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Ph.D. International Thesis

Development and characterization of an ischaemia/reperfusion model for the study of muscle signalling induced by free radicals and physical exercise

Ángel Gallego Sellés

Modelo Anexo I - tesis R:D: 99/11

D. ALBERTO MONTOYA ALONSO,
COORDINADOR/A DEL PROGRAMA DE DOCTORADO DE INVESTIGACIÓN EN BIOMEDICINA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

INFORMA,

De que la Comisión Académica del Programa de Doctorado, en su sesión en septiembre de 2022 tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada "Desarrollo y caracterización de un modelo de isquemia/reperfusión para el estudio de la señalización muscular inducida por radicales libres y ejercicio físico." presentada por el doctorando D. Ángel Gallego Sellés y dirigida por la Doctora Dª Cecilia Dorado García.

Y para que así conste, y a efectos de lo previsto en el Artº 11 del Reglamento de Estudios de Doctorado (BOULPGC 04/03/2019) de la Universidad de Las Palmas de Gran Canaria, firmo la presente en Las Palmas de Gran Canaria.

Modelo Anexo II - tesis R:D: 99/11

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
ESCUELA DE DOCTORADO

- Programa de doctorado: Investigación en Biomedicina
- Título de la Tesis: Desarrollo y caracterización de un modelo de isquemia/reperfusión para el estudio de la señalización muscular inducida por radicales libres y ejercicio físico.
- Tesis Doctoral presentada por D. Ángel Gallego Selles.
- Dirigida por la Dra. Dª Cecilia Dorado García.
- Codirigida por el Dr. D. Joaquín Sanchís Moysi.

La Directora, El Codirector, El Doctorando,

INTERNATIONAL PH.D. THESIS

Ph.D. Tesis Internacional

Development and characterization of an ischemia/reperfusion model for the study of muscle signalling induced by free radicals and physical exercise

Desarrollo y caracterización de un modelo de isquemia/reperfusión para el estudio de la señalización muscular inducida por radicales libres y eiercicio físico

Human Performance, Physical Exercise,
& Health Research Group; Department of Physical Education
Ph.D. program in biomedicine research

Grupo de investigación de Rendimiento Humano,
Ejercicio Físico y Salud; Departamento de Educación Física
Doctorado en Investigación en Biomedicina

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Dedicatoria

Esta tesis está dedicada a mi abuelo, quien a lo largo de su vida caminó 6 km diarios para trabajar su tierra con el único objetivo de alimentar a su familia. Incluso meses antes de morir por múltiples patologías, entre ellas Alzheimer, y sin conocimiento científico, se pasaba los días recorriendo (a su ritmo) un pequeño carril de ida y vuelta de 50m que tenía a la entrada de su casa. Él estaba convencido de que permanecer físicamente activo era lo único que le permitiría mantenerse con vida, físicamente independiente y mentalmente saludable. Como experimento casero, cada vuelta que daba al carril dejaba una pequeña piedra de lado para tener una referencia de los metros que había caminado ese día e intentar mejorar al día siguiente. Esta tesis aspira a ser una de esas piedras que él dejaba, una referencia personal del trabajo de estos últimos 4 años que intentaré superar en el futuro con los valores que aprendí de mi abuelo: trabajo duro, constancia y humildad. Sin duda, la memoria de mi abuelo me sigue motivando en el día a día más que los posibles éxitos que pueda llegar a alcanzar. Si bien he disfrutado de este "viaje" en la investigación, espero continuar agregando "pequeñas piedras" sobre los beneficios del ejercicio físico en las personas, e intentar demostrar que él tenía razón.

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PREFACE

“A journey of a thousand miles begins with a single step”.

1. PREFACE

1.1. Thesis impact statement

The findings of this thesis are part of a larger project initially designed **to determine the mechanisms that limit performance during exercise in humans** (1). In addition, complementary studies were undertaken to determine muscle signalling responses.

In this thesis, we have used the muscle biopsies (seven biopsies in eleven subjects) to carry out **one of the most comprehensive analyses ever conducted on the intramuscular protein regulation of three transcription factors** and the exercise-induced responses of upstream and downstream signals in the human skeletal muscle, which required **more than four years of intense laboratory work**.

The thesis **is unique due to the technical difficulty** of doing this type of experiment in humans and because we have studied two exercise conditions: normoxia and severe acute hypoxia. The level of hypoxia used is close to the limit that humans can tolerate without acclimatization to altitude.

This thesis provides insights into the development of **a novel method for the rapid and sensitive identification of human skeletal muscle signalling**, as we have shown by using immediate post-exercise ischaemia to observe that these signalling pathways change extremely rapidly in the human skeletal muscle. It is important to note that most of the signals disappear 60 s after the end of the exercise. The latter must be considered when interpreting the literature. Finally, we would like to highlight the novelty of the study and the new mechanistic insight with **more than ten new findings in human physiology**.

1.2. Main Scientific publications

Original contributions arising from this thesis are listed below:

Article 1 (Gallego-Selles et al. 2020)

- Authors: Angel Gallego-Selles, Marcos Martin-Rincon, Miriam Martinez-Canton, Mario Perez-Valera, Saul Martin-Rodriguez, Miriam Gelabert-Rebato, Alfredo Santana, David Morales-Alamo, Cecilia Dorado, Jose A.L. Calbet.
- Title: 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.
- Journal: Redox Biology; Volume 36, September 2020, 101627
- DOI: 10.1016/j.redox.2020.101627

Article 2 (Gallego-Selles et al. 2022)

- Authors: Angel Gallego-Selles, Victor Galvan-Alvarez, Miriam Martinez-Canton, Eduardo Garcia-Gonzalez, David Morales-Alamo, Alfredo Santana, Juan Jose Gonzalez-Henriquez, Cecilia Dorado, Jose A.L. Calbet, Marcos Martin-Rincon.
- Title: Fast regulation of the NF-κB signalling pathway in human skeletal muscle revealed by high-intensity exercise and ischaemia at exhaustion: role of oxygenation and metabolite accumulation.
- Journal: Redox Biology; Volume 55, September 2022, 102398
- DOI: 10.1016/j.redox.2022.102398

1.3. Other scientific contributions

Additional publications during the thesis period are cited below, but are not included in this thesis:

Article 3 (Perez-Valera et al. 2021)

- Authors: Mario Perez-Valera, Miriam Martinez-Canton, **Angel Gallego-Selles**, Victor Galván-Alvarez, Miriam Gelabert-Rebato, David Morales-Alamo, Alfredo Santana, Saul Martin-Rodriguez, Jesus Gustavo Ponce-Gonzalez, Steen Larsen, Jose Losa-Reyna, Ismael Perez-Suarez, Cecilia Dorado, David Curtelin, Juan Jose Gonzalez-Henriquez, Robert Boushel, Jostein Hallen, Pedro de Pablos Velasco, Jorge Freixinet-Gilart, Hans-Christer Holmberg, Jorn W. Helge, Marcos Martin-Rincon, Jose A. L. Calbet
- Title: Angiotensin-Converting Enzyme 2 (SARS-CoV-2 receptor) expression in human skeletal muscle.
- Journal: Scandinavian Journal of Medicine & Science in Sports.
- DOI: 10.1111/sms.14061

Article 4 (Martinez-Canton et al. 2020)

- Authors: Miriam Martinez-Canton, **Angel Gallego-Selles**, Miriam Gelabert-Rebato, Marcos Martin-Rincon, Fernando Pareja-Blanco, David Rodriguez-Rosell, David Morales-Alamo, Joaquin Sanchis-Moysi, Cecilia Dorado, Juan Jose Gonzalez-Badillo, Jose A. L. Calbet.
- Title: Role of CaMKII and sarcolipin in muscle adaptations to strength training with different levels of fatigue in the set.
- Journal: Scandinavian Journal of Medicine & Science in Sports.
- DOI: 10.1111/sms.13828

Article 5 (Martin-Rincon et al. 2020)

- Authors: Marcos Martin-Rincon, Miriam Gelabert-Rebato, Victor Galvan-Alvarez, **Angel Gallego-Selles**, Miriam Martinez-Canto, Laura Lopez-Rios, Julia C. Wiebe, Saul Martin-Rodriguez, Rafael Arteaga-Ortiz, Cecilia Dorado, Sergio Perez-Regalado, Alfredo Santana, David Morales-Alamo, Jose A. L. Calbet.
- Title: Supplementation with a Mango Leaf Extract (Zynamite®) in Combination with Quercetin Attenuates Muscle Damage and Pain and Accelerates Recovery after Strenuous Damaging Exercise.
- Journal: Nutrients.
- DOI: 10.3390/nu12030614

Article 6 (Gelabert-Rebato et al. 2019)

- Authors: Miriam Gelabert-Rebato, Marcos Martin-Rincon, Victor Galvan-Alvarez, **Angel Gallego-Selles**, Miriam Martinez-Canton, Tanausú Vega-Morales, Julia C. Wiebe, Constanza Fernandez-del Castillo, Elizabeth Castilla-Hernandez, Oriana Diaz-Tiberio, Jose A. L. Calbet.
- Title: A Single Dose of The Mango Leaf Extract Zynamite® in Combination with Quercetin Enhances Peak Power Output During Repeated Sprint Exercise in Men and Women.
- Journal: Nutrients.
- DOI: 10.3390/nu11112592

1.4. Congress presentations

International congress presentations of results from this thesis are listed below:

Presentation 1: Oral defence for the YIA competition.

- Authors: **Angel Gallego-Selles**; Marcos Martin-Rincon; Miriam Martinez-Canton; Alfredo Santana-Rodriguez; Víctor Galvan-Alvarez; David Morales-Alamo; Cecilia Dorado; Jose A. L. Calbet.
- Title: Post-exercise ischaemia maintains the exercise-induced activating phosphorylation of Nrf2: role of metabolites and PO_2.
- Congress: European College of Sport Science.
- Date: from 03 to 06/07/2019.

Presentation 2: YIA competition in poster category.

- Authors: **Angel Gallego-Selles**; Marcos Martin-Rincon; Miriam Martinez-Canton; Miriam Gelabert-Rebato; Víctor Galvan-Alvarez; Sergio Perez-Regalado; Alfredo Santana; Saul Martin-Rodriguez; David Morales-Alamo, Cecilia Dorado; Jose A. L. Calbet.
- Title: High-intensity exercise combined with post-exercise ischaemia induces the activation of Nrf2 in human skeletal muscle.
- Congress: International Sport Forum.
- Date: from 15 to 16/11/2019.

Presentation 3: awarded with a ***Young Investigator Award***.

- Authors: **Angel Gallego-Selles**; Miriam Martinez-Canton; Marcos Martin-Rincon; Sergio Perez-Regalado; Saul Martin-Rodriguez; Alfredo Santana; David Morales-Alamo; Cecilia Dorado; Jose A. L. Calbet.

- Title: Application of ischaemia reveals an important role of Pi and PO_2 in the regulation of Nrf2 and NF-κB signalling in human skeletal muscle.
- Congress: European College of Sport Science.
- Date: from 28 to 30/10/2020.

Presentation 4: oral defence for the YIA competition.

- Authors: **Angel Gallego-Selles**, Victor Galvan-Alvarez, Miriam Martinez-Canton, Sergio Perez-Regalado, Alfredo Santana Rodriguez, Cecilia Dorado García, Saul Martin-Rodriguez, Giovani Garcia-Perez, David Morales-Alamo, Marcos Martin-Rincon, Jose A. L. Calbet.
- Title: Fast activation/deactivation of the NFκB signalling pathway in human skeletal muscle: role of oxygenation and metabolite accumulation.
- Congress: European College of Sport Science.
- Date: from 08 to 10/09/2021.

Presentation 5: oral defence for the YIA competition.

- Authors: **Angel Gallego-Selles**, Victor Galvan-Alvarez, Miriam Martinez-Canton, Eduardo Garcia-Gonzalez, Miriam Gelabert-Rebato, Giovani Garcia-Perez, Alfredo Santana, David Morales-Alamo, d1, Benjamin Fernandez-García, Robert Boushel, Jostein Hallén, Jose A. L. Calbet, Marcos Martin-Rincon.
- Title: Nrf2 and NF-kB signalling, and antioxidant enzyme adaptations to sprint interval training are potentiated by brief ischaemia application during the recovery periods.
- Congress: European College of Sport Science.
- Date: from 30/08/2022 to 02/09/2022.

1.5. Funding sources

- Candidate Funding:

The development of this thesis has been possible thanks to obtaining the competitive predoctoral research contract financed by the University of Las Palmas de Gran Canaria to be part of the University Predoctoral Research Staff in (Personal Docente e Investigador en formación).

- Economic resources used for this thesis:

The research for this thesis was financed in a project previously awarded with grants from Ministerio de Economía y Competitividad (DEP2015-71171-R; DEP2017-86409-C2-1-P), University of Las Palmas de Gran Canaria (ULPGC 2015/05), and ACIISI (ProID2017010106).

1.6. Research Stay Abroad

A description of the research stay carried out during this thesis can be found below:

- Traineeship title: Development of an in vitro model of ischaemia reperfusion using cultured human myotubes to study Nrf2 activation/deactivation mechanisms.
- Receiving Organisation: The Swedish School of Sport and Health Sciences (GIH).
- Mentor: Filip Larsen.
- Affiliation: Åstrand Laboratory; Physiology, Nutrition, and Biomechanics.
- Address: Lidingövägen 1, 114 33 Stockholm, Sweden; Website: https://www.gih.se/
- Traineeship period: from 01/11/2020 to 01/05/2021.
- Duration of the traineeship: 6 months (4.344 Total hours).

During the research stay I learned about the experiments with myotubes in the cell culture laboratory. My experience began with purification of satellite cell cultures, maintenance, and proliferation of isolated muscle cell cultures, followed by testing of different human culture media and analysis of specific biomarkers. After different experiments with culture differentiation media and coating plates, I worked on *in vitro* experiments with human myotube cultures in the development of a cell stimulation model that mimics exercise by using different electrostimulation protocols in the cultures. The effect of exposure to different concentrations of antioxidant reagents (such as sulforaphane or Iberine) separately and accompanied by electrostimulation was also studied. Finally, the effects of antioxidant treatment and electrostimulation on mitochondrial respiration and the generation of cellular protein signalling responses induced by reactive oxygen and nitrogen species (Nrf2, catalase, SOD, etc.) were examined. Additionally, I participated as a subject and collaborator in different projects.

2. SYMBOLS, TERMS, AND ABBREVIATIONS

ADP, adenosine diphosphate

AMPK, AMP-activated protein kinase

ANOVA, analysis of variance

ARE, antioxidant response element

ATP, adenosine triphosphate

BCA, bicinchoninic acid

BMI, body mass index

BSA, bovine serum albumin

Ca^{2+}, calcium ion

CaMKII, calcium/calmodulin-dependent protein kinase II

CK, creatine kinase

Cr, creatine

DEXA, dual-energy x-ray absorptiometry

DNA, deoxyribonucleic acid

ERK, extracellular-signal-regulated kinase

F_IO_2, inspired oxygen fraction

GPx, glutathione peroxidase

GR, glutathione reductase

GSH, reduced glutathione

GSSG, oxidised glutathione

H^+, hydrogen ion

H_2O_2, hydrogen Peroxide

HR, heart rate

HRmax, maximal heart rate

Hyp, hypoxia

IE, incremental exercise to exhaustion

IKK, IκB kinase

IκB, inhibitor of nuclear factor κappa B

kDa, kilodalton

Keap1, Kelch-like ECH-associated protein 1

MAPK, mitogen activated protein kinase

mRNA, messenger ribonucleic acid

NAD(P)H or NOX, nicotinamide adenine dinucleotide phosphate

$NADH^{+}$, nicotinamide adenine dinucleotide reduced

NF-κB, nuclear factor κappa-light-chain-enhancer of activated B cells

$Nrf2^{-/-}$, Nrf2-null mice

Nrf2, nuclear factor erythroid-derived 2-like 2

Nx, normoxia

Oxidization: the transfer of a negatively charged electron from one organic compound to another organic compound or to oxygen.

p105, p105 subunit of NF-κB

p38 MAPK, p38 mitogen-activated protein kinases

p50, p50 subunit of NF-κB

p62/SQSTM1, Sequestosome 1

p65, p65 (RelA) subunit of NF-κB

PCr, phosphocreatine

Phosphorylation, a biochemical process that involves the addition of phosphate to an organic compound.

Pi, inorganic phosphate

P_IO_2, partial pressure of inspired O_2

PO_2, oxygen pressure

PVDF, polyvinylidene fluoride

Redox reaction, a process in which one molecule is reduced and another is oxidized.

Reduction, a half-reaction in which a chemical species decreases its oxidation number, usually by gaining electrons.

RNS, reactive nitrogen species

RONS, reactive oxygen and nitrogen species

ROS, reactive oxygen species

Ser, serine

SOD, superoxide dismutase

Thr, threonine

Trx1, Thioredoxin 1

TrxR1, Thioredoxin Reductase 1

Tyr, tyrosine

VO_2, oxygen uptake

VO_2max, maximal O_2 uptake

VO_2peak, peak O_2 uptake

Wmax, maximal power output at exhaustion during the incremental exercise

ABSTRACT

3. ABSTRACT

3.1. Abstract of the thesis

Introduction: unaccustomed, prolonged, and exhaustive exercise can generate excess production of reactive oxygen and nitrogen species (RONS), which, accompanied by an insufficient antioxidant response, cause oxidative stress, triggering tissue damage, impaired muscle contractility and even muscle degenerative pathologies. RONS have traditionally been considered harmful for causing oxidative damage; however, recent discoveries have shown their importance as essential modulators for proper cell function by regulating signalling pathways essential for skeletal muscle adaptation. Redox-sensitive Nrf2 and NF-κB signalling pathways are activated by extracellular signals and intracellular changes, regulating more than 150 genes involved in inflammation, antioxidant response and muscle metabolism. Although their activation is required for the adaptive response to exercise *in vitro* or in rodents, whether Nrf2 and NF-κB signalling pathways are activated during exercise in human skeletal muscle remains unknown. The application of hypoxia could affect the activation of these transcription factors since the low oxygen levels characteristic of hypoxia seem to alter the antioxidant response and the production of RONS. Furthermore, the instantaneous application of complete ischaemia after exercise could prevent early recovery and help the study of exercise-induced redox changes due to the short in vivo duration of RONS and the rapid recovery of energy metabolism at the end of exercise.

Objectives: the main objective of this study was to determine the regulation of Nrf2 and NF-κB by acute exercise in human skeletal muscle, and the role of muscle oxygenation and metabolite accumulation in this process. Another objective was to determine the time

course of Nrf2 and NF-κB signalling during early recovery and to determine whether these signalling pathways were activated by the application of post-exercise ischaemia.

Hypothesis: we hypothesized that acute exercise would activate Nrf2 and NF-κB signalling, more markedly during exercise in severe acute hypoxia and during post-exercise ischaemia. This activation would be accompanied by an increase in antioxidant enzymes. Furthermore, we hypothesized that these changes would return to pre-exercise levels one minute after exercise completion when the muscles recover without circulatory occlusion.

Methods: eleven men performed incremental exercise to exhaustion (IE) in normoxia (P_IO_2: 143 mmHg) and hypoxia (P_IO_2: 73 mmHg). After IE, circulation to one leg was instantly occluded (300 mmHg). Muscle biopsies of m. vastus lateralis were taken before (Pre), and 10s (from the occluded leg) and 60s after the exercise simultaneously from both legs, the occluded and the leg with free circulation.

Results: Nrf2 and NF-κB signalling pathways were activated by exercise to exhaustion with similar responses in normoxia and severe acute hypoxia. CaMKII and AMPKα phosphorylation were similarly induced in both conditions. Enhanced Nrf2 signalling was achieved by elevating total Nrf2 protein and Ser^{40} Nrf2 phosphorylation, accompanied by a reduction in Keap1. Keap1 protein degradation was facilitated by Ser^{349} phosphorylation of p62/SQSTM1. NF-κB activation was reflected in increased levels of p105, p50, IKKα, IκBβ, and GR, and the activation of the main kinases involved, particularly $pSer^{176/180}$ IKKα/ß and CaMKII δD, while IKKβ was not affected. The ratio of Nrf2 to Keap1 was markedly elevated and was closely associated with a 2-fold increase in catalase. While SOD2 did not change significantly during exercise or ischaemia, SOD1 protein expression was slightly down- and up-regulated during exercise in normoxia and

hypoxia, respectively. Trx1 expression was reduced immediately after IE and after 1 min of occlusion, while Gpx1 and TrxR1 expression levels were unaffected. Post-exercise ischaemia maintained most of the changes by preventing muscle reoxygenation. Changes were rapidly reversed at the end of exercise when the muscles recovered with free circulation.

Conclusions: Nrf2 signalling is increased after incremental exercise to exhaustion to a similar degree in normoxia and severe acute hypoxia. This increase seems to occur through a mechanism related to the decrease in the amount of Keap1 protein. At the same time, this thesis shows a strong activation of NF-κB signalling with exercise to exhaustion that, similar to Nrf2 activation, is not magnified by severe acute hypoxia and remains stimulated by the application of post-exercise ischaemia. Exercise-induced activation of the Nrf2 and NF-κB signalling pathways seems to regulate the expression levels of the antioxidant enzymes catalase and GR in human skeletal muscle. Interestingly, these changes are reversed in less than 60 seconds by an O_2-dependent mechanism, as suggested by a rapid return to pre-exercise levels as muscles recover with free circulation. These findings show the importance of obtaining muscle biopsies as close as possible to the end of exercise, and the usefulness of applying post-exercise ischaemia to capture these rapid response signals. Finally, these results indicate that a delay of as little as one minute in obtaining muscle biopsies can significantly affect the results and interpretation of exercise-induced activation of these signalling pathways in human skeletal muscle.

3.2. Abstract of Article 1

The Nrf2 transcription factor is induced by reactive oxygen and nitrogen species and is necessary for the adaptive response to exercise in mice. It remains unknown whether Nrf2 signalling is activated by exercise in human skeletal muscle. Here we show that Nrf2 signalling is activated by exercise to exhaustion with similar responses in normoxia (P_iO_2: 143 mmHg) and severe acute hypoxia (P_iO_2: 73 mmHg). CaMKII and AMPKα phosphorylation were similarly induced in both conditions. Enhanced Nrf2 signalling was achieved by raising Nrf2 total protein and Ser^{40} Nrf2 phosphorylation, accompanied by a reduction of Keap1. Keap1 protein degradation is facilitated by the phosphorylation of p62/SQSTM1 in Ser^{349} by AMPK, which targets Keap1 for autophagic degradation. Consequently, the Nrf2-to-Keap1 ratio was markedly elevated and closely associated with a 2-3-fold increase in Catalase protein. No relationship was observed between Nrf2 signalling and SOD1 and SOD2 protein levels. Application of ischaemia immediately at the end of exercise maintained these changes, which were reverted within one minute of recovery with free circulation. While SOD2 did not change significantly during either exercise or ischaemia, SOD1 protein expression was marginally downregulated and upregulated during exercise in normoxia and hypoxia, respectively. We conclude that Nrf2/Keap1/Catalase pathway is rapidly regulated during exercise and recovery in human skeletal muscle. Catalase emerges as an essential antioxidant enzyme acutely upregulated during exercise and ischaemia. Post-exercise ischaemia maintains Nrf2 signalling at the level reached at exhaustion and can be used to avoid early post-exercise recovery, which is O_2-dependent.

3.3. Abstract of Article 2

The NF-κB signalling pathway plays a critical role in inflammation, immunity, cell proliferation, apoptosis, and muscle metabolism. NF-κB is activated by extracellular signals and intracellular changes in Ca^{2+}, Pi, H^{+}, metabolites and reactive oxygen and nitrogen species (RONS). However, it remains unknown how NF-κB signalling is activated during exercise and how metabolite accumulation and PO_2 influence this process. Eleven active men performed incremental exercise to exhaustion (IE) in normoxia and hypoxia (P_IO_2: 73 mmHg). Immediately after IE, the circulation of one leg was instantaneously occluded (300 mmHg). Muscle biopsies from m. *vastus lateralis* were taken before (Pre), and 10s (Post, occluded leg) and 60s after exercise from the occluded (Oc1m) and free circulation (FC1m) legs simultaneously together with femoral vein blood samples. NF-κB signalling was activated by exercise to exhaustion, with similar responses in normoxia and acute hypoxia, as reflected by the increase of p105, p50, IKKα, IκBβ and glutathione reductase (GR) protein levels, and the activation of the main kinases implicated, particularly IKKα and CaMKII δD, while IKKβ remained unchanged. Postexercise ischaemia maintained and stimulated further NF-κB signalling by impeding muscle reoxygenation. These changes were quickly reverted at the end of exercise when the muscles recovered with open circulation. Finally, we have shown that Thioredoxin 1 (Trx1) protein expression was reduced immediately after IE and after 1 min of occlusion while the protein expression levels of glutathione peroxidase 1 (Gpx1) and thioredoxin reductase 1 (TrxR1) remained unchanged. These novel data demonstrate that exercising to exhaustion activates NF-κB signalling in human skeletal muscle and regulates the expression levels of antioxidant enzymes in human skeletal muscle. The fast regulation of NF-κB at exercise cessation has implications for the interpretation of published studies and the design of new experiments.

RESUMEN

4. RESUMEN (SUMMARY IN SPANISH)

Introducción: el estrés oxidativo ha sido redefinido como un término utilizado para describir el estado prooxidante causado por el desequilibrio celular entre la producción de oxidantes y las propiedades antioxidantes. El ejercicio excesivamente prolongado y/o exhaustivo, especialmente practicado sin un periodo de adaptación, pueden generar un exceso de especies reactivas de oxígeno y nitrógeno (colectivamente llamadas RONS). Acompañadas por una insuficiente respuesta antioxidante, RONS podrían llegar a provocar estrés oxidativo, y, por consiguiente, daño oxidativo. De hecho, el estrés oxidativo severo puede conllevar la muerte celular, al inducir modificaciones en componentes celulares como el ADN, los lípidos y las proteínas. Además, el estrés oxidativo favorece el deterioro de la función contráctil muscular, e incluso promueve patologías musculares degenerativas. En el músculo esquelético sano, RONS son constantemente producidas y contrarrestadas, manteniendo el equilibrio redox. Aunque la principal fuente de producción de radicales libres en el músculo esquelético durante el ejercicio continúa siendo materia de estudio, varios candidatos han sido identificados: cadena de transporte de electrones mitocondrial, NAD(P)H-oxidasa, xantina oxidasa, óxido nítrico sintasa, entre otros.

Experimentos in vitro sugieren que la actividad contráctil muscular durante el ejercicio podría aumentar hasta 3 veces los niveles intracelulares de RONS. Los principales radicales libres producidos por el músculo esquelético son el óxido nítrico y el superóxido, cuya transformación química puede producir entre otras especies reactivas, el radical hidroxilo, el peróxido de hidrógeno o el peroxinitrito. A pesar de que la medición directa de RONS en experimentos in vivo es todavía compleja debido a su alta reactividad y su corta vida media, la evaluación de marcadores de estrés oxidativo (p. ej.,

peroxidación lipídica, niveles de antioxidantes o activación de proteínas redox-dependientes) permite estimar y evaluar cambios en el estado redox.

Tradicionalmente RONS han sido considerados nocivos por ser causantes del daño oxidativo, sin embargo, recientes descubrimientos han evidenciado su importancia como moduladores para la correcta funcionalidad celular. Por ejemplo, cierto grado de RONS en el músculo esquelético es necesario para una adecuada producción de fuerza muscular, mientras que el desequilibrio redox conduce a una pérdida de producción de fuerza asociada a la fatiga. Además del estrés oxidativo, tanto factores metabólicos como mecánicos podrían dañar el músculo esquelético debido a un entrenamiento excesivamente intenso y/o prolongado. Consecuentemente, los niveles de metabolitos del músculo esquelético (p. ej., creatina quinasa o lactato deshidrogenasa) junto con los marcadores del estado redox (p. ej., glutatión reductasa o catalasa) son comúnmente examinados como indicadores del estado funcional de tejido muscular. Además, RONS actúan como inductores que modulan vías de señalización esenciales para el correcto funcionamiento y adaptación celular.

Dos de los principales factores de transcripción implicados en la regulación de la expresión génica mediada por RONS son Nrf2 y NF-κB. Nrf2 se acumula y se traslada al núcleo, donde se une a los elementos de respuesta antioxidante para regular la transcripción de más de 250 genes implicados en la respuesta antioxidante, el metabolismo, y la inflamación. Por su parte, NF-κB regula más de 150 genes implicados en la inflamación, la respuesta antioxidante, la apoptosis y el metabolismo muscular. Ambas vías de señalización, Nrf2 y NF-κB, son sensibles al estado redox celular y se activan tanto mediante señales extracelulares como intracelulares. Aunque la activación de estos factores de transcripción parece ser necesaria para la respuesta adaptativa al ejercicio, aún no se ha confirmado que estas vías de señalización se activan en el músculo

esquelético humano durante el ejercicio, y se desconoce cómo la acumulación de metabolitos y la PO_2 podrían influir en su activación. Teniendo en consideración que experimentos *in vitro* y/o en animales sugieren que las vías de señalización dependiente de Nrf2 o NF-κB podrían activarse en respuesta a las alteraciones redox provocadas por el ejercicio, la utilización de un protocolo de ejercicio hasta el agotamiento que alcance el consumo máximo de oxígeno (VO_2max), provocando una activación marcada de la glucólisis y posiblemente un alto grado de estrés oxidativo, parece un modelo óptimo para el estudio de estos cambios redox en el musculo esquelético humano. Además, la aplicación de hipoxia severa aguda podría afectar la activación de estos factores de transcripción, puesto que los bajos niveles de oxígeno característicos de la hipoxia parecen alterar la respuesta antioxidante y la producción de RONS. Asimismo, debido a la corta duración *in vivo* de RONS y la rápida recuperación del metabolismo energético al final del ejercicio, la aplicación instantánea de isquemia completa posteriormente al ejercicio podría prevenir la recuperación temprana y ayudar al estudio de los cambios redox inducidos por el ejercicio. Para el modelo experimental propuesto, se utilizaría un manguito neumático para ocluir la circulación en una sola pierna, usando la pierna contralateral como control.

Objetivos: el propósito principal de este estudio fue determinar la regulación de Nrf2 y NF-κB por el ejercicio agudo en el músculo esquelético humano, y el papel que tiene la oxigenación muscular y la acumulación de metabolitos en este proceso. El siguiente objetivo era determinar el curso temporal de la señalización de Nrf2 y NF-κB durante la recuperación temprana, y determinar si estas vías de señalización permanecen activadas por la aplicación de isquemia posterior al ejercicio.

Hipótesis: hipotetizamos que el ejercicio agudo activaría la señalización de Nrf2 y de NF-κB, más intensamente durante el ejercicio en hipoxia aguda severa y durante la

isquemia posterior al ejercicio. Esta activación estaría acompañada por el aumento de las enzimas antioxidantes. Además, planteamos la hipótesis de que estos cambios volverían a los niveles previos al ejercicio dentro del minuto posterior a la finalización del ejercicio cuando los músculos se recuperan sin oclusión de la circulación.

Métodos: después de la realización de pretest y familiarizaciones, once hombres realizaron ejercicio incremental hasta el agotamiento (IE) en normoxia (F_IO_2: 143 mmHg) e hipoxia aguda severa (F_IO_2: 73 mmHg). Después del IE, la circulación de una pierna se ocluyó instantáneamente (300 mmHg). Biopsias musculares del m. vastus lateralis se tomaron antes (Pre), 10s después del ejercicio de la pierna ocluida, y 60s después del ejercicio simultáneamente tanto de la pierna ocluida como de la que tenía libre circulación. Los niveles de expresión de las proteínas estudiadas y sus fosforilaciones reguladoras se analizaron mediante Western Blot. El análisis estadístico fue principalmente realizado con SPSS ($p < 0.05$).

Resultados: las vías de señalización de Nrf2 y NF-κB fueron activadas por el ejercicio hasta el agotamiento similarmente en normoxia e hipoxia aguda severa. De igual manera, la fosforilación de CaMKII y AMPKα se indujo también en ambas condiciones. El aumento en la señalización de Nrf2 se logró elevando la proteína Nrf2 total y la fosforilación en Ser^{40} de Nrf2, acompañadas de una reducción en Keap1. La degradación de la proteína Keap1 fue facilitada por la fosforilación en Ser^{349} de p62/SQSTM1. La activación de NF-κB se reflejó en niveles aumentados de p105, p50, IKKα, IκBβ y GR, y la activación de las principales quinasas involucradas, particularmente la fosforilación en $Ser^{176/180}$ de IKKα/ß y CaMKII δD, mientras que IKKβ no cambió significativamente. La ratio de Nrf2/Keap1 aumentó notablemente y estuvo estrechamente asociada con el aumento en 2 veces de Catalasa. Aunque SOD2 no cambió significativamente durante el ejercicio o la isquemia, la expresión de la proteína SOD1 se reguló ligeramente a la baja

y al alza durante el ejercicio en normoxia e hipoxia, respectivamente. La expresión de Trx1 se redujo inmediatamente después del IE y después de 1 min de oclusión, mientras que los niveles de expresión de Gpx1 y TrxR1 no se vieron afectados por la intervención. La isquemia posterior al ejercicio mantuvo la mayoría de los cambios inducidos por el ejercicio al impedir la reoxigenación muscular. Estos cambios se revirtieron rápidamente al final del ejercicio cuando los músculos se recuperaron con libre circulación.

Conclusiones: la señalización de Nrf2 aumenta después del ejercicio incremental hasta el agotamiento en un grado similar en normoxia y en hipoxia aguda severa. Este aumento parece realizarse a través de un mecanismo relacionado con la disminución de la cantidad de proteína Keap1. Al mismo tiempo, esta investigación muestra una fuerte activación de la señalización de NF-κB con el ejercicio hasta el agotamiento que, de manera similar a Nrf2, no se magnifica con la hipoxia aguda severa y permanece estimulada por la isquemia. La activación inducida por el ejercicio de las vías de señalización de Nrf2 y NF-κB parece regular los niveles de expresión de las enzimas antioxidantes Catalasa y GR en el músculo esquelético humano. Curiosamente, estos cambios se revierten en menos de 60 segundos por un mecanismo dependiente de O_2, puesto que vuelven rápidamente a niveles previos al ejercicio cuando los músculos se recuperan con libre circulación. Estos hallazgos muestran la importancia de obtener las biopsias musculares lo más cerca posible de la finalización del ejercicio, y la utilidad de la aplicación de isquemia posterior al ejercicio para captar estas señales de respuesta rápida. Finalmente, se debe considerar que un retraso de tan solo un minuto en la obtención de biopsias musculares puede afectar significativamente a los resultados y la interpretación de la activación inducida por el ejercicio en estas vías de señalización en el músculo esquelético humano.

