-Alves, J. Magalhães, A. Ascensão, T. Pagès, G. Viscor, J.R. Torrella, Modulation of mitochondrial biomarkers by intermittent hypobaric hypoxia and aerobic exercise after eccentric exercise in trained rats, Appl. Physiol. Nutr. Metabol. 42 (7) (2017) 683–693, https://doi.org/10.1139/apnm-2016-0526.