PH.D.
THESIS

MIRIAM MARTÍNEZ CANTÓN

Molecular and physiological effects of the polyphenolic mango leaf extract (Zynamite®) on muscle fatigue and mitochondrial respiration during exercise and ischaemia-reperfusion in humans

MOLECULAR AND PHYSIOLOGICAL EFFECTS OF THE POLYPHENOLIC MANGO LEAF EXTRACT (ZYNAMITE®) ON MUSCLE FATIGUE AND MITOCHONDRIAL RESPIRATION DURING EXERCISE AND ISCHAEMIA-REPERFUSION IN HUMANS

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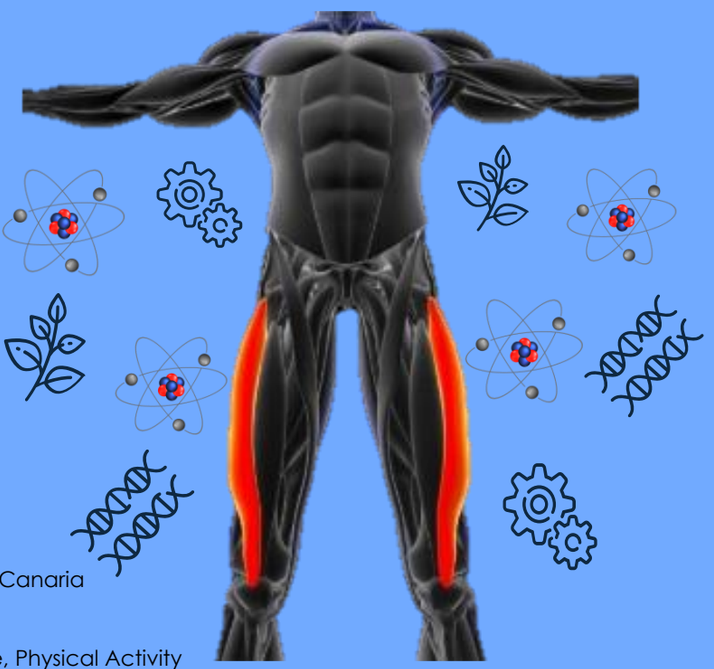

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Efectos moleculares y fisiológicos del extracto polifenólico de hoja de mango (Zynamite®) sobre la fatiga muscular y la respiración mitocondrial durante el ejercicio y la isquemia-reperfusión en seres humanos

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DEDICATION/DEDICATORIA

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1. Article 1 in Free Radical Biology & Medicine

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3. Article 3 in Scandinavian Journal of Medicine & Science in Sports

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4. Article 4 in Free Radical Biology & Medicine

Martinez-Canton M, Galvan-Alvarez V, Gallego-Selles A, Gelabert-Rebato M, Garcia-Gonzalez E, Gonzalez-Henriquez JJ *et al.* Activation of macroautophagy and chaperone-mediated autophagy in human skeletal muscle by high-intensity exercise in normoxia and hypoxia and after recovery with or without post-exercise ischemia. *Free Radic Biol Med* 2024; **222:** 607-624. (Q1, Biochemistry & Molecular Biology; IF: 7.1).

5. Article 5 in Nutrients

Martinez-Canton M, Galvan-Alvarez V, Garcia-Gonzalez E, Gallego-Selles A, Gelabert-Rebato M, Garcia-Perez G *et al.* A Mango Leaf Extract (Zynamite®) Combined with Quercetin Has Exercise-Mimetic Properties in Human Skeletal Muscle. *Nutrients* 2023; **15**(13). (Q1, Nutrition & Dietetics; IF: 4.8).

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Conference 5: Oral format (YIA competition). International Sport Forum on Strength, Training and Nutrition Madrid 2019. *Sarcolipin expression increases with strength training depending on the level of fatigue allowed in the set.*

Awards

Award 1: Study 5 was awarded first place at 2024 NutraIngredients Awards USA in the "Nutrition Research Project" category (see: https://nutraingredientsusa-awards.com/live/en/page/2024-winners-and-finalists), together with Nektium Pharma S.L.