INTRODUCTION

5. INTRODUCTION

5.1. General introduction

5.1.1. Reactive Oxygen and Nitrogen Species.

Atoms or molecules that contain one or more unpaired electrons that are capable of existing independently are known as "free radicals". However, there are non-radical reactive derivatives of oxygen and nitrogen molecules that can easily cause free radical reactions in living organisms. Reactive oxygen species (ROS) is a general term that refers as a group to oxygen-centred radicals (such as superoxide (O_2^-) and hydroxyl (HO^-)) and non-radical but reactive derivatives of oxygen (such as hydrogen peroxide (H_2O_2), singlet oxygen ($^-O_2$) and ozone (O_3)). Similarly, reactive nitrogen species (RNS) are nitrogen radicals (such as nitric oxide (NO^-) and nitrogen dioxide (NO_2^-)) and non-radical reactive nitrogen molecules (such as peroxynitrite ($ONOO^-$), nitrous acid (HNO_2) and dinitrogen trioxide (N_2O_3)).

Collectively, ROS and RNS are known as reactive oxygen and nitrogen species (RONS). RONS arise from chain reactions that include three steps: initiation, propagation, and termination. After oxidative signal initiation, primary free radicals (O_2^- and NO^-) tend to "steal" a surrounding electron, triggering signal propagation through the formation of secondary RONS. After that, newly formed RONS seek to steal electrons from cellular structures or molecules. Cellular antioxidants are a group of molecules capable of slowing or terminating the oxidation of other molecules. Therefore, antioxidants are oxidized to prevent cell damage, stopping these chain reactions by removing RONS intermediates and preventing subsequent oxidation reactions.

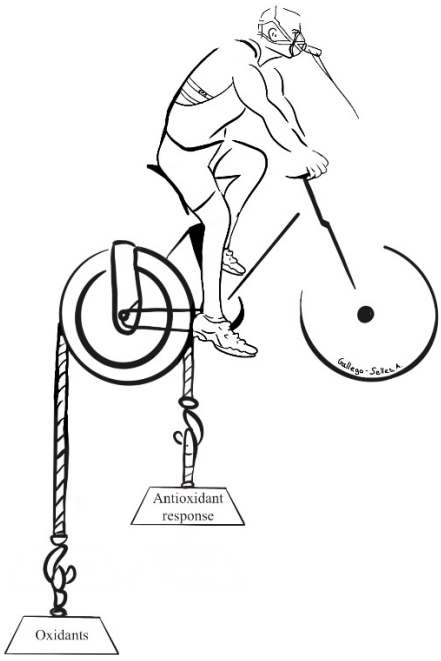
5.1.2. Cellular consequences of oxidative stress.

Incidents during World War II exhibited the pathological effects (mutations and diseases) on humans exposed to prominent levels of radiation. Consequently, a new biological field of research focused on free radicals and cellular redox balance began to develop. In 1954, Gerschman et al. (2) asserted that cell damage caused by exposure to ionizing radiation was due to free radicals, while Commoner et al. (3) showed that free radicals are present in living organisms. The same year, *free radical theory of aging* stated that organisms age due to the accumulation of free radical damage over time (4). Expansion of this idea in the 1970s led to the discovery of mitochondrial damage caused by free radicals (5) while the identification of superoxide dismutase enzyme (specifically SOD1) evidenced the functioning of antioxidant enzymes against free radicals in mammals (6). In the late 1970s, consideration of free radicals shifted towards direct modifiers of enzyme activation and necessary inducers of signalling pathways (7).

Controlled amounts of RONS are required for normal cellular function, acting as regulatory signalling mediators in physiological processes. However, overproduction of RONS accompanied by insufficient antioxidant capacity leads to a damaging oxidative environment called oxidative or nitrosative stress. As shown in Supplementary Fig. 1, oxidative stress has been redefined as a term used to describe the pro-oxidant state caused by the imbalance between the production of oxidants and the antioxidant properties (8). The concept of oxidative stress involves different aspects, such as overproduction of RONS, inadequate antioxidant response, alteration of cellular redox balance, and oxidative damage of cellular components. Different classifications of the degree of oxidative stress have been proposed depending on the redox state of the cell, for example, basal, low-intensity, intermediate-intensity, and high-intensity oxidative stress (9).

Adaptive cellular response against oxidative stress restores reduction-oxidation (redox) homeostasis. Since the cellular redox state is determined not only by the balance between the rate of RONS production but also by the rate of RONS removal by antioxidants, a complex unit of RONS-regulating enzyme antioxidants (SOD, catalase, GPx) and antioxidants non-enzymatic (vitamin C, E, carotenoids, etc.) are strategically compartmentalized in the cell to protect from oxidative damage during periods of increased production of reactive radicals (e.g. muscle fibers during extremely intense or prolonged exercise) (10). The extremely high reactivity of RONS implies that they only diffuse over short distances, reacting with molecules close to their site of production (11).

Oxidative stress can result in severe cell oxidative damage, producing cell death and leading to tissue injury (12) by modifying cellular components such as DNA (13), lipid (14), and proteins (15). In fact, strong evidence suggests that oxidative stress biomarkers are associated with the primary or secondary pathophysiological mechanisms of multiple acute and chronic human diseases (16).



Supplementary Figure 1. Schematic representation of oxidative stress, defined as the pro-oxidant state caused by the imbalance between the production of oxidants and the antioxidant properties (8).

5.1.3. Oxidative stress effect on gene expression.

The intracellular redox state plays an essential role in the regulation of gene expression. Oxidative stress can cause damage to genetic information due to increased DNA base degradation, DNA-strand breaks, DNA binding modifications, and DNA proteins cross-linking (17). Mitochondrial DNA may be especially susceptible to RONS-induced damage due to its location close to the electron transport chain (18). Changes in the base composition of DNA binding sites for some transcription factor disrupt transcription factor binding and thus the expression of related genes (19). Regulated levels of RONS function as physiological regulators of gene expression mediated through specific redox-sensitive signal as transcription factors, such as Nrf2 and NF-κB signalling pathways (20). The regulation of signalling pathways sensitive to redox changes is studied to formulate hypotheses about the cellular redox state, for example, assessing the activation of Nrf2 likely induced against exercise-induced oxidative stress in human skeletal muscle.

5.1.4. Oxidative stress mediated lipid damage and protein modifications.

The structure of the lipid bilayer present in all biological membranes is composed of lipids and proteins. Modifications in the biological properties of the membrane are triggered by oxidative stress-induced peroxidation of membrane lipids (21), which disrupts normal membrane function by propagating oxidative damage by creating pores in the membrane and inactivating membrane-bound proteins. This modification alters the permeability and fluidity of the membrane (14, 22), leading to the immediate depolarization of the membrane potential, swelling of the matrix, rupture of the external mitochondrial membrane, which implies the release of pro-apoptotic molecules to the cytosol (23). Therefore, lipid peroxidation products, such as 4-hydroxy-2-nonenal (HNE), are investigated as secondary biomarkers of cellular oxidative damage (24).

Comparably, RONS induce oxidation of protein amino acids, cause breakage of peptide chains, modify electrical charge, increase proteolysis through specific proteases, cause loss of protein function and alter protein structure (25). RONS-induced oxidative damage to amino acids produces protein carbonyl groups, considered a marker of RONS-mediated protein oxidation (26). Furthermore, RONS can induce post-translational modifications, these are reversible and non-reversible and could change the function, localization, or stability of various proteins. Protein phosphorylation is the most studied post-translational modification, as one-third of mammalian proteins can be phosphorylated, often modulating protein function.

In short, oxidative stress compromises cellular integrity and viability through reacting with intracellular macromolecules and generating oxidative stress biomarkers. Excessive oxidative stress leads to dysregulation of DNA cross-linking (18), attack double bonds of phospholipids in cell membranes (14), and impair protein functions (27). In addition, oxidative stress triggers the deterioration of muscle contractility and even degenerative muscle pathologies.

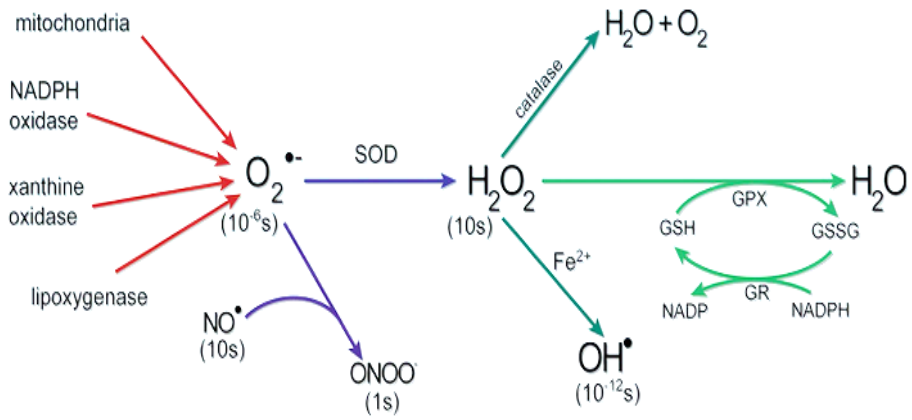
5.1.5. Free radical production in skeletal muscle.

In skeletal muscle, RONS are continuously produced and counteracted in several subcellular compartments both at rest and during muscle contractions of exercise (28). Periods of muscle contractile activity during exercise increase intracellular RONS levels by 1- to 3-fold (29). The main free radicals produced by skeletal muscle are nitric oxide (NO^-) and superoxide (O_2^-) (29) (see supplementary Fig. 2).

Nitric oxide synthase (NOS) regulates the conversion of the amino acid L-arginine to citrulline using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor (30, 31), producing a free radical known as NO^- (32). The NO^- radical generates highly

reactive species (such as peroxynitrite) when combined with O_2^- (33). Although iNOS isoform is expressed in skeletal muscle, nNOS isoform is considered the major source of NO release, especially in fast-twitch muscle fibers (34). Increased intracellular calcium concentration during muscle contraction facilitates phosphorylation of Ca/calmodulin-dependent protein kinase II (CaMKII), an interactive protein identified from nNOS transcription in neuronal cells (35). In fact, higher nNOS protein content has been detected as an adaptation in skeletal muscle to intense period of training (36).

Considered the most potent oxidant generated from the one-electron reduction of O_2, the negatively charged free radical superoxide arises through incomplete reduction of O_2 in the electron transport chain or as a product of enzymatic reactions. The O_2^- radical can be either protonated to produce a hydroperoxyl radical (HO^-) or depleted by a dismutation reaction to produce hydrogen peroxide (H_2O_2) (29). Moreover, O_2^-, release ferrous iron by damaging aconitase and dismutates to form hydrogen peroxide (H_2O_2) (37, 38), which is ultimately converted into a water molecule H_2O and a molecular oxygen (O_2). H_2O_2 may reacts either with ferrous iron to form hydroxyl (HO^-) or with nitric oxide (NO^-) to form peroxynitrite (39), contributing to the increase of RONS mitochondrial and the cytosolic cellular locations (40).



Supplementary Figure 2. The major reactive oxygen species in muscle. Numbers in brackets indicate approximate lifetimes of various species. GPX, glutathione peroxidase. Extracted from Allen et al. (2007) (41).

5.1.6. RONS production during exercise in skeletal muscle.

Although the main source of free radicals production in skeletal muscle remains unclear (29), several sources of RONS have been proposed (10, 42): mitochondrial electron transport chain (mETC) (43), nicotinamide adenine dinucleotide phosphate oxidase (NAD(P)H-oxidase or NOX) (44, 45), xanthine oxidase (XO) (46), nitric oxide synthase (NOS). To a lesser extent, other additional sources have also been considered for the production of RONS during exercise (47, 48), such as the loss of cytochrome oxidase activity induced by high temperatures (49), exercise-induced activation of phospholipase A(2) isoform (50) and exercise-produced catecholamine metabolites (51). However, identifying RONS sources during exercise is challenging as RONS formation can further stimulate RONS production (RONS-induced release of RONS) (52).

I. Mitochondrial RONS production.

According to the endosymbiotic theory, mitochondria evolved by endosymbiosis from a bacterial parent within a eukaryotic host cell millions of years ago (53). Mitochondria are organelles necessary for cellular metabolism that generate energy stored in an energized molecule called adenosine triphosphate (ATP) through biochemical reactions through the process called oxidative phosphorylation system (OXPHOS). Although considered the "powerhouse" of the cell, mitochondria are also involved in different cellular processes, including cell cycle control, innate immunity, autophagy, redox balance, and calcium homeostasis (54, 55). This organelle is continually remodelled by opposing but balanced processes called mitochondrial dynamics, changing its morphology, function, and location in response to different physiological stimuli (56). Pathological alterations in mitochondrial dynamics are associated with neurodegenerative diseases, cardiomyopathies, cancer and inflammatory diseases (56, 57).

Oxygen is used as an electron acceptor and reduced to form H_2O during mETC (between complexes I and IV). H^+ are released especially in complexes I and III through the inner mitochondrial membrane, creating an electrochemical gradient of H^+, called the proton motive force (58). According to Mitchel's chemiosmotic theory (59), the proton motive force is used by ATP synthase (or mitochondrial complex V) to transfer H^+ back into the mitochondrial matrix, resulting in the release of energy used to convert phosphorylated adenosine 5'-diphosphate (ADP) and inorganic phosphate into adenosine 5'-triphosphate (ATP). The increase of RONS production during exercise has traditionally been considered as a side-effect of the increased oxidative metabolism in mitochondria.

Although the formation and removal of RONS are well adjusted under normal conditions, the rapid electron transfer during mETC can lead to the production of free radicals due to electron leakage (60). Although complexes I and III have classically been considered the main mitochondrial sources of O_2^- (61), the RONS-generating contributions of additional mitochondrial sites (such as monoamine oxidase, aconitase, and $p66^{shc}$) in vivo and during exercise are still doubtful (52, 62). In contrast to previous hypotheses, isolated skeletal muscle mitochondria experiments simulating physiological conditions at rest, and low and intense aerobic exercise intensities indicated low mitochondria RONS production (62). In fact, only between 0.15% and 5% of the O_2 consumed by the mitochondria forms O_2^-, depending on the mitochondrial isolation technique applied (63, 64). Interestingly, the relative contributions of different mitochondrial sites of RONS production may change depending on cellular bioenergetic condition (65). Although OXPHOS provides energy during aerobic exercise, mitochondrial superoxide supply may represent less during exercise than at rest in skeletal muscle, since mitochondrial RONS production in state 3 (ADP-stimulated) is lower than in state 4 (baseline) (49). Finally, the release of O_2^- is regulated by the $NADH/NAD^+$ ratio

(61), which decreases along with the proton motive force during situations of high ATP demand, such as muscle contraction during exercise (66).

II. NOX activity in skeletal muscle.

Although mitochondria were one of the first identified sources of free radicals in the cell, additional sources must be considered during exercise in skeletal muscle. Measurements of relative free radical concentrations in skeletal muscle fibers showed predominant non-mitochondrial sources in RONS production, emphasizing the importance of NOX and XO enzymes (28). Indeed, experiments of muscle fibers stimulated by mimicking muscle contraction increased cytosolic RONS production without altering mitochondrial redox status (67). Interestingly, administration of NOX inhibitor abolished RONS production, targeting this enzyme as an important source of RONS during skeletal muscle contraction (67). These findings are consistent with increased cytosolic O_2^- production during contractile activity associated with stimulation of NOX activity in muscle fibers (68). Although there is only indirect evidence in skeletal muscle, cellular redox status may mediate crosstalk between NOX and mitochondria that modifies RONS production (69).

Skeletal muscle NOX family members are essential modulators of redox homeostasis that using either NADH or NADPH as electron donors (44, 45). Therefore, the term "NAD(P)H oxidase" is used for the expression of NOX isoforms in skeletal muscle. As has been demonstrated in several tissues, NOX is an enzyme system of multiple isoforms that reduces O_2 and generates O_2^- and H_2O_2 (70, 71). NOX isoforms are distributed in the sarcolemma, the transverse tubules, the sarcoplasmic reticulum, and even in the mitochondria (28). NOX2 and NOX4 are considered by some authors as the main sources of RONS during exercise (29), which are located in the sarcoplasmic reticulum, the transverse tubules and the plasma membrane (10) likely depending on the

muscle fibers type (72). Rapidly activated by various stimuli (e.g., mechanical forces, hypoxic environment, and cytokines), regulation of NOX activity is essential to maintain adequate levels of RONS (73). Moreover, crosstalk between NOX and XO has been observed since NOX inhibition prevented XO activation in pig heart muscle (74).

III. XO-mediated RONS production.

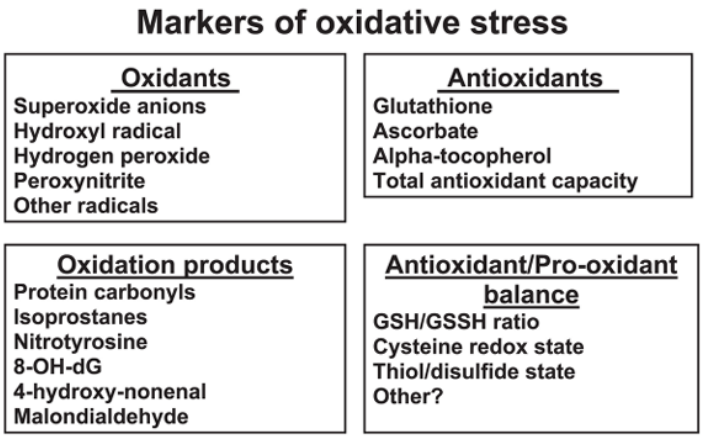
The enzyme XO catalyses in the presence of O_2 the oxidation of hypoxanthine to xanthine and of xanthine to uric acid during purine catabolism and generates O_2^- and H_2O_2 (75). Intense exercise may increase XO activity by breaking down ATP and triggers XO-induced activations of skeletal muscle signalling pathways (76). Application of XO inhibitors (77) or decreased XO-mediated oxidative stress (78) prevents exercise-induced activation of redox-sensitive MAPK and NF-κB signalling pathways. However, further research is required to determine the role XO plays in exercise induced RONS production.

5.1.7. RONS: direct and indirect measures.

Unfortunately, direct measurement of RONS sources represent a great challenge due to their high reactivity and short half-life. Therefore, human evidence is limited by the lack of identified biomarkers, available measuring techniques or potential tissue auto-oxidation during sample handling (10). Novel techniques such as fluorescent probes or electron spin resonance (ESR) spectroscopy allow obtaining structural information and kinetic information on the formation and decline of generated reactive radicals (8). ESR accurately detects the presence of unpaired electrons, but the accumulation of reactive species is too low to be measured under in vivo conditions. Although the use of these techniques is unsafe in humans since it interferes with the biological system under investigation, there are animal experiments that add "trap" agents to react with reactive radicals and generate stable radicals that can be detected. However, increased RONS production in isolated measurements does not necessarily lead to oxidative stress.

Therefore, indirect measurement of RONS overproduction (such as decreased antioxidant agents or increased oxidatively modified molecules) or cellular redox balance (e.g., GSH/GSSH ratio) are used to overcome these difficulties and assess cellular oxidative stress (See supplementary Fig. 3).

Since metabolic and mechanical factors can damage skeletal muscle as a result of intense and prolonged training, skeletal muscle metabolites (e.g., creatine kinase or lactate dehydrogenase) and markers of oxidative stress (e.g., superoxide dismutase or catalase) are collectively analysed as indicators of the state of muscle tissue (101). Finally, to avoid the individual limitations of each type of measurement and to obtain a complete picture of skeletal muscle status, the use of more than one of these signals is recommended to provide a better estimate of skeletal muscle oxidative stress.



Supplementary Figure 3. Classes of biomarkers used to assess cellular oxidative stress in tissues. These categories include the measurement of oxidant production, cellular levels of antioxidants, oxidation products, and the antioxidant/pro-oxidant balance. 8-OH-dG, 8hydroxydeoxyguanosine; GSH/GSSG, ratio of reduced glutathione to oxidized glutathione. Extracted from Powers and Jackson (2008) (10)

5.1.8. Antioxidant administration on redox adaptations and sports performance.

The late 1970s witnessed the discovery that muscular exercise increases oxidative damage in animals (79) and humans models (80). Maintenance of RONS levels occurs through the antioxidant system, including enzymatic (i.e., catalase, superoxide dismutase

(SOD), glutathione peroxidase (GPX)) and non-enzymatic antioxidants (i.e., vitamin C, vitamin E, Glutathione, carotenoids, a-Lipoic acid, Bilirubin, acid uric, Coenzyme Q10) (81). Although RONS have essential roles in normal cell function and homeostasis, oxidative stress occurs when RONS levels exceed the ability to be neutralized by the antioxidant system. Since the 1980s, some pioneering studies have suggested that the administration of antioxidants such as vitamin E could minimize muscle damage caused by exercise (82).

Although the evidence in humans is conflicting, recent studies have indicated that supporting endogenous antioxidant systems with additional oral doses of antioxidants is a non-invasive strategy to interfere with exercise-induced changes by affecting RONS-mediated cell signalling, altering adaptations such as vasodilation, insulin signalling, mitochondrial biogenesis (83, 84). Exposure of RONS-scavenging dietary antioxidants vitamins C and E in isolated muscle fibers or intact animals muscle improves performance delaying muscle fatigue (41), but hampered redox cellular adaptations in exercised human skeletal muscles without affecting exercise performance (85, 86). Similarly, the intake of antioxidants (α-lipoic acid, vitamins C and E) decreased the glycolytic rate, mitigating the increase in the AMP/ATP ratio and the reduction in the NAD^+/$NADH.H^+$ ratio without effects on performance in hypoxia (87).

Administration of the antioxidant N-acetylcysteine (NAC) appears to increase the time to voluntary fatigue, improving human performance at submaximal intensity, although these findings are inconsistent during maximal intensity (10). In fact, supplementation with NAC antioxidants interrupts the inflammatory response of skeletal muscle probably by attenuated activation of redox-sensitive signalling pathways (88). In most studies, researchers administered a single antioxidant rather than a cocktail of antioxidants. However, our research group have observed that a polyphenols antioxidants

cocktail exert a notable ergogenic effect, increasing muscle power during fatigue, enhancing peak VO_2 and brain oxygenation (89). The effect of different isolated antioxidant supplements or cocktails on exercise-mediated adaptations requires further investigation in humans. In addition, antioxidant consumption could also improve post-exercise recovery (90), which could be induced by increased AMPK-mediated signalling after sprint exercise in human skeletal muscle (91).

As previously mentioned, a certain degree of oxidative stress could be necessary to induce the hormetic response, since RONS act as mediators for the correct functioning of the cell signalling process (84). Interestingly, this concept was stated by Hippocrates over 2.000 years ago: "If we could give each individual the proper amount of nutrition and exercise, not too little and not too much, we would find the surest path to health". Evidence has accumulated over the years indicating that redox signalling plays a role in some of the health benefits of training, however the mechanism of action remains unclear.

5.1.9. Landmark studies on RONS, exercise and skeletal muscle.

During the 1980s, the discovery that physical exercise increased oxidation biomarkers in humans (79, 80) led to the redox biology research on skeletal muscle and the concept oxidative stress was defined for the first time (92). Importantly, skeletal muscle was identified as a source of free radicals (93) capable of adapting antioxidant enzymes capacity with training (94).

Similar to previous animal findings, the contribution of reactive oxidants species to muscle fatigue during exercise in humans was reported in the 1990s (95). This was confirmed by O'Neill et al. (96) observation that HO^- produced by skeletal muscle contraction increased in a dependent manner of maximal force produced. In recent decades, research in redox biology applied to exercise has focused on reactive radicals as

molecules necessary for muscle adaptations, redefining the concept of oxidative stress (97). Overwhelming evidence has revealed that RONS play important regulatory roles in skeletal muscle for the regulation of cell signalling pathways (98), gene expression (99), and physical performance (10, 100). Therefore, physical exercise represents an optimal model to study redox biology, allowing the investigation of RONS-mediated cellular processes.

5.1.10. Incremental exercise to exhaustion and maximal oxygen consumption.

The analysis of the human cardiovascular and pulmonary response to physiological stress caused by exercise has been in constant evolution since pioneering studies more than a century ago established the use of exercise tests accompanied by blood pressure measurements to assess adequate cardiac function (101). Using this test, A. V. Hill established the relationship between oxygen consumption and load intensity, defining terms such as "maximal O_2 intake", "O_2 requirement" and "steady-state exercise" by performing spirometry measurements during incremental exercises (102). Although other criteria have been proposed to consider the plateau observed as VO_2max, the concept of a “plateau” in the VO_2/intensity relationship was defined as an increase in VO_2 of less than 150 mL/min with increasing exercise intensity (103). For this finding, Taylor et al. (1955) used a discontinuous protocol of 3 min of constant-intensity exercise bouts on successive days. Continuous exercise protocols were developed for the combined assessment of VO_2max and cardiorespiratory fitness, but differences were observed between the results obtained using continuous and discontinuous protocols (104).

Decades later, technological progress led to the improvement of continuous tests up to the subject's tolerance limit (volitional exhaustion), which highlighted the importance of this criterion for the correct performance of this test (105, 106). In addition, the duration of the continuous protocols has been considered a critical variable to achieve

real VO_2max values. One factor that determines the duration of the test is the rate at which the intensity increases over time or the slope of the ramp, usually expressed in watts/min. The classically proposed ideal selection of work rate should increase to bring subjects to their tolerance limit in approximately 10 min (106). However, a more recent review of data indicate that even durations between 5 and 26 minutes could be optimal for reaching VO_2max in healthy subjects when an adequate warm-up is performed (107). Contrary to the VO_2peak observed at a certain intensity, observing a plateau in VO_2 during incremental tests to exhaustion was classically associated with reaching VO_2max. However, difficulties in detecting plateau have been reported depending on the specific exercise protocol, population studied, subject experience, and data processing (108). Therefore, including a verification test a few minutes after finishing the incremental test has become essential to guarantee that VO_2max has been reached during this test (108).

The most widely used test to examine locomotor and cardiopulmonary fitness is the incremental exercise to exhaustion (109), accepted as the "gold standard" for determining maximal oxygen uptake (VO_2max) in humans (110). Although general international guidelines are available from the ACSM (111), accurate measurement of VO_2max requires the performance of procedures that ensure quality control of the measurements. This includes combustion tests, the use of standardized incremental exercise protocols with a verification phase preceded by an adequate familiarization test, and the correct processing and interpretation of the data (108). Determining the maximum consumption of O_2 allows knowing and guaranteeing the appropriate functioning of the person's external and internal respiration. External respiration refers to the ventilatory movement of air in and out of the lungs accompanied by the exchange of O_2 and CO_2 between the alveoli and capillaries. On the other hand, the exchange between capillary blood and active muscle of O_2 and CO_2 is known as internal respiration. In addition, this test allows

the evaluation of the circulatory system, which acts as an intermediary transporting O_2 and CO_2 in the blood in both processes. Maximal oxygen consumption (VO_2max) has predictive value for clinical outcomes and all-cause mortality.

Reaching VO_2max implies that the individual's physiological cardiopulmonary limit (also called maximal aerobic capacity) has been reached, explained by Fick's equation in 1870 as the product of the amount of blood the heart pumps in a minute, known as cardiac output (heart rate x stroke volume), and arteriovenous oxygen difference ($C_{(a-v)}$ O_2) at maximal effort during exercise. During exercise, cardiac output increases, facilitating increased O_2 consumption. Energy needs increases the extraction of O_2 in the muscles, widening the arteriovenous difference in oxygen concentration. In addition, blood flow is redistributed to active skeletal muscles from inactive tissues, increasing O_2 supply to facilitate O_2 consumption.

In short, VO_2max is the maximum amount of O_2 that the body can uptake, transport, and use in a given amount of time. The measurement of the difference between the inhaled and exhaled oxygen content allows knowing the amount of oxygen consumed in a certain time, commonly expressed in absolute (litres per minute) or relative (in relation to weight) values. In the field of sports science, VO_2max is considered the best predictor of specific sport performance in trained non-elite athletes (112). Moreover, having a high VO_2max is necessary to compete in high categories in different sports, although the ability of VO_2max to predict performance decreases in homogeneous groups of athletes (for example, elite level) (113, 114). The reduction in P_IO_2 at altitude reduces the oxygen saturation in the blood, oxygen delivery and VO_2max, affects muscle and heart metabolism and decreases sports performance (115).

5.1.11. Fatigue-induced task failure in skeletal muscle.

Exercise-related muscle contraction generates electrostatic events associated with motor neuron activation, calcium release-reuptake in muscle fibers, mechanical stress on bone, decreased energy availability in the cell and increased production of RONS (116, 117). Exercise-induced fatigue is defined as a reversible reduction in force- or power-generating capacity and has classically been elicited by "central" and/or "peripheral" mechanisms. The repeated and intense use of the muscles leads to a decrease in physical performance that is recognized as muscle fatigue, changing muscle properties such as the action potential and the accumulation of extracellular and intracellular metabolites. Although the mechanisms leading to fatigue during incremental testing to exhaustion are still being studied, fatigue increases in parallel with exercise intensity and is exacerbated when O_2 demand exceeds O_2 delivery (118), requiring a greater supply of energy from glycolysis and phosphagens. This lead to partial energy depletion, accumulation of metabolites, electrolyte alterations and augmented production of RONS (91, 119).

Classically, lactate accumulation and muscle acidification have been pointed out as the main mechanisms eliciting muscle fatigue (120) by reducing muscle ability to generate tension, depressing both aerobic and anaerobic metabolism (121). However, lactic acid has been found to have beneficial effects on the performance of fatigued rat muscles, preserving muscle excitability when muscles become depolarized (122). Human experiments suggested that the main mechanism causing task failure during incremental exercise to exhaustion is not lactate and H^+ accumulation, nor PCr and ATP depletion (117). In fact, a higher mean power output can be achieved after 60s than 10s of post-exercise ischaemia despite hypothetically worse metabolic conditions (lower PCr and pH, and higher muscle lactate).

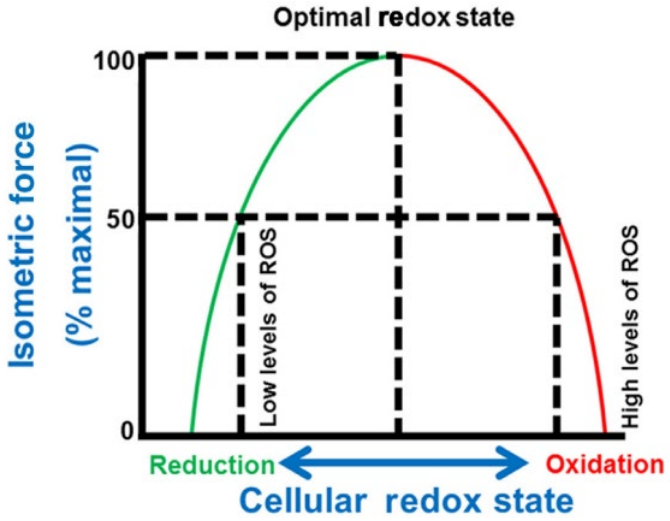
Consequently, greater importance of central versus peripheral mechanisms was demonstrated by observing that at the end of incremental exercise to exhaustion (when reaching fatigue) a functional reserve remains in the muscles to generate power, regardless of the fraction of inspired oxygen ($F_IO_{2)}$ (117). Furthermore, the increase in plasma lactate concentration after incremental exercise to exhaustion have been linked to increased systemic total oxidative status (123). Therefore, exercise-caused fatigue has been considered as a mechanism to avoid exceeding a critical threshold of peripheral muscle fatigue to safeguard organ systems from damage (124).

5.1.12. Effect of oxidative stress on muscle fatigue.

Knowledge of muscle redox status is crucial in understanding muscle fatigue during exercise. The conventional explanation of fatigue caused by the accumulation of intracellular lactate and hydrogen ions that alters contractile proteins has lost relevance due to recent findings. Recent evidence of decreased performance due to repeated or intense use of muscle fibers has increased interest in RONS-mediated adaptations. Muscle fatigue has been approached from a mechanistic point of view as a multifactorial consequence characterized by ionic changes in the action potential, calcium regulation mechanisms, and cellular adaptations induced by RONS (reviewed elsewhere (41)).

Certainly, remarkable advances have been made in understanding the effects of RONS during skeletal muscle fatigue induction on myofibrillar function and Ca^{2+} regulation (119). In fact, RONS-mediated loss of skeletal muscle function is associated with decreased Ca^{2+} sensitivity of muscle fibers (125). RONS have a major impact on force production in skeletal muscle, as an optimal redox state of skeletal muscle is necessary for adequate muscular force production, and redox imbalance leads to a loss of force production observed in muscle fatigue (126) (see supplementary Fig. 4). Force production is an example of RONS-mediated hormesis in skeletal muscle, as moderate

increases in RONS enhance force generation, while force production is decreased by excessive RONS levels or by the use of RONS scavengers. Although exhaustive physical exercise, such as exercise to exhaustion, causes oxidative stress, exercise performed at a medium-low relative intensity does not produce oxidative muscle damage (127).



Supplementary Figure 4. Cellular redox state and skeletal muscle force production. Maximal force production in skeletal muscle requires an optimal redox state. Movement away from the optimal redox state (an increase in reduction or oxidation) results in a decrease in maximal isometric force production. Extracted from Powers et al. (2020) (128).

5.1.13. Effect of hypoxia on performance and adaptations to exercise.

The use of prolonged exposure to hypoxia in sports was popularized after the observation of upregulation of the hormone erythropoietin (commonly known as EPO) that triggers the proliferation and differentiation of red blood cells, improving the performance in certain sports (129). Exposure to hypoxia promotes several adaptations, such as increased ventilation, boosted utilization of anaerobic rather than aerobic metabolism, and increases the O_2-carrying capacity of the blood (130). Reductions in the F_IO_2 reduce arterial oxygen content and impair exercise capacity and VO_2max (115). Several mechanisms explain the one-third reduction in VO_2max observed in severe acute hypoxia, namely as reduced P_IO_2, impaired pulmonary gas exchange, and reduced maximal cardiac output and maximal muscle blood flow (131). The lower VO_2max

achieved in severe hypoxia implies that the same absolute intensity is perceived as a higher relative intensity. In fact, when exercise-induced fatigue is reached the muscle activation is lower in hypoxia than in normoxia (116).

