

Early heart and skeletal muscle mitochondrial response to a moderate hypobaric hypoxia environment

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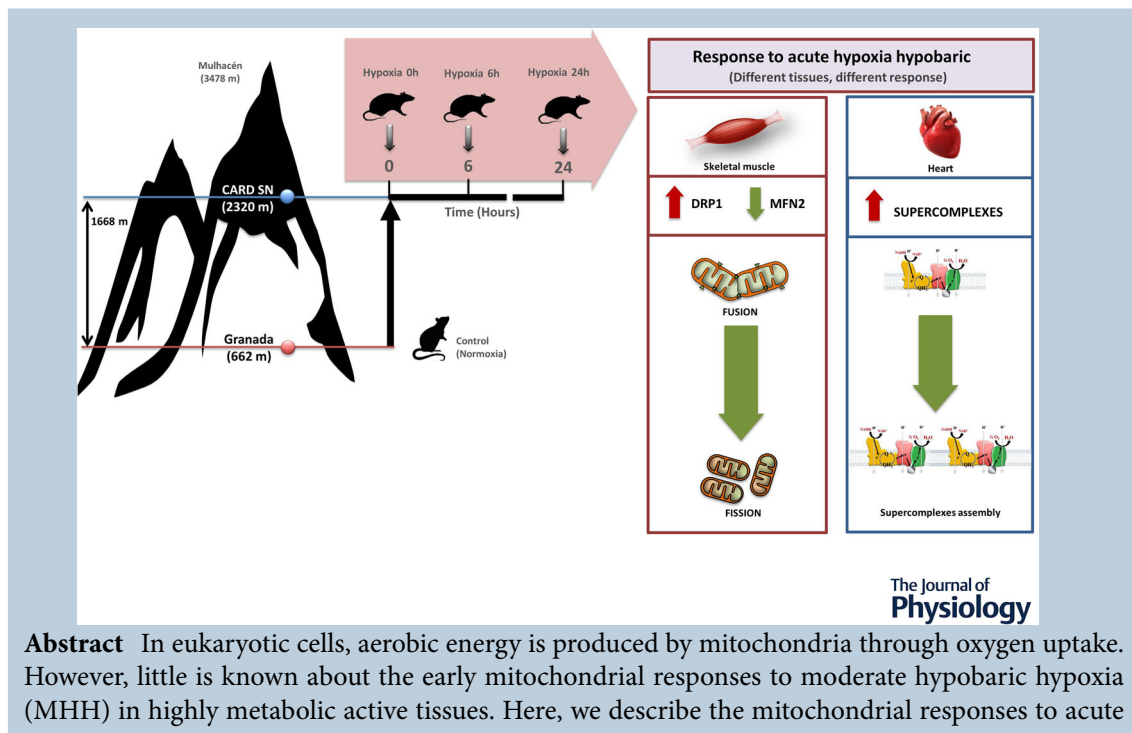
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MHH in the heart and skeletal muscle. Rats were randomly allocated into a normoxia control group ($n = 10$) and a hypoxia group ($n = 30$), divided into three groups (0, 6, and 24 h post-MHH). The normoxia situation was recapitulated at the University of Granada, at 662 m above sea level. The MHH situation was performed at the High-Performance Altitude Training Centre of Sierra Nevada located in Granada at 2320 m above sea level. We found a significant increase in mitochondrial supercomplex assembly in the heart as soon as the animals reached 2320 m above sea level and their levels are maintained 24 h post-exposure, but not in skeletal muscle. Furthermore, in skeletal muscle, at 0 and 6 h, there was increased dynamin-related protein 1 (Drp1) expression and a significant reduction in Mitofusin 2. In conclusion, mitochondria from the muscle and heart respond differently to MHH: mitochondrial supercomplexes increase in the heart, whereas, in skeletal muscle, the mitochondrial pro-fission response is triggered. Considering that skeletal muscle was not actively involved in the ascent when the heart was beating faster to compensate for the hypobaric, hypoxic conditions, we speculate that the different responses to MHH are a result of the different energetic requirements of the tissues upon MHH.

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Abstract figure legend After acute exposure to moderate hypobaric hypoxia, rats were killed at the indicated time points, and mitochondria from skeletal muscle and the heart were analysed. The results showed greater mitochondrial fragmentation in skeletal muscle, whereas heart mitochondria demonstrated an increase in supercomplex assembly.

Key points

- The heart and the skeletal muscle showed different mitochondrial responses to moderate hypobaric hypoxia.
- Moderate hypobaric hypoxia increases the assembly of the electron transport chain complexes into supercomplexes in the heart.
- Skeletal muscle shows an early mitochondrial pro-fission response following exposure to moderate hypobaric hypoxia.