Award 2: Study 3 was awarded a Young Investigator Award (YIA) at the International Sports Forum on Strength, Training and Nutrition Madrid 2019. *Sarcolipin expression increases with strength training depending on the level of fatigue allowed in the set.*

LIST OF SYMBOLS AND ABBREVIATIONS

Akt, Protein kinase B (PKB)

ARE, Antioxidant response elements

ATP5A, ATP synthase F1 subunit alpha or Complex V

BECN1, Beclin-1

β-TrCP, β-transducin repeat-containing protein

Ca^{2+}, Calcium ion

CaMKII, Calcium/calmodulin-dependent protein kinase II

CMA, Chaperone-mediated autophagy

CUL-E3, Ubiquitin E3 ligase complex

eIF2α, Eukaryotic initiation factor 2 alpha

EEF2, Eukaryotic elongation factor 2

EEF2K, Eukaryotic elongation factor 2 kinase

EIMD, Exercise-induced muscle damage

FOXO, Forkhead Box O1

GPx, Glutathione peroxidase

GR, Glutathione reductase

GSH, Reduced glutathione

GSK3β, Glycogen synthase kinase 3 beta

GSSG, Oxidised glutathione

H^+, Hydron

H_2O_2, Hydrogen peroxide

HIF1α, Hypoxia-inducible factor 1-alpha

HSPA8/HSC70, Heat shock protein family A [Hsp70] member 8

Hyp, Hypoxia

IE, Incremental exercise to exhaustion

IRES, Internal ribosome entry site

Keap1, Kelch-like ECH-associated protein 1

KIR, Keap1 interacting region

LAMP2A, Lysosome-associated membrane protein 2A

LC3B, Microtubule-associated protein 1A/1B-light chain 3

MHC, Myosin heavy chain

MHC-IIa, Myosin heavy chain type IIa

MHC-IIx, Myosin heavy chain type IIx

mTOR, Mammalian target of rapamycin

MW, Molecular weight

NOX, NAD(P)H oxidase

NOX2, NAD(P)H oxidase 2

NDUFB8, NADH:ubiquinone oxidoreductase subunit B8 or Complex I

Nrf2, Nuclear factor (erythroid-derived 2)-like 2

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

Nx, Normoxia

O_2^-, Superoxide radical

OXPHOS, Oxidative phosphorylation (mitochondrial protein expression)

p38 MAPK, p38 mitogen-activated protein kinase

p70S6K, p70 ribosomal S6 kinase, also S6K1

PHAF/MYTHO, Phagophore assembly factor 1/macro-autophagy and youth optimizer

P_i, Inorganic phosphate

P_IO_2, Partial inspiratory pressure of oxygen

PKA, Protein kinase A

PKC, Protein kinase C

PGC1α, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

RNS, Reactive nitrogen species

RONS, Reactive oxygen and nitrogen species

ROS, Reactive oxygen species

SDHB, Succinate dehydrogenase complex iron sulfur subunit B or Complex II

SERCA, Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase

SLN, Sarcolipin

SODs, Superoxide dismutase

SQSTM1/p62, Sequestosome 1

Trx1/Trx2, Thioredoxin isoforms 1 and 2

ULK1, Unc-51 like autophagy activating kinase 1

VO_2 max, Maximal oxygen uptake

XO, Xanthine oxidase

I

SUMMARY

1. SUMMARY

Introduction

This thesis was initially designed to determine the molecular effects of the oral ingestion of Zynamite®, a polyphenolic extract rich in mangiferin and quercetin, on skeletal muscle signalling under unstressed conditions and in response to exercise in humans. Since the two main compounds of Zynamite® are potent antioxidants and modulators of the main enzymes implicated in reactive oxygen species (ROS) production during exercise, we first studied how redox balance is sensed and regulated in human skeletal muscle, with particular emphasis on calcium (Ca^{2+})/calmodulin-dependent protein kinase II (CaMKII) and nuclear factor erythroid 2-related factor 2 (Nrf2). Thus, the thesis starts with a review of Nrf2 (**Study 1**), a pleiotropic transcription factor which is mainly activated by ROS. This is followed by a methodological study centred on the optimisation of CaMKII isoforms detection in human skeletal muscle (**Study 2**). The next study addresses the effects of strength training on CaMKII basal isoform expression (**Study 3**). The fourth study is centred on the regulation of autophagy during acute high-intensity exercise and ischaemia in human skeletal muscle (**Study 4**). Lastly, the final study deals with the main focus of the thesis, which inspired its title (**Study 5**).