Although low oxygen levels could presumably be favourable for low RONS production, exposure to hypoxia may alter the antioxidant response and RONS-sources, leading to oxidative damage (132) and subsequent induction of the main regulators of the antioxidant response (133). The paradox of increased RONS production during hypoxia was demonstrated by applying high-precision cellular redox state assessment methods and observing accumulation of oxidation products (134). Indeed, exposure to lower oxygen levels can increase RONS production in a hypoxic dose-dependent manner (intensity and duration) (135). Enhanced hypoxia-induced oxidative stress is mediated by increased RONS production by several RONS-generating systems (mitochondrial electron transport chain, XO, and NOS) and impaired antioxidant (enzymatic and non-enzymatic) capacity (135, 136).

Severe hypoxia exacerbated 30s all-out sprint exercise-induced changes in metabolite accumulation and RONS production (137, 138). Compared to normoxia (sea level), high-intensity sprint exercise in severe acute hypoxia (e.g., 5.300 m above sea level) elicited a higher glycolytic rate, greater reductions in the $NAD/NADH^{+}$ ratio, lower muscle pH, and increased protein carbonylation, suggesting greater RONS production in hypoxia (138). The increased lactate accumulation observed during sprint exercise in severe acute hypoxia may be explained by a different mechanism of activation of the pyruvate dehydrogenase (137). In short, high intensity exercise performed in severe acute hypoxia modify different skeletal muscle signalling pathways (138). Accordingly, exercise and post-exercise recovery in hypoxia alter exercise adaptations to oxidative stress compared to normoxic conditions (139, 140). However, the mechanism behind the increased

oxidative stress is still under scrutiny, as exercise to exhaustion could produce similar changes in metabolites (muscle lactate, phosphocreatine, and ATP) in normoxia and severe acute hypoxia, although remarkably lower femoral vein PO_2 (and probably intracellular PO_2) during exercise observed in severe acute hypoxia (117). Nevertheless, oxidative stress seems to be exacerbated by exercise as indicated by the fact that exercise to exhaustion in hypoxia exerts greater increase in markers of oxidative stress than exposure to hypoxia without exercise (141).

5.1.14. Redox balance during ischaemia-reperfusion.

Ischaemia is the reduction of blood flow (hypoperfusion) to certain organs/tissues of the body that causes a decrease in the amount of oxygen (hypoxic ischaemia) and nutrients in the affected area followed by a burst of RONS produced upon reperfusion. Ischaemia may also impair the elimination of metabolites from the affected tissues. The production of RONS after an ischaemia-reperfusion (IR) phenomenon observed in different conditions (e.g., atherosclerosis) causes tissue damage (for example, heart, liver, and brain) that can lead to serious complications in organ transplants, extremity injuries and myocardial infarction, among others. In skeletal muscle, a prolonged ischaemia followed by reperfusion can lead to cell apoptosis (142) and decrease myofibrillar sensitivity to Ca^{2+}, thus compromising muscle contractile function (143). Then, early re-opening of blood flow to the ischaemic area helps to preserve tissue function.

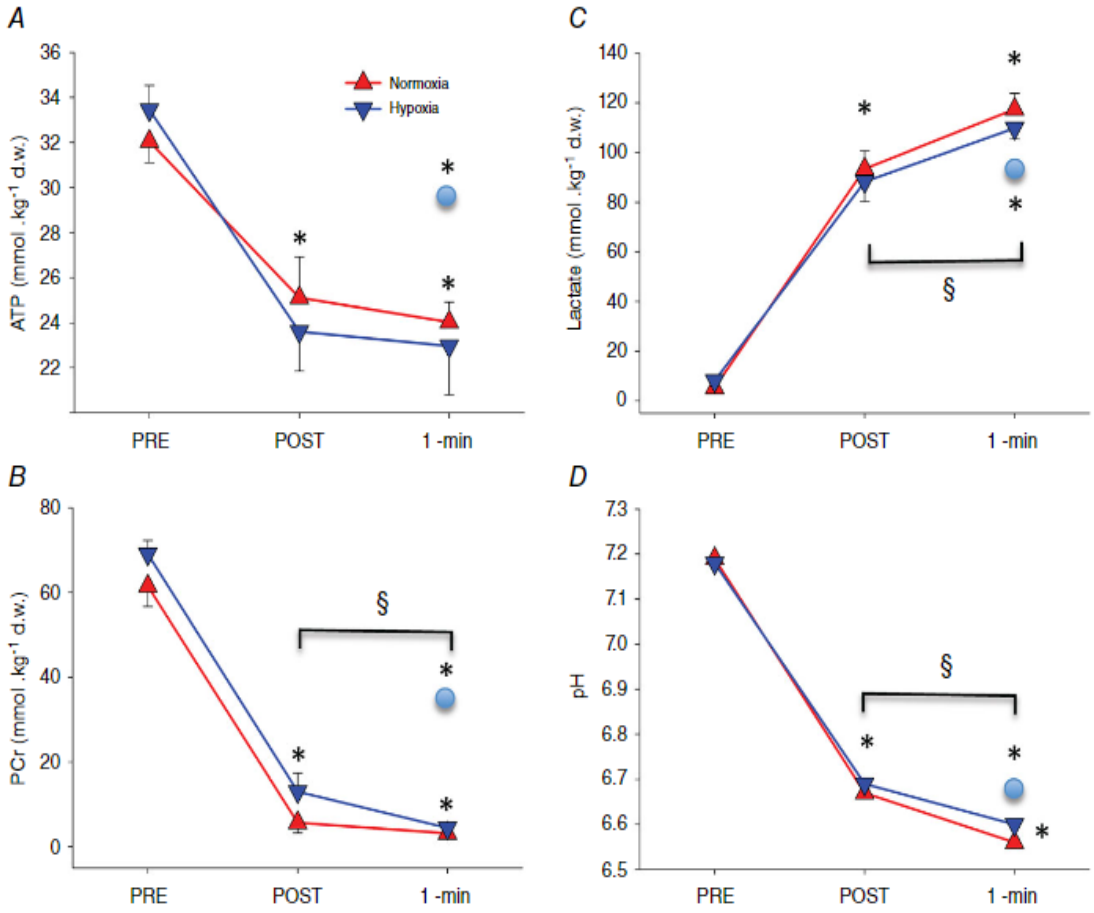
Decreased O_2 supply due to arterial blood flow obstruction induces the use of anaerobic metabolism and leads to decreased ATP production, probably due to impaired enzyme activity by osmotic and cellular pH changes (Ca^{2+} and H^+ accumulation) due to dysfunction in the electron transport chain in mitochondria and in sodium/potassium and calcium pumps (a detailed review can be found elsewhere (75)). Subsequent reperfusion of ischaemic tissue after redox alteration increases the generation of RONS and induces

oxidative stress and a local inflammatory response causing damage to cellular structures due to the so-called ischaemia-reperfusion phenomenon. The intake of antioxidants has been proposed for cardio-protection against oxidative stress caused by ischaemia-reperfusion injury (143, 144). RONS overproduction after ischaemia reperfusion seems to peak during the first 5 minutes (145). Furthermore, oxygen does not immediately drop to zero when ischaemia is applied, and in fact the little O_2 trapped during the ischaemia period is used and can generate RONS. Indeed, Electron paramagnetic resonance (EPR) experiments have shown that free radicals are generated in mammals not only during the reperfusion phase, but also in small amounts during ischaemia (146). However, the levels of RONS generated during ischaemia appear to be low in relation to reperfusion and the physiological importance of these remains uncertain.

Although not fully elucidated in skeletal muscle, mechanisms of oxidative stress induction from different sources of RONS (XO, NOX, NOS, mitochondrial electron transport chain, etc.) during ischaemia-reperfusion have been reviewed elsewhere (147). For example, proteolytic attack triggered by calcium dysregulation on xanthine dehydrogenase and limited levels of O_2 and ATP during ischaemia in skeletal muscle induce hypoxanthine accumulation and diminish the activity of xanthine oxidase to convert hypoxanthine into xanthine (148). Upon reperfusion, O_2 availability increase, and hypoxanthine is abruptly converted by the reaction of xanthine oxidase, producing even greater RONS formation during reperfusion than during ischaemia.

Even during the high intensity exercise in severe hypoxia muscle blood flow is not completely interrupted, muscle O_2 reserves are not completely exhausted, oxidative phosphorylation is not inhibited, the ATP is not completely consumed, and mitochondrial respiration is not impaired (149). The complete interruption of muscle blood flow produced during ischaemia limits oxygen consumption and increase RONS production,

likely leading to mitochondrial dysfunction by increased mitochondrial permeability transition pore (150). Greater reduction of blood flow (greater intensity of ischaemia) could increase the production of free radicals (146). Especially after post-exercise ischaemia, insufficient production of ATP to meet the demand for ATP impairs the activities of the sodium-potassium and calcium pumps, and an increase in intracellular lactate and H^+ occurs (117). In fact, only one minute of ischaemia after incremental exercise to exhaustion increased muscle lactate and decreased phosphocreatine and pH (117) (see supplementary Fig. 5). The adaptative regulation of metabolism generates metabolites that alter gene expression, modifying chromatin and regulating transcription and translation processes. Metabolome can dynamically adjust gene activity due to metabolism-dependent changes in chromatin structure, metabolite-induced changes in the activity of transcription factors and cofactors, and small molecule feedback loops at the molecular level of RNA transcription (151). When ischaemia ends and reperfusion begins, a burst of RONS (primarily superoxide) arises from different RONS production sources, such as the mitochondria (152) or XO activity (153). Antioxidants administration may protect proper skeletal muscle contractile function after IR injuries (154).



Supplementary Figure 5. Muscle metabolites after incremental exercise to exhaustion. Muscle ATP (A), phosphocreatine (PCr) (B), lactate (C) and pH (D) under resting conditions before (PRE) exercise, and 10 s (POST) and 60 s (1-min) after the end of an incremental exercise to exhaustion either in normoxia (P_IO_2 = 143 mmHg) or hypoxia (P_IO_2 = 73 mmHg) performed in random order. At exhaustion, a cuff was instantaneously inflated at 300 mmHg around the thigh of one leg to impede recovery. A muscle biopsy was obtained 10 and 60 s after the end of the sprint, while the occlusion was maintained, from the musculus vastus lateralis of the occluded leg. A 60 s biopsy was also obtained simultaneously from the non-cuffed leg (circles in the graphs); ∗ P<0.05, compared with PRE; §ANOVA time effect POST vs.1-min occlusion; P<0.05. Extracted from Morale-Alamo et al. (2015) (117)

5.1.15. Application of ischaemic preconditioning in redox research.

At certain levels that are not harmful to the cell, RONS functions as a mediator for different cell signalling cascades related to the adaptive response. Ischaemic preconditioning (IPC) is an experimental technique that involves the application of repeated cycles of IR at a non-injurious intensity to elicit an adaptive response that confers effective protection to tissues affected by IR injury (e.g., prolonged coronary occlusion).

For example, application of IPC to the myocardium decreases oxidative damage during prolonged pathologic exposure typical of IR injury (155), resulting in cardio-protection by reduced infarct size, attenuated arrhythmias, and preserved cardiac function (143, 156). The underlying hormesis mechanisms of IPC involve moderate levels of RONS (157), opening of mitochondrial permeability transition pore and ATP-sensitive K+ channels (158), optimal regulation of Ca^{2+} (156). The application of IPC in skeletal muscle could provide protective effects such as reduction in infarct size (159) through decreased consumption of ATP during ischaemia (160), lower generation of oxidative products and mediators of the inflammatory response (161). In addition, direct IPC (in the target tissue) and remote IPC (in a remote tissue) may increase the expression of endogenous antioxidant enzymes in skeletal muscle (162).

Apart from IPC, other clinical alternatives have been proposed for the treatment of ischaemia-reperfusion injury. The advance of other clinical treatments and the lack of practical application of IPC due to the difficulty in anticipating myocardial infarctions or extremity injuries has led to the use of ischaemic postconditioning (IPostC), which is more likely than preconditioning to be feasible as a clinical application to patients undergoing acute myocardial infarction (163). IPostC involves a series of brief cycles of ischaemia and reperfusion applied immediately at initiation of reperfusion in the organ previously subjected to ischaemia, also reducing infarct size and preserving endothelial function (164) likely by similar mechanisms than IPC (165). On the other hand, some pharmacological strategies such as the intake of antioxidants or NOX and XO inhibitors could improve the prognosis of RONS-related I/R injury (155). Finally, regular exercise provides a well-documented cardioprotective effect as a reducer of risk factors for cardiovascular diseases such as obesity and hypertension (166). As the sources of RONS production could be similar during ischaemia and exercise (153), the implementation of

short-term ischaemia after exercise could boost the signals produced during exercise to induce a greater accumulation of metabolites and RONS generation, enhancing exercise-induced signalling and augmenting the adaptive exercise-mediated response.

5.1.16. Sedentary lifestyle, metabolic syndrome, and oxidative stress.

Physical inactivity is one of the most important public health problems of the 21st century (167), becoming the 4th leading risk factor for mortality. Prolonged physical inactivity leads to cardiovascular disease (CVD), diabetes, cancer, hypertension, obesity, depression, and osteoporosis (168, 169). Physical inactivity induces oxidative stress by multifactorial mechanisms leading to muscle atrophy through decreased protein synthesis and increased protein degradation (170). The coexistence of several important CVD risk factors is a characteristic of the metabolic syndrome (171), which is associated with a higher risk of CVD and all-cause mortality, morbidity, and hospital stay (172, 173). Regular exercise markedly impacts the function of many tissues that affect metabolic homeostasis throughout the body. In general, physically active and fit persons have approximately 20% to 35% lower relative risk of all-cause death compared inactive and unfit persons (168). The identification of signals induced by the high production of RONS produced in skeletal muscle during strenuous exercise could significantly improve our understanding of muscle redox regulation, helping to combat oxidative stress-related diseases and optimize athletic performance.

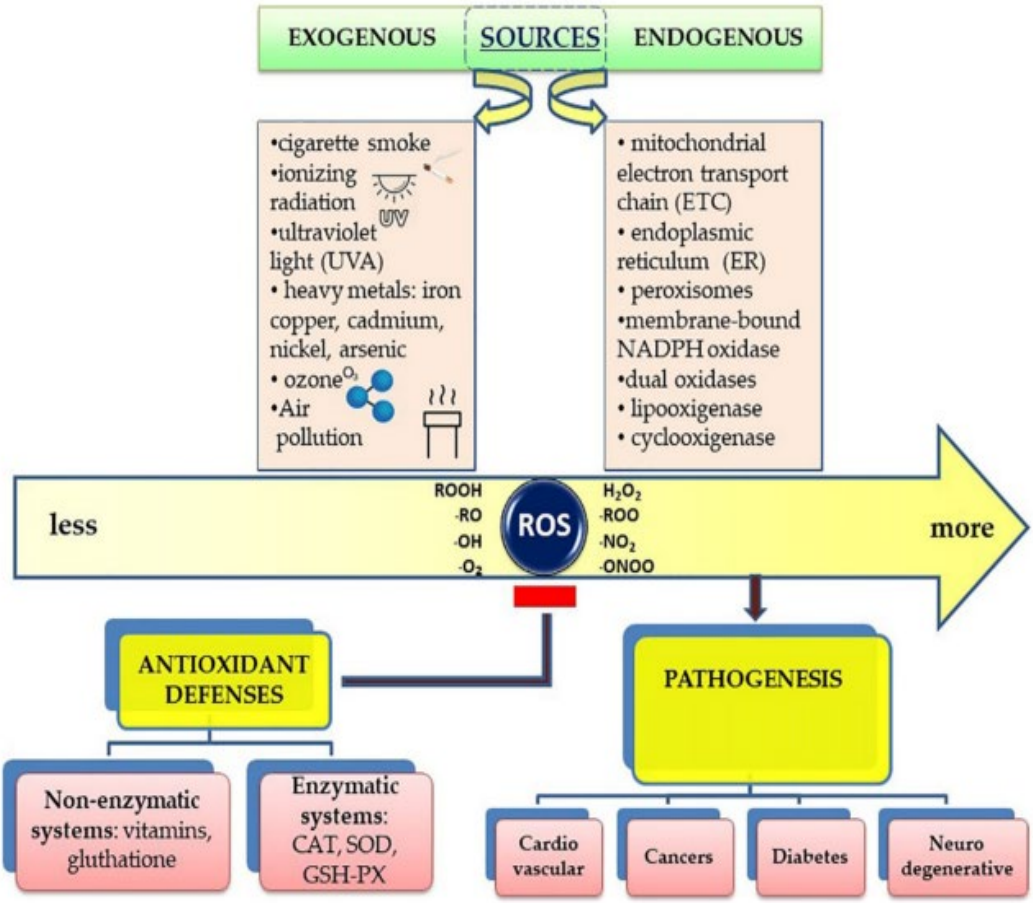
Recent government recommendations highlight the importance of improving physical fitness for better health, staying physically active and reducing sedentary behaviour (169). Moreover, sedentary time increases the likelihood of developing different risk factors involved in the metabolic syndrome, independently of the fitness level (174). Three abnormal factors out of five characterize a person with metabolic syndrome: elevated blood pressure (≥ 130/85 mm Hg systolic/ diastolic blood pressure), elevated triglycerides

(≥ 150 mg/dL) and low high-density lipoprotein cholesterol (< 40/50 mg/dL in men/women), elevated fasting glucose (≥ 100 mg/dL) and central obesity (waist circumference ≥ 102/88 cm in men/women) (175). This cluster of risk factors increase the risk for cerebral and cardiovascular events (176). Oxidative stress is considered to play an important role in the pathogenesis of metabolic syndrome (177, 178). For a comprehensive description of the effect of oxidative stress on the human body, see supplementary Figure 6.

A blunted ability to resist and repair oxidative damage and high levels of prooxidants have been related to the pathogenesis of diseases such as cancer, atherosclerosis, neurodegenerative diseases, hypertension, diabetes mellitus, cardiovascular diseases and aging (179). Furthermore, inflammation associated with chronic diseases is closely related to oxidative stress (179). Metabolic syndrome further potentiates elevated oxidative stress in obese adults determined by higher levels of RONS and lower antioxidant capacity, accompanied by higher systemic inflammation (178).

Increased systemic oxidative stress by advanced oxidation protein products are associated with increased presence of metabolic syndrome risk factors (177) and lower aerobic capacity and impaired skeletal muscle energy metabolism (180). Additional free radical production is generated from endogenous (inflammation, infection, cancer, etc.) and exogenous (poor diet, alcohol consumption, smoking, etc.) sources. Physical training is an effective treatment to reduce oxidative stress and risk factors associated with metabolic syndrome (181), reducing inflammation, blood pressure, and improving muscle metabolism (182). Additional effects of regular physical exercise are improved dyslipidemia and lipid profile (183), increased bone density (184), enhanced weight loss and maintenance (185), and extended life expectancy (186). Regular practice of exercise modifies the basal state and the response to an acute session of exercise, reducing the

overregulation caused immediately after exercise of oxidative stress that targets lipids, increasing antioxidant defences and decreasing protein oxidation processes (187, 188). The model used in this thesis is incremental exercise to exhaustion with the aim of producing oxidative stress in skeletal muscle and possibly in other tissues, allowing the study of the activation of various signals mediated by RONS.



Supplementary Figure 6. Sources of free radicals and their effects on the human body. Extracted from Sharifi-Rad et al. (2020) (189).

5.1.17. RONS generation and aging.

The free radical theory of aging was originally described in the 1950s by Denham Harman (4). Since then, the accumulation of damage induced by oxidative stress has been highlighted as the main responsible for the progressive loss of function of tissues and organs associated with age (190). Back in 1990, Zerba et al. identified that advancing age

produces greater susceptibility to muscle injuries in a mechanism associated with the increased production of RONS (191). The benefit of exercise are also observed when aging occurs under active lifestyle, presenting lower levels of oxidative stress at rest and a higher antioxidant response to physical exercise (192, 193).

Improving cardiovascular fitness even in sedentary middle-aged people increases resistance to oxidative stress (194). Moreover, regular exercise in old people increase the activity of antioxidant enzymes (195) and slows the age-related decline in antioxidant capacity, reaching antioxidant levels comparable to those seen in inactive youth (196). Furthermore, ensuring adequate levels of endogenous antioxidants through diet could reduce not only oxidative damage due to exercise, but also alter the threshold for age-related reduction in muscle mass and exercise capacity (197).

5.1.18. RONS-mediated hormetic adaptation induced by exercise.

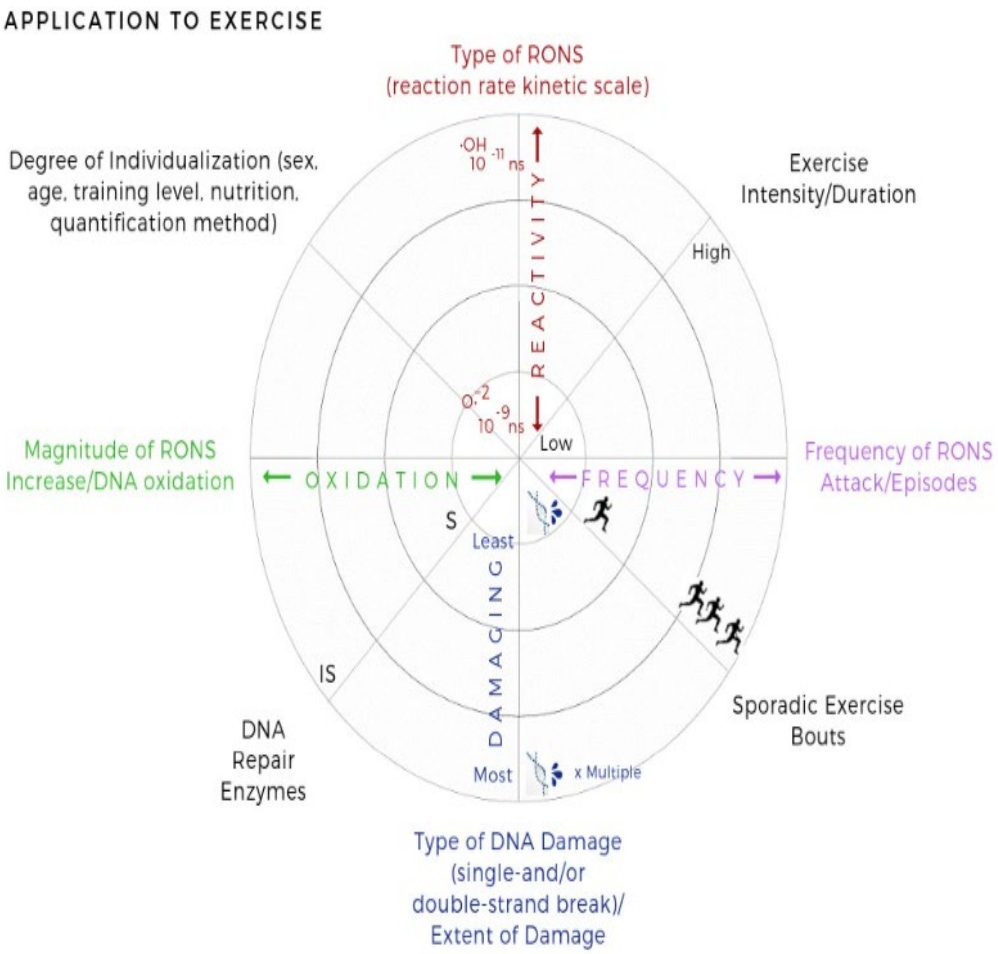
As an easily accessible, lack of adverse effects, and low-cost polypill, many people start exercising with the goal of improving health and gaining the benefits of exercise to prevent and/or treat almost every chronic disease and improving fitness (198). However, despite decades of research, controversy still exists as to whether exercise-induced RONS production is a double-edged sword for health (128). Therefore, the development and characterization of an ischaemia/reperfusion model is necessary for the study of muscle signalling induced by free radicals and physical exercise, the objective of this thesis.

Moderate levels of RONS production during exercise promote positive physiological adaptation in active skeletal muscles, whereas damage to macromolecular structure associated with high degrees of oxidative stress may be achieved during prolonged, high-intensity exercise. Exercise modulates any beneficial or detrimental effects mediated by RONS, in a relationship from physical inactivity to overtraining. For example, DNA

damage can occur after exhaustive exercise, especially if the exercise is performed at a high intensity (199). However, this transient DNA damage is also repaired by exercise-stimulated antioxidant systems (199). In fact, there is no consistent evidence to support that prolonged high-intensity exercise results in tissue damage and impaired physiological function (128). Although physical exercise per se induces the production of RONS, regular practice of physical activity leads to a better training status, reduces oxidative damage markers and increase antioxidants agents (as reviewed elsewhere (200)). As mentioned above, excessive antioxidant capacity could excessively reduce RONS production after exercise, impairing the exercise-induced hormesis response and inhibiting adaptations. The repeated production of RONS during exercise sessions paradoxically acts as mild stressors that trigger a biomolecular hormetic response through redox-sensitive signalling pathways (See supplementary Fig. 7). Therefore, identifying and understanding the major signalling pathways and antioxidant enzymes stimulated by exercise could lead to their use as targets to regulate the hormetic adaptive response to exercise.

Repeatedly facing situations of non-pathological oxidative stress through regular physical exercise elicits a beneficial effect of adaptation by hormesis, which induces a beneficial adaptive response in the antioxidant response observed in more trained subjects (187, 196, 201). Therefore, long-term regular practice of physical exercise increases antioxidant systems and decreases protein oxidation processes at rest, and attenuates the immediate response to oxidative stress that occurs in an acute exercise session (187) and likely in pathological situations of oxidative. Moreover, higher levels of physical activity have been associated with reduced all-cause mortality, with increased risk reduction per time for vigorous exercise compared to moderate-intensity activities (202). In agreement, recent evidence suggests that vigorous exercise produces a greater decrease in the level

of oxidative stress, which is likely to provide even greater health benefits (128). Consequently, more evidence is needed to understand the effects of high-intensity exercise on the adaptive hormetic response and the production of oxidative stress markers (199).



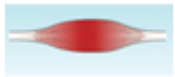
Supplementary Figure 7. Multi-dimensional model showing multiple factors to be considered when assessing the degree of oxidative damage when applied to the exercise model. IS, insufficient; RONS, reactive oxygen and nitrogen species; S, sufficient. Extracted from Tryfidou et al. (2020) (199)

5.1.19. Oxidative stress, myokine and exercise-induced adaptations.

More than 1000 post-translational signalling events are altered in skeletal muscle with a single exercise session in human skeletal muscle (203). Acute exercise has an impact on the control of metabolism, mRNA transcription, and protein translation (204). The effect of integrating successive sets of acute exercise leads to chronic molecular changes

produced by long-term training (204). Part of the well-recognized health benefits are mediated by exercise-mediated release of cytokines (called exerkines). Cytokines are released from many organs (such as adipose tissue, the heart, and the brain), but there has been particular interest in cytokines released from skeletal muscle for their exercise-associated health effect (205). The health benefits of exercise occur not only in skeletal muscle in an autocrine manner, but also affect different organs/tissues, producing endocrine adaptations in the body. In fact, certain cytokines produced by skeletal muscle (called myokines (206)) play a fundamental role in the communication of skeletal muscle with numerous organs. This is of particular interest as emerging evidence suggests that plasma redox status (at the systemic level) does not reflect skeletal muscle redox-sensitive protein signalling (207). The advancement of knowledge in this matter is due to the approach with a multifocal approach (in vitro and in vivo) to solve possible inconveniences and individual methodological problems to know what happens in human skeletal muscle during exercise (see supplementary Fig. 8)

Although muscles are known to release more than 300 myokines (208) that drive adaptations throughout the body, the number of identified myokines continues to increase (209). Some of the adaptations produced by myokines in the body are associated with the regulation of metabolism, induction of signalling pathways, alteration of enzymatic activity, modification of hormonal regulation and moderation of gene expression (210). Myokines are still under study due to their recent identification, but RONS have been identified as import regulators of numerous myokines (211).

Muscle in vivo 	Advantages	All physiological mechanisms present Fatigue can be central or peripheral All types of fatigue can be studied Stimulation patterns appropriate for fiber types and stage of fatigue
	Disadvantages	Mixture of fiber types Complex activation patterns Produces correlative data; hard to identify mechanisms Experimental interventions very limited
Isolated muscle 	Advantages	Central fatigue eliminated Dissection simple
	Disadvantages	Mixture of fiber types Inevitable extracellular gradients of O_2, CO_2, K^+, lactic acid Mechanisms of fatigue biased by presence of extracellular gradients Drugs cannot be applied rapidly because of diffusion gradients
Isolated single fiber	Advantages	Only one fiber type present Force and other changes (ionic, metabolic) can be unequivocally correlated Fluorescent measurements of ions, metabolites, membrane potential, etc. possible Easy and rapid application of extracellular drugs, ions, metabolites, etc.
	Disadvantages	Dissection difficult Environment different to in vivo K^+ accumulation and other in vivo changes absent Prone to damage at physiological temperatures Small size makes analysis of metabolites difficult
Skinned fiber	Advantages	Precise solutions can be applied Possible to study myofibrillar properties, SR release and uptake, AP/Ca^{2+} release coupling Metabolic and ionic changes associated with fatigue can be studied in isolation
	Disadvantages	Relevance to fatigue can be questionable May lose important intracellular constituents Relevant metabolites to study must be identified in other systems

Supplementary Figure 8. Advantages and disadvantages of various approaches to the study of fatigue. Extracted from Allen et al. (2007) (41)

5.2. Introduction of article 1

During exercise reactive oxygen (ROS) and nitrogen species (RNS) (collectively called RONS) are produced depending on the fitness level, the energy substrates oxidized and the characteristics of exercise (212-215). Although in some circumstances, RONS may cause oxidative damage, RONS also stimulate signalling pathways essential for the adaptive response to exercise (212, 216). One of the main transcription factors involved in RONS-mediated regulation of gene expression is the nuclear factor erythroid-derived 2-like 2 (Nrf2), as shown in Nrf2-null mice ($Nrf2^{-/-}$) (217-219). In mice skeletal muscle, total Nrf2 protein expression has been reported to increase after 90 min of continuous running (220) and nuclear Nrf2 protein content after 6 h of continuous running (221). In humans, unchanged and reduced Nrf2 mRNA levels have been reported in skeletal muscle biopsied 3-4 hours after exercise (222-225). However, the changes in Nrf2 protein levels and associated signalling events in response to acute exercise and recovery have not been determined in human skeletal muscle. This is relevant because reduced Nrf2 expression has been associated with lower exercise performance in animal models of chronic disease (226).

The RONS produced during exercise are accompanied by intramuscular changes in oxygen pressure (PO_2), metabolites and signalling molecules (Ca^{2+}, P_i, Cr, PCr, H^+, $NADH.H^+$, etc.), which return to pre-exercise levels with different time courses (227, 228). Such changes in metabolite accumulation and RONS production are exacerbated when the exercise is performed in hypoxia (229, 230), leading to specific adaptations (231-233). Animal and cell culture experiments indicate that skeletal muscle Nrf2 signalling is upregulated by hypoxia (234, 235). Nevertheless, it remains unknown whether metabolite accumulation and muscle oxygenation influence Nrf2 signalling in response to acute exercise.

Nrf2 signalling is principally regulated by Kelch-like ECH-associated protein 1 (Keap1), which under basal conditions binds to Nrf2 promoting its ubiquitination and proteasomal degradation (236). Keap1 is a cysteine-rich protein sensitive to modification by electrophiles and oxidants, which cause conformational changes of Keap1 that stabilize the Keap1-Nrf2 interaction, preventing Nrf2 proteasomal degradation. Under lower availability of free Keap1, the newly formed Nrf2 accumulates and translocates to the nucleus where it binds with antioxidant response elements (AREs) to regulate the transcription of more than 250 genes involved in the xenobiotic and antioxidant response, mitochondrial biogenesis, metabolism, detoxification, cytoprotection, inflammation, autophagy, and cell differentiation (236). Although it is well established that exercise increases in the gene expression of some antioxidant enzymes (222, 228, 237), the acute effects of exercise on the protein levels of Keap1, superoxide dismutase isoenzyme 1 (SOD1), superoxide dismutase isoenzyme 2 (SOD2), and Catalase in skeletal muscle remain unknown. Moreover, the process of activation/deactivation of Nrf2 signalling in skeletal muscle with contractile activity has not been investigated.

Given the intrinsic difficulty in assessing RONS production in human skeletal muscle and the low specificity and sensitivity of the oxidative markers at use, we examined potential changes in RONS production by assessing the phosphorylation changes known to be mediated by RONS. This is the case of the phosphorylation of Nrf2 at its Serine 40 by protein kinase Cδ (PKCδ), a ROS-sensitive kinase (238). Likewise, we determined the phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) at its threonine 187. CaMKII is activated by oxidation and autophosphorylation (239), and effect likely amplified by ROS-induced inhibition of phosphatases (240). During high-intensity exercise, Thr^{287} CaMKII phosphorylation is blunted by the administration of antioxidants before exercise (216). Likewise,

overexpression of antioxidant enzymes prevents Thr^{287} CaMKII phosphorylation in other experimental models (241, 242). As downstream indicators of Nrf2 signalling, we determined the protein expression levels of Catalase, and SOD1 and SOD2. Animal data and cell culture experiments indicate that the gene expression of Catalase (236), SOD1 (243), and SOD2 (244) are stimulated by Nrf2, while the physiological ROS-induced expression of Catalase is blunted in $Nrf2^{-/-}$ mice (217, 245). We also measured Thr^{172} AMPKα phosphorylation as a marker of metabolic stress, since this enzyme is activated principally depending on the AMP/ATP ratio (246), and is necessary to enhance the expression of SOD2 in response to training (247). Besides, due to the short half-live of RONS and the fast recovery of the energy metabolism upon cessation of exercise, a new experimental model was developed in humans, to impede early recovery through the instantaneous application of complete post-exercise ischaemia with a pneumatic cuff in one leg only, using the contralateral leg as a control.