Introduction

Currently, there are different groups frequently exposed to moderate intermittent hypoxia, including working cohorts such as miners and soldiers. Indeed, as a result of this exposure, these groups are more prone to suffer cardiac disorders (Aragón-Vela et al., 2020). Conversely, pilots and flight attendants are not affected

by this exposure, possibly because their work routine is exempt from any type of high-intensity physical activity (Aragón-Vela et al., 2020). Furthermore, it is common for athletes to stay at high altitude (≥ 2500 m) to improve physical performance (Bejder & Nordsborg, 2018). Indeed, altitude training in an hypobaric hypoxic environment facilitates the increase in haemoglobin

Jerónimo Aragón-Vela is interested in the mitochondrial adaptations generated after chronic and acute exposure to moderate hypobaric hypoxia. He received his PhD at the University of Granada where he described oxidative damage and enzymatic and non-enzymatic antioxidant responses in swimming at different intensities under moderate hypoxia. Subsequently, Jerónimo Aragón-Vela joined the University of Copenhagen as a postdoctoral fellow aiming to detect possible molecular markers for the prevention of ischemic pathologies when working at high altitude. **Rafael A. Casuso** is interested in the metabolism of skeletal muscle in response to exercise and nutrition. He earned his PhD in Health Sciences from the University of Jaén (Spain), where he investigated mitochondrial adaptations to exercise and the effects of polyphenols in various tissues. Currently, he is a researcher at Universidad Loyola Andalucía (Spain), where his work explores the sexual dimorphisms of muscle mitochondria in response to exercise.



mass, resulting in an enhanced oxygen-carrying capacity, and therefore improves the energy conversion rate (Bonne et al., 2014). However, this condition usually requires a previous period of adaptation to avoid undesirable physiological consequences such as pulmonary hypertension, especially for those individuals who have never previously been exposed to this condition (Aragón-Vela et al., 2020). However, the optimal duration of the acclimatization period to avoid side effects and benefit from the altitude training program has not been determined.

The main benefit of the altitude training program is the transitory production of erythropoietin stimulated by the hypoxia-induced protein factor-1 (HIF-1) complex (Haase, 2013) to counteract the oxygen deficiency that inhibits mitochondrial activity and induces mitochondrial autophagy (Dehne & Brüne, 2014; Horscroft & Murray, 2014). Therefore, energy supply would be limited by ATP depletion (Chitra & Boopathy, 2014). However, to maintain energetic homeostasis in hypoxia situations, cells can respond by inducing mitochondrial biogenesis (Zou et al., 2015). The peroxisome proliferator activated receptor gamma coactivator-1 α (PGC-1 α) has been shown to be a master regulator of mitochondrial biogenesis (Fernandez-Marcos & Auwerx, 2011), with sirtuin 1 (SIRT1) playing an important role in its regulation (Gerhart-Hines et al., 2007). In addition to mitochondrial biogenesis, the co-ordination of mitochondrial function depends on the dynamic nature of mitochondria, which is controlled by two opposing processes: mitochondrial fission and mitochondrial fusion (Suen et al., 2008). In mammals, mitofusin 2 (MFN2) and Optic atrophy 1 (OPA1) proteins control the process of mitochondrial fusion, whereas dynamin-related protein 1 (DRP1) is involved in mitochondrial fission (Feng et al., 2020; Kitaoka et al., 2015; Silva Ramos et al., 2016). Indeed, in an *in vivo* study, exposure to hypobaric hypoxia induced an imbalance in mitochondrial dynamics, as demonstrated by increased fission (mediated by DRP1) and decreased fusion (mediated by MFN2) (Chitra & Boopathy, 2013). To improve the electron transport efficiency and limit the production of reactive oxygen species (Genova & Lenaz, 2014; Maranzana et al., 2013; Vartak et al., 2013), mitochondria organize the complexes of the respiratory chain into supercomplexes (Calvo et al., 2020; Lenaz & Genova, 2010).

However, most of the mitochondrial data upon hypoxia originate from *in vitro* experiments and from rodents submitted to hypobaric chambers, thus limiting the conclusions to an *in vivo* and natural exposure to environmental hypobaric hypoxia. Indeed, in the hypobaric chamber, all that occurs is a decrease in oxygen concentration, whereas MHH induces a decrease in barometric pressure as well as a change in humidity, temperature and climatic conditions (Morabito et al., 2016). In addition, we reproduce a passive way of ascent

(vehicle transportation) in which muscles do not perform any work out when the heart is compensating for the changes in pressure and oxygen levels by increasing the beats. Therefore, the present study aimed to understand the mitochondrial response to acute but natural MHH in both heart and skeletal muscle (SKM).

Methods

Ethical approval

All experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee of the University of Granada (procedures Granada, Spain. no. 203-CEEA-OH-2017) and in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (CETS #123), directive 2010/63/EU on the protection of animals used for scientific purposes and the Spanish law (R.D. 53/2013). In addition, all of the investigators confirm that the experiments described in the present study conform to the guidelines and regulations described in Grundy (2015). The rats were maintained in a well-ventilated room under standard conditions ($22 \pm 2^\circ\text{C}$), with a reversed 12:12 h light/dark photoperiod, and had free access to food and water. Animal transportation was conducted by authorized transporters who ensure consistent standards to minimize stress on the animals. Rats were killed by intraperitoneal injection with an overdose of anaesthetic (ketamine–xylazine, $100\text{--}10\text{ mg kg}^{-1}$, respectability). All efforts were made to protect animal welfare and to minimize suffering at each step of the protocol.