Most chronic diseases, cancer, inflammation, mutagenesis, neurodegenerative diseases, and ageing processes are characterised by elevated levels of ROS, which cause oxidative stress. Several conditions, including exercise, increase ROS production. While excessive ROS levels can cause cellular damage, regular exercise enhances antioxidant capacity in skeletal muscle, mitigating ROS-induced damage and contributing to adaptive responses. Nrf2 is primarily activated by elevated ROS levels and

orchestrates the adaptive response to oxidative stress during exercise, mainly by mediating the transcription of antioxidant genes.

CaMKII is a protein induced by ROS and Ca^{2+} transients, regulating muscle phenotype, mitochondrial biogenesis, autophagy, and antioxidant signalling. The precise characterisation of CaMKII isoforms (δ_D, γ/δ, and β_M) in human skeletal muscle is crucial for understanding their specific functions and responses to exercise. Thus, in **Study 2**, we developed an immunoblotting analysis based on the incubation of muscle extracts with several antibodies directed against specific CaMKII isoforms to facilitate the identification of those more responsive to exercise and Zynamite PX®. In a third study (**Study 3**), we determined whether the basal expression of CaMKII depends on the level of fatigue elicited during the strength training program and whether CaMKII changes with training were associated with the adaptive responses in muscle mass (hypertrophy), myosin heavy chain (MHC) composition, and mitochondrial protein expression (OXPHOS). **Study 3** also assessed whether sarcolipin (SLN) changes with strength training. Sarcolipin is a protein that, when overexpressed, diminishes the Ca^{2+} coupling to the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), resulting in increased energy expenditure, oxidative stress, fast-to-slow muscle fibre type shift and mitochondrial biogenesis.

Excessive ROS production can cause oxidative stress and cellular damage, which may impair cellular function. Autophagy is a cellular process that degrades damaged cellular components and misfolded proteins. This process is essential for maintaining muscle health and is required for a physiological adaptive response to exercise. In skeletal muscle, two main types of autophagy have been described: macroautophagy and chaperone-mediated autophagy (CMA). Macroautophagy is a process where double-membrane vesicles, known as

autophagosomes, capture and degrade damaged organelles and proteins to maintain cellular homeostasis. Chaperone-mediated autophagy is a similar process involving the selective degradation of specific proteins recognised by chaperone proteins, subsequently transported to the lysosome for degradation. It remains unknown how autophagy signalling pathways are regulated by acute exercise to exhaustion and how different levels of muscle oxygenation and metabolite accumulation in human skeletal muscle influence macroautophagy and CMA (**Study 4**). For this purpose, healthy males performed an incremental exercise to exhaustion (IE) in normoxia and hypoxia, followed by ischaemia-reperfusion. Biopsies were taken from the *vastus lateralis.* The primary aim was to determine skeletal muscle autophagy signalling in responses to IE with different levels of inspiratory partial pressure of oxygen (P_IO_2) and ischaemia-reperfusion. Moreover, we also aimed to analyse the regulation of FOXO, Akt-mTOR and GSK3β under high metabolic stress and its influence on skeletal muscle autophagy signalling.

Natural polyphenols and xanthones, such as mangiferin and quercetin, possess antioxidant and anti-inflammatory properties that may enhance exercise performance by inducing Nrf2-mediated signalling, scavenging free radicals, and inhibiting xanthine oxidase (XO) and NADPH oxidase (NOX), the primary enzymes producing ROS during exercise. Zynamite®, an extract from mango leaves rich in mangiferin, has been studied in combination with different formulations, demonstrating its ability to enhance exercise performance and to accelerate recovery after exercise-induced muscle damage (EIMD). However, no human study has determined whether Zynamite PX®, a polyphenolic extract combining the mango leaf extract with quercetin, exerts effects on muscle signalling in resting skeletal muscle and in response to high-intensity exercise with or without post-exercise ischaemia-reperfusion (**Study 5**).

Material and methods

Within the scope of this thesis, four experimental studies were conducted (**Studies 2-5)**. Western Blot was used as the main technique to assess the protein expression in skeletal muscle biopsies from young healthy males. Muscle lysates were prepared with ~10 mg of tissue ground by stainless steel balls homogenised in urea lysis buffer with protease and phosphatase inhibitor cocktails.

Study 1: This article is a contribution to the special issue entitled "Unlocking Athletic Potential: Exploring Exercise Physiology from Mechanisms to Performance", published in Free Radical Biology & Medicine 2024. To conduct this review, all articles containing the terms "Nrf2," "Keap1," and "skeletal muscle" were searched on Web of Science (WOS). Additionally, a search was performed using the terms "glutathione" and "skeletal muscle." Further searches were conducted based on the references cited within these articles if they had not been previously identified.