Since Nrf2/Keap1 signalling is expected to be activated by exercise models eliciting redox perturbations, we used an exercise protocol that allows the achievement of maximal oxygen uptake (VO_2max) in 10-15 minutes and elicits a marked activation of the glycolysis close to exhaustion (213, 248), resulting in oxidative stress (249).

5.3. Introduction of article 2

The transcription factor nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) regulates over 150 genes involved in inflammation, immunity, cell proliferation, apoptosis (250-253), and muscle metabolism (253-256). NF-κB is activated by extracellular signals, mostly cytokines, as well as intracellular changes in calcium (257) and reactive oxygen and nitrogen species (RONS) (258-260). Although these signals are present in contracting muscles, contradicting findings have been reported regarding the effect of exercise on NF-κB activation and signalling. Exercise activates NF-κB in rodents, although this response is not homogeneous in all exercised muscles (261) and associated with muscle damage (262, 263). In humans, increased (264, 265), unchanged (266) and decreased (267) NF-κB signalling has been reported after acute endurance (264, 266) and resistance (265, 267) exercise. Part of these discrepancies could be accounted for by differences in exercise protocol, characteristics of the subjects and collection timing of the muscle biopsies, which may influence metabolite accumulation and redox balance. It remains unknown whether a certain level of metabolite accumulation and RONS production is necessary to trigger an acute signalling response by NF-κB.

RONS production and metabolite build-up is exacerbated when the exercise is performed in hypoxia (229, 230). Cell culture experiments indicate that NF-κB (254, 268-270) is stimulated by hypoxia and RONS. Nevertheless, whether metabolite accumulation and muscle oxygenation influence the NF-κB signalling response to exercise remains unknown. There is some experimental evidence in rodent muscle indicating that the exercise activation of NF-κB is produced through phosphorylation and activation of IKK by the RONS-sensitive upstream kinases ERK1/2 and p38 mitogen-activated protein kinase (p38 MAPK) (261, 271). However, data in humans are not

conclusive (266). In turn, NF-κB activation has been shown to induce the expression of some antioxidant enzymes, like Gpx1 and Trx1 (272-274), although information about this effect in human skeletal muscle *in vivo* is lacking. Glutathione reductase (GR) catalyses the reduction of the oxidised glutathione (GSSG) to reduced glutathione (GSH), and its activity is increased by oxidative stress in skeletal muscle (275) and by activation of Nrf2 signalling (276). GR expression may be increased to facilitate the restoration of GSH during exercise conditions eliciting oxidative stress, and hence and increased expression of GR could be used as a biomarker of oxidative stress. Nevertheless, no previous study has determined the effects of intense exercise on the protein levels of GR in humans.

It has been reported that thioredoxin reductase 1 (TrxR1) may facilitate NF-κB signalling (277). TrxR1 has been shown to be unchanged in human skeletal muscle after prolonged aerobic exercise (278) and increased after repeated sprint exercise (279). Whether skeletal muscle TrxR1 expression increases during incremental exercise to exhaustion in normoxia and hypoxia remains unknown.

AIMS & HYPOTHESES

6. AIMS AND HYPOTHESIS

6.1. Aims and hypothesis of article 1.

Therefore, the primary purpose of this study was to determine whether Nrf2 is upregulated by acute exercise in human skeletal muscle and the role that Keap1 protein plays in this process. Another aim was to determine whether the level of oxygenation during the exercise influences the Nrf2 signalling response, as well as the role played by muscle oxygenation and metabolite accumulation in the early recovery after exercise.

We hypothesised that Nrf2 protein amount and its downstream-regulated proteins Catalase, SOD1, and SOD2 would be increased in response to exhaustive exercise, and more markedly during exercise in hypoxia than normoxia. We also hypothesised that these changes would revert to pre-exercise levels within one minute of the cessation of exercise in the leg recovering with free circulation. At the same time, Nrf2-depending signalling would increase further in the ischaemic leg due to the additional accumulation of metabolites and the reduction of PO_2 to anoxic levels.

6.2. Aims and hypothesis of article 2.

The primary aim of this study was to determine whether NF-κB signalling is activated by acute exercise to exhaustion in human skeletal muscle and whether muscle oxygenation and metabolite accumulation play a role in this process. Another aim was to determine the time course of NF-κB signalling during the early recovery and ascertain whether NF-κB signalling remains activated by post-exercise ischaemia application.

We hypothesized that NF-κB signalling is more markedly activated during exercise in severe acute hypoxia and further activated during post- exercise ischaemia and would be accompanied by upregulation of antioxidant enzymes regulated by NF-κB. We also hypothesized that NF-κB signalling would return to pre-exercise levels within one minute of the termination of exercise when the muscles recover without occlusion.

METHODS

7. METHODS

7.1. Subjects.

Eleven young men volunteered to participate in this study (means ± SD; age: 21.5 ± 2.0 years, body mass: 72.3 ± 9.3 kg, height: 174 ± 8 cm, and body fat: 16.1 ± 4.9%). The inclusion criteria were: a) age between 18 and 35 years, b) sex: male, c) body mass index: < 30 $kg.m^{-2}$ all, d) normal 12-lead electrocardiogram, and e) having a physically active lifestyle exercising regularly 2-4 times a week, but without following a specific training program; and the exclusion criteria: a) smoking, b) any disease o allergy, c) any medical contraindication for exercise, d) being under any medical treatment (280). All volunteers signed a written consent after receiving information about the aims and potential risk of the study. The study commenced after approval by the Ethical Committee of the University of Las Palmas de Gran Canaria and was carried out according to the Declaration of Helsinki. Subjects were asked to avoid ingesting caffeine and taurine-containing drinks, alcohol and exercise 24 h before the experiments. Besides, they recorded their dinner on the day before the first experimental session to repeat a similar diet on subsequent experimental sessions. Subjects were asked to maintain their usual diet until the end of the study.

7.2. Study design.

Although this research was initially designed to determine the mechanisms that limit performance during whole-body exercise in humans previously published (1, 281-283), it was also planned to analyse the main signalling pathways activated by cellular stress during exercise and post-exercise ischaemia. In a recent paper, we focussed on Nrf2 mechanisms of activation/deactivation during exercise and recovery (280). The present paper contains novel results regarding the mechanisms regulating NF-κB signalling during exercise in normoxia and severe acute hypoxia.

7.3. Pre-test and familiarization.

Anthropometric and DEXA body composition assessments were performed (Hologic QDR-1500, software version 7.10, Hologic Corp., Waltham, MA, USA) (1) during the first visit to the laboratory, followed by familiarization with the exercise protocol. This was continued by two sessions to determine their maximal power at exhaustion (Wmax), the peak oxygen consumption (VO_2peak), and maximal heart rate (HRmax) in normoxia (Nx; F_IO_2 = 0.21; P_IO_2 ~143mmHg) and hypoxia (Hyp; F_IO_2 = 0.104; P_IO_2 ~73mmHg) using a ramp incremental exercise test to exhaustion on a Lode Excalibur Sport 925900 (Groningen, The Netherlands) (1). VO_2 was measured breath-by-breath with a metabolic cart (Vmax N29; Sensormedics, Yorba Linda, CA, USA) which was calibrated according to the manufacturer's instructions, using high-grade calibration gases (Carburos Metálicos, Las Palmas de Gran Canaria, Spain) (1). The accuracy and precision of the metabolic cart was determined using a butane combustion test as previously described (284). The highest 20s-averaged VO_2 registered during the test was taken as the VO_2peak (285).

7.4. Main experiments.

Two main experimental sessions including one incremental exercise to exhaustion (Lode Excalibur Sport 925900, Groningen, The Netherlands), one performed in normoxia (Nx; F_IO_2 = 0.21; barometric pressure 735–745 mmHg) and another in hypoxia (Hyp; F_IO_2 = 0.104; barometric pressure 735–745 mmHg) were carried out on separate days and random order (Fig. 1). During the tests, subjects were requested to maintain a pedalling rate close to 80 rpms. In both sessions, exhaustion (also task failure hereafter) was defined by the subject stopping pedalling suddenly or a pedalling rate below 50 rpm despite strong verbal encouragement for 5 s. The duration of the incremental exercise test to exhaustion was 15±3 min in normoxia and 12±4 min in hypoxia.

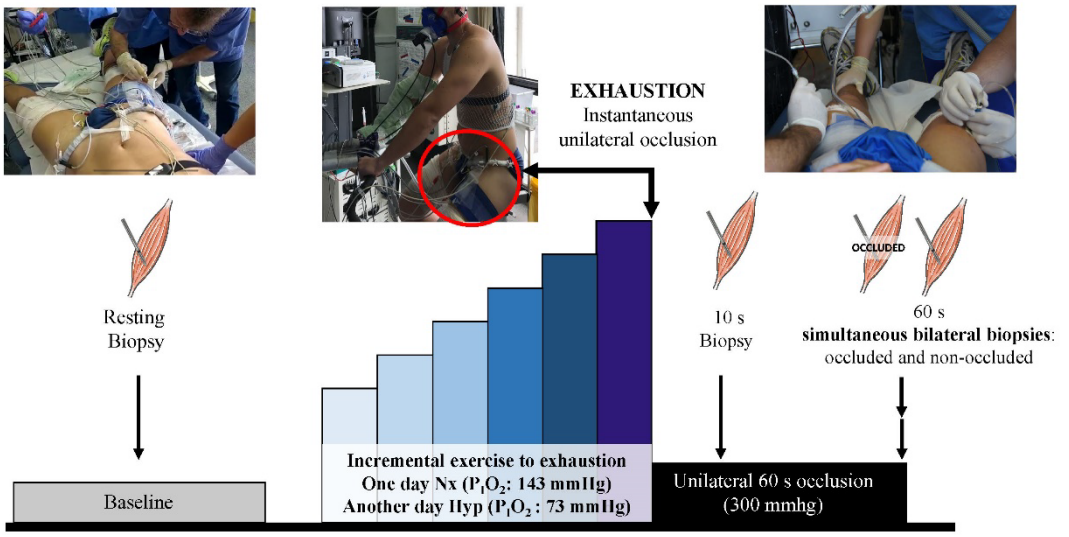


Figure 1. Schematic illustration of the experimental protocol. Eleven subjects performed an incremental exercise to exhaustion either in normoxia (Nx; $F_IO_2 = 0.21$) or in severe normobaric hypoxia (Hyp; $F_IO_2 = 0.104$) in random order. A resting skeletal muscle biopsy was obtained from the m. *vastus lateralis* before warm-up, followed by an incremental exercise test until exhaustion. Immediately at exhaustion, one leg was occluded at 300 mmHg and maintained during 60 s. Subsequent biopsies were taken from the occluded leg at 10 s and 60 s of occlusion in both trials (Nx and Hyp). In the test performed in hypoxia, the biopsies were taken bilaterally from the occluded leg and the leg recovering with free circulation 60 s after exercise cessation, while the subjects recovered breathing normoxic air.

On the main experimental days, volunteers reported to the laboratory at 08.00 h, following an overnight fast. On the Nx day, a first basal muscle biopsy was obtained from the m. *vastus lateralis* of one of the two thigs, assigned randomly. This biopsy was labelled as Pre Nx. The needle was directed distally for the first biopsy, with 45° inclination (286). Then a 5 mm incision was performed in the contralateral leg to obtain fast post-exercise muscle biopsies from both legs. Both incisions were covered with temporary plasters easy to remove at exhaustion. After that, a cuff (SCD10, Hokanson, Bellevue, WA, USA) connected to a rapid cuff inflator (Hokanson, E20 AG101) was placed around the thigh biopsied first and taped as close as possible to the inguinal crease. Then, the subjects moved to the cycle ergometer, and after verification of proper connections and readings from the instruments, and a two-min data collection phase, the

exercise test in normoxia was started at 80 W for two min and increased by 30 W every 2 min until task failure. At this moment, the cuff was inflated instantaneously at 300 mmHg, and a countdown started to obtain a second biopsy (labelled as Post Nx, second biopsy) exactly 10 s after exhaustion, i.e., after 10 s of complete ischaemia. For this second biopsy, the needle was introduced perpendicular to the thigh. Then, the subject rested quietly on the cycle ergometer while maintaining the cuff inflated, and exactly 60 s after the end of the exercise, the needle was introduced with 45° inclination towards the head to obtain the third biopsy (named as Oc1m Nx) (286). This last biopsy allowed assessing muscle signalling changes during 60-s ischaemia, while metabolites from the anaerobic metabolism build-up and mitochondrial PO_2 decreased to zero (1).

On the Hyp day, the first muscle biopsy was obtained while the subjects were breathing normoxic room air (Pre Hyp biopsy). The exercise test in hypoxia began with a 2-min recording period at rest (P_IO_2 ~73 mmHg; AltiTrainer200, SMTEC, Nyon, Switzerland), followed by 2 min at 60 W, and increments of 20 W every 2 min until task failure. At this point, the cuff was instantaneously inflated, and the subjects switched to breath normoxic room air for the rest of the test. On the 10^{th} s after the end of the exercise, the second biopsy was obtained (Post Hyp biopsy). Thereafter, the volunteers were moved to a stretcher while maintaining the cuff inflated to obtain the third muscle biopsy (Oc1m Hyp biopsy) exactly after 60 s of ischaemia. Simultaneously with the third, a fourth biopsy was taken from the contralateral thigh (FC1m), recovering with free circulation in normoxia during 60 s. This means that one leg recovered for 60 s in ischaemia and the other did so with an intact circulation. All biopsies were immediately frozen in liquid nitrogen and stored at −80°C. We failed to obtain the biopsy corresponding to OC1M in two volunteers. In addition, due to scarce biopsy material, some assessments could not be done at all points for all subjects.

7.5. Muscle Metabolites, Protein extraction and Western blotting.

Muscle metabolites and protein extracts were analysed as reported elsewhere (1), and total protein content was quantified using the bicinchoninic acid assay (287). Briefly, ~10 mg of muscle were ground by stainless steel balls during one minute in a Mikro-Dismembrator S (Sartorius, Goettingen, Germany) and immediately homogenised in urea lysis buffer (6 M urea, 1% SDS) and 50X Complete protease inhibitor (Cat. #11697498001) and 10X PhosSTOP phosphatase inhibitor (Cat. #4906837001) cocktails (Roche, Basel, Switzerland). Almost equal final concentration in all muscle protein extracts was acquired by following an individual adjustment of the extract volume using a volume calibration curve. Then, the lysate was centrifuged for 12 min at 25,200 g at 16° C. The resulting supernatant was diluted with electrophoresis loading buffer (160 mM Tris-HCl, pH 6.8, 5.9 % SDS, 25.5% glycerol, 15% β-mercaptoethanol-bromophenol blue).

The optimal amount of total protein to be loaded and the antibody concentration for each assay was determined by loading protein from control and experimental samples in different amounts ranging from 2 to 35 μg. After verification of linearity within this range, equal amounts of protein of each sample (5-30 ug) were electrophoresed on SDS-PAGE gels using the system of Laemmli and transferred to Immun-Blot polyvinylidene fluoride (PVDF) membranes for protein blotting (Bio-Rad Laboratories, Hercules, CA, USA) (Supplementary Table 1). Control samples (whole skeletal muscle lysates from healthy young men) were prepared and run as the experimental samples. A total protein staining technique (Reactive Brown 10, Sigma Aldrich, St. Louis, MO, USA) was used to accurately quantify the variability of the assays and ensure optimal loading and transfer efficiency. For protein expression determination, the samples from each subject were run together onto the same gel intercalated with four control samples.

Membranes were blocked for one hour in either 4% bovine serum albumin or 2.5-5% non-fat dried milk powder (blotting grade blocker) diluted in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) (BSA-or Blotto-blocking buffer) and incubated overnight for 12-15 h at 4ºC with primary antibodies. Antibodies were diluted in 4% BSA-blocking buffer, 2.5 or 5 % Blotto-blocking buffer. After incubation with primary antibodies, the membranes were incubated with an HRP-conjugated anti-rabbit or anti-mouse antibody (diluted 1:5000 to 1:20000 in 5% Blotto blocking buffer) and subsequent chemiluminescent visualization using Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) using a ChemiDoc^{TM} Touch Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). Finally, band densitometric data were quantified in an exposition prior to saturation of the signal with the Image Lab © software 6.0.1 (Bio-Rad Laboratories, Hercules, CA, USA) as arbitrary units (a.u). Since loading was homogeneous in all membranes, no further corrections were performed. Representative immunoblots are depicted in Fig. 2.

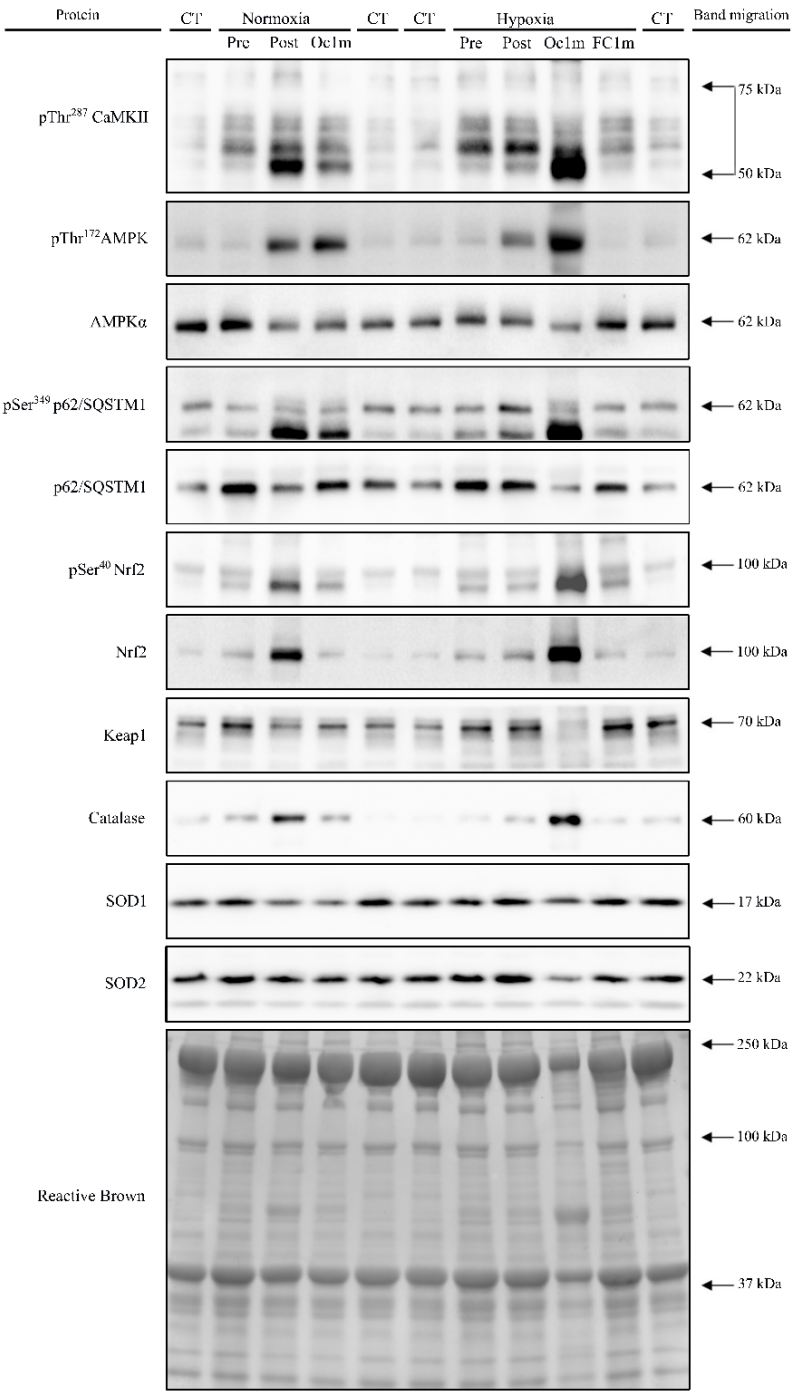


Figure 2A. Representative Western Blot Images from Study 1. Protein expression levels for studied proteins and their regulatory phosphorylations and the total amount of protein loaded (Reactive Brown staining) from a single participant. From top to bottom: pThr^{287} CaMKII, pThr^{172} AMPKα, AMPKα total, pSer^{349} p62 / SQSTM1, p62 / SQSTM1 total, pSer^{40} Nrf2, Nrf2, Keap1, Catalase, SOD1, SOD2 and Reactive Brown (as protein loading control). Nx; test performed in normoxia (F_IO_2 = 0.21, P_IO_2: 143 mmHg), Hyp; test performed in severe acute normobaric hypoxia (F_IO_2 = 0.104, P_IO_2: 73 mmHg); Pre, before exercise; Post, 10 s after the end of exercise with ischaemic recovery; Oc1m, 60 s after the end of exercise with ischaemic recovery; FC1m, 60 s after the end of exercise without ischaemic recovery (free circulation); CT, control sample. Arrows indicate estimated molecular weights.

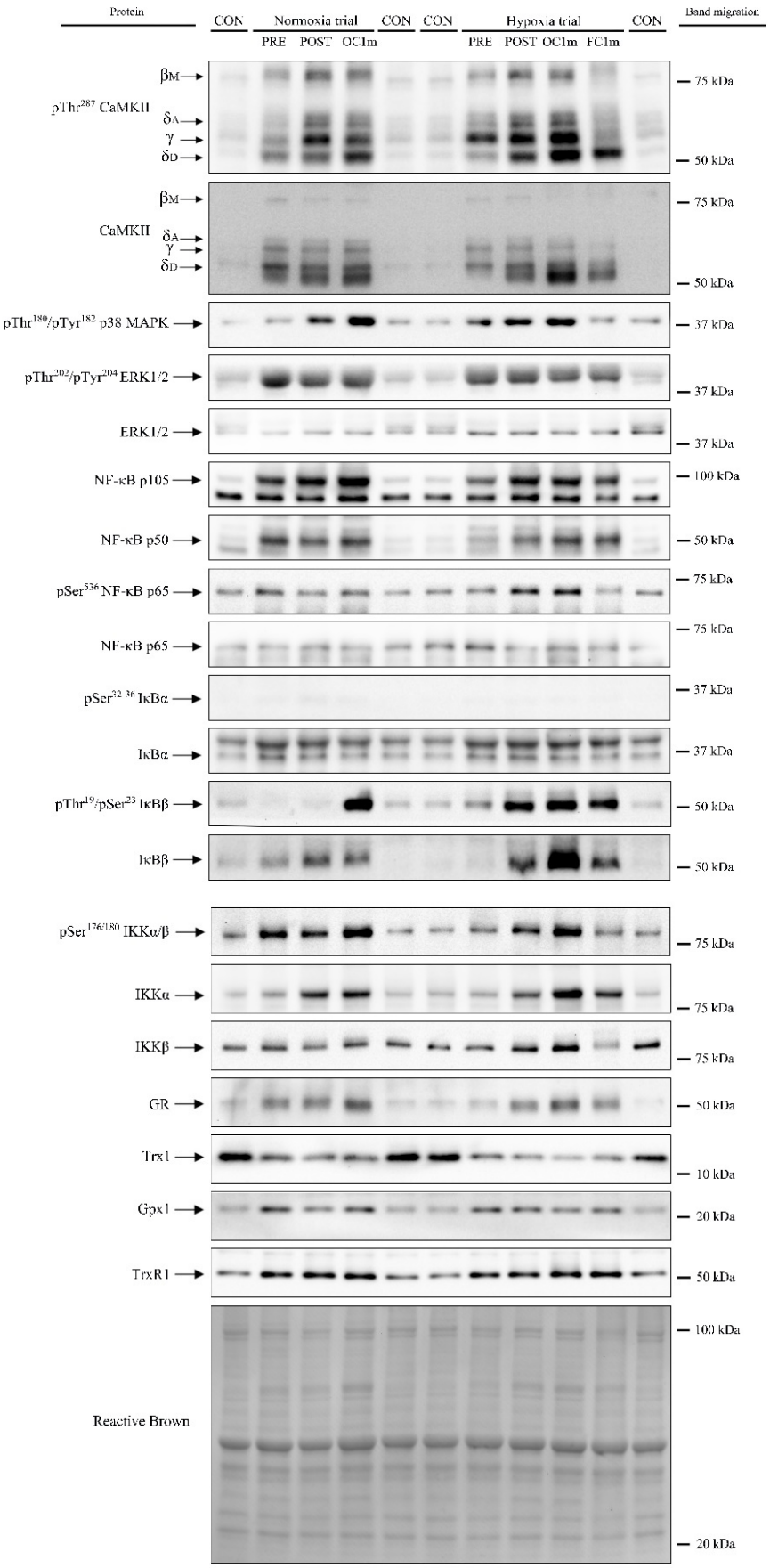

Protein
CON
Normoxia trial
PRE POST OC1m
CON
CON
Hypoxia trial
PRE POST OC1m FC1m
CON
Band migration
pThr287 CaMKII
βM
δA
γ
δD
CaMKII
pThr180/pTyr182 p38 MAPK
pThr202/pTyr204 ERK1/2
ERK1/2
NF-κB p105
NF-κB p50
pSer536 NF-κB p65
NF-κB p65
pSer32-36 IκBα
IκBα
pThr19/pSer23 IκBβ
IκBβ
pSer176/180 IKKα/β
IKKα
IKKβ
GR
Trx1
Gpx1
TrxR1
Reactive Brown
75 kDa
50 kDa
37 kDa
100 kDa
10 kDa
20 kDa

Figure 2B. Representative Western Blot Images from Study 2. Immunoblots of all proteins studied, their regulatory phosphorylations and total amount of protein loaded (Reactive Brown Staining) for a single study participant. Images from top to bottom: pThr^{287} CaMKII, Total CaMKII, pThr^{180}/Tyr^{182} p38 MAPK, pThr^{202}/Tyr^{204} ERK1/2, NF-κB p105, NF-κB p50, NF-κB p65, pSer^{536} NF-κB p65, pSer$^{32/36}$ IκBα, Total IκBα, pThr^{19}/Ser^{23} IκBß, Total IκBß, pSer$^{176/180}$ IKKα/β, Total IKKβ, Total IKKα, GR, Txr1, Gpx1, TrxR1 and Reactive Brown (as total protein loading control). Detailed description of experimental phases is included in Figure 1. CON, non-intervention healthy human sample included in quadruplicate onto each gel as a loading control. Normoxia; test performed with F_IO_2 = 0.21, Hypoxia; test performed with F_IO_2 = 0.104; Pre, before exercise; Post, 10 s after the end of exercise with ischaemic recovery; Oc1m, 60 s after the end of exercise with ischaemic recovery; FC1m, 60 s after the end of exercise without ischaemic recovery. The molecular weight standard markers closest to the migration of the band are indicated on the right side of the panel.

7.6. Materials

The Protein Plus Precision All Blue Standards were acquired from Bio-Rad Laboratories (Hemel Hempstead Hertfordshire, UK). The antibodies employed in this investigation were obtained from different manufacturers.

The corresponding catalogue numbers from Abcam (Cambridge, USA) were as follows: pSer^{40} Nrf2 (no. ab76026), Nrf2 (no. ab62352), Keap1 (no. ab119403), SOD1 (no. ab16831), Sequestosome 1 (SQSTM1 / p62) (no. ab56416) and SQSTM1 / p62 (pSer^{349}) (no. ab211324), IκB beta total (no. ab109509) and Gpx1 (no. ab108429). The antibodies purchased from Cell Signalling Technology (Danvers, MA, USA) were: Thr^{287} CaMKII (no. 12716), AMPKα (no. 2532), Thr^{172} AMPKα (no. 2535), Catalase (no.14097) and SOD2 (no. 13141), pThr^{287} CaMKII (no. 12716), Total CaMKII (no. 4436), pThr^{180}/Tyr^{182} p38 MAPK (no. 9211), pThr^{202}/Tyr^{204} ERK 1/2 (no. 9106), Total ERK 1/2 (no. 9102), NF-κB p105 and p50 (no. 13586), Total NF-κB p65 (no. 3034), pSer^{536} NF-κB p65 (no. 3033), pSer$^{32/36}$ IκBα (no. 9246), Total IκBα (no. 9242), pThr^{19}/Ser^{23} IκBß (no. 4921), pSer$^{176/180}$ IKKα/β (no. 2697), Total IKKα (no. 2682), Total IKKβ (no. 2370) and Trx1 (no. 2429).

Other antibodies were purchased from Proteintech (Rosemont, USA): GR (no. 18257-1-AP) and TxrR1 (no. 11117-1-AP). The secondary HRP-conjugated goat anti-rabbit (no. 111-035-144) and the HRP-conjugated goat anti-mouse (no.115-035-003) antibodies were acquired from Jackson ImmunoResearch (West Grove, PA, USA). A CaMKII δ isoform-specific antibody (anti- CaMKII delta isoform no. A010-55AP; Badrilla) was employed to distinguish between the γ and δ isoforms, as previously described (288). For more details, check supplementary Table 1.

7.7. Statistical analysis

The Gaussian distribution of variables was determined with the Shapiro–Wilks test, and when required, data were transformed logarithmically before further analysis. The main effects and interactions were assessed using a two-way 3 x 2 repeated-measures ANOVA with time (Pre, Post, and Oc1m) and F_IO_2 (Normoxia and hypoxia) as within-subject factors. Additionally, when no significant differences were observed between the postexercise conditions, the average of the means of the two Pre conditions was compared with those of post-exercise conditions (Post normoxia, Oc1m normoxia, Post hypoxia and Oc1m. For this purpose, a contrast analysis in a two way within repeated measures analysis was performed using R (R Foundation for Statistical Computing, Vienna, Austria). The differences between the occluded and non-occluded leg were determined using a paired t-test. The Mauchly's test of sphericity was applied before the ANOVAs.

In the case of violation of the sphericity assumption, the degrees of freedom were adjusted according to the Huynh and Feldt test. When significant main or interaction effects were detected, pairwise comparisons at specific time points were adjusted for multiple comparisons using the Holm-Bonferroni procedure. Linear relationships between variables were examined using a linear mixed model, and the Likelihood Ratio Test for the random effects (LRT) was computed and reported with the marginal and

conditional r-squared values. Unless otherwise stated, results are reported as the mean ± standard deviation (SD). Statistical significance was set at $p < 0.05$. Statistical analyses were performed using IBM SPSS Statistics v.21 for Mac (SPSS Inc., Chicago, IL, USA) and jamovi v1.8.1. (Jamovi project, 2021).

Supplementary Table 1: Detailed description of Western blotting antibodies and procedures.

Antibody	Manufacturer company	Catalog number	Protein molecular weight (kDa)	Gel %	Protein amount loaded (µg)	Electro-phoresis running time (min)	Blotting transfer time (min)	Blocking reagent	Primary antibody concentration	Secondary antibody concentration
Thr^{287} CamKII	Cell Signaling	12716	50-75	10	20	98	90	BSA 4%	1:2000	1:5000
Thr^{172} AMPK	Cell Signaling	2535	62	10	25	100	90	BSA 4%	1:2000	1:5000
AMPKα	Cell Signaling	2532	62	10	25	100	90	BSA 4%	1:2000	1:5000
Ser^{349} SQSTM1/p62	Abcam	ab211324	62	10-12.5	20	90	90	BSA 4%	1:2000	1:5000
SQSTM1/p62	Abcam	ab56416	62	10	25	40	90	Blotto 5%	1:3000	1:5000
Catalase	Cell Signaling	14097	60	10	10	120	90	Blotto 5%	1:2000	1:5000
SOD1	Abcam	ab16831	17	10-12.5	20	90	90	BSA 4%	1:2000	1:5000
SOD2	Cell Signaling	13141	22	10-12.5	20	90	90	BSA 4%	1:20000	1:5000
Ser^{40} Nrf2	Abcam	ab76026	100	4-20%	15	75	90	BSA 4%	1:5000	1:5000
Nrf2	Abcam	ab62352	100	10	15	75	90	BSA 4%	1:500	1:5000
Keap1	Abcam	ab119403	70	4-20%	15	75	90	BSA 4%	1:2000	1:5000

Supplementary Table 1: Detailed description of Western blotting antibodies and procedures.

Antibody	Manufacturer company	Catalog number	Gel %	Protein amount loaded (µg)	Blotting transfer time (min)	Blocking reagent	Primary antibody concentration	Secondary antibody concentration
Thr^{287} CaMKII	Cell Signaling	12716	10	20	90	BSA 4%	1:2000	1:5000
Total CaMKII	Cell Signaling	4436	10	12.5	90	BSA 4%	1:1000	1:5000
$pThr^{180}/Tyr^{182}$ p38 MAPK	Cell Signaling	9211	7.5-10	10	90	Blotto 5%	1:3000	1:20.000
$pThr^{202}/Tyr^{204}$ ERK 1/2	Cell Signaling	9106	10	12.5	90	BSA 4%	1:1000	1:20.000
Total ERK 1/2	Cell Signaling	9102	10	10	90	Blotto 1%	1:1000	1:20.000
NFκB p105	Cell Signaling	13586	4-20	30	90	Blotto 5%	1:2000	1:5.000
NFκB p50	Cell Signaling	13586	4-20	30	90	Blotto 5%	1:2000	1:5.000
Total NFκB p65	Cell Signaling	3034	10	22.5	90	BSA 4%	1:2000	1:10.000
$pSer^{536}$ NFκB p65	Cell Signaling	3033	4-20	15	90	BSA 4%	1:5000	1:5.000
$pSer^{32/36}$ IκBα	Cell Signaling	9246	10	10	90	BSA 4%	1:2000	1:10.000

Total IκBα	Cell Signaling	9242	4-20	7.5	90	BSA 4%	1:2000	1:10.000
pThr^{19}/Ser^{23} IκBß	Cell Signaling	4921	7.5-10	10	90	BSA 4%	1:1000	1:10.000
Total IκBß	Abcam	ab109509	10	10	90	Blotto 5%	1:3000	1:20.000
pSer$^{176/180}$IKKα/ß	Cell Signaling	2697	4-20	12.5	90	BSA 4%	1:3000	1:20.000
Total IKKß	Cell Signaling	2370	4-20	12.5	90	BSA 4%	1:3000	1:10.000
Total IKKα	Cell Signaling	2682	4-20	15	90	BSA 4%	1:3000	1:20.000
GSR	ProteinTech	18257-1-AP	10	5	90	Blotto 2.5%	1:2000	1:10.000
TXR1	Cell Signaling	2429	15	12.5	90	Blotto 2.5%	1:2000	1:10.000
GPX1	Abcam	ab108429	15	12.5	90	Blotto 2.5%	1:2000	1:10.000
TXNRD1	ProteinTech	11117-1-AP	10	5	90	Blotto 2.5%	1:2000	1:10.000

RESULTS

8. RESULTS

8.1. Muscle metabolites.

During the incremental exercise to exhaustion, subjects reached 287.3 ± 39 and 177.3 ± 36.4 W in normoxia and hypoxia, respectively ($p < 0.001$). The effects of metabolite accumulation in both conditions have been reported previously (1). Briefly, muscle lactate, phosphocreatine (PCr) and ATP changed similarly after IE. Muscle lactate increased only at Oc1m (25%; $p < 0.05$), and PCr was reduced by a 94 and 48% in Oc1m and FC1m, respectively ($p < 0.005$). Femoral vein PO_2 was 21.1± 2.0 and 10.6±2.8 mmHg at Wmax, in Nx and Hyp, respectively ($p < 0.001$).