Animals

An *a priori* sample size was performed using G*Power (<http://www.gpower.hhu.de>). The parameters selected were a moderate effect size ($F = 0.5$), an α level of 0.10, a power level of 0.90, four groups, 23 measurements, a correlation among the repeated measures of 0.5 and critical $F = 2.290$ for the analysis of variance that repeated measures within–between the interaction. The sample size was determined to be at least 32 rats. Male Wistar rats were purchased from Janvier Labs (Le Genest-Saint-Isle, France) at 7 weeks of age. The rats initially weighed $202 \pm 13.4\text{ g}$. The rats were randomly allocated into a normoxia group ($n = 10$) as a control group and a hypoxia group ($n = 30$), divided into three groups.

Experimental design

The animals were exposed to MHH at different time points. In a normoxia situation, eight rats were killed as

a control group in the animal facility of the Biomedical Research Centre of the University of Granada, at 662 m above sea level. In a hypobaric hypoxia situation, 30 rats were killed at different time points after arrival at the High-Performance Altitude Training Centre of Sierra Nevada located in Granada, 2320 m above sea level. The hypoxia groups were divided into a 0 h group ($n = 10$), 6 h group ($n = 10$) and 24 h group ($n = 10$) (Fig. 1).

Tissue collection

The rats were killed by an overdose of anaesthesia. Once permanent cessation of circulation was achieved, both left and right gastrocnemius and soleus and the heart were extracted. The organs were stored at -80°C until use.

Western blotting

The heart and soleus samples were lysed in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol and a protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA), in ice for 20 min (Huertas et al., 2017). After centrifugation (13,000 g for 30 min at 4°C), the protein content in the supernatant was determined using a Protein Assay Kit II (Bio-Rad Laboratories, Hercules, CA, USA). Samples containing 30 μg of protein were

mixed with $3 \times$ SDS-PAGE sample buffer (100 mM Tris-HCl, pH 6.8, 25% SDS, 0.4% bromophenol blue, 10% β -mercaptoethanol and 2% glycerol), separated via SDS-PAGE using TGX Any kD gel (Bio-Rad) and then transferred onto a nitrocellulose membrane (Bio-Rad). After incubation in blocking buffer (5% non-fat milk and 1% Tween 20 in Tris-buffered saline), the membranes were probed with the following antibodies: mouse anti-PGC1 α (4A8), mouse anti-HSP-70 (B-6) (internal control, dilution of 1:1000 in 5% non-fat milk), mouse SIRT1 (B-7) and rabbit anti-MFN2 (H-68) (dilution of 1:500 in 5% non-fat milk) obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); mouse anti-OPA1 (612 606; dilution of 1:1000 in 5% non-fat milk) acquired from BD Biosciences (Franklin Lakes, NJ, USA); rabbit DRP1 antibody (#8570; dilution of 1:1000 in 5% non-fat milk), rabbit anti-HIF-1 α antibody (#3716) and rabbit anti-hydroxy-HIF-1 α (Pro564; both at a dilution of 1:500 in 5% non-fat milk) purchased from Cell Signaling Technologies (Beverly, MA, USA); anti-Vinculin Recombinant rabbit (3J7P3) MA5-42795 (Life Technologies) rabbit anti-UQCRC2 (CORE2) antibody (14742-1-AP) acquired from ProteinTech (Rosemont, IL, USA); mouse anti-MTCO1 (CO1) monoclonal antibody (1D6E1A8) acquired from Invitrogen (Thermo Fisher Scientific, Waltham, MA USA); and rabbit anti-NDUFB8 antibody (ab110242) acquired from Abcam (Cambridge, UK). Fluorescence secondary