Study 2: This study was designed to optimise the detection of the different CaMKII isoforms by Western blot and stripping-reprobing procedures using eight different CaMKII commercial antibodies in human skeletal muscle. Human skeletal muscle from a healthy participant obtained under resting conditions was used to test eight CaMKII commercial antibodies: Aldrich oxidized-Met$^{281/282}$ (no. 07-1387, Sigma Aldrich), CST pThr^{287} (no. 12716, Cell Signaling Technology), BD Total (no. 611292, BD Biosciences), CST Total (no. 4436, Cell Signaling Technology), Abcam δ (no. 181052, Abcam), Badrilla δ (no. A010-55AP, Badrilla), Proteintech γ (no. 12666-2-AP, Proteintech), SCBT β (no. 376828, Santa Cruz Biotechnology). As an additional assay to validate the banding pattern, *vastus lateralis* biopsies were obtained before and after a strength training intervention from 3 subjects [1] and a bout of acute high-intensity exercise

from 1 subject [2], which underwent incubation (pThr^{287}-CaMKII and ox-Met$^{281/282}$-CaMKII, respectively), stripping and subsequent reprobing with the antibody for the total CaMKII. The Lanes and Bands and MW Analysis Tools within the Image Lab© software 6.0.1 (Bio-Rad) were used to determine the experimental molecular weights.

Study 3: This study was an extension of a previous study [3]. The aim of this study was to determine whether CaMKII and SLN were involved in muscle phenotype and performance changes elicited by strength training. For this purpose, twenty-two young males followed an 8-week velocity-based strength training program using the full squat exercise while monitoring repetition velocity. Subjects were randomly assigned to two resistance training programs differing in the repetition velocity loss allowed in each set: 20% (VL20) vs 40% (VL40). *Vastus lateralis* biopsies were taken before and after training. Protein expression of CaMKII, SLN and OXPHOS was determined by Western blot. Previously published data [3] regarding the improvements in maximal strength (1RM) and vertical jump performance, the number of repetitions carried out during the training program, the degree of muscle hypertrophy, and the change in myosin heavy chain IIx (MHC-IIx) percentage were used for the interpretation of muscle signalling responses. Statistical analyses were performed using a two-way 2 x 2 repeated-measures ANOVA with SPSS software version v.18 for Windows (SPSS Inc). Pearson's correlation analysis was applied to examine the associations between variables.

Study 4: This investigation was conducted to determine the changes in protein expression of macroautophagy and CMA signalling pathways after high-intensity exercise and ischaemia-reperfusion, including signals potentially regulated by Zynamite®. Forkhead box O (FOXO) signalling and protein elongation (GSK3β and EEF2), hypoxic (HIF1α) and protein synthesis (Akt and mTOR) markers were determined. To achieve these

aims, eleven males performed an incremental exercise to exhaustion in normoxia (Nx, P_IO_2:143 mmHg) and hypoxia (Hyp, P_IO_2:73 mmHg, AltiTrainer200), in random order. At exhaustion, the circulation of one leg was instantaneously occluded for 60 s with a rapid inflator (300 mmHg, Hokanson) connected to a cuff (SC10D Hokanson) while the subjects were carefully moved to a stretcher. *Vastus lateralis* biopsies were taken before (Pre), 10 s after (Post, only occluded leg), and 60 s after IE in the occluded (Oc1m) and non-occluded (FC1m) legs. The FC1m biopsies were only taken after the hypoxic test, obtained from the leg that had been recovering with free circulation in normoxia for 60 s. Oxygen uptake was measured breath-by-breath using a metabolic cart (Vmax N29; Sensormedics, Yorba Linda, CA, USA). Muscle metabolites were previously assessed and reported [4]. Statistical analyses were performed using a two-way 3 x 2 repeated-measures ANOVA. The occluded and non-occluded legs were compared using a paired two-tailed *t*-test. Linear relationships between proteins were determined by applying a linear mixed model. The likelihood Ratio Test for the random effects (LRT) was calculated and reported with the marginal and conditional R-squared values. All statistical analyses were performed with IBM SPSS software, v.29.0, for Mac (IBM, New York, NY, USA) and Jamovi v2.4.8. (The jamovi project 2023).