8.2. Results of article 1.

8.2.1. Muscle signalling.

I. CaMKII, AMPK and p62.

$pThr^{287}$ CaMKII expression was increased by 1.7-fold after IE, remaining at this level after one minute of occlusion (1.9-fold above Pre), with a similar response in Nx and Hyp (ANOVA F_IO_2 effect $p = 0.83$, time effect $p = 0.001$, F_IO_2 by time interaction $p = 0.9$) (Fig. 3A). Solely in the leg recovering with free circulation, $pThr^{287}$ CaMKII levels returned to pre-exercise values one minute after the end of the IE.

Compared to Pre, $pThr^{172}$ AMPKα expression was increased by 2.4 and 3.0-fold at Post and Oc1m (Post vs Oc1m $p = 0.07$; ANOVA time effect $p < 0.001$), with a similar response in Nx and Hyp (ANOVA F_IO_2 effect $p = 0.71$, F_IO_2 by time interaction $p = 0.68$, Fig. 3B). After the IE performed in Hyp, no significant differences were observed between the occluded and non-occluded leg one min after exercise ($p = 0.19$) (Fig. 3B).

Compared to Pre, AMPKα total expression was reduced by 25 and 27 % at Post and Oc1m (ANOVA time effect p = 0.007), with a similar response in Nx and Hyp (F_IO_2 effect p = 1.0, ANOVA F_IO_2 by time interaction p = 0.72, Fig. 3C). One minute after the end of the IE, AMPKα total expression returned to the pre-exercise levels only in the non-occluded leg (Fig. 3C).

The ratio $pThr^{172}$ AMPKα / AMPKα total was increased by 4.5 and 4.0-fold at Post and Oc1m (Post vs Oc1m p = 0.38; ANOVA time effect p < 0.004), with a similar response in Nx and Hyp (ANOVA F_IO_2 effect p = 0.71, F_IO_2 by time interaction p = 0.42, Fig. 3D). After the IE performed in Hyp, the $pThr^{172}$ AMPKα / AMPKα total ratio tended to be 52 % lower in the leg recovering without occlusion (p = 0.057) (Fig. 3D).

The protein expression of $pSer^{349}$ p62 showed a tendency to increase with time (ANOVA time effect p = 0.051). When the mean of the two Pre conditions was compared to the mean of Post and Oc1m conditions, $pSer^{349}$ p62 protein was 26 % higher after exercise (p = 0.04). Following the IE performed in Hyp, $pSer^{349}$ p62 protein was 41 % lower in the leg recovering without occlusion compared with the occluded leg (p = 0.026), and similar to that observed at Pre (Fig. 3E). No significant changes in p62 total protein were detected by the with raw data ANOVA (F_IO_2 effect p = 0.84, time effect p = 0.07, F_IO_2 by time interaction p = 0.50). A secondary analysis was carried out to compare the mean of the two Pre conditions with the mean of the two post-exercise conditions (Post and Oc1m), which showed that p62 was reduced by ~20% after exercise (p = 0.02) (Fig. 3F). The ratio $pSer^{349}$ p62 / p62 total was increased by 1.8 and 1.5-fold at Post and OC1m (Post vs Oc1m p = 0.17; ANOVA time effect p = 0.029), with a similar response in Nx and Hyp (F_IO_2 effect p = 0.34, ANOVA F_IO_2 by time, p = 0.19, Fig. 3G). Following the IE performed in Hyp, the ratio $pSer^{349}$ p62 / p62 total was 43 % lower in the leg recovering without occlusion (p = 0.017), and similar to that observed before the exercise (Fig. 3G).

After the IE performed in Hyp, the pSer^{349} p62 / p62 total ratio increased by 1.5-fold during the period of ischaemia (p = 0.026). There was a strong association between the ratio pThr^{172} AMPKα / AMPKα total and the ratio pSer^{349} p62 / p62 total across conditions (r = 0.83, p = 0.02, n = 8, each point represents the mean of the subjects studied) (Fig. 3H).

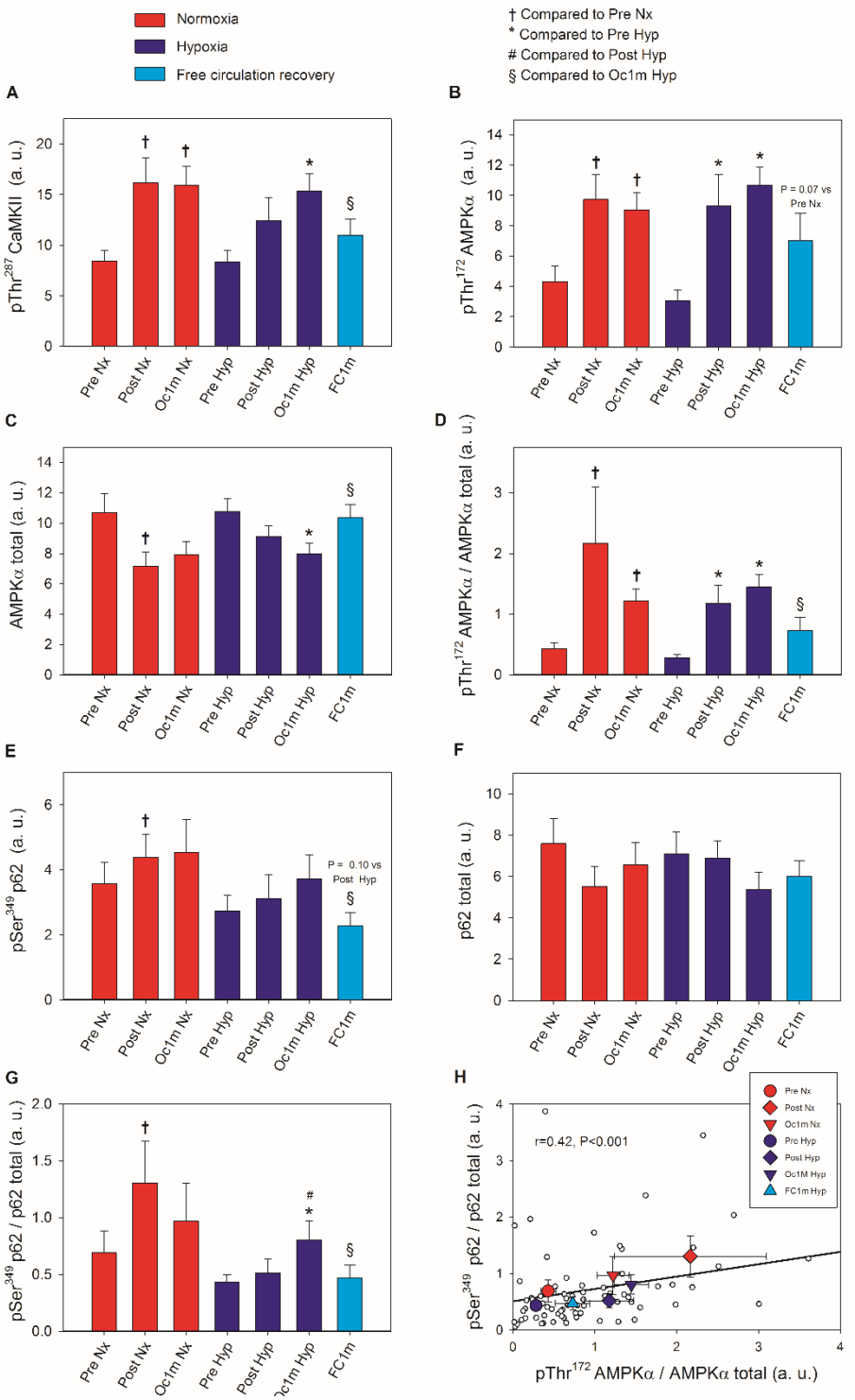


Figure 3. Skeletal muscle intracellular CaMKII, AMPKα and p62 signalling in response to incremental exercise to exhaustion in normoxia and severe hypoxia and the application of immediate ischaemic or non-ischaemic recovery. Protein expression levels of $pThr^{287}$ CaMKII **(A)**, $pThr^{172}$ AMPKα **(B)**, AMPKα total **(C)**, $pThr^{172}$ AMPKα / AMPKα total ratio **(D)**, $pSer^{349}$ p62 **(E)**, p62 total (**F**), $pSer^{349}$ p62 / p62 total ratio (**G**), and association between the ratio of $pThr^{172}$ AMPKα / AMPKα total and the ratio of $pSer^{349}$ p62 / p62 total **(H)**. Nx; test performed in normoxia (F_IO_2 = 0.21, P_iO_2: 143 mmHg), Hyp; test performed in severe acute normobaric hypoxia (F_IO_2 = 0.104, P_iO_2: 73 mmHg); Pre, before exercise; Post, 10 s after the end of exercise with ischaemic recovery; Oc1m, 60 s after the end of exercise with ischaemic recovery; FC1m, 60 s after the end of exercise in leg recovering without occlusion (free circulation); p62 / SQSTM1 (shortened to p62). n = 11 in all conditions except for Oc1m Nx (n = 9), Post Hyp (n = 10), and Oc1m Hyp (n = 10). In (**H**), large symbols represent the mean of the subjects studied in each condition. The correlation coefficients and regression line has been calculated using the individual values (small white circles, n = 73). A detailed description of the experimental phases is explained in Fig. 1. The statistical analysis was performed with logarithmically transformed data for the $pThr^{172}$ AMPKα / AMPKα total ratio and p62 total. The values shown are means ± standard errors and expressed in arbitrary units (a.u.). † $p < 0.05$ vs Pre Nx; * $p < 0.05$ vs Pre Hyp; $^{\#}$ $p < 0.05$ vs Post Hyp; § $p < 0.05$ vs Oc1m Hyp.

II. Catalase, SOD1 and SOD2 antioxidant enzymes.

Catalase protein expression was increased by 2.3 and 2.8-fold immediately after IE (Post) and after one minute of ischaemic recovery (Oc1m), respectively, with a similar response in Nx and Hyp (ANOVA time effect p = 0.001, F_IO_2 by time interaction p = 0.12). Since the level of Catalase tended to be lower in the Pre value obtained the day of the experiment in Hyp (p = 0.10), we additionally analysed the experiment performed in Hyp separately. During the IE in Hyp, compared to Pre, Catalase expression levels were elevated 1.9-fold and 3.3-fold immediately after the IE (Post) and after one minute of occlusion (Oc1m), respectively. From the end of the IE in Hyp, the level of Catalase doubled during the occlusion (Post vs Oc1m, p = 0.018) (Fig. 4A). One minute after the IE in Hyp, Catalase protein levels were reduced by 56 % in the non-occluded leg (FC1m), remaining 1.5-fold above Pre levels (p = 0.048) (Fig. 4A).

Although no significant changes were observed in SOD1 protein levels with exercise nor ischaemia, SOD1 tended to decrease and increase after the IE in Nx and Hyp, respectively (ANOVA F_IO_2 effect p = 0.58; time effect p = 0.86, F_IO_2 by time interaction p = 0.053). To reduce variability between starting values on the testing day, we also repeated the analysis for fold changes regarding the pre-exercise value of each day. This analysis showed that SOD1 was reduced and increased after the IE in Nx and Hyp, respectively (ANOVA F_IO_2 effect p = 0.002, time effect p = 0.90, F_IO_2 by time interaction p = 0.044). The mean value of the Post and Oc1m for each subject after the IE in Nx and Hyp was compared using a paired Student´s *t*-test. This analysis found a 30 % higher SOD1 protein content after exercise + occlusion in Hyp than in Nx (p = 0.001). In both experiments, SOD1 remained unchanged from the 10^{th} to 60^{th} s of ischaemia. One min after IE in Hyp, no significant differences were observed in SOD1 expression between the occluded and the non-occluded leg from 10^{th} to 60^{th} s (p = 0.11) (Fig. 4B). No significant changes were observed in SOD2 protein levels with exercise nor ischaemia (ANOVA F_IO_2 effect p = 0.34; time effect p = 0.45, F_IO_2 by time interaction p = 0.22) (Fig. 4C). The protein expression of SOD1 and SOD2 were positively associated (r = 0.84, p = 0.02, n = 8, with each point representing the mean of the subjects studied).

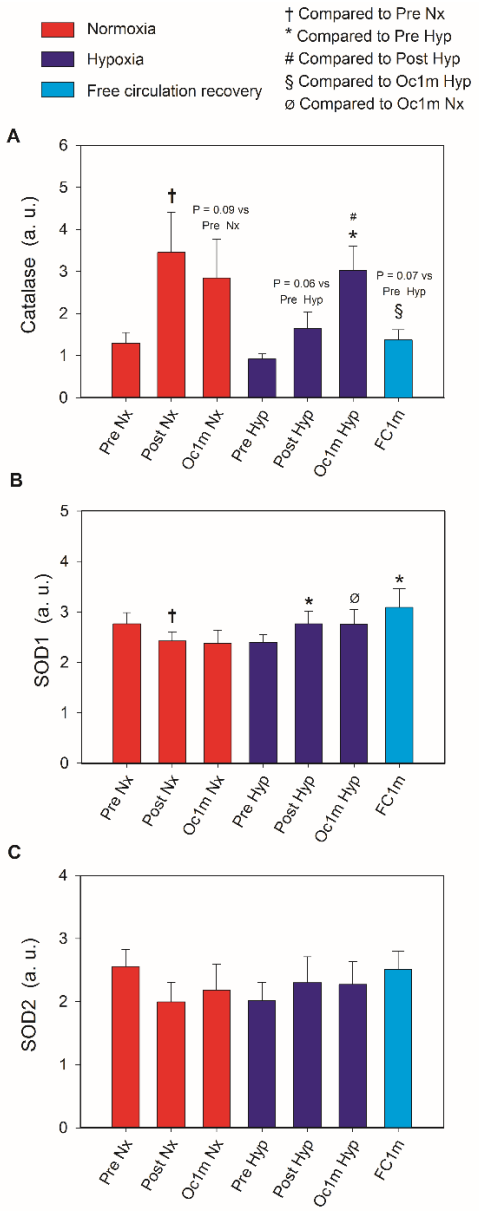


Figure 4. Skeletal muscle intracellular Catalase, SOD1 and SOD2 protein levels in response to incremental exercise to exhaustion in normoxia and severe hypoxia and the application of immediate ischaemic or non-ischaemic recovery. Protein expression levels of Catalase **(A)**, SOD1 **(B)**, and SOD2 **(C)**. Nx; test performed in normoxia (F_IO_2 = 0.21, P_iO_2: 143 mmHg), Hyp; test performed in severe acute normobaric hypoxia (F_IO_2 = 0.104, P_iO_2: 73 mmHg); Pre, before exercise; Post, 10 s after the end of exercise with ischaemic recovery; Oc1m, 60 s after the end of exercise with ischaemic recovery; FC1m, 60 s after the end of exercise in leg recovering without occlusion (free circulation). A detailed description of the experimental phases is explained in Fig. 1. The statistical analysis was performed with logarithmically transformed data for Catalase and SOD2 and with fold-change data for SOD1. The values shown are means ± standard errors and expressed in arbitrary units (a.u.). n = 11 in all conditions except for Oc1m Nx (n = 9), Post Hyp (n = 10), and Oc1m Hyp (n = 10). † $p < 0.05$ vs Pre Nx; * $p < 0.05$ vs Pre Hyp; $^{\#}$ $p < 0.05$ vs Post Hyp; § $p < 0.05$ vs Oc1m Hyp; ^{Ø} $p < 0.05$ vs Oc1m Nx.

III. Nrf2/Keap1 signalling.

The levels of phosphorylated Nrf2 at Ser^{40} were increased in Post and Oc1m compared to Pre by 1.5 and 1.6-fold, respectively, with a similar response in Nx and Hyp (ANOVA F_IO_2 by time, $p = 0.7$; ANOVA time effects $p < 0.01$, Fig. 5A). The exercise-elicited increase of $pSer^{40}$ Nrf2 was maintained at the same level after one min of ischaemia, while it recovered to pre-exercise values in the non-occluded leg. Similar changes were observed in the expression of Nrf2 total protein (Fig. 5B). Although no statistically significant changes were observed in the pSer40 Nrf2 / Nrf2 total ratio between Nx and Hyp, 1 min after IE in Hyp, this ratio was higher in the occluded compared to the non-occluded leg ($p = 0.02$) (Fig. 5C).

Compared to Pre, Keap1 expression was diminished by 23 and 29 % at Post and Oc1m, respectively (ANOVA time effects $p = 0.015$; ANOVA F_IO_2 by time interaction $p = 0.52$). One minute after exercise, Keap1 recovered pre-exercise values in the non-occluded leg (Fig. 5D).

The Nrf2 total protein / Keap1 ratio was augmented by 3.3-fold ($p = 0.02$) at Post, remaining at this level (3.4-fold above Pre) after one min of occlusion. This response was similar for the exercise performed in Nx and Hyp (ANOVA time effects $p = 0.002$; F_IO_2 by time interaction $p = 0.45$). One minute after the end of the IE, Nrf2 total protein / Keap1 ratio returned to the pre-exercise levels in the non-occluded leg (Fig. 5E).

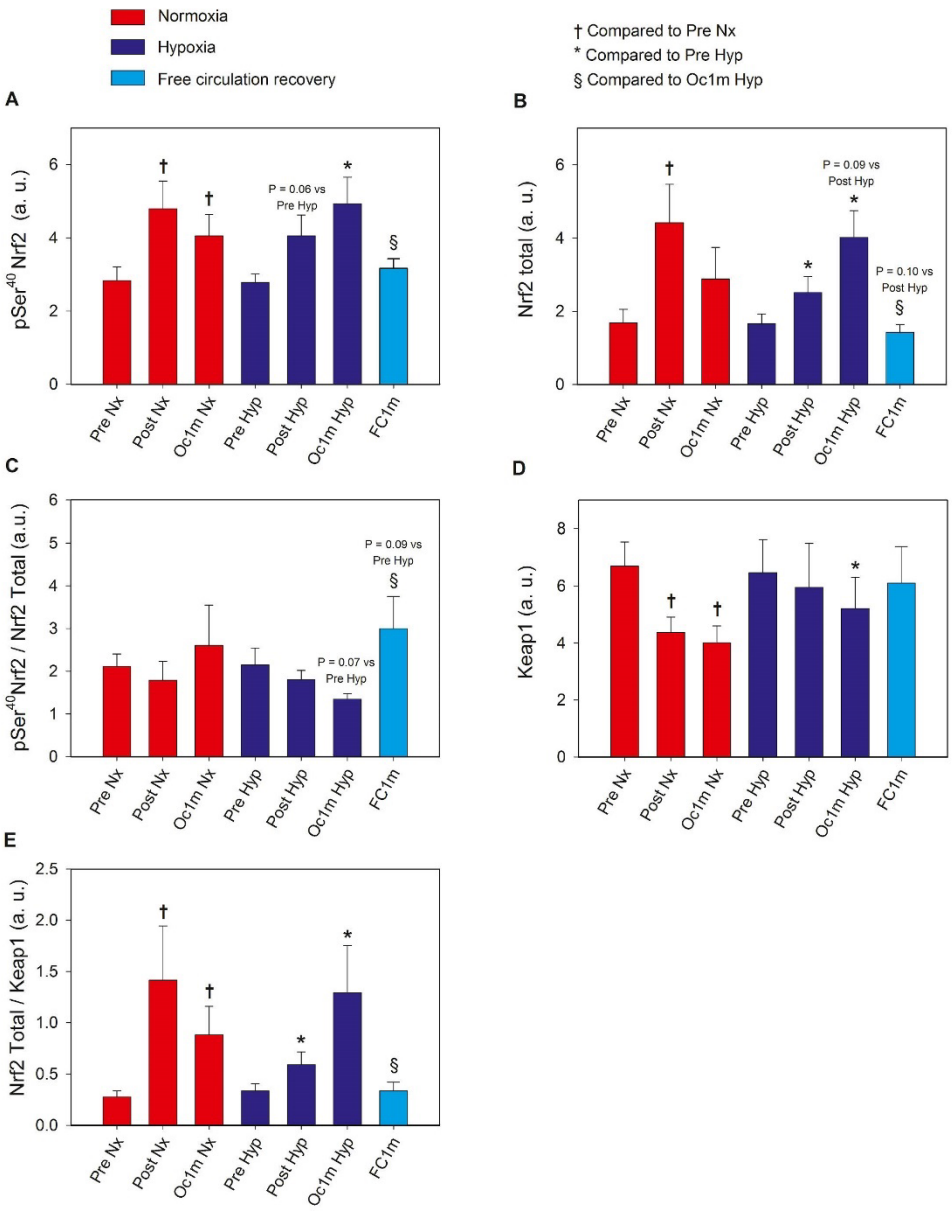


Figure 5. Skeletal muscle intracellular Nrf2 and Keap1 signalling in response to incremental exercise to exhaustion in normoxia and severe hypoxia and the application of immediate ischaemic or non-ischaemic recovery. Protein expression levels of pSer^{40} Nrf2 **(A)**, Nrf2 total **(B),** pSer^{40} Nrf2 / Nrf2 total ratio (**C**), Keap1 **(D),** Nrf2 Total / Keap1 ratio (**E**). Nx; test performed in normoxia (F_IO_2 = 0.21, P_iO_2: 143 mmHg), Hyp; test performed in severe acute normobaric hypoxia (F_IO_2 = 0.104, P_iO_2: 73 mmHg); Pre, before exercise; Post, 10 s after the end of exercise with ischaemic recovery; Oc1m, 60 s after the end of exercise with ischaemic recovery; FC1m, 60 s after the end of exercise in leg recovering without occlusion (free circulation). A detailed description of the experimental phases is explained in Fig. 1. The statistical analysis was performed with logarithmically transformed data for all proteins except for Keap1. The values shown are means ± standard errors and expressed in arbitrary units (a.u.). n = 11 in all conditions except for Oc1m Nx (n = 9), Post Hyp (n = 10), and Oc1m Hyp (n = 10). † $p < 0.05$ vs Pre Nx; * $p < 0.05$ vs Pre Hyp; # $p < 0.05$ vs Post Hyp; § $p < 0.05$ vs Oc1m Hyp.

8.2.2. Linear associations

Phosphorylated CaMKII at Thr287 is closely associated with Nrf2 and Keap1 proteins. As illustrated in Fig. 6, pThr287 CaMKII protein levels were positively associated with those of pSer40 Nrf2 expression ($r = 0.93$, $p = 0.003$), Nrf2 total protein ($r = 0.87$, $p = 0.01$), and the Nrf2 total protein/Keap1 ratio ($r = 0.90$, $p = 0.005$, and negatively with Keap1 ($r = -0.93$, $p = 0.003$) across conditions ($r = 0.90$, $p = 0.005$, $n = 8$, in all instances each point represents the mean of the subjects studied). There was a close negative association between the protein expression levels of Nrf2 and Keap1 across conditions ($r = -0.81$, $p = 0.03$, $n = 8$, each point represents the mean of the subjects studied). Nrf2 signalling during exercise is closely associated with Catalase protein expression but not with SOD1 or SOD2 protein levels

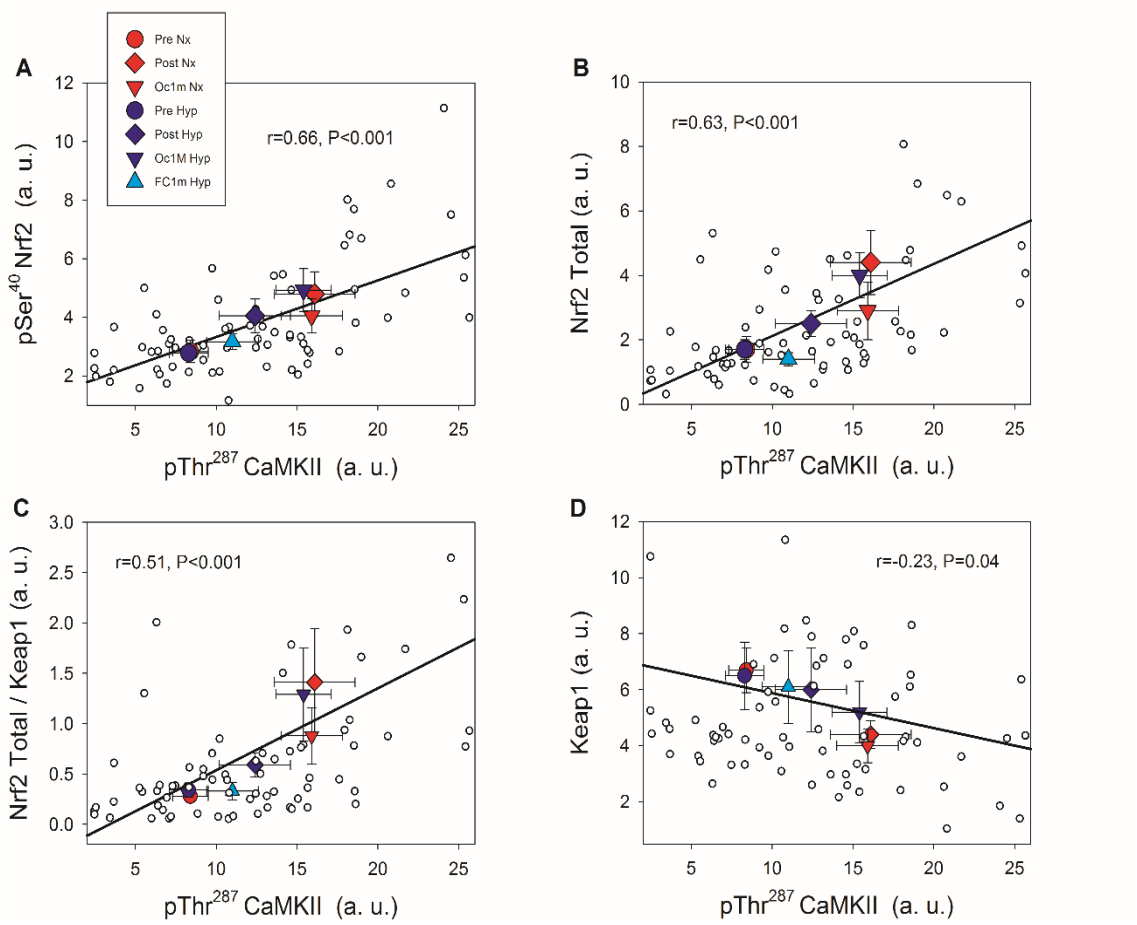


Figure 6. Associations between levels of phosphorylated CaMKII, Nrf2 and Keap1 protein expression across experimental phases. pThr^{287} CaMKII and pSer40 Nrf2 **(A)** pThr^{287} CaMKII and Nrf2 total **(B)**, pThr^{287} CaMKII and Nrf2 / Keap1 ratio **(C)**, pThr^{287} CaMKII and Keap1 **(D)**. A description of the experimental phases is explained in Fig. 1. Nx; test performed in normoxia (F_IO_2 = 0.21, P_iO_2: 143 mmHg), Hyp; test performed in severe acute normobaric hypoxia (F_IO_2 = 0.104, P_iO_2: 73 mmHg); Pre, before exercise; Post, 10 s after the end of exercise with ischaemic recovery; Oc1m, 60 s after the end of exercise with ischaemic recovery; FC1m, 60 s after the end of exercise in leg recovering without occlusion (free circulation). n = 11 in all conditions except for Oc1m Nx (n = 9), Post Hyp (n = 10), and Oc1m Hyp (n = 10). Large symbols: each point is representing the mean of the subjects studied in each condition. Correlation coefficients and regression lines have been calculated using the individual values (small white circles, n = 73). The values shown are means ± standard errors and expressed in arbitrary units (a.u.). Statistical significance was set at $p < 0.05$.

Catalase protein expression was closely associated with Nrf2 total protein expression (r = 0.94, p = 0.002), pSer40 Nrf2 (r = 0.92, p = 0.04), Keap1 (r = -0.90, p = 0.005), and the Nrf2 total protein / Keap1 ratio (r = 0.96, p < 0.001, in the four cases n = 8, with each point representing the mean of the subjects studied) (Fig. 7).

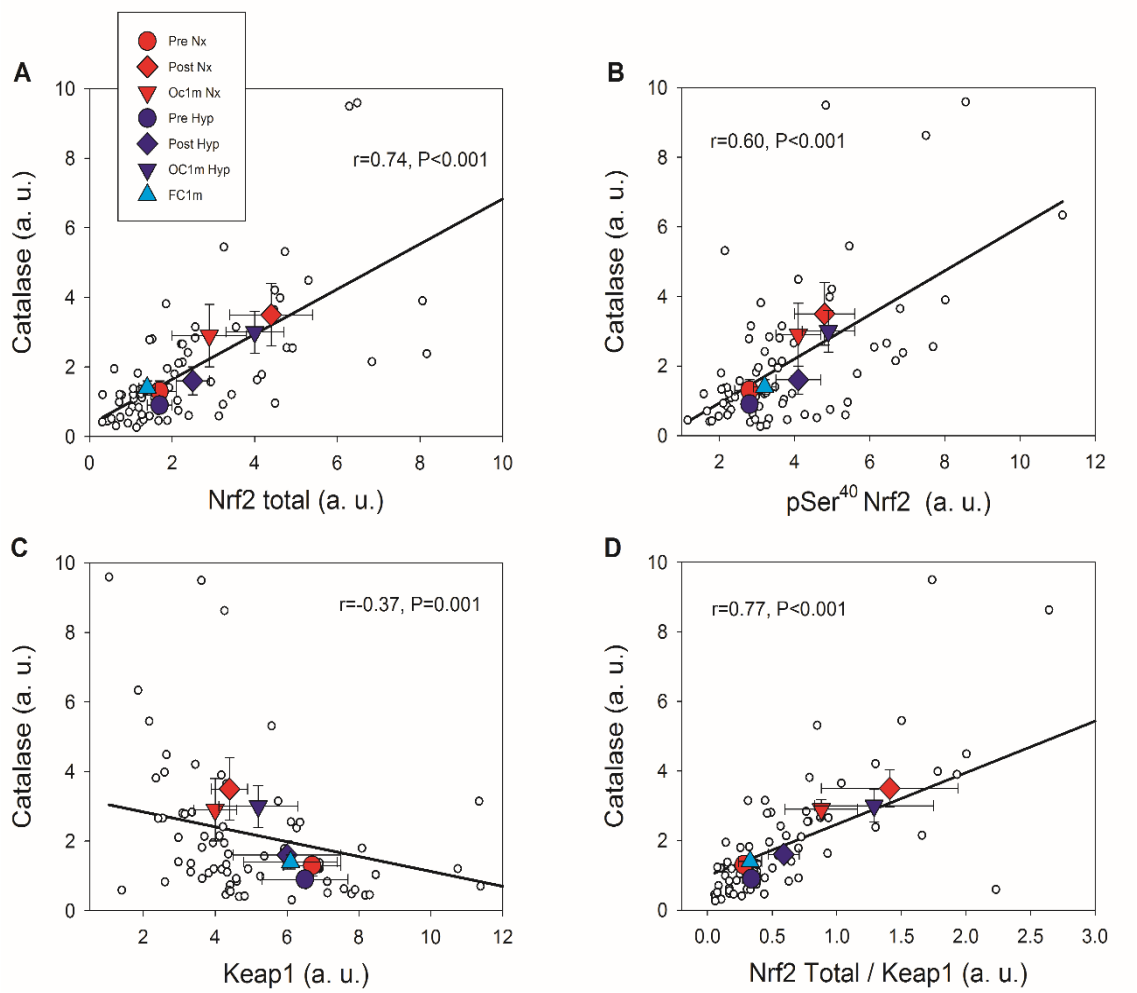


Figure 7. Associations between levels of Catalase, Nrf2 and Keap1 protein expression across experimental phases. Nrf2 total and Catalase **(A),** pSer^{40} Nrf2 and Catalase **(B)**, Keap1 and Catalase **(C)** and Nrf2 / Keap1 ratio and Catalase (**D**). A description of the experimental phases is explained in Fig. 1. Nx; test performed in normoxia (F_IO_2 = 0.21, P_iO_2: 143 mmHg), Hyp; test performed in severe acute normobaric hypoxia (F_IO_2 = 0.104, P_iO_2: 73 mmHg); Pre, before exercise; Post, 10 s after the end of exercise with ischaemic recovery; Oc1m, 60 s after the end of exercise with ischaemic recovery; FC1m, 60 s after the end of exercise without ischaemic recovery (free circulation). n = 11 in all conditions except for Oc1m Nx (n = 9), Post Hyp (n = 10), and Oc1m Hyp (n = 10). Large symbols: each point is representing the mean of the subjects studied in each condition. Correlation coefficients and regression lines have been calculated using the individual values (small white circles, n = 73). The values shown are means ± standard errors and expressed in arbitrary units (a.u.). Statistical significance was set at p < 0.05.

No associations were observed between Nrf2 total protein and SOD1 (r = -0.39, p = 0.38); pSer40 Nrf2 protein and SOD1 (r = -0.19, p = 0.67), Nrf2 total protein / Keap1 ratio and SOD1 (r = -0.37, p = 0.41), and pThr287 CaMKII and SOD1 (r = -0.28, p = 0.54, in the four cases n=8, with each point representing the mean of the subjects studied). No associations were observed between Nrf2 total protein and SOD2 (r = -0.53, p = 0.22), pSer40 Nrf2 protein and SOD2 (r = -0.39, p = 0.39), Nrf2 total protein / Keap1 ratio and SOD2 (r = -0.50, P=0.22), and pThr287 CaMKII and SOD2 (r = -0.39, p = 0.38, in the four cases n=8, with each point representing the mean of the subjects studied).