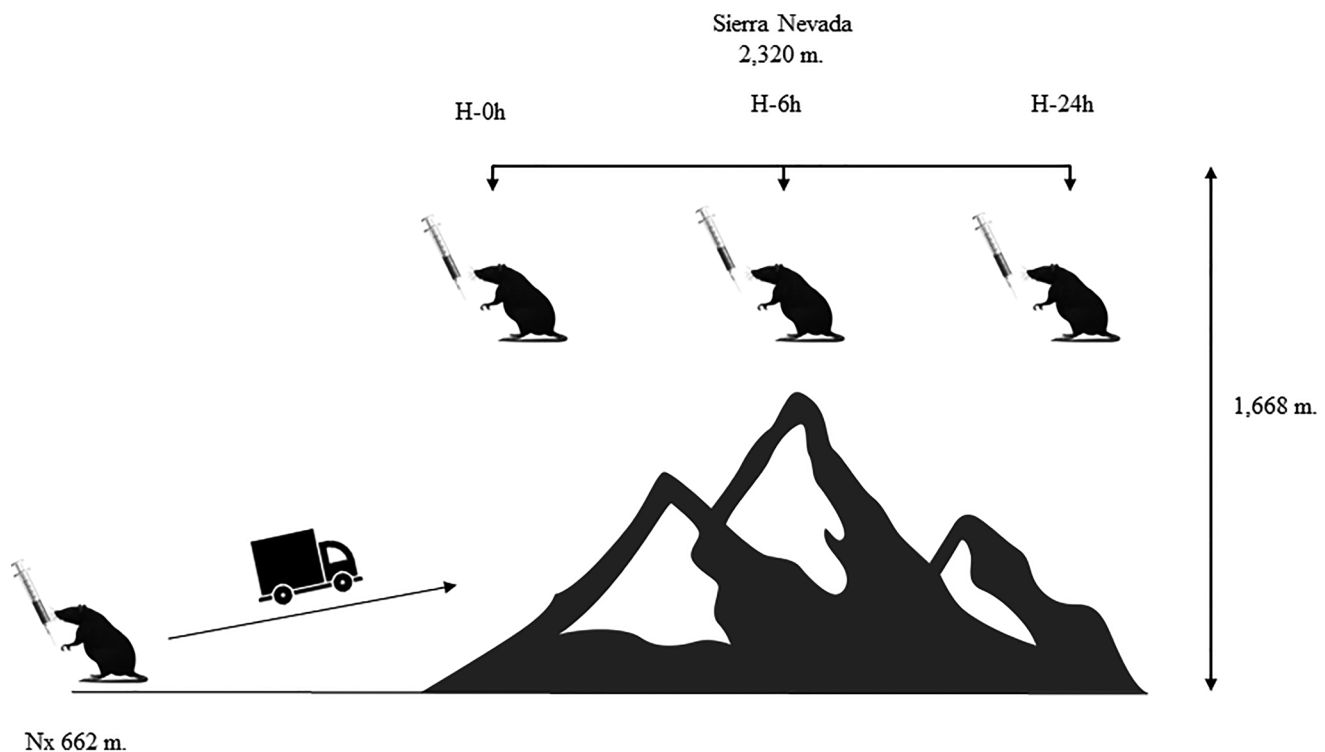


Figure 1. Experimental design

Summary of the experimental plan of the study. Nx, normoxia; H, hypoxia. Syringe represents the time of killing.

antibodies comprising anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor™ 680 (A21076), and anti-mouse IgG (H+L) cross-adsorbed secondary antibody, DyLight™ 800 (SA5-10176), were acquired from Invitrogen and used at dilution of 1:5000 in 5% non-fat milk. Signals were detected and quantified using Odyssey CLX (LI-COR, Lincoln, NE, USA). For chemiluminescence detection, secondary antibodies anti-mouse Ig, human ads-HRP (1010-05), and anti-rabbit Ig, human ads-HRP (4010-05), were purchased from BioNova (Fremont, CA, USA). Immunoreactive signals were detected via enhanced chemiluminescence (Super-Signal West Dura Chemiluminescent Substrate, 34075; Thermo Fisher Scientific) and the membranes were digitally imaged and quantified through densitometry using ImageJ (NIH, Bethesda, MD, USA). The results are represented as fold-change (Fc) in expression relative to the control. The graph shows a representative crop blot.

Evaluation of supercomplex formation in SKM by Blue Native- Page (BN-PAGE) electrophoresis

Blue native Page (BN-PAGE) electrophoresis was performed on crude mitochondrial fractions from the heart and gastrocnemius of rats. Mitochondrial isolation was performed as previously described (Huertas

et al., 2017). One aliquot of the crude mitochondrial fraction was used for protein determination. The remaining samples were then centrifuged at 13,000 g for 3 min at 4 °C. The mitochondrial pellets were suspended in an appropriate volume of medium C (1 M aminocaproic acid, 50 mM Bis-Tris-HCl, pH 7.0) to create a protein concentration of 10 mg mL⁻¹ and the membrane proteins were solubilized with digitonin (4 g g⁻¹) and incubated for 10 min in ice. After 30 min of centrifugation at 13,000 g at 4 °C, the supernatants were collected and 3 μ L of 5% Brilliant Blue G dye prepared in 1 M aminocaproic acid was added. Mitochondrial proteins (100 μ g) were then loaded and run on a 3–13% gradient native gel as previously described (Luna-Sánchez et al., 2015). After electrophoresis, the samples were electroblotted onto polyvinylidene fluoride membranes and sequentially tested with specific antibody against UQCRC2 (CORE2) subunit antibody (14742-1-AP) acquired from ProteinTech (Rosemont, IL, USA).

Statistical analysis

Statistical analyses were performed using SPSS, version 22 (IBM Corp., Armonk, NY, USA). The data are presented as the mean \pm SD. $P < 0.05$ was considered statistically significant. The normality of distribution was assessed via the Shapiro–Wilk test. Homogeneity

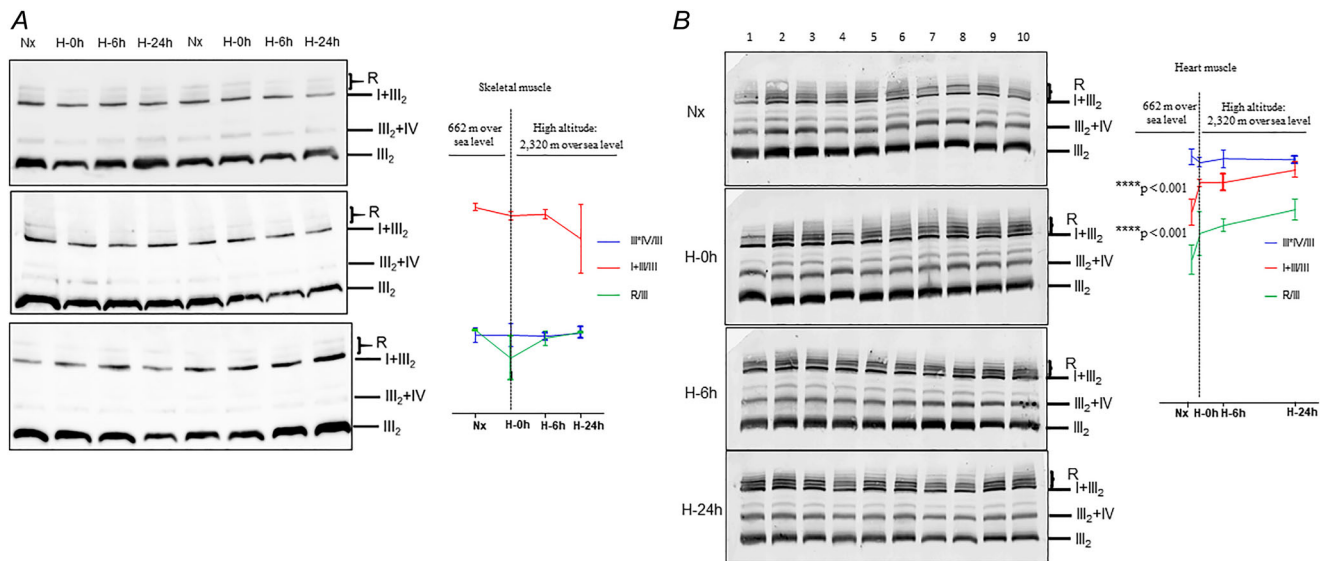


Figure 2. Effects of MHH on supercomplexes (SCs) assembly

A, immunodetection of core2 (complex III) after BN-PAGE electrophoresis of digitonin solubilized mitochondria from SKM after the indicated time points after MHH and the correspondent densitometric quantification of the proportion of complex III assembled into supercomplexes III₂+IV, I+III₂ and I+III₂+IV (R) ($n = 6$ rats). B, immunodetection of core2 (complex III) after BN-PAGE electrophoresis of digitonin solubilized mitochondria from SKM after the indicated time points after MHH and the correspondent densitometric quantification of the proportion of complex III assembled into supercomplexes III₂+IV, I+III₂ and I+III₂+IV (R) ($n = 9$ rats). Data are shown as the mean \pm SD. **** $P < 0.001$ vs. moderate hypobaric hypoxia. * $P < 0.05$ vs. moderate hypobaric hypoxia. Nx, normoxia; H, hypoxia

of variance was analysed through via the Levene test. Analysis of variance with repeated measures was used to analyse differences between the normoxia group and hypoxia groups. Multiple comparisons were performed via the Bonferroni *post hoc* test.

Results

Heart but not SKM assembles mitochondrial supercomplexes after MHH

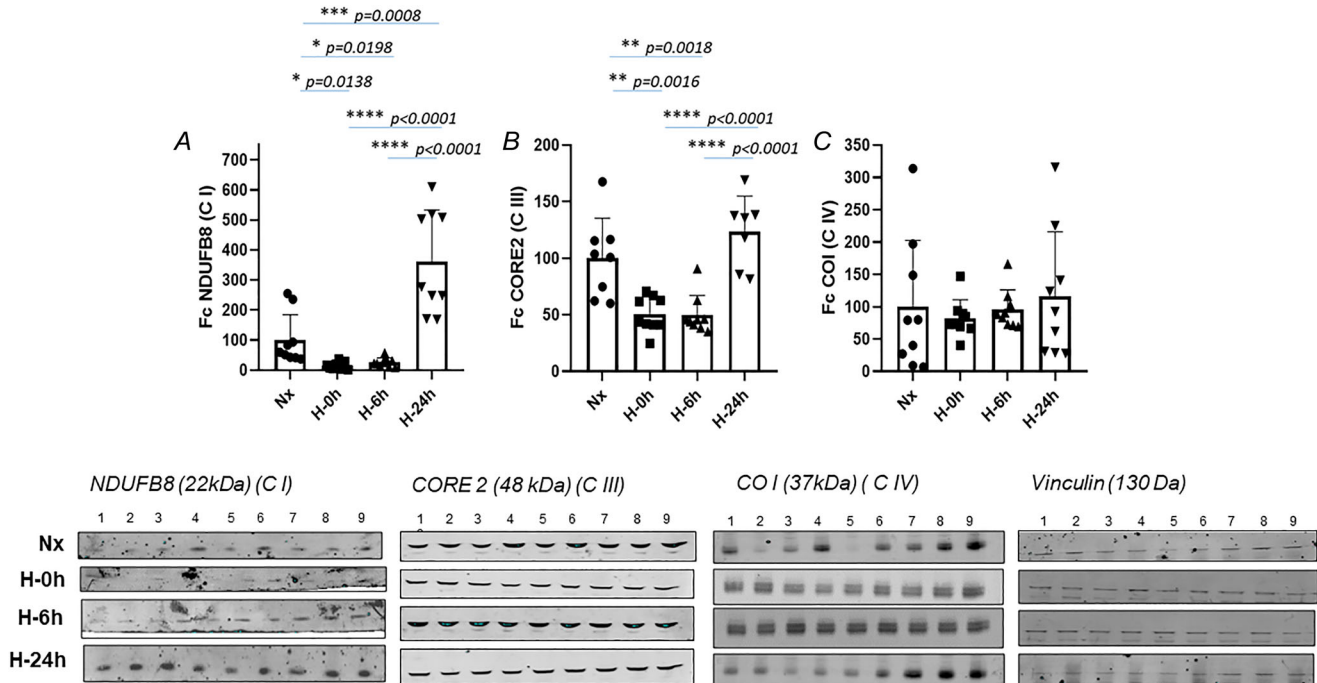
Because mitochondria assemble supercomplexes to decrease reactive oxygen species production (Calvo et al., 2020; Huertas et al., 2017), we aimed to address the hypothesis that, after exposure to MHH, the supercomplex assembly could change. Accordingly, we performed a BN-PAGE analysis with both rat heart and SKM mitochondria at different time points after MHH. The results indicate that mitochondrial supercomplexes assembly does not change in SKM after MHH (Fig. 2A). By contrast, we observed an increase in supercomplex assembly in the heart immediately after reaching 2500 m above sea level (Fig. 2B). In particular, there is a significant increase in mitochondrial supercomplex assembly (I+III₂) and respirasome assembly (I+III₂+IV) but not the III₂+IV supercomplexes. Therefore, acute

exposure to MHH induces mitochondrial supercomplex assembly only in the heart.

Because changes in the levels of mitochondrial supercomplexes may be influenced by the level of individual subunits, we also evaluated those parameters by SDS-PAGE in heart mitochondria after MHH. The results suggested that there is an initial decrease (at 0 and 6 h) in response to MHH in the levels of the subunit NDUFB8 of complex I (Fig. 3A), as well as of CORE2, subunit of complex III (Fig. 3B). Both subunits increase at 24 h after MHH (Fig. 3A and B). The subunit of complex IV COI remains stable over time (Fig. 3C). Because the assembly is evident immediately upon arrival to MHH, it can be suggested that the supercomplexes formation is initially the result of a rearrangement of the existing complexes, whereas, later, new subunits synthesis will be engaged.

Heart and SKM modulate mitochondrial dynamics signalling differently after MHH

One of the factors related with the metabolic response to hypoxia is HIF-1 α . In our experimental approach, the HIF-1 α H/HIF-1 α ratio decreases in response to MHH exposure at 0 h (Fig. 4A) ($P = 0.0323$) in SKM. Because HIF1 α has an important regulatory role in mitochondrial



a different adaptation with longer times. Furthermore, our data do not support the activation of a mitochondrial biogenesis pathway in SKM and the heart. Specifically, under our experimental conditions, PGC-1 α , the master regulator of this process (Cantó et al., 2009), does not increase.

We also observed a drop of MFN2 levels (from 0 h to 6 h) concomitantly with an up-regulation of DRP that is probably indicative of an early mitochondrial fission in response to MHH in SKM. Taken together, these results suggest that, in response to MHH, SKM mitochondria may activate a pro-fission mechanism. By contrast, heart mitochondria show a decrease in DRP1 following 6 h of MHH exposure. However, this was not accompanied by alterations in fusion-related proteins such as OPA1. Those results suggest that the fission–fusion–biogenesis cycle in the heart does not change upon MHH. As is the case under hypoxia, a reduction in respiratory activity as a consequence of fission processes has been described, which ultimately helps to maintain reactive oxygen species at physiological levels (Fuhrmann & Brüne, 2017). These observations further support that the idea that SKM can more easily shift ATP production from oxidative to glycolytic sources (Kierans & Taylor, 2021; Wang et al., 2022).

Within SKM, we found an increase in SIRT1 content in response to MHH. This is somewhat surprising because the SIRT–PGC-1 α axis controls mitochondrial

responses to physiological stress such as during exercise (Cantó et al., 2009, 2010). Therefore, SIRT1 may be activating a non-mitochondrial pathway within SKM. Indeed, studies in which cells were exposed to a low oxygen (1%) environment showed that both HIF-1 α and HIF-2 α increase SIRT1 expression and this ultimately leads to enhanced activity of HIF-2 α but not HIF-1 α (Chen et al., 2011). Therefore, HIF signalling promotes SIRT1 expression, which partially potentiates the hypoxia-induced transcriptional response. The same response was confirmed in the liver of mice exposed to 2 h of a 6% oxygen environment (Chen et al., 2011). It has also been suggested, in mice exposed for 4 weeks to a 11% oxygen environment, that an increment in SIRT1 expression results in attenuated HIF-1 α activation in adipose tissue (Wu et al., 2023). Because our data show that SIRT1 concomitantly increases as HIF-1 α became activated, this may suggest a regulatory loop between SIRT1 and HIF-1 in response to environmental hypobaric hypoxia within SKM. Although its physiological role within SKM is yet unclear, it might serve as regulatory switch between oxidative and glycolytic metabolism.

The present study has several limitations that need to be mentioned. First, supercomplexes were assessed in the gastrocnemius muscle, whereas western blots were performed in the soleus. These muscles may differ in their oxidative capacity because of a different fibre type distribution. In rats, it has been suggested

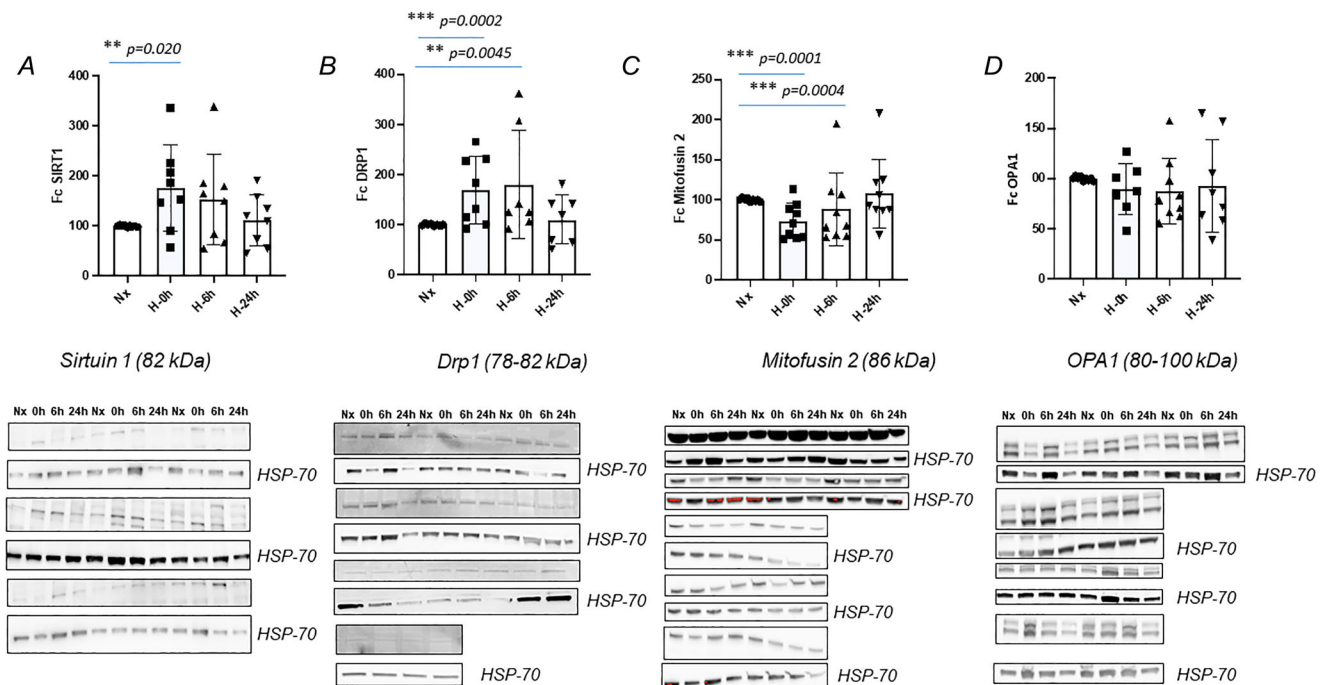


Figure 5. MHH triggers mitochondrial fission in SKM

Densitometric quantification (top) of the immunoblots (bottom) of (A) SIRT1, (B) Drp1, (C) Mitofusin 2, (D) OPA1 at the indicated time points. The results are represented as fold change compared to normoxia values and normalized by the HSP-70 value ($n = 8$ rats). Data are shown as the mean \pm SD. * $P < 0.05$ vs. moderate hypobaric hypoxia.

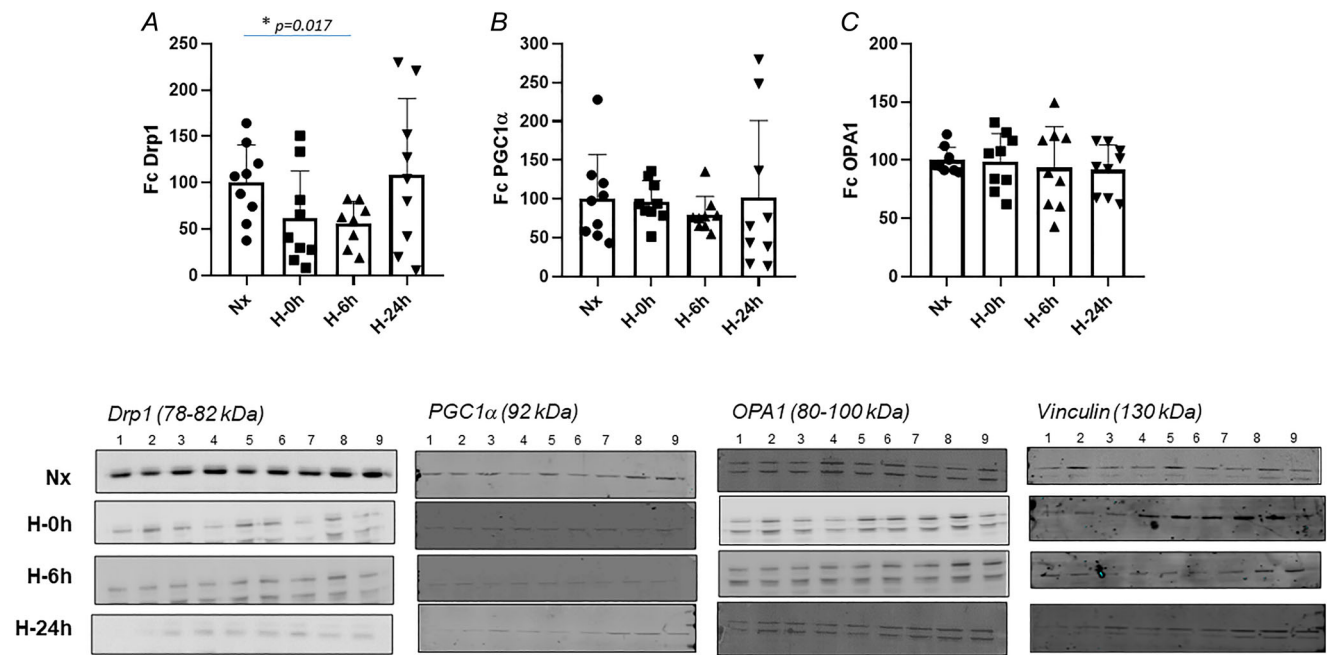


Figure 6. The fusion-fission pathways do not change upon MHH

Densitometric quantification (top) of the immunoblots (bottom) of (A) DRP1, (B) PGC-1 α , (C) OPA1 at the indicated time points ($n = 9$ rats). The results are represented as fold change compared to normoxia values and normalized by the vinculin value. Data are shown as the mean \pm SD.

that different oxidative capacities between the white gastrocnemius and the soleus are probably a result of differences in mitochondrial volume density and sub-cellular distribution (Philippi & Sillau, 1994). In humans, recent human data suggest a different intrinsic respiratory capacity as well as a different content of proteins controlling cristae between fast-twitch and slow-twitch fibres (Edman et al., 2024). Therefore, we cannot rule out the possibility that supercomplex assembly in response to MHH can differ in the two muscles. Second, we have been unable to measure several proteins in heart tissue such as MFN2, SIRT1 and HIF-1. Similarly, we have not measured mitochondrial morphology via transmission electron microscopy to directly compare heart and SKM. Furthermore, these results should be considered with caution because they have been obtained from animal studies; therefore, the data need to be confirmed in humans.

Conclusions

In the present study, we show that the heart and the SKM have different mitochondrial responses to MHH. Specifically, we show that supercomplexes increase in the heart but not in the SKM. Moreover, we show that the SKM has an early mitochondrial pro-fission response that is not evident for the heart. These observations are in line with other evidence suggesting that fission in SKM is probably involved in the hypoxia signalling and in the switch

towards a glycolytic metabolism (Fuhrmann & Brüne, 2017), whereas the heart changes its mitochondrial supra-molecular organization to optimize oxygen metabolism.

Perspectives

To the best of our knowledge, this is the first study to describe the mitochondrial responses to acute natural MHH within SKM and the heart. These results should be taken into consideration when aiming to avoid any type of training that implies a maximal use of aerobic metabolism during the early hours of exposure. Therefore, if similar adaptations occur in humans, it is recommended that, during the early hours of exposure to MHH, athletes should perform an activity focused on physical conditioning, aiming to achieve the correct mitochondrial acclimatization for a successful start of the training period.

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Additional information

Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding authors.

Competing interests

The authors declare that they have no competing interests.

Author contributions

A.R.-R., A. H.-G. and R.-C.A. conceived the experiments, collected the tissues and performed the experiments. A.S.A. performed the experiments and the statistical analysis with heart tissues. J.P.-D. and J.R.H. performed the statistical analysis. J.A.-V., R.A.C. and S.C. wrote the manuscript and supervised the experiments. L.C.L. revised the manuscript critically. All authors

read, edited an approved the final version of the manuscript submitted for publication.

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Keywords

heart, mitochondria, moderate hypobaric hypoxia, skeletal muscle, supercomplexes

Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

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