Study 5: This research was designed to determine the effects of 48 h of Zynamite PX® supplementation on skeletal muscle Nrf2 protein levels and Nrf2-induced signalling under basal conditions and in response to high-intensity exercise combined with ischaemia-reperfusion in humans. The main regulatory proteins of Nrf2 levels were also determined, namely CaMKII, GSK3β, p38 MAPK and Keap1, as well as the protein expression of the antioxidant enzymes GR and catalase. To achieve these aims, 25 young males (17 Control group; 8 Zynamite PX® group) performed incremental exercise (IE) to exhaustion, followed by instantaneous

unilateral leg occlusion for 60 s with a rapid inflator (300 mmHg, Hokanson) connected to a cuff (SC10D Hokanson). Upon cuff release, subjects sprinted maximally for 30 s (Wingate test) with the ergometer set in isokinetic mode (80 RPMs), and thereafter, the same leg was instantaneously occluded for 90 s. Immediately upon occlusion, the subjects were moved carefully to a stretcher, where they rested in the supine position while the circulation of the leg biopsied remained fully occluded. Supplementation consisted of 140 mg of Zynamite® (standardised to 60% mangiferin, an aqueous extract from *Mangifera indica*) in combination with 140 mg of quercetin (provided as 280 mg *Sophora japonica* extract, standardised to 50% quercetin), marketed as Zynamite PX®, every 8 h for a total of six doses 48 h before the resting biopsy. A last dose of the supplement was ingested ~60 min before the start of IE. *Vastus lateralis* biopsies were taken at baseline (before ingesting the last dose of Zynamite PX®, Pre – B1), 20 s after IE (Post, occluded leg – B2) and 10 s after Wingate (OcW, occluded leg – B3), and from the occluded and non-occluded leg at 90 s (Oc90 and nOc90 – B4) and 30 min post-Wingate (Oc30m and nOc30m – B5). Oxygen uptake was measured breath-by-breath using a metabolic cart (Vyntus, Jaeger-CareFusion, Höchberg, Germany). An unpaired two-tailed *t*-test was used to study the changes in protein expression under resting conditions between the supplemented and the control group. A paired *t*-test assessed differences between the ischaemic and free circulation legs at 90 s and 30 min post-exercise. Due to similar responses in both legs at each time point, the 90 s biopsies were averaged, as were the 30 min biopsies, and these averages were used to represent the post-exercise responses at 90 s and 30 min, respectively. Statistical analyses were performed using a two-way 5 x 2 repeated-measures ANOVA with IBM SPSS software, v. 29.0, for Mac (IBM, New York, NY, USA).

Results

Study 1 provides an overview of the Nrf2 role in enhancing exercise performance and training adaptations in humans. Nrf2 signalling is primarily activated in response to perturbation of redox balance by ROS or electrophiles in skeletal muscle [5, 6]. Studies in Nrf2 knockout mice ($Nrf2^{-/-}$) reported less force and more fatigability due to less basal mitochondrial respiratory capacity and mitochondrial adaptability to exercise training [7]. In humans, Nrf2 basal expression in skeletal muscle has been associated with maximal oxygen uptake (VO_2max) [8] and high-intensity performance [9, 10].

During exercise, ROS are produced mainly by NAD(P)H oxidase 2 (NOX2) and XO [11-13], as well as by the mitochondrial electron transport chain. The contribution of each ROS source varies with the exercise intensity, duration, substrate oxidation, training state, oxygenation, level of fatigue, and age [11, 14-16]. The ROS are counteracted by enzymatic, such as superoxide dismutases (SODs), glutathione reductase (GR), catalase, glutathione peroxidase (GPx), and non-enzymatic antioxidants. The superoxide radical (O_2^-) is reduced to hydrogen peroxide (H_2O_2) by SODs. Hydrogen peroxide, which is a less reactive oxidant, is reduced by catalase and GPx. GPx enzymatic activity consumes reduced glutathione (GSH) and produces oxidised glutathione (GSSG), being the GSH:GSSG ratio a theoretical indicator of redox buffering capacity [17]. Nevertheless, studies assessing GSH:GSSG ratios in human skeletal muscle show high variability, indicating that it may not be a reliable biomarker of the antioxidant buffering capacity of the cell unless accurately measured [18].