8.3. Results of Article 2.

8.3.1. Muscle signalling.

I. pThr^{287} CaMKII muscle isoforms.

Compared to Pre, pThr^{287} CaMKII β_M was increased by 1.6 and 2.0-fold after IE and one minute of occlusion, respectively, with a similar response in Nx and Hyp (F_IO_2 effect p = 0.96, time effect p = 0.005, F_IO_2 x time interaction p = 0.92). pThr^{287} CaMKII β_M returned to pre-exercise values after one-minute recovery with open circulation (Fig. 8A). Compared to Pre, pThr^{287} CaMKII δ_A was increased by 1.3 and 1.5-fold after IE and one minute of occlusion, respectively, with a similar response in Nx and Hyp (F_IO_2 effect p = 0.74, time effect p = 0.014, F_IO_2 x time interaction p = 0.93). pThr^{287} CaMKII δ_A returned towards pre-exercise values after one-minute recovery with open circulation (p = 0.24, compared to Pre levels) (Fig. 8B). Compared to Pre, pThr^{287} CaMKII γ was increased by 1.3 and 1.4- fold after IE and one minute of occlusion, respectively (F_IO_2 effect p = 0.16, time effect p = 0.022, F_IO_2 x time interaction p = 0.72). One minute after IE, the level of pThr^{287} CaMKII γ was similarly elevated in both legs (p = 0.10) (Fig. 8C). Compared to Pre, pThr^{287} CaMKII δ_D was increased by 2.0 and 2.5-fold after IE and one minute of occlusion, respectively, with a similar response in Nx and Hyp (F_IO_2 effect p = 0.75, time effect p < 0.001, F_IO_2 x time interaction p = 0.79). pThr^{287} CaMKII δ_D returned to pre-exercise values after recovery for one min with open circulation (p = 0.73 compared to Pre) (Fig. 8D).

Total CaMKII δ_D was increased by 1.6 and 2.4-fold after IE and one minute of occlusion, respectively (time effect p < 0.001), with a similar response in Nx and Hyp (F_IO_2 effect p = 0.09, F_IO_2 x time interaction p = 0.62) (Fig. 8E). Total CaMKII δ_D returned to pre-exercise values after one-minute recovery with open circulation (p = 0.13 compared to Pre). One min after exercise, total CaMKII δ_D was 46% lower in the leg with

free circulation compared with the occluded leg (p = 0.014) (Fig. 8E). No significant changes were observed in the total expression of the other CaMKII isoforms (Fig. 9).

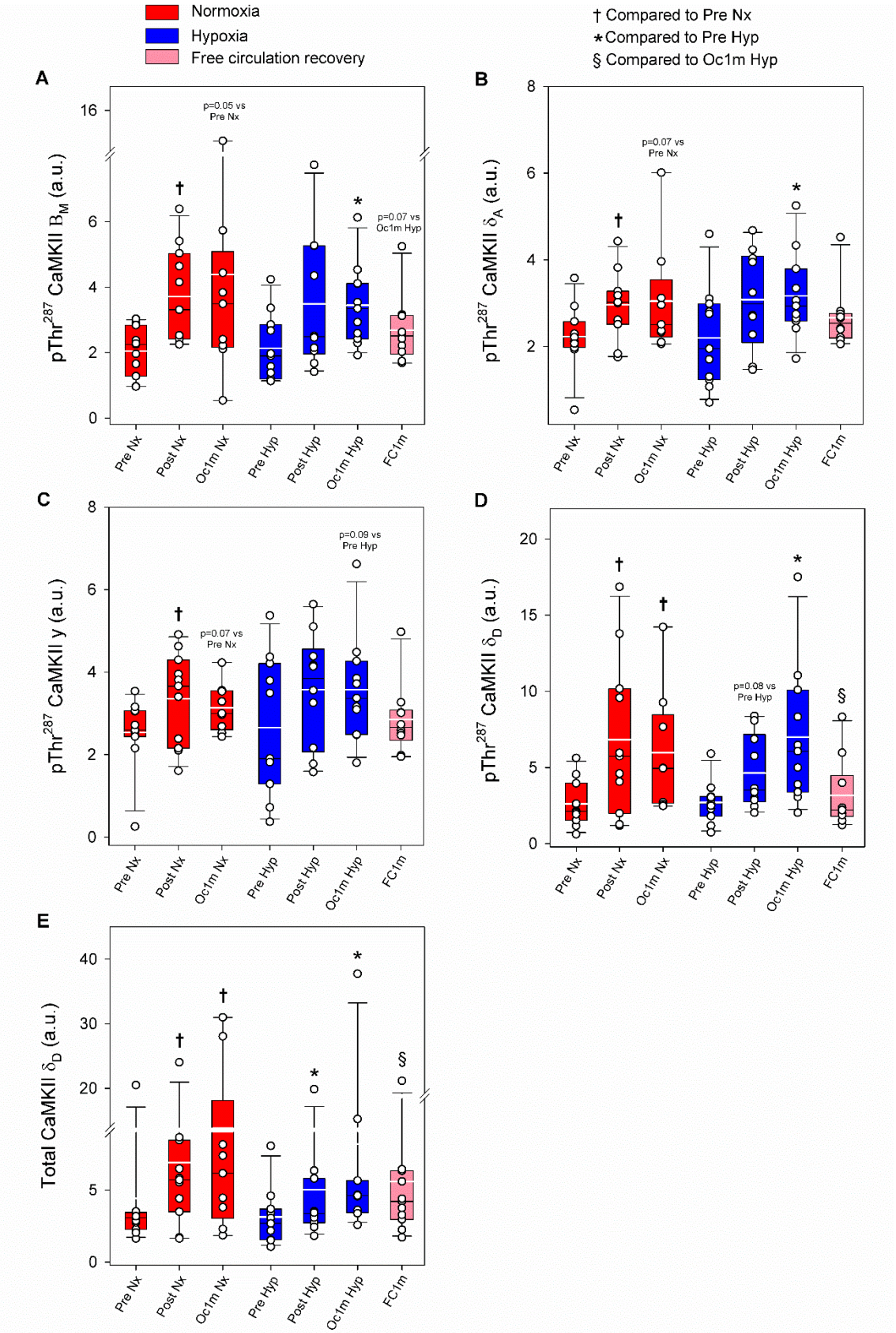


Figure 8. CaMKII isoforms phosphorylation and total CaMKII δD signalling in human skeletal muscle in response to incremental exercise to exhaustion in normoxia and severe hypoxia, and post-exercise ischaemia. Levels of protein expression of (A) pThr^{287} CaMKII β_M, (B) pThr^{287} CaMKII γ, (C) pThr^{287} CaMKII δ_A, (D) pThr^{287} CaMKII δ_D and (E) Total CaMKII δ_D. Nx: normoxia session (F_IO_2 = 0.21, P_IO_2 = 143mmHg); Hyp: severe normobaric hypoxia session (F_IO_2 = 0.104, P_IO_2 = 73 mmHg); Pre: before exercise; Post: 10 s after exercise cessation during ischaemic recovery; Oc1m: 60 s after exercise cessation during ischaemic recovery; FC1m: 60 s after exercise cessation during recovery with free circulation. n = 11 for all conditions except for Oc1m Nx (n = 9), Post Hyp (n =10) and FC1m (n=10). See Fig. 1 for a detailed description of the experimental phases. The statistical analysis was performed with logarithmically transformed data for pThr^{287} CaMKII β_M, pThr^{287} CaMKII δ_A and Total CaMKII δ_D. Values presented are means ± standard errors and expressed in arbitrary units (a.u.). † $p < 0.05$ vs. Pre Nx; * $p < 0.05$ vs. Pre Hyp; § $p < 0.05$ vs. Oc1m Hyp.

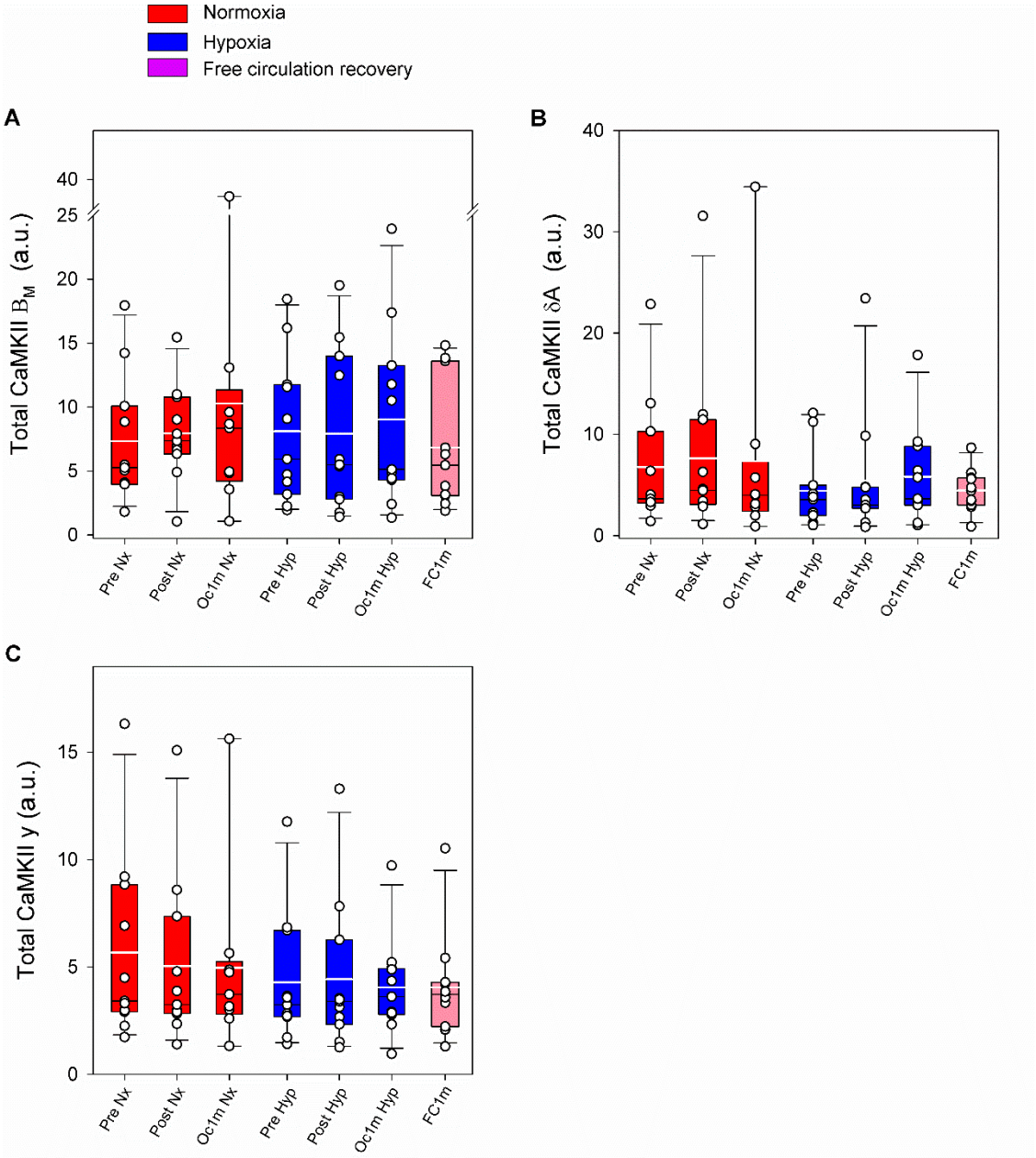


Figure 9. Total CaMKII isoforms signalling in human skeletal muscle in response to incremental exercise to exhaustion in normoxia and severe hypoxia, and post-exercise ischaemia. Levels of protein expression of (A) Total CaMKII β_M, (B) Total CaMKII δ_A, and (C) Total CaMKII γ. Total CaMKII δ_D is included in Fig. 2E. Nx: normoxia session (F_IO_2 = 0.21, P_IO_2 = 143 mmHg); Hyp: severe normobaric hypoxia session (F_IO_2 = 0.104, P_IO_2 = 73 mmHg); Pre: before exercise; Post: 10 s after exercise cessation during ischaemic recovery; Oc1m: 60 s after exercise cessation during ischaemic recovery; FC1m: 60 s after exercise cessation during recovery with free circulation. n = 11 for all conditions except for Oc1m Nx (n = 9). See Fig. 1 for a detailed description of the experimental phases. Values presented are means ± standard errors and expressed in arbitrary units (a.u.). No statistically significant effects were detected.

II. p38 MAPK, ERK1/2 signalling.

No significant changes were observed in p38 MAPK phosphorylation at Thr^{180}/Tyr^{182} (F_IO_2 effect p = 0.95, time effect p = 0.54, F_IO_2 x time interaction p = 0.25) (Fig. 10A). Compared to Pre, phospho-Thr^{202}/Tyr^{204} ERK1/2 was reduced 17 and 24 % after IE, and after one minute of occlusion (time effect p = 0.007), with a similar response in normoxia and hypoxia (F_IO_2 effect p = 0.92, F_IO_2 x time interaction p = 0.62). After one min of recovery, phospho-Thr^{202}/Tyr^{204} ERK1/2 was similar in both legs, regardless of the recovery with open or occluded circulation (p = 0.71) (Fig. 10B).

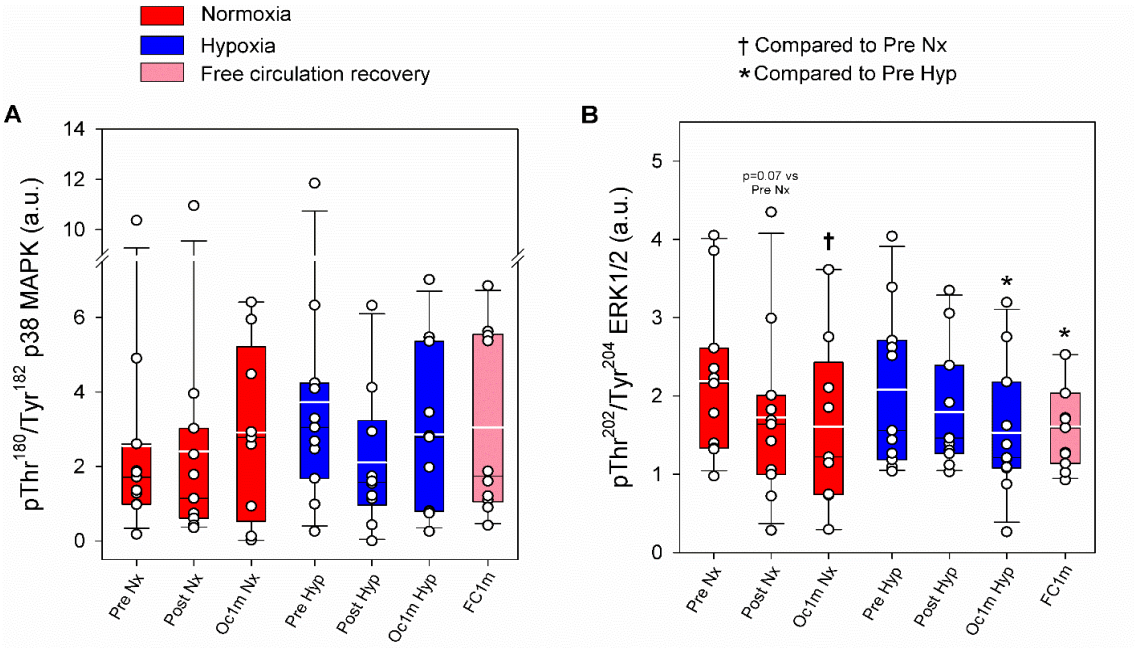


Figure 10. p38 MAPK and ERK1/2 phosphorylation signalling in human skeletal muscle in response to incremental exercise to exhaustion in normoxia and severe hypoxia, and post-exercise ischaemia. Levels of protein expression of (A) pThr^{180}/Tyr^{182} p38 MAPK and (B) pThr^{202}/Tyr^{204} ERK1/2. Nx: normoxia session (F_IO_2 = 0.21, P_IO_2 = 143mmHg); Hyp: severe normobaric hypoxia session (F_IO_2 = 0.104, P_IO_2 = 73 mmHg); Pre: before exercise; Post: 10 s after exercise cessation during ischaemic recovery; Oc1m: 60 s after exercise cessation during ischaemic recovery; FC1m: 60 s after exercise cessation during recovery with free circulation. For panel (A), n = 11 for all conditions except for Oc1m Nx (n = 9), Post Hyp (n =10) and FC1m (n=10) and for panel (B), n = 11 for all conditions except for Oc1m Nx (n = 9). See Fig. 1 for a detailed description of the experimental phases. The statistical analysis was performed with logarithmically transformed data for pThr^{180}/Tyr^{182} p38 MAPK. Values presented are means ± standard errors and expressed in arbitrary units (a.u.). † $p < 0.05$ vs. Pre Nx; * $p < 0.05$ vs. Pre Hyp; # $p < 0.05$ vs. Post Hyp; § $p < 0.05$ vs. Oc1m Hyp.

III. NF-κB signalling.

Compared to Pre, p105 was increased by 1.9 and 2.1-fold after IE, and after one minute of occlusion (time effect $p < 0.001$), with a similar response in normoxia and hypoxia (F_IO_2 effect $p = 0.91$, F_IO_2 x time interaction $p = 0.58$) (Fig. 11A). p105 returned to pre-exercise values after one minute of recovery with open circulation ($p = 0.44$, Fig. 11A). Consequently, one min after exercise p105 was 47% lower in the leg with free circulation compared with the occluded leg ($p = 0.011$). p50 followed a similar pattern, increasing by 1.3 and 1.5-fold after IE, and after one-minute occlusion (time effect $p = 0.005$), respectively, with a similar response in normoxia and hypoxia (F_IO_2 effect $p = 0.72$, F_IO_2 x time interaction $p = 0.32$) (Fig. 11B). p50 returned to pre-exercise values after one minute of recovery with open circulation ($p = 0.45$, Fig. 11B). Compared to the occluded leg, p50 was 33% lower in the leg recovering with free circulation ($p = 0.003$). The total amount of p65 was unchanged immediately after IE, increasing by 11 % during ischaemia, compared to the immediate post-exercise value ($p = 0.014$); time effect $p = 0.032$), with a similar response in normoxia and hypoxia (F_IO_2 effect $p = 0.52$, F_IO_2 x time interaction $p = 0.14$) (Fig. 11C). p65 returned to pre-exercise values after one-minute

recovery with open circulation (p = 0.057, Fig. 11C). One min after exercise, p65 was similar in the leg recovering with free circulation and the ischaemic leg (p = 0.087).

The p65+p50 was increased 1.2 and 1.4-fold after IE, and after one minute of occlusion (time effect p = 0.006), respectively, with a similar response in normoxia and hypoxia (F_IO_2 effect p = 0.73, F_IO_2 x time interaction p = 0.37) (Fig. 11D). p65+p50 returned to pre-exercise values after one-minute recovery with open circulation (p = 0.80, Fig. 11D). Compared to the occluded leg, p65+p50 was 29 % lower in the leg recovering with free circulation (p < 0.001). During the one min of ischaemia, p65+p50 was increased by 17 % (p = 0.046).

Phospho-Ser^{536} p65 was unchanged immediately after IE and was increased 1.6-fold in the leg recovering with ischaemia, compared to the immediate post-exercise value (p = 0.023); time effect p = 0.006), with a similar response in normoxia and hypoxia (F_IO_2 effect p = 0.97, F_IO_2 x time interaction p = 0.55) (Fig. 11E). One min after exercise, Phospho-Ser^{536} p65 was similar in the leg recovering with free circulation and the ischaemic leg (p = 0.19) (Fig. 11E).

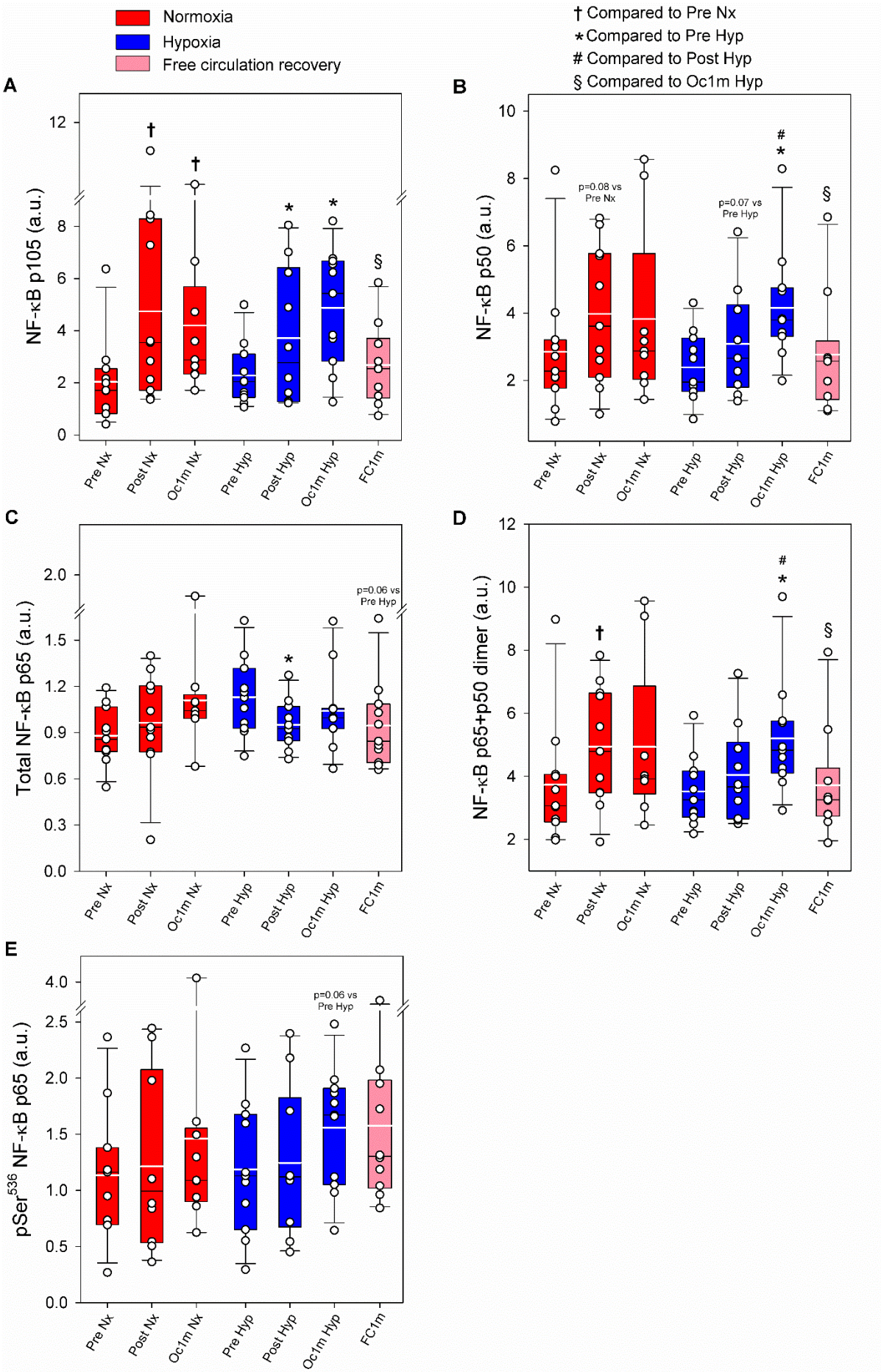

Normoxia
Hypoxia
Free circulation recovery
† Compared to Pre Nx
* Compared to Pre Hyp
# Compared to Post Hyp
§ Compared to Oc1m Hyp
A
NF-κB p105 (a.u.)
B
NF-κB p50 (a.u.)
p=0.08 vs Pre Nx
p=0.07 vs Pre Hyp
C
Total NF-κB p65 (a.u.)
p=0.06 vs Pre Hyp
D
NF-κB p65+p50 dimer (a.u.)
E
pSer536 NF-κB p65 (a.u.)
p=0.06 vs Pre Hyp
Pre Nx
Post Nx
Oc1m Nx
Pre Hyp
Post Hyp
Oc1m Hyp
FC1m

Figure 11. NF-κB signalling in human skeletal muscle in response to incremental exercise to exhaustion in normoxia and severe hypoxia, and post-exercise ischaemia. Levels of protein expression of (A) NF-κB p105, (B) NF-κB p50, (C) Total NF-κB p65, (D) NF-κB p65+p50 dimer and (E) $pSer^{536}$ NF-κB. Nx: normoxia session (F_IO_2 = 0.21, P_IO_2 = 143mmHg); Hyp: severe normobaric hypoxia session (F_IO_2 = 0.104, P_IO_2 = 73 mmHg); Pre: before exercise; Post: 10 s after exercise cessation during ischaemic recovery; Oc1m: 60 s after exercise cessation during ischaemic recovery; FC1m: 60 s after exercise cessation during recovery with free circulation. For panels (A), (B), (D) n = 11 for all conditions except for Oc1m Nx (n = 9), Post Hyp (n = 10) and FC1m (n = 10), for panel (C) n = 11 for all conditions except for Oc1m Nx (n = 9) and for panel (E) n = 11 for all conditions except for Post Nx (n=10), Oc1m Nx (n = 9), Post Hyp (n = 10) and FC1m (n = 10), See Fig. 1 for a detailed description of the experimental phases. The statistical analysis was performed with logarithmically transformed data for NF-κB p105, Total NF-Kb p65 and NF-κB p65+p50 dimer. Values presented are means ± standard errors and expressed in arbitrary units (a.u.). † $p < 0.05$ vs. Pre Nx; * $p < 0.05$ vs. Pre Hyp; # $p < 0.05$ vs. Post Hyp; § $p < 0.05$ vs. Oc1m Hyp.

IV. <u>IκB signalling.</u>

No significant changes were observed in the total amount of IκBα protein (F_IO_2 effect p = 0.83, time effect p = 0.07, and F_IO_2 x time interaction p = 0.47) (Fig. 12A), while its phosphorylation remained below the detection levels in all conditions. However the average of the post-exercise conditions was 20% lower than the average of the two Pre conditions (p = 0.008). During the one-min ischaemia the total amount of IκBα protein was reduced by 13 % when compared to POST (p = 0.019, t-test) (Fig. 12A).

Compared to Pre, IκBβ Thr^{19}/Ser^{23} phosphorylation was increased by 2.8 and 3.1-fold immediately after IE and after one minute of occlusion, respectively, (time effect p = 0.01) (Fig. 12B). The response was similar in normoxia and hypoxia (F_IO_2 effect p = 0.78, F_IO_2 x time interaction p = 0.73). IκBβ Thr^{19}/Ser^{23} phosphorylation returned to pre-exercise values after one min of recovery with free circulation (p = 0.26, Fig. 12B). Compared to the occluded leg, IκBβ Thr^{19}/Ser^{23} phosphorylation was 54% lower in the leg recovering with free circulation (p = 0.026). The total amount of IκBβ protein was increased by 4.5 and 5.8-fold immediately after IE and after one minute of occlusion, respectively, (time effect $p < 0.001$) (Fig. 12C). The response was similar in normoxia

and hypoxia (F_IO_2 effect p = 0.78, F_IO_2 x time interaction p = 0.68). Compared to the leg recovering in ischaemia, IκBβ total protein was slightly reduced by 63% in the leg recovering with a free circulation (p = 0.015, Fig. 12C). However, one minute after the end of exercise, the total amount of IκBβ protein was 3.9-fold higher than pre-exercise values in the leg recovering with free circulation (p = 0.005).

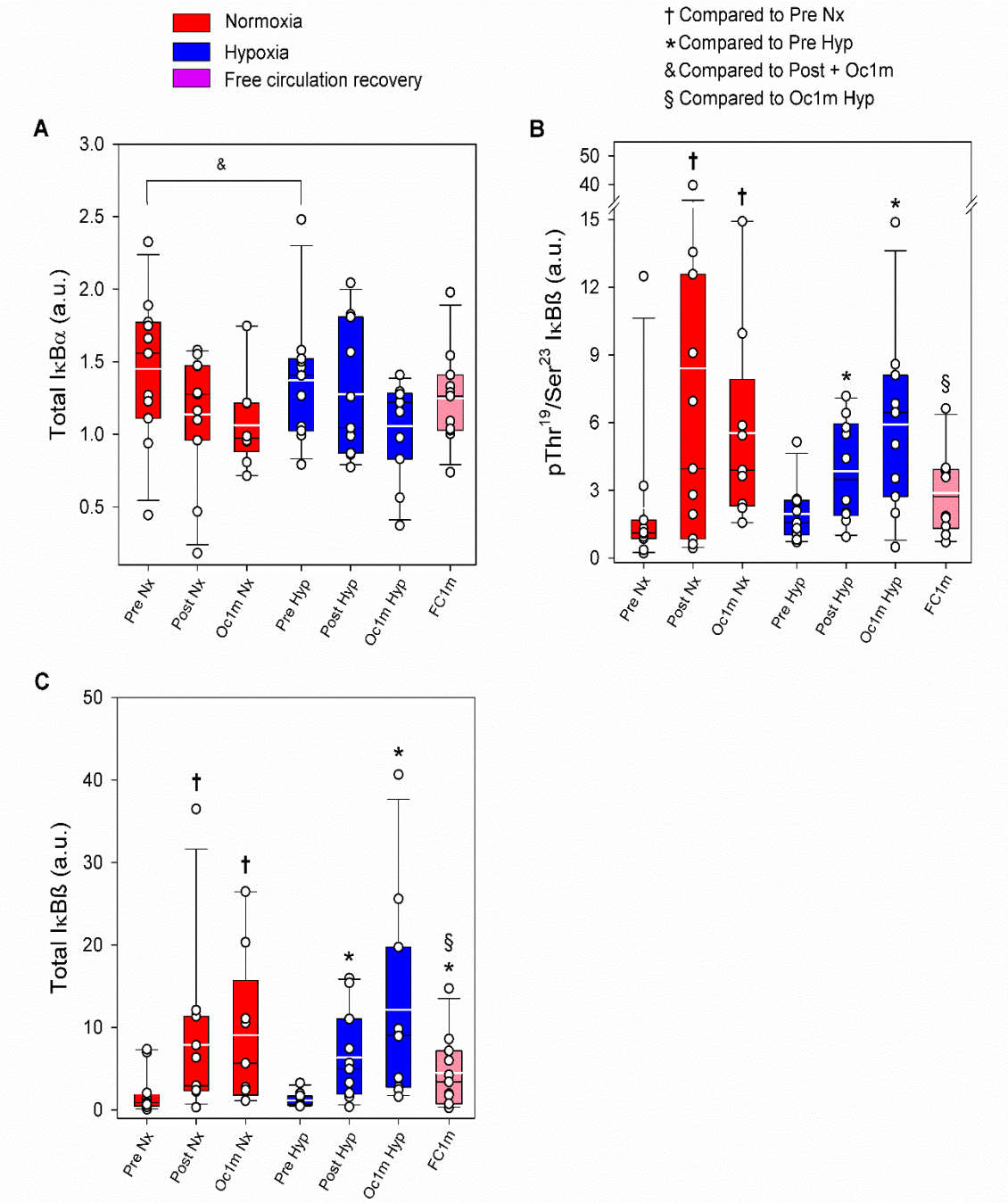


Figure 12. IκB signalling in human skeletal muscle in response to incremental exercise to exhaustion in normoxia and severe hypoxia, and post-exercise ischaemia. Levels of protein expression of (A) Total IκBα, (B) pThr^{19}/Ser^{23} IκBß, and (C) Total IκBß. Nx: normoxia session (F_IO_2 = 0.21, P_IO_2 = 143mmHg); Hyp: severe normobaric hypoxia session (F_IO_2 = 0.104, P_IO_2 = 73 mmHg); Pre: before exercise; Post: 10 s after exercise cessation during ischaemic recovery; Oc1m: 60 s after exercise cessation during ischaemic recovery; FC1m: 60 s after exercise cessation during recovery with free circulation. For panels (A) and (C), n = 11 for all conditions except for Oc1m Nx (n = 9) and for panel (B), n = 11 for all conditions except for Oc1m Nx (n = 9), Post Hyp (n =10) and FC1m (n = 10). See Fig. 1 for a detailed description of the experimental phases. The statistical analysis was performed with logarithmically transformed data for Total IκBß. Values presented are means ± standard errors and expressed in arbitrary units (a.u.). † $p < 0.05$ vs. Pre Nx; * $p < 0.05$ vs. Pre Hyp; # $p < 0.05$ vs. Post Hyp; § $p < 0.05$ vs. Oc1m Hyp.

V. <u>IKK signalling.</u>

Compared to Pre, IKKα/β Ser$^{176/180}$ phosphorylation was increased by 1.8 and 2.0-fold immediately after IE and after one minute of occlusion, respectively, (time effect p = 0.04) (Fig. 13A). The response was similar in normoxia and hypoxia (F_IO_2 effect p = 0.67, F_IO_2 x time interaction p = 0.34). IKKα/β Ser$^{176/180}$ phosphorylation returned to pre-exercise values after one min of recovery with a free circulation (p = 0.87, Fig. 13A). Compared to the occluded leg, IKKα/β Ser$^{176/180}$ phosphorylation was 61% lower in the leg recovering with free circulation (p = 0.019, Fig. 13A). IKKβ total protein did not change significantly (F_IO_2 effect p = 0.67, time effect p = 0.34, and F_IO_2 x time interaction p = 0.81) (Fig. 13B). Compared to Pre, IKKα total protein was increased by 2.6 and 3.5-fold immediately after IE and after one minute of occlusion, respectively, (time effect p < 0.001) (Fig. 13C). The response was similar in normoxia and hypoxia (F_IO_2 effect p = 0.24, F_IO_2 x time interaction p = 0.40). IKKα total protein returned to pre-exercise values after one min of recovery with free circulation remaining 1.9-fold above Pre (p = 0.008, Fig. 13C). Compared to the occluded leg, IKKα total protein was 44% lower in the leg recovering with free circulation (p = 0.03).

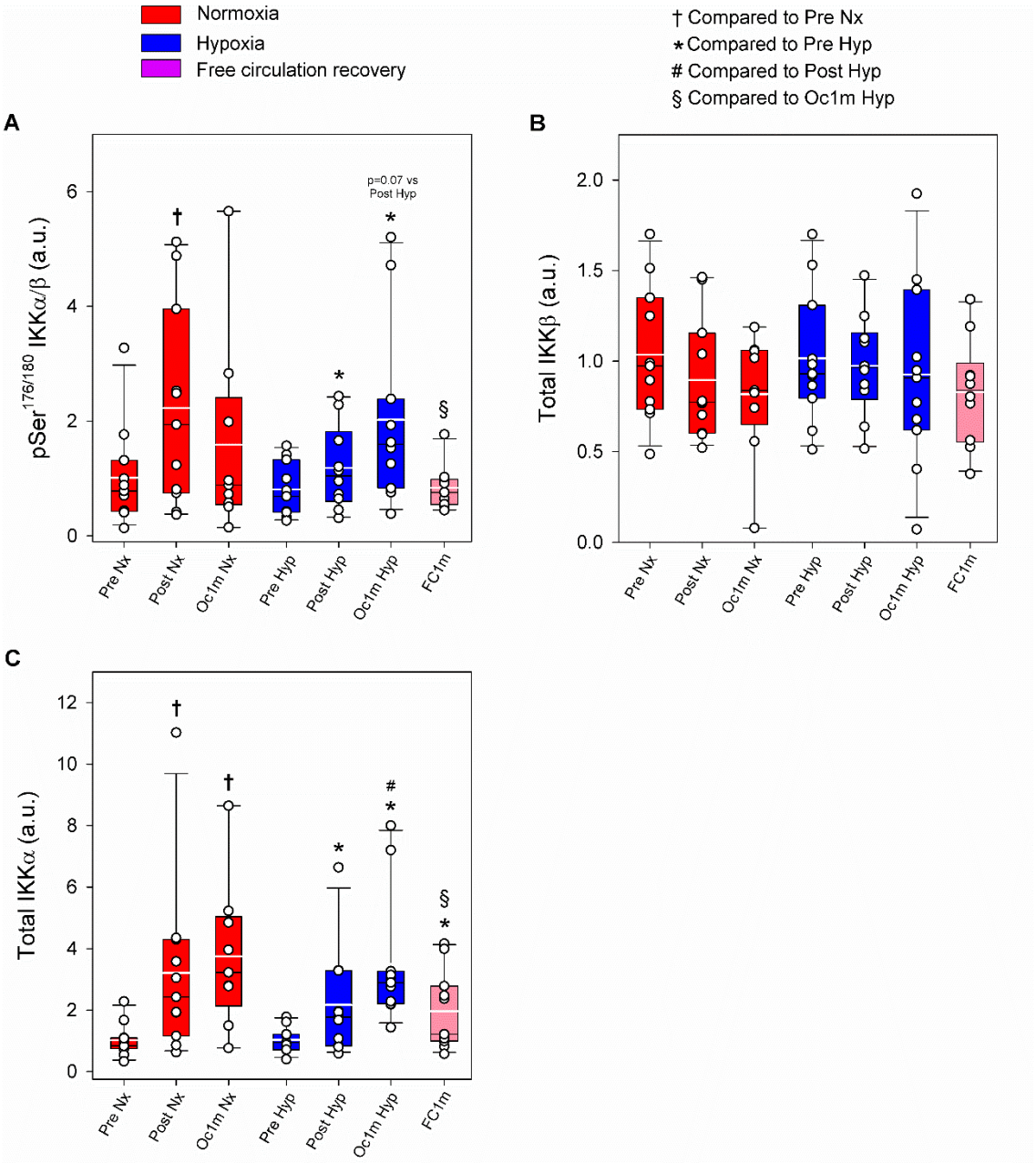


Figure 13. IKK signalling in human skeletal muscle in response to incremental exercise to exhaustion in normoxia and severe hypoxia, and post-exercise ischaemia. Levels of protein expression of (A) pSer$^{176/180}$ IKKα/ß, (B) Total IKKß, and (C) Total IKKα. Nx: normoxia session (F_IO_2 = 0.21, P_IO_2 = 143mmHg); Hyp: severe normobaric hypoxia session (F_IO_2 = 0.104, P_IO_2 = 73 mmHg); Pre: before exercise; Post: 10 s after exercise cessation during ischaemic recovery; Oc1m: 60 s after exercise cessation during ischaemic recovery; FC1m: 60 s after exercise cessation during recovery with free circulation. For panels (A) and (B), n = 11 for all conditions except for Oc1m Nx (n = 9), Post Hyp (n =10) and FC1m (n = 10) and for panel (C), n = 11 for all conditions except for Oc1m Nx (n = 9). See Fig. 1 for a detailed description of the experimental phases. The statistical analysis was performed with logarithmically transformed data for Total IKKα. Values presented are means ± standard errors and expressed in arbitrary units (a.u.). † $p < 0.05$ vs. Pre Nx; * $p < 0.05$ vs. Pre Hyp; # $p < 0.05$ vs. Post Hyp; § $p < 0.05$ vs. Oc1m Hyp.

VI. GR, Trx1, Gpx1 and TrxR1 antioxidant enzymes.

Compared to Pre, glutathione reductase (GR) protein expression was increased by 2.1 and 2.2-fold immediately after IE and after one minute of occlusion, respectively, (time effect $p = 0.002$) (Fig. 14A). The response was similar in normoxia and hypoxia (F_IO_2 effect $p = 0.46$, F_IO_2 x time interaction $p = 0.53$). GR protein expression returned to pre-exercise values after one min of recovery with a free circulation ($p = 0.15$, Fig. 14A). Compared to the occluded leg, GR protein expression was 52% lower in the leg recovering with free circulation ($p = 0.059$, Fig. 14A).

Compared to Pre, thioredoxin 1 (Trx1) protein expression was reduced by 10 and 17% immediately after IE and after one minute of occlusion, respectively, (time effect $p = 0.012$) (Fig. 14B). The response was similar in normoxia and hypoxia (F_IO_2 effect $p = 0.51$, F_IO_2 x time interaction $p = 0.77$). After one min of recovery, Trx1 protein expression was similar in the legs recovering with ischaemia and free circulation ($p = 0.083$, Fig. 14B).

No significant changes were observed in protein expression levels of glutathione peroxidase 1 (Gpx1) (F_IO_2 effect $p = 0.11$, time effect $p = 0.52$; F_IO_2 x time interaction $p = 0.50$) (Fig. 14C). No significant changes were observed in protein expression levels of thioredoxin reductase 1 (TrxR1) (F_IO_2 effect $p = 0.27$, time effect $p = 0.99$; F_IO_2 x time interaction $p = 0.48$) (Fig. 14D).

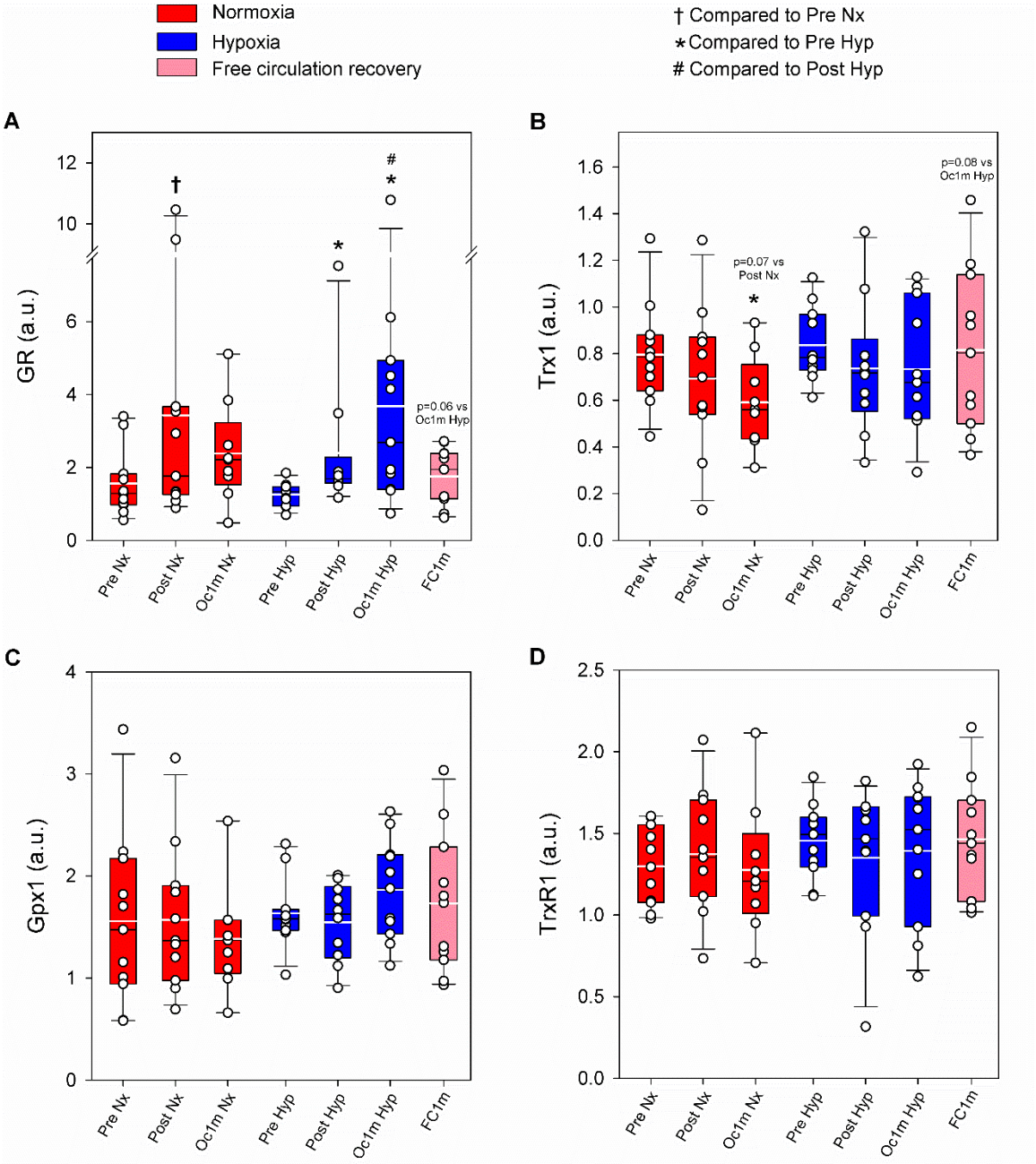


Figure 14. Skeletal muscle intracellular GR, Trx1, Gpx1 and TrxR1 protein levels in response to incremental exercise to exhaustion in normoxia and severe hypoxia, and post-exercise ischaemia. Levels of protein expression of (A) GR, (B) Trx1, (C) Gpx1 and (D) TrxR1. Nx: normoxia session (F_IO_2 = 0.21, P_IO_2 = 143mmHg); Hyp: severe normobaric hypoxia session (F_IO_2 = 0.104, P_IO_2 = 73 mmHg); Pre: before exercise; Post: 10 s after exercise cessation during ischaemic recovery; Oc1m: 60 s after exercise cessation during ischaemic recovery; FC1m: 60 s after exercise cessation during recovery with free circulation. For panel (A), n = 11 for all conditions except for Oc1m Nx (n = 9) and Post Hyp (n = 10) and for panels (B), (C) and (D), n = 11 except for Oc1m Nx (n = 9). See Fig. 1 for a detailed description of the experimental phases. The statistical analysis was performed with logarithmically transformed data for GR. Values presented are means ± standard errors and expressed in arbitrary units (a.u.). † $p < 0.05$ vs. Pre Nx; * $p < 0.05$ vs. Pre Hyp; # $p < 0.05$ vs. Post Hyp.

8.3.2. Linear associations

Positive linear associations were observed between $pThr^{287}$ CaMKII δ_D and $pSer^{176/180}$ IKKα/β (R^2 marginal = 0.52, R^2 conditional = 0.74, intercept and slope random effect LRT $p < 0.001$), Total IKKα (R^2 marginal = 0.58, R^2 conditional = 0.92, intercept and slope random effect LRT $p < 0.001$), NF-κB p105 (R^2 marginal = 0.55, R^2 conditional = 0.83, intercept and slope random effect LRT $p = 0.001$), Total IκB β (R^2 marginal = 0.46, R^2 conditional = 0.79, intercept and slope random effect LRT $p < 0.001$), Phospho-Ser^{536} p65 (R^2 marginal = 0.07, R^2 conditional = 0.46, intercept and slope random effect LRT $P<0.001$) p50+p65 (R^2 marginal = 0.40, R^2 conditional = 0.84, intercept and slope random effect LRT $p = 0.004$) and GR (R^2 marginal = 0.42, R^2 conditional = 0.90, intercept and slope random effect LRT $p < 0.001$). (Figure 15A, B, C, D, E, F and G).

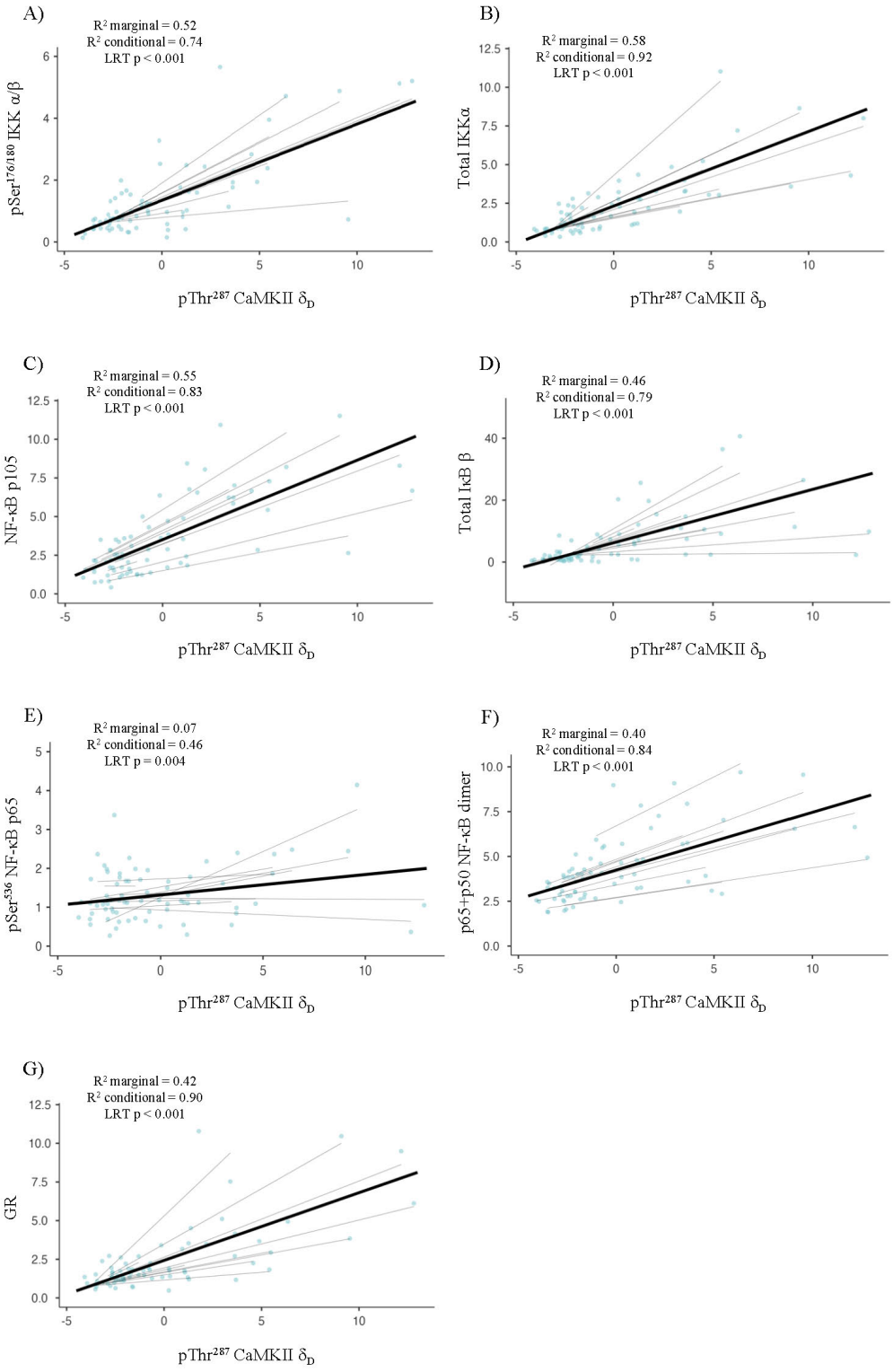


Figure 15. Linear relationships between pThr287 CaMKII δD and protein markers. (A) pThr^{287} CaMKII δ_D and pSer$^{176/180}$ IKK α/β, (B) pThr^{287} CaMKII δ_D and Total IKKα, (C) pThr^{287} CaMKII δ_D and NF-κB p105, (D) pThr^{287} CaMKII δ_D and Total IκB ß, (E) pThr^{287} CaMKII δ_D and pSer^{536} NF-κB p65, (F) pThr^{287} CaMKII δ_D and p65+p50 NF-κB, and (G) pThr^{287} CaMKII δ_D and GR. Linear relationships were assessed using a linear mixed model. The Likelihood Ratio Test for the random effects (LRT) was calculated and reported with the marginal (R^2 marginal) and conditional (R^2 conditional) r-squared values (n = 73-75).

A positive linear association was observed between p50 and its precursor p105 (R^2 marginal = 0.45, R^2 conditional = 0.85, intercept and slope random effect LRT p = 0.04), while $pSer^{176/180}$ IKKα/β was linearly associated with Total IκBβ with GR (R^2 marginal = 0.30, R^2 conditional = 0.74, intercept and slope random effect LRT p < 0.001) (Figure 16A and B).

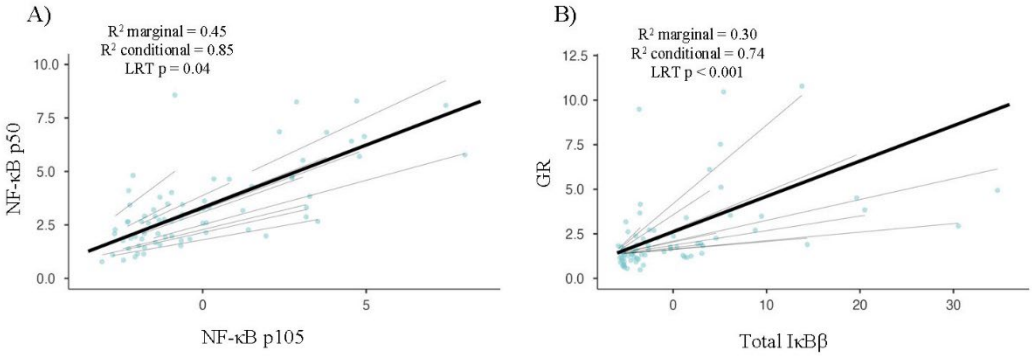


Figure 16. Additional linear relationships between (A) NF-κB p105 and NF-κB p50, and (B) Total IκBß and GR. Linear relationships were assessed using a linear mixed model. The Likelihood Ratio Test for the random effects (LRT) was computed and reported with the marginal (R^2 marginal) and conditional (R^2 conditional) r-squared values (n = 73-75).

DISCUSSION

9. DISCUSSION

9.1. Discussion of Article 1.

This study shows that Nrf2 signalling is activated by exercise to exhaustion in human skeletal muscle. To our knowledge, this is the first study examining the response of the Nrf2 signalling to intense acute exercise in human skeletal muscle and its relationship with metabolite accumulation and the level of F_IO_2. In contrast with our hypothesis, the degree of activation of Nrf2 signalling was essentially similar in normoxia and hypoxia, despite a 50 % lower femoral vein PO_2 during the exercise in severe acute hypoxia. Increased Nrf2 signalling was achieved by enhancing the total Nrf2 protein content while reducing that of Keap1, the main inhibitor of Nrf2 signalling. The combination of the increase and reduction of Nrf2 and Keap1, respectively, resulted in a substantial elevation of the Nrf2-to-Keap1 ratio, facilitating the nuclear translocation of Nrf2 and subsequent upregulation of the antioxidant enzyme Catalase, whose expression was closely associated to that of Nrf2. No association was observed between Nrf2 signalling and SOD1 and SOD2 protein expressions. While SOD2 did not change significantly during either exercise or ischaemia, SOD1 protein expression was downregulated and upregulated during exercise in normoxia and hypoxia, respectively (see Fig. 17 for a graphical summary).

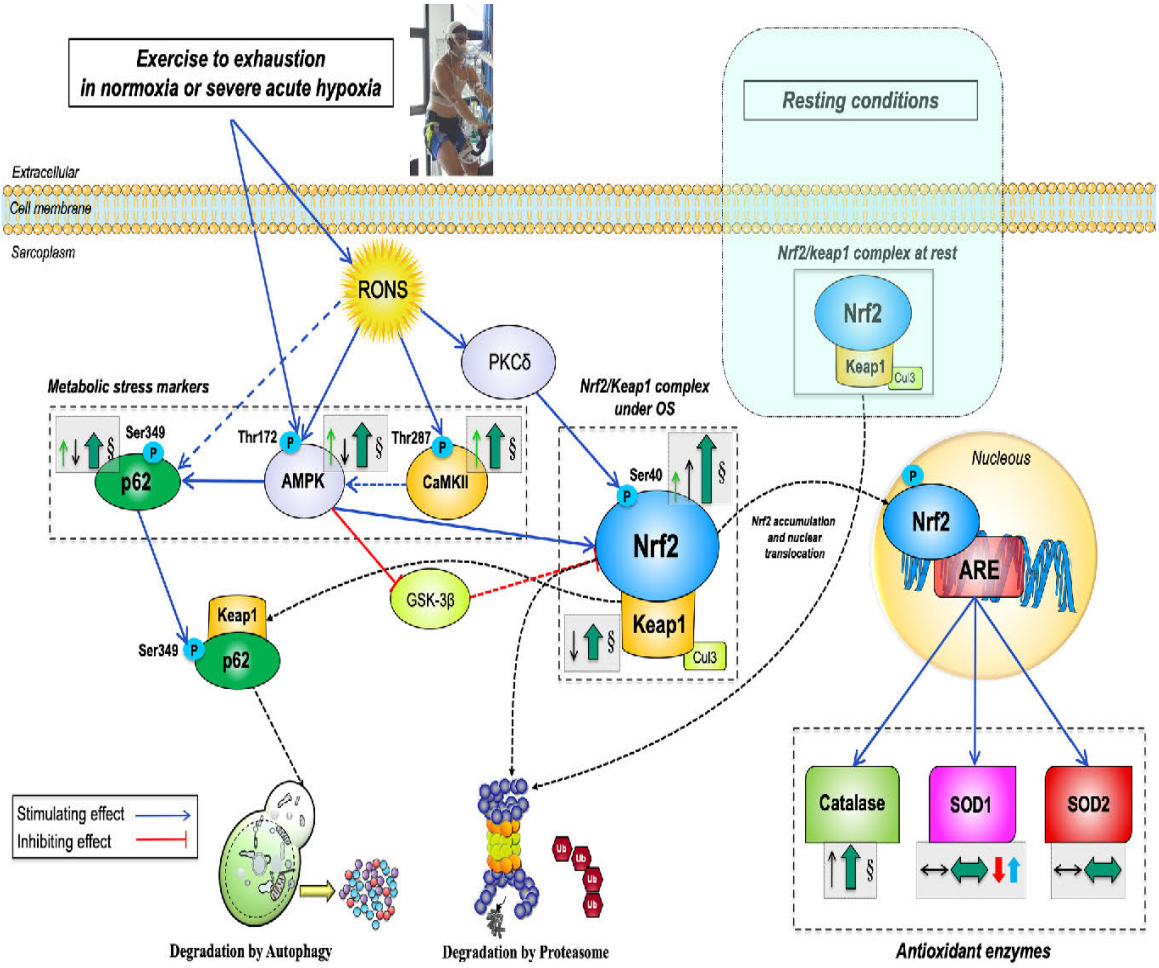


Figure 17. Schematic model of the regulation of Nrf2 and Keap1 signalling in human skeletal muscle immediately after incremental exercise to exhaustion in normoxia and severe hypoxia. Under basal conditions, Keap1 continuously targets Nrf2 for ubiquitination and degradation, allowing for minimal levels of Nrf2. The production of RONS during exhaustive exercise stimulates the activation of AMPK and CaMKII. Concomitantly, CaMKII acts indirectly as an upstream AMPK activator (by an unknown mechanism). AMPK promotes the increase of Nrf2 levels by two main mechanisms. Firstly, by phosphorylating p62 at Ser^{349}, which stimulates the p62-mediated degradation of Keap1 via autophagy; secondly, by phosphorylating and blocking its GSK3-β, which activates β-TrCP (an E3 ubiquitin-protein ligase) which tags Nrf2 for proteasomal degradation (not measured here). RONS may also activate PKCδ which phosphorylates Nrf2 at its Serine 40 promoting its nuclear translocation and genes transactivation. The lowered levels of Keap1 and the reduced amount of p62 observed here are suggestive of co-degradation following exercise. Overall, the augmented Nrf2 total and phosphorylated protein expression together with the rise in the Nrf2-to-Keap1 ratio elicited by exhausting exercise should be sufficient to enhance the Nrf2-mediated antioxidant response. A central role of Catalase is manifested by a remarkable increase in its protein content following exercise, which was exacerbated during exercise in severe acute hypoxia, likely as a response to increased H_2O_2 production, by superoxide dismutases. This process is facilitated in hypoxia due to the upregulation of SOD1. No acute changes in SOD2 protein expression

were observed. Most changes evoked by the exhaustive exercise bout were almost entirely reverted to baseline in less than 60 s by an O_2-dependent mechanism. Activating / inhibiting actions are represented by blue / red connecting lines (dashed if the effect is indirect). Changes on cellular locations are presented with black dashed lines. The arrows and symbols depicted inside dashed grey boxes and located beside the specific markers illustrate the overall protein expression changes in this investigation, as follows: Thin arrows in green (phosphorylated form) and black (total form) depict the overall direction of the outcomes (increase / decrease) for the particular muscle protein; thick arrows in darker green represent the overall effect on stimulation / inhibition of Nrf2 signalling; the symbol § indicates a significant difference between the biopsies taken 60 s after the end of the exercise, i.e., between the legs recovering with and without ischaemia. A differential modulation due to F_IO_2 is illustrated by the presence of arrows in red (normoxia) and blue (severe hypoxia). The size of each arrow is commensurate with the magnitude of the change. Abbreviations not defined in the text: ARE, antioxidant response element.

Contrary to our hypothesis, the application of immediate ischaemia at exhaustion did not amplify the changes elicited by the bout of intense exercise until exhaustion in pThr^{287} CaMKII, pThr^{172} AMPKα, Nrf2 nor Keap1. Nonetheless, Catalase protein expression was further increased after the application of ischaemia at exhaustion in hypoxia. By using a novel experimental approach in humans, we have demonstrated that Nrf2, Keap1 and Catalase have a fast turnover in exercised human skeletal muscle, recovering pre-exercise levels within one minute after the end of a bout of intense exercise by an O_2-dependent mechanism. Interestingly, during recovery with free circulation after exhausting exercise in hypoxia, SOD1 increased while Catalase was reduced, revealing a different regulation of these two critical antioxidant enzymes in response to exercise.

9.1.1. Skeletal muscle Keap1 protein levels are reduced during intense exercise.

The present investigation demonstrates for the first time that exhaustive exercise reduces the amount of Keap1 protein in human skeletal muscle. Keap1 is a substrate adaptor protein for the Cul3 RING-box 1 (RBX1) E3 ubiquitin ligase which ubiquitinates Nrf2 for proteasomal degradation in response to oxidants and electrophiles (289-291). Heavy metals and several oxidative and electrophilic agents may induce thiol

modifications in Keap1 which impair Nrf2 ubiquitination, resulting in Nrf2 protein accumulation (236). These type of Keap1 modifications have been observed in cells treated with oxidised lipids (292), H_2O_2 (293), and nitric oxide (293, 294).

The drop in Keap1 levels could be due to reduced synthesis of the protein, increased degradation, or both. Our results provide evidence for increased proteasomal and autophagic degradation of Keap1 during exercise, likely triggered by its oxidative modification by RONS (212-214). Although oxidative stress markers were not directly assessed in this investigation, the increased Thr^{287} CaMKII phosphorylation supports this explanation (216). Likewise, the observed augmented levels of Ser^{40} Nrf2 phosphorylation combined with the reduction of Keap1 levels and the close negative association between Thr^{287} CaMKII phosphorylation and Keap1 are also compatible with increased RONS-mediated signalling.

Due to the experimental limitations imposed by the small amount of tissue available, the rate of Keap1 transcription and translation were not measured here. Nevertheless, a reduction of the Keap1 mRNA expression with the exercise is unlikely, given that the amount of Nrf2 protein and Ser^{40} Nrf2 phosphorylation was increased and the fact that Nrf2 is a positive modulator of Keap1 gene expression (295). Although several microRNAs (miRs) have been reported to downregulate Keap1 mRNA in cancer cells (miR-23a (296); miR-141 (297, 298), miR-421 (299), miR-432 (300), and miR-200a (301)), myocardium (miR-200a (302); miR-26b (303)), retinal pigment epithelium cells (miR-626 (304)), and oligodendrocyte precursor cells (miR-146b-5p (305)), only miR-23a has been reported to be altered by exercise in skeletal muscle (306). D'Souza et al. (306) reported a reduction of *vastus lateralis* miR-23a expression levels in muscle samples obtained within 5 min after the end of a high-intensity intermittent exercise session in men. In any case, a potential reduction of miR-23a would facilitate rather than

repress Keap1 translation. Nevertheless, the fast dynamics of Nrf2 and Keap1 protein levels within the first minute after the exercise complicates the interpretations of D'Souza et al. (306) findings in light of the current investigation. Given the substantial number of miRs with potential inhibitory effects on Keap1 mRNA translation, further investigations should address this issue.

9.1.2. Skeletal muscle total Nrf2 and its Ser^{40} phosphorylation are increased by exhaustive exercise in humans with similar responses in normoxia and severe acute hypoxia.

Nrf2 abundance was elevated at exhaustion suggesting increased *de novo* synthesis or reduced proteasomal degradation during exercise. The observed activation of AMPKα may have prevented the degradation of Nrf2 by stimulating the p62-mediated autophagy of Keap1 (307-309) or by phosphorylating and inhibiting glycogen synthase kinase-3-β (GSK3-β) / β-transducin repeat-containing E3 ubiquitin-protein ligase (β-TrCP) which tags Nrf2 for proteasomal degradation (310, 311). In agreement with p62-mediated autophagy of Keap1 elicited by AMPK phosphorylation of its serine 349 (309), the fractional phosphorylation of p62 was closely associated with that of Thr^{172} AMPKα in the present investigation. Besides, p62 protein was significantly reduced after exercise, suggesting co-degradation with Keap1 (312). Although AMPK may phosphorylate several serine residues in Nrf2, the role of these phosphorylations in regulating *in vivo* Nrf2 stabilization, nuclear translocation and transactivation properties remains unknown (313). The fact that Keap1 protein content was lowered supports a reduction of Nrf2 proteasomal degradation during intense exercise, as indicated by the inverse association between the two observed here. Nevertheless, the marked increase of the Nrf2-to-Keap1 ratio also indicates stimulation of Nrf2 *de novo* synthesis.

It must be emphasized that the level of hypoxia utilized here is close to the limit that humans can tolerate without altitude acclimatization (314). RONS production in skeletal muscle is exacerbated by exercise in hypoxia, likely due to higher activation of the anaerobic metabolism (230). In fact, we have previously shown increased protein carbonylation in human skeletal muscle during prolonged sprint exercise performed at this level of hypoxia (230). Despite the latter, and in contrast with our hypothesis, Nrf2 accumulation and Keap1 reduction were similar at exhaustion in normoxia and hypoxia, indicating that the stimulation of Nrf2 signalling was already maximal in normoxia or that the additional reduction in cellular PO_2 during exercise in hypoxia was not sufficient as to stimulate Nrf2 accumulation or Keap1 reduction further.

Serine^{40} phosphorylation of Nrf2 by the ROS-sensitive protein kinase Cδ (PKCδ) (238) is thought to facilitate its nuclear translocation (315) and gene transactivation (316), although experimental evidence is not conclusive (236). Nrf2 is also phosphorylated in other serine residues by AMPK (313). AMPK is principally activated by the increase of the AMP/ATP ratio (317), but both PKC and AMPK may be activated by RONS during intense exercise (238, 248). The present investigation shows that pSer^{40} Nrf2 expression is increased during high-intensity exercise to a similar extent when the exercise is performed in normoxia and severe acute hypoxia. This may have facilitated nuclear translocation and gene transactivation, as supported by the increased protein expression of Catalase.

9.1.3. Catalase protein expression is increased during incremental exercise to exhaustion.

No previous study has determined acute changes in Catalase protein expression with acute exercise in humans. In agreement with our results, an increase in protein Catalase expression has been reported in mice skeletal muscle immediately after 60 min treadmill

running at moderate and high intensity (247). The few studies measuring this protein in humans have focussed on basal levels, reporting either an increased expression of the protein (318) or no change (247). Three days after a 20 min high-intensity intermittent exercise session, basal levels of Catalase and SOD2 protein expression were increased in the human *vastus lateralis* muscle (319). Likewise, increased SOD2 mRNA expression has been reported in human skeletal muscle 3 hours after high-intensity and prolonged continuous exercise for 50 min (228).

Catalase is localized principally in peroxisomes but is also present in mitochondria (320). The fast increase in Catalase protein expression (within minutes of exercise and within seconds during ischaemia after the incremental exercise in hypoxia) is likely necessary to counteract an increased H_2O_2 produced during exercise and ischaemia. Interestingly, cardiomyocyte overexpression of either Catalase or SOD2 results in increased lethality when transgenic mice are submitted to a forced-swimming program (321). In these mice, the overexpression of SOD2 increases H_2O_2 production exceeding the detoxifying capacity of Catalase and peroxidases during exercise, leading to pathological levels of oxidative stress (321). Overexpression of Catalase may result in hampering of signalling events necessary for the normal adaptation to exercise (322), causing maladaptation and increased dead in transgenic mice submitted to repeated forced swimming (321). Here we have observed a transient increase of Catalase expression partly reverted within seconds after exercise and no significant changes of SOD2. Thus, our results indicate that the level of Catalase in finely tuned in human skeletal muscle depending on redox conditions. This experimental observation concurs with the proposed role of H_2O_2 as a crucial signal driving the skeletal muscle adaptations to exercise (322).

Nevertheless, SOD2 protein content is increased after endurance training in human skeletal muscle (247, 323), but in similar proportion to the gain in mitochondrial

volume. Mice overexpressing Catalase and SOD2, have a much larger abundance of these proteins per gram of mitochondrial protein. This likely hampers the physiological response to exercise-induced oxidative stress and maladaptation, and death of a large proportion of these mice is seen when submitted to a rigorous swimming program (323).

Our results indicate that skeletal muscle can increase the amount of critical antioxidant enzymes acutely, likely via RONS-stimulated Nrf2 activation. When the exercise is stopped the excess antioxidant capacity build-up during the exercise is to a large extent, if not wholly, reversed to restore the redox balance to pre-exercise avoiding the risks of excessive reductive capacity (324).

9.1.4. Catalase and SOD1 are differentially regulated in response to exercise.

Previous studies indicate that SOD1 is constitutively expressed with limited regulation by external stimuli (325). SOD1 is located in the cytoplasm, nucleus and outer mitochondrial membrane, while Catalase is a predominantly an extramitochondrial protein, but also found in the mitochondrial matrix (321, 325). SOD1 was slightly increased during exercise in hypoxia, facilitating the dismutation of superoxide generated by extramitochondrial oxidases (326). This response concurs with the observed increased RONS production during high-intensity exercise in hypoxia (214), which has a significant cytoplasmatic component (322, 326). In the presence of higher levels of SOD1, the production of H_2O_2 is likely increased during exercise in severe hypoxia, requiring a higher amount of Catalase to avoid unchecked oxidative damage. In agreement with this explanation, it has been reported that H_2O_2 may induce SOD1 gene transcription by an Nrf2-independent mechanism (327). Besides, it has been shown that H_2O_2 promotes SOD1 nuclear localization, where it acts as a transcription factor promoting the expression of antioxidant enzymes (328). The remarkable acute increase of atalase expression after exercise in hypoxia and during ischaemia is likely necessary to

counteract an excessive H_2O_2 production during exercise and after the application of ischaemia (329, 330). The fact that the antioxidant enzymes increased in response to exercise and ischaemia are located mostly outside the mitochondria is compatible with a predominant extramitochondrial production of RONS during exercise (326), which may be even more marked during exercise hypoxia and ischaemia.

9.1.5. Keap1 levels recover rapidly after the cessation of contractile activity in an O_2-dependent mechanism.

In the present study, subjects performed exercise until their limit, and upon exhaustion, a pneumatic cuff was instantaneously inflated to 300 mmHg to fully occlude the circulation in less than 2 seconds in one leg, while the other leg recovered without circulatory restraints. During the first 3-5 s of the occlusion, the O_2 stores (O_2 trapped in capillary blood and bound to myoglobin) are depleted by oxidative phosphorylation, which is strongly stimulated (1, 213). This was evidenced by the fast reduction and plateauing of muscle oxygenation measured by near-infrared spectroscopy (NIRS), as previously reported (1). The first post-exercise muscle biopsy was obtained at the 10^{th} second after the end of the exercise, preventing potential effects of early oxygenation at exhaustion on muscle signalling. Then, the leg biopsied first remained occluded and, at the 60^{th} s another two muscle biopsies were obtained from the occluded and non-occluded leg simultaneously. This allowed a direct comparison of the occluded and non-occluded leg. During the 60 s of occlusion, the energy metabolism remained active in the occluded leg, utilizing the energy supplied by the glycolysis, leading to a higher accumulation of lactate, H^+, Pi and Cr. In contrast, the concentration of ATP remained at the same level reached at exhaustion, i.e. ~80% of the concentration observed before exercise (1). Despite the increased build-up of glycolytic metabolites during the 60 s occlusion, no further increase of Nrf2 or reduction of Keap1 was detected in the occluded leg. Although

we cannot rule out some RONS production from the 10th to the 60th of ischaemia (329, 330), its magnitude should have been small as indirectly indicated by stability during this period of both pThr^{287} CaMKII, pSer^{40} Nrf2 and Keap1, which are sensitive to RONS.

In the leg recovering with free circulation, Nrf2, Keap1 and the Nrf2-to-Keap1 ratio returned to pre-exercise levels within 60 s after the end of exercise, even though muscle lactate and H^+ remained at the same level reached at exhaustion (1). In contrast, Pi and Cr were reduced, and PCr increased during the 60 s of recovery with free circulation, without reaching pre-exercise values. This also indicates that the glycolytic metabolites accumulated during exercise do not play an essential role in either eliciting or maintaining Nrf2 activation. Nevertheless, the massive increase of Pi, which led to almost depletion of PCr during ischaemia may have inhibited the phosphatases (240), keeping the phosphorylation levels during ischaemia.

The principal difference between ischaemic and free circulation recoveries is the presence of O_2. Femoral vein PO_2, a surrogate of mean capillary PO_2, is rapidly increased after the cessation of contractile activity as it was reflected by the NIRS signal captured at the end of exercise in the perfused leg (1). The production of ATP by oxidative phosphorylation is likely mandatory to reactivate the *de novo* synthesis of Keap1. Despite the shortage of energy during ischaemia, mainly when applied at exhaustion following exercise in severe acute hypoxia, Catalase protein content was almost doubled after the IE in Hyp, indicating, that even in ischaemia the synthesis of some proteins is still active (331). The reason why Nrf2 levels were not reduced to pre-exercise values during ischaemia may be, in part, explained by the attenuation of global protein degradation in anoxia (332). In the leg recovering with free circulation, Nrf2 was reduced to the pre-exercise level already after one minute, likely through proteasomal and autophagy degradation facilitated by the fast increase of Keap1 protein expression during recovery.

Our results do not support an elevated production of RONS during the early recovery period following exhaustive exercise; otherwise, Nrf2 signalling would not have returned to pre-exercise values in the leg recovering with free circulation. Nevertheless, we cannot rule out an increased mitochondrial ROS production several hours after the end of the exercise, as observed in mice (333).

It has been suggested that once modified by oxidants or electrophiles, Keap1 is committed to p62-mediated autophagy (312). During recovery following exercise, Keap1 is likely newly translated by Nrf2-mediated induction of Keap1 which has an ARE gene promoter (236, 295). Besides, part of the oxidized Keap1 may have been regenerated by thioredoxin reductase 1 (236) during the one-minute recovery with open circulation. It remains to be determined whether the application of post-exercise ischaemia could be used to enhance Nrf2-mediated adaptation, as observed in tissues submitted to repeated episodes of ischaemia-reperfusion (331, 334).

9.2. Discussion of article 2.

This study shows that during incremental exercise to exhaustion, NF-κB signalling is activated to a similar extent in normoxia and severe acute hypoxia in human skeletal muscle. Importantly, NF-κB signalling remains stimulated during post-exercise ischaemia. However, most components of the NF-κB signalling pathway return to pre-exercise levels within one minute after the finalization of the exercise when the muscles recover with a free circulation, demonstrating the O_2-dependency of this process. These responses are closely associated with the activating phosphorylation of CaMKII δ_D and involve an increase of the protein expression of IKKα, IκBβ, and glutathione reductase protein levels in skeletal muscle (Fig. 18). These findings highlight the importance of obtaining the muscle biopsies as close as possible to exhaustion and the usefulness of applying immediate post-exercise ischaemia to impede the recovery of this signalling cascade with the cessation of muscle contractile activity.

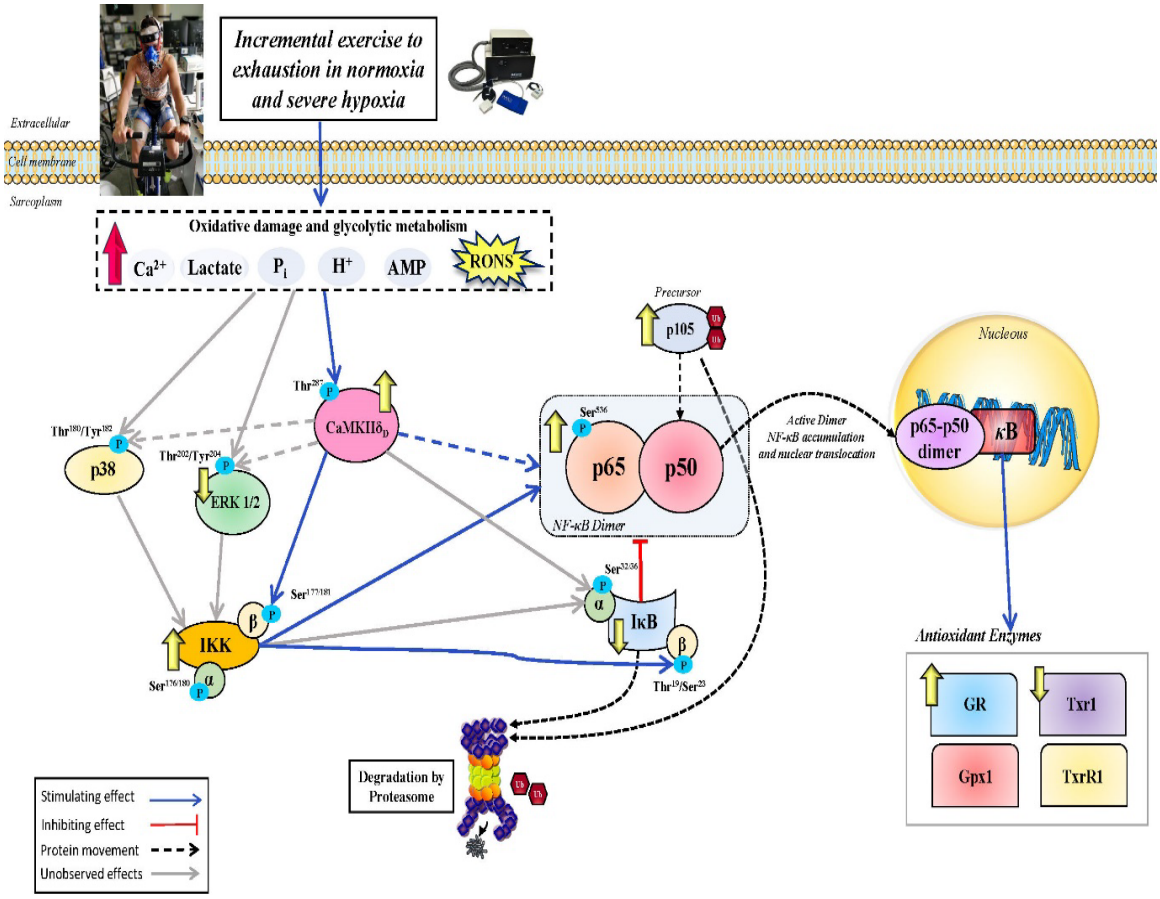


Figure 18. Schematic model of the measured mechanisms regulating NF-κB signalling in human skeletal muscle in response to exhaustive exercise in normoxia and severe hypoxia. Extracellular and intracellular signals such as Ca^{2+}, lactate, H^+, Pi, AMP and RONS evoked by an incremental exercise to exhaustion largely activated CaMKII. CaMKII activation reduces the inhibitory action of IκB proteins via phosphorylation, which targets them for proteasomal degradation by a direct or indirect mechanism (through $CaMKII\delta_D$-mediated activation of IKKβ). This was accompanied by an increase in the total levels of IKKα, which should favour the nuclear translocation of the p65-p50 heterodimer and transcriptional regulation of NF-κB-responsive genes. The phosphorylation levels of the RONS-sensitive upstream kinases ERK1/2 and p38 MAPK were not elevated by the exhausting exercise test.. Overall, the activation in NF-κB signalling was associated with increased and decreased GR and Trx1protein content, respectively. No changes were found for Gpx1 and TrxR1 after incremental exercise. Several key markers increased by exercise were rapidly downregulated within 60 s when the leg recovered with free circulation, demonstrating a fast regulation of NF-κB at exercise cessation which depends on muscle reoxygenation. None of the proteins studied were differentially modulated by performing exercise either in normoxia or severe hypoxia. Stimulating/inhibiting effects are represented by blue/red connecting lines (dashed if the effect is indirect). Known actions not observed in the present investigation are shown in grey. Changes in cellular locations are depicted with black dashed lines. The arrows in yellow shown beside the specific markers, illustrate the magnitude of the overall protein expression changes (increase/decrease) in this investigation.

9.2.1. NF-κB signalling is activated during exercise to exhaustion in human skeletal muscle.

NF-κB proteins consist of five members, including p65 protein (or RelA), RelB, c-Rel, p50 protein (or mature NF-κB1), and p52 protein (or mature NF-κB2), which form dimeric complexes that transactivate several target genes via binding to the κB enhancer (251, 260). NF-κB may be activated through the canonical and noncanonical pathways (335). Canonical NF-κB activity depends on the heterodimer p65-p50 that consists of the transcriptional activator (p65 protein) and the protein p50, which is produced by constitutive proteasomal processing of the precursor p105 (or NF-κB1 precursor protein) (336). The present investigation shows that the protein levels of p50 and its precursor p105 are elevated in human skeletal muscle by intense exercise indicating that exercise promotes upregulation of the transcription and translation of p105 and its subsequent proteasomal processing to produce p50. Interestingly, p50 and p105 increases with exercise were similar when the exercise was performed in normoxia and a simulated altitude of 5300 m above sea level. In contrast with our results, no changes in p50 and IκBα proteins were observed after 40 min of bicycling exercise at 70% of VO_2max (264). This disagreement is likely explainable by the fact that in Tantiwong et al. (264), the exercise was of moderate-intensity and not carried out until exhaustion or that the post-exercise muscle biopsy was slightly delayed since the subjects were moved from the cycle ergometer to a stretcher before taking the post-exercise muscle biopsy. Thus, it is critical to consider the timing of the post-exercise biopsies when interpreting skeletal muscle signalling responses, which should be reported in all studies.

Under resting conditions, NF-κB is bound to an inhibitor of κB proteins (IκBα, β, and ε), which keep NF-κB in the cytosol. Upon stimulation, IκBs are phosphorylated by IκB kinase (IKK), a trimeric enzyme constituted by two catalytic (IKKα and IKKβ) and

one regulatory subunit (IKKγ). Their phosphorylation targets IκBs, and particularly IκBα, for proteasomal degradation, releasing its inhibitory action on NF-κB (337, 338). In agreement, IκBα protein was reduced at the end of the incremental exercise and further reduced during ischaemia. However, in contrast with our hypothesis, these effects were not exacerbated when the exercise was carried out in severe acute hypoxia.

As a novelty, we have measured exercise-induced IκBβ Thr^{19}/Ser^{23} phosphorylation changes in human skeletal muscle. We have shown that IκBβ Thr^{19}/Ser^{23} phosphorylation increases remarkably in response to exercise to exhaustion, parallel with its upstream kinase IKKα/β, which was also phosphorylated and activated in response to exercise. The level of phosphorylation at $Ser^{176/180}$ of IKKα/β and Thr^{19}/Ser^{23} of IκBβ were maintained in the leg recovering with ischaemia while it returned within 1 min after the cessation of exercise to pre-exercise levels in the leg recovering with free circulation. As expected, this response was paralleled by the changes in phospho-Thr^{19}/Ser^{23} IκBβ and phospho-Ser^{536} p65, which are known targets of IKKα (339).

Our results concur in part with two previous studies (265, 266). Firstly, in partial agreement with our results, Vella et al. (265) observed a reduction of total IκBα and increase of phospho-Ser^{536} p65, but their first post-exercise biopsy was done 2 h after a single bout of resistance exercise. This finding by Vella et al. (265) could arise from the increased mitochondrial ROS production observed during the first hours after exercise (279). Secondly, Petersen et al. (266) obtained muscle biopsies from eight well-trained men (VO_2max 65 $ml.kg^{-1}.min^{-1}$) after 45 min of exercise at 71 % of VO_2max and after exhaustion, since their subjects resumed exercise at 92% of VO_2max until exhaustion immediately after the withdrawal of the 45 min biopsy. Opposed to Vella et al., (265) Petersen et al. did not see significant changes in p65 Ser^{536} phosphorylation immediately after exercise (in agreement with our findings), while IκBα was reduced by 14% after 45

min of exercise and by 7% at exhaustion (the reduction observed at exhaustion was not statistically significant p = 0.06). Also in agreement with our results, Parker et al. (340) reported decreased IκBα immediately after acute sprint interval exercise compared to less intense exercise modalities. Overall, the present findings and previous studies indicate that both the intensity of exercise and exhaustion favour the reduction of the inhibitor protein IκBα, facilitating the activation of NF-κB.

Exercise-related skeletal muscle changes in IκBβ protein levels have not been previously reported, and the role that this NF-κB inhibitor may play in skeletal muscle physiology remains unknown. Here we have demonstrated a differential regulation of IκBα and IκBβ in human skeletal muscle: while the total amount of IκBα is reduced with exercise and ischaemia, IκBβ increases. A differential temporal regulation of IκBs has been reported in cell cultures (341) and ageing hearts in mice (342). In the present investigation, we have shown that both the phosphorylated and total form of IκBβ protein are remarkably increased during high-intensity exercise, remaining elevated during ischaemic recovery. While phospho-Thr^{19}/Ser^{23} IκBβ returns to pre-exercise levels within 1 min of recovery with free circulation, the total amount of IκBβ remained elevated 1 min after IE. This increase of IκBβ with exercise may act as a negative feedback loop to impede excessive NF-κB activation. In addition, the increased expression of IκBβ may contribute to the transcriptional specificity of NF-κB through the formation of the appropriate homo/heterodimers and the subsequent gene regulation (339, 343). In this regard, cell experiments have also shown that IκBβ is a crucial mediator of the mitochondrial stress response (344) and is essential for the antioxidant response (343). Moreover, overexpression of IκBβ protects the liver against the ischaemia-reperfusion injury (345). Thus, the linear association observed in the present investigation between

IκBβ and GR protein expression is compatible with a role of IκBβ in the enhancement of skeletal muscle antioxidant capacity with regular, intense exercise (318, 346-348).

9.2.2. The total amount of IKKα but not IKKβ is acutely increased in response to exercise with a similar response in normoxia and severe acute hypoxia.

The effect of exercise on the protein levels of IKKα and IKKβ has not been previously studied in human skeletal muscle. Here we have observed differential regulation of these two catalytic subunits. In addition, we have detected a marked increase in the level of phosphorylation of IKKα/β $Ser^{176/180}$, which was reverted within one min after the end of exercise. The latter may explain why no significant changes in IKKα/β $Ser^{176/180}$ phosphorylation were observed immediately after a session of strength training (349). In agreement with our results, increased IKKα/β $Ser^{176/180}$ phosphorylation has been reported immediately after a single session of resistance, but not endurance exercise (2h at 60% of VO_2max) (350).

The activation of IKKs is necessary for the canonical stimulation of NF-κB signalling. In turn, IKKs phosphorylate p65 at Ser^{536} (351), which is necessary for its nuclear localization and protein stability and transcriptional activity (352, 353). The present investigation shows that post-exercise ischaemia promotes Ser^{536} p65 phosphorylation.

IKKs may be activated by autophosphorylation (339) and several upstream IKKs (339), among which only p38 and ERK1/2 have been mechanistically associated with contraction-induced NF-κB signalling in rodent muscle by inhibiting the two kinases pharmacologically (261). In agreement with our results, it has been shown that ERK1/2 does not seem essential for NF-κB activation in cultured skeletal muscle cells (270). Here, no increase in p38 MAPK phosphorylation was detected, in agreement with previous studies (354, 355). Nevertheless, increased p38 phosphorylation has been observed after

repeated sprints (228, 355) or prolonged continuous exercise (228, 266, 356, 357), high-intensity repeated exercise (358), and intermittent exercise of moderate-intensity (359).

CaMKII has been shown to reduce IκB and activate NF-κB signalling (360). More recently, direct phosphorylation of IKKβ by the delta isoform of CaMKII has been shown in cardiac fibroblasts (361). In agreement, the present work shows a linear association between CaMKIIδ_D and the phosphorylated form of IKKα/β, supporting that CaMKII plays a similar role in skeletal muscle as reported in heart (360) and isolated cardiomyocytes (362).

9.2.3. Most of the NF-κB signalling induced by incremental exercise to exhaustion is quickly reverted to pre-exercise levels at exercise cessation unless metabolic recovery and re-oxygenation are prevented by the immediate application of ischaemia.

All exercise-induced changes in NF-κB signalling, except the reduction in total IκBα, were reverted to pre-exercise values within one minute from the end of exercise, showing that the deactivation of this signalling pathway is extremely fast, as previously shown in the heart (360). In the present study, we used a novel experimental design to specifically determine whether muscle contractions are necessary to maintain NF-κB signalling. Immediately at the end of exercise, a pneumatic cuff was swiftly inflated at 300 mmHg to completely occlude the circulation in less than 2 seconds in one leg, while the contralateral leg recovered normally, i.e., with an intact circulation. At exhaustion, the muscle oxygenation was about 30 % lower in hypoxia than normoxia (1). Nonetheless, PCr and ATP were reduced, and lactate and H^+ increased with similar responses at exhaustion in normoxia and hypoxia (1). Since no significant differences were observed in muscle metabolites at exhaustion between normoxia and hypoxia, our

results indicate that a lower oxygenation level *per se* does not elicit more NF-κB signalling.

The main differences between the contracting muscle at exhaustion and the muscle recovering under ischaemia were the interruption of Ca^{2+} transients due to the cessation of contractile activity, the absence of O_2 during the ischaemic recovery and the lack of metabolic recovery. During the following 50s of ischaemia, lactate, H^+, Pi and free creatine were increased, while no changes were observed in the concentration of ATP, which remained ~20% below the pre-exercise values (1). The Ca^{2+} transients elicited by muscle contractions are stopped at exhaustion in both legs. Since the increase of cytosolic Ca^{2+} has been shown to elicit NF-κB signalling in cells (257), it has been suggested that Ca^{2+}-induced signalling could mediate the activation of NF-κB signalling in contracting muscles (261). This is supported by the linear association between CaMKII δ_D phosphorylation and several critical molecules involved in NF-κB signalling in the present investigation.

Thus, the present findings indicate that the metabolites accumulated during the exercise and/or the lack of O_2 may contribute to the maintenance of NF-κB signalling, likely by keeping CaMKII active. Interestingly, it has been reported that NF-κB contributes to stimulate glycolysis in C2C12 cells through activation of the glycolytic regulator hypoxia-inducible factor-1α (HIF-1α) (254). Thus, the acute activation of NF-κB signalling during exercise and ischaemia may have contributed to upregulating the glycolytic energy production close to exhaustion and during the 60 s of ischaemia when the glycolysis provided more than 90% of the energy consumed (1).

In contrast, the recovery of ATP, PCr and the abundance of oxygen during recovery with open circulation may have facilitated CaMKII deactivation by the

phosphatases, leading to downregulation of part of NF-κB signalling within seconds after the cessation of contractile activity.

9.2.4. RONS and NF-κB signalling during exercise and ischaemia in human skeletal muscle.

Cell experiments have shown that NF-κB signalling may be stimulated by RONS (258, 259, 363-366) and hypoxia (268-270). RONS are produced in skeletal during exercise depending on exercise characteristics, the energy substrates oxidized, and fitness status (212-215, 275). This process is facilitated by exercise conditions eliciting a robust stimulation of the glycolysis (212, 216 , 367), as it occurs during exercise at high intensity and in hypoxia (212, 213, 367). Using data from the same research project, we have reported a strong activation of the nuclear factor erythroid-derived 2-like 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1) signalling (280), which is activated by redox changes. The latter was associated with a remarkable increase of the antioxidant enzyme catalase, but not of superoxide dismutase 1 (Sod1) and Sod2 in the same biopsies studied here (280). Thus, both Nrf2/Keap1 and NF-κB pathways are activated by exhausting exercise and postexercise ischaemia, leading to an immediate increase of the antioxidant enzymes catalase (previously reported) (280) and GR, which is necessary to efficiently counteract superoxide and H_2O_2 (368). In contrast, Trx1 content in skeletal muscle was decreased in the present investigation. This concurs with the secretion of Trx1 by C2C12 myotubes (369), and the observation of increased plasma levels of TRX1 60 min and 48 h following high-intensity exercise (370).

In cells, hypoxia (1% O_2) inhibits prolyl hydroxylase-1 (PDH-1), which results in IKKβ activation, leading to IκBα phosphorylation and subsequent degradation (268). Interestingly, hypoxia also facilitates the cellular response to cytokine-mediated stimulation of NF-κB (268). Despite a remarkably lower femoral vein PO_2 (and

presumably in intracellular PO_2) during exercise in severe acute hypoxia (1), no significant differences were observed between normoxia and hypoxia in any of the NF-κB signalling molecules assessed here.

In agreement with a RONS-dependent stimulation of NF-κB signalling during exercise, it has been reported that the administration before the exercise of allopurinol (a xanthine oxidase inhibitor) blunts NF-κB signalling by reducing RONS production in exercising rodents (271). However, in humans, only one study has determined the effect of antioxidants (N- acetylcysteine infusion) administered before prolonged aerobic exercise on NF-κB signalling in skeletal muscle and no significant interactions were observed compared with placebo (266). Thus, it remains to be determined whether antioxidants may prevent NF-κB signalling in exercising human skeletal muscles.

The present experiments demonstrate that ischaemia contributes to maintaining NF-κB signalling by impeding metabolite recovery and muscle re-oxygenation. Nevertheless, we have also observed that p65+p50 and $pSer^{536}$ NF-κB p65 were increased and IκBα reduced during ischaemia, indicating that post-exercise ischaemia stimulates NF-κB signalling further. The latter might have been facilitated by reducing cellular PO_2 to anoxic levels. Thus, it seems that during incremental exercise to exhaustion in normoxia and severe acute hypoxia, NF-κB signalling is activated almost maximally and that further activation would require the application of post-exercise ischaemia to reduce muscle PO_2 further or elicited a higher accumulation of metabolites. This finding implies that post-exercise ischaemia could be used to prolong the exercise-induced activation of NF-κB and the associated adaptive responses.

9.2.5. NF-κB activation is associated with the fast increase of glutathione reductase.

NF-κB activation has been associated with the induction of antioxidant enzymes in several experimental models. For example, in TNF-α treated Ewing's sarcoma cells,

NF-κB activation increased both thioredoxin and MnSOD levels (272). Likewise, Glutathione S-transferase Pi, Metallothionein-3, NAD(P)H dehydrogenase [quinone]1, heme oxygenase-1 and glutathione peroxidase-1 have been shown to be induced by NF-κB (274). Here, we show that the activation of NF-κB is positively associated with the protein expression levels of GR in human skeletal muscle. However, no similar association was observed for the other antioxidant enzymes tested.

CONCLUSIONS

10. CONCLUSIONS

I. In human skeletal muscle, Nrf2 signalling is increased to a similar degree in normoxia and severe acute hypoxia after incremental exercise to exhaustion, through a mechanism related to Keap1 protein downregulation. This may have facilitated the nuclear translocation of Nrf2 and subsequent upregulation of the antioxidant enzyme Catalase, whose expression is closely associated to that of Nrf2.

II. Similarly, NF-κB signalling is also activated after exercise to exhaustion in human skeletal muscle, regardless of F_IO_2. This activation is closely associated with the activating phosphorylation of CaMKII δ_D and involve an increase of the protein expression of IKKα, IκBβ, and glutathione reductase in skeletal human muscle.

III. Exercise-induced activation of Nrf2 and NF-κB signalling pathways regulates the expression levels of antioxidant enzymes in human skeletal muscle. Indeed, Catalase and glutathione reductase emerge as acutely upregulated essential antioxidants during exercise and post-exercise ischaemia.

IV. Nrf2 and NF-κB signalling pathways remain stimulated by the application of post-exercise ischaemia. These changes are almost completely reversed to pre-exercise levels in less than 60 seconds by an O_2-dependent mechanism, as these changes are rapidly reversed at the end of the exercise when muscles recover with free circulation.

V. This thesis highlights the importance of obtaining muscle biopsies as close as possible to the end of exercise and the usefulness of post-exercise ischaemia to capture these rapid response signals. A delay of only one minute in obtaining muscle biopsies can significantly affect the interpretation of whether Nrf2 and NF-κB signalling activation is induced by exercise in human skeletal muscle.

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APPENDIX

13. APPENDIX

Original publications.

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Research Paper

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

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ABSTRACT

The Nrf2 transcription factor is induced by reactive oxygen and nitrogen species and is necessary for the adaptive response to exercise in mice. It remains unknown whether Nrf2 signalling is activated by exercise in human skeletal muscle. Here we show that Nrf2 signalling is activated by exercise to exhaustion with similar responses in normoxia (P_IO_2: 143 mmHg) and severe acute hypoxia (P_IO_2: 73 mmHg). CaMKII and AMPKα phosphorylation were similarly induced in both conditions. Enhanced Nrf2 signalling was achieved by raising Nrf2 total protein and Ser^{40} Nrf2 phosphorylation, accompanied by a reduction of Keap1. Keap1 protein degradation is facilitated by the phosphorylation of p62/SQSTM1 at Ser^{349} by AMPK, which targets Keap1 for autophagic degradation. Consequently, the Nrf2-to-Keap1 ratio was markedly elevated and closely associated with a 2-3-fold increase in Catalase protein. No relationship was observed between Nrf2 signalling and SOD1 and SOD2 protein levels. Application of ischaemia immediately at the end of exercise maintained these changes, which were reverted within 1 min of recovery with free circulation. While SOD2 did not change significantly during either exercise or ischaemia, SOD1 protein expression was marginally downregulated and upregulated during exercise in normoxia and hypoxia, respectively. We conclude that Nrf2/Keap1/Catalase pathway is rapidly regulated during exercise and recovery in human skeletal muscle. Catalase emerges as an essential antioxidant enzyme acutely upregulated during exercise and ischaemia. Post-exercise ischaemia maintains Nrf2 signalling at the level reached at exhaustion and can be used to avoid early post-exercise recovery, which is O_2-dependent.

1. Introduction

During exercise reactive oxygen (ROS) and nitrogen species (RNS) (collectively called RONS) are produced depending on the fitness level, the energy substrates oxidized and the characteristics of exercise [1–4]. Although in some circumstances, RONS may cause oxidative damage, RONS also stimulate signalling pathways essential for the adaptive response to exercise [1,5]. One of the main transcription factors involved in RONS-mediated regulation of gene expression is the nuclear factor erythroid-derived 2-like 2 (Nrf2), as shown in Nrf2-null mice ($Nrf2^{-/-}$) [6–8]. In mice skeletal muscle, total Nrf2 protein expression has been reported to increase after 90 min of continuous running [9] and nuclear Nrf2 protein content after 6 h of continuous running [10]. In humans, increased, unchanged and reduced Nrf2 mRNA levels have been reported in skeletal muscle biopsied 3–4 h after exercise [11–14]. However, the changes in Nrf2 protein levels and associated signalling events in response to acute exercise and recovery have not been determined in human skeletal muscle. This is relevant because reduced Nrf2 expression has been associated with lower exercise performance in animal models of chronic disease [15].

The RONS produced during exercise are accompanied by

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Fast regulation of the NF-κB signalling pathway in human skeletal muscle revealed by high-intensity exercise and ischaemia at exhaustion: Role of oxygenation and metabolite accumulation

Angel Gallego-Selles [a,b], Victor Galvan-Alvarez [a,b], Miriam Martinez-Canton [a,b], Eduardo Garcia-Gonzalez [a,b], David Morales-Alamo [a,b], Alfredo Santana [a,b,c], Juan Jose Gonzalez-Henriquez [b,d], Cecilia Dorado [a,b], Jose A.L. Calbet [a,b,e,*,1], Marcos Martin-Rincon [a,b,1]

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ABSTRACT

The NF-κB signalling pathway plays a critical role in inflammation, immunity, cell proliferation, apoptosis, and muscle metabolism. NF-κB is activated by extracellular signals and intracellular changes in Ca^{2+}, P_i, H^+, metabolites and reactive oxygen and nitrogen species (RONS). However, it remains unknown how NF-κB signalling is activated during exercise and how metabolite accumulation and PO_2 influence this process. Eleven active men performed incremental exercise to exhaustion (IE) in normoxia and hypoxia (P_IO_2:73 mmHg). Immediately after IE, the circulation of one leg was instantaneously occluded (300 mmHg). Muscle biopsies from m. *vastus lateralis* were taken before (Pre), and 10s (Post, occluded leg) and 60s after exercise from the occluded (Oc1m) and free circulation (FC1m) legs simultaneously together with femoral vein blood samples. NF-κB signalling was activated by exercise to exhaustion, with similar responses in normoxia and acute hypoxia, as reflected by the increase of p105, p50, IKKα, IκBβ and glutathione reductase (GR) protein levels, and the activation of the main kinases implicated, particularly IKKα and CaMKII δ_D, while IKKβ remained unchanged. Postexercise ischaemia maintained and stimulated further NF-κB signalling by impeding muscle reoxygenation. These changes were quickly reverted at the end of exercise when the muscles recovered with open circulation. Finally, we have shown that Thioredoxin 1 (Trx1) protein expression was reduced immediately after IE and after 1 min of occlusion while the protein expression levels of glutathione peroxidase 1 (Gpx1) and thioredoxin reductase 1 (TrxR1) remained unchanged. These novel data demonstrate that exercising to exhaustion activates NF-κB signalling in human skeletal muscle and regulates the expression levels of antioxidant enzymes in human skeletal muscle. The fast regulation of NF-κB at exercise cessation has implications for the interpretation of published studies and the design of new experiments.

1. Introduction

The transcription factor nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) regulates over 150 genes involved in inflammation, immunity, cell proliferation, apoptosis [1–4], and muscle metabolism [4–7]. NF-κB is activated by extracellular signals, mostly cytokines, as well as intracellular changes in calcium [8] and reactive oxygen and nitrogen species (RONS) [9–11]. Although these signals are present in contracting muscles, contradicting findings have been reported regarding the effect of exercise on NF-κB activation and signalling. Exercise activates NF-κB in rodents, although this response is not

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