In basal conditions, Nrf2 forms a complex with kelch-like ECH-associated protein 1 (Keap1) [19, 20], which acts as an adaptor protein facilitating the action of a ubiquitin E3 ligase complex (CUL-E3), which ubiquitinates Nrf2 [20]. The latter causes the dissociation of Nrf2 from Keap1 and subsequent proteasomal degradation of the ubiquitinated Nrf2 [21, 22].

Upon oxidative stress, ROS modify Keap1, disabling its adaptor function for CUL E3, impeding the ubiquitination of Nrf2 [21, 23, 24] and stabilising the Keap1-Nrf2 complex. The first newly synthesised Nrf2 is sequestered by the remaining free Keap1, and only once free Keap1 is depleted, Nrf2 translocates to the nucleus [19, 25], where it binds to antioxidant response elements (AREs) to activate antioxidant enzyme expression [7, 26-32]. Moreover, Keap1 degradation is also mediated by sequestosome 1 (p62/SQSTM1), which directly binds to Keap1 through a specific sequence, the Keap1 interacting region (KIR) [2, 33, 34], leading to Keap1 lysosome degradation, promoting Nrf2 signalling [35]. Nrf2 regulation upon oxidative stress may also be independent of Keap1. Nrf2 nuclear retention is modulated by Nrf2 multiple potential phosphorylation sites, which are regulated by exercise-induced kinases, i.e. p38 MAPK at $pSer^{40}$-Nrf2 [36 37]. Additionally, the upregulation of the exercise-induced kinases (AMPK, Akt, PKA, and PKC) [38-40] may produce a drop in the activity of GSK3β, which blunts Nrf2 Ser^{338} and Ser^{342} phosphorylation, impeding Nrf2 proteasome degradation. Inhibition of GSK3β may also facilitate Nrf2 nuclear retention by impeding phosphorylation of Nrf2 at Ser^{344} and Ser^{347} [41-43] and at Ser^{550} [44]. Moreover, hydrogen peroxide and sulforaphane may stimulate Nrf2 cap-independent translation via an internal ribosome entry site ($IRES_{Nrf2}$)[45, 46], a process that requires phosphorylation at eukaryotic initiation factor 2 alpha (eIF2α) by stress-induced kinases [47-49]. Acetylation of Nrf2 promotes nuclear translocation[50]. Nuclear export and subsequent ubiquitination and degradation are facilitated by Fyn kinase by phosphorylating Nrf2 at Tyr^{568} [51].

Polyphenols may reduce Keap1 availability, enhance Nrf2 nuclear translocation, induce Nrf2 transcription and translation [52 46], and reduce proteasomal Nrf2 degradation [52]. Although it has been suggested that antioxidant ingestion before exercise may blunt some of the adaptations to

exercise, this remains controversial in healthy humans [53] and may be observed only after the intake of high doses of vitamin C and E [54, 55]. In turn, the intake of fruit-derived polyphenols is considered positive for enhancing performance and recovery in athletes [56].

Study 2 determined the molecular weights of CaMKII isoforms in human skeletal muscle based on SDS-PAGE mobility, which correspond to: δ_D, at 54.2 ± 2.1 kDa; γ/δ, at 59.0 ± 1.2 kDa and 61.6 ± 1.3 kDa; and β_M isoform, at 76.0 ± 1.8 kDa. We also assessed the specificity of CaMKII antibodies. The β CaMKII antibody (no. 376828, Santa Cruz Biotechnology) recognised a single band, precisely aligned at the molecular weight (MW) corresponding to a similar band marked by the antibodies directed against the total, phospho- and oxidised CaMKII. Moreover, CaMKII antibody (no. 181052, Abcam) showed high specificity for the δ_D, the most responsive isoform to ROS and intracellular Ca^{2+} transients in human skeletal muscle. By cons, the γ CaMKII antibody (no. 12666-2-AP, Proteintech) displayed cross-reactivity with all CaMKII isoforms, not allowing distinguishing between γ/δ isoforms.

Study 3 showed that the amount of fatigue allowed in each set of the strength training intervention critically determined muscle basal CaMKII, basal SLN protein expression and muscle phenotype changes. Strength training elicited muscle hypertrophy, improved 1RM and increased total CaMKII, which was mainly driven by an increase in total CaMKII δ_D. The group that trained with higher intra-set fatigue (VL40) experienced greater muscle hypertrophy and reduction of MHC-IIx percentage (which could limit the improvement of muscle power with training) and an upregulation of CaMKII δ_D phosphorylation at Thr^{287}. In contrast, the group that trained with lower intra-set fatigue (VL20) increased SLN expression and attenuated the IIx-to-IIa MHC shift without changes in CaMKII phosphorylation. The changes in the CaMKII δ_D

phosphorylation levels were positively associated with muscle hypertrophy and the number of repetitions during training and negatively with the changes in MHC-IIx. SLN expression was not associated with changes in muscle hypertrophy, MHC-IIx percentage, the number of repetitions, and improvements in vertical jump performance and 1RM, while it was negatively associated with pThr^{287}-CaMKII δ_D, SDHB (Complex II), and ATP5A (Complex V). Most OXPHOS proteins remained unchanged except for NDUFB8 (Complex I), which was reduced after training in both groups. No association was seen between pThr^{287}-CaMKII and OXPHOS, indicating that a higher CaMKII activation did not induce mitochondrial protein expression.

Study 4 showed that an acute bout of high-intensity exercise with ischaemia application at exhaustion induced macroautophagy and chaperone-mediated autophagy similarly in normoxia (Nx) and hypoxia (Hyp). Low levels of AMP:ATP ratio and metabolic stress activated macroautophagy signalling through AMPKα-UKL1. This was followed by the phosphorylation at Ser^{15} BECN1, crucial for phagophore formation. Increased pSer^{349}-SQSTM1/p62 levels, reduced total SQSTM1/p62 and higher LC3B-II:LC3B–I ratio suggested enhanced autophagosome degradation. This signalling was accompanied by the marked upregulation of PHAF1/ MYTHO, a novel macroautophagy biomarker, which was positively associated with LC3B-II:LC3B-I ratio. Increased LAMP2A protein expression indicated activation of chaperone-mediated autophagy in response to high-intensity exercise, regardless of post-exercise HSPA8/HSC70 downregulation. Additionally, pSer^{40}-Nrf2 and LAMP2A were positively associated, suggesting ROS-dependent CMA stimulation. FOXOs did not appear to regulate macroautophagy and CMA in healthy skeletal muscle, as their signalling was inhibited after high-intensity exercise. Diminished levels of pThr^{56}-EEF2, which is inhibited by EEF2K,

were indicative of downregulated protein elongation signalling after exercise. EEF2K is activated by active AMPKα and GSK3β. Moreover, decreased Akt activation may indicate reduced protein synthesis under high metabolic stress. The upregulation of hydroxy-Pro^{564} HIF1α with exercise in normoxia was not further stimulated by hypoxia or ischaemia. The positive association between pSer^{571}-PGC1α and pThr^{172}-AMPKα suggested increased mitochondrial biogenesis and transcription of macroautophagy genes. Despite increased glycolytic metabolism, severe acute hypoxia did not exacerbate the autophagy signalling response, indicating that autophagy levels were almost maximally stimulated with exercise in normoxia. Most of the signalling changes reverted within 1 min of recovery with free circulation, while the application of immediate post-exercise ischaemia impeded recovery.

Study 5 demonstrated that compared to the controls, the Zynamite PX® supplemented group had increased basal protein expression of pThr^{287}-CaMKII δ_D, which phosphorylates GSK3β at Ser^{9}, inhibiting its activity. The upregulated inhibition of GSK3β may downregulate the proteasomal Nrf2 degradation rate, and consequently, a non-significant increase of total Nrf2 ($p = 0.099$) and pSer^{40}-Nrf2 ($p = 0.061$) was seen. No changes in basal antioxidant enzymes (catalase and GR) were observed, suggesting that higher doses may be needed or that the antioxidant enzyme system is tightly regulated. In the control group, exercise combined with ischaemia-reperfusion and recovery led to the activation of stress kinases (pThr^{287}-CaMKII δ_D and pThr^{180}/Tyr^{182}-p38 MAPK) and the antioxidant signalling response (total Nrf2, Nrf2/Keap1, catalase, and GR). However, this signalling was partly blunted in the Zynamite PX® group, suggesting that Zynamite PX® may reduce metabolic stress and ROS-induced signalling by enhancing basal Nrf2 signalling after Zynamite PX® ingestion.

Conclusions

The regulation of the Nrf2 transcription factor in skeletal muscle during exercise involves a complex interplay between redox balance, cellular stress, and adaptive responses. Nrf2 activates the expression of various antioxidant enzymes and pathways in response to oxidative stress and is regulated through Keap-1 and through several posttranslational modifications, which control its nuclear localisation, protein translation, and proteasomal degradation, independent of Keap1-mediated regulation. Additionally, dietary polyphenols have emerged as potential modulators of Nrf2 activity (**Study 1**).

The molecular weights of CaMKII isoforms characterised by immunoblotting are 54.2 ± 2.1 kDa (δ_D), 59.0 ± 1.2 kDa and 61.6 ± 1.3 kDa (γ/δ), and 76.0 ± 1.8 kDa (β_M), with δ_D being the most responsive to ROS and intracellular Ca^{2+} transients (**Study 2**).

Total CaMKII δ_D increases in response to strength training and executing repetitions within each set close to failure is associated with elevated basal $pThr^{287}$-CaMKII δ_D phosphorylation levels. The increase in $pThr^{287}$-CaMKII δ_D phosphorylation is positively associated with muscle hypertrophy and negatively with changes in MHC-IIx expression. Sarcolipin protein expression increases in response to strength training, but this effect is only observed when the level of fatigue allowed in the set is low. An inverse association has been shown between the changes in basal $pThr^{287}$-CaMKII δ_D phosphorylation and those of sarcolipin, which is compatible with the existence of a negative loop limiting an excessive expression of SLN after fatiguing muscle contractions (**Study 3**).

Macroautophagy and CMA are similarly upregulated during exercise to exhaustion in normoxia and severe acute hypoxia. ROS produced during incremental exercise and ischaemia may activate macroautophagy and

CMA but not FOXOs. There is an increased expression of the novel autophagy PHAF1/MYTHO biomarker in human skeletal muscle in response to exercise in normoxia, hypoxia, and post-exercise ischaemia. There is concurrent activation of autophagy with inhibition of protein elongation and synthesis (**Study 4**).

The oral intake of Zynamite PX® increases basal pThr^{287}-CaMKII δ_D and GSK3β inhibitory phosphorylation in human skeletal muscle, which may elicit muscle adaptations like those elicited by exercise. Consequently, the stress kinases' responses to exercise and ischaemia-reperfusion are partly blunted after Zynamite PX® supplementation. This can be explained by 1) the enhanced Nrf2 signalling prior to exercise by 48 h Zynamite PX® supplementation and 2) the dose of Zynamite PX® taken 60 min before exercise, which may have contributed to attenuating part of the Nrf2/ROS-induced signalling response (**Study 5**).

Overall, this thesis provides valuable insights into skeletal muscle redox regulation and Zynamite PX® effects on human skeletal muscle. This knowledge is of significant interest for identifying new molecular targets to counteract ROS-derived diseases and ischaemic injuries. It also offers molecular insights into how polyphenols regulate skeletal muscle signalling, which may prove useful for developing new applications for Zynamite PX®.

II

RESUMEN

(SUMMARY IN SPANISH)

2. RESUMEN

Introducción

Esta tesis fue inicialmente diseñada para determinar los efectos moleculares de la ingesta oral de Zynamite®, un extracto polifenólico rico en mangiferina y quercetina, sobre la señalización muscular en condiciones basales y en respuesta al ejercicio en seres humanos. Dado que los dos principales compuestos de Zynamite® son potentes antioxidantes y moduladores de las principales enzimas implicadas en la producción de especies reactivas de oxígeno (ROS) durante el ejercicio, primero estudiamos cómo se regula el equilibrio redox en el músculo esquelético humano, con énfasis en la Ca^{2+}/calmodulina proteína cinasa II (CaMKII) y el factor nuclear eritroide similar al factor 2 (Nrf2). Por ello la tesis comienza con una revisión centrada en Nrf2 (**Estudio 1**), un factor de transcripción pleiotrópico activado principalmente por ROS. Seguidamente, se llevó a cabo un estudio metodológico centrado en la optimización de la detección de las isoformas de CaMKII en el músculo esquelético humano (**Estudio 2**). El siguiente estudio aborda los efectos del entrenamiento de fuerza sobre la expresión basal de las isoformas de CaMKII (**Estudio 3**). El cuarto estudio se centra en la regulación de la autofagia durante el ejercicio agudo de alta intensidad y la isquemia en el músculo esquelético humano (**Estudio 4**). Finalmente, el último estudio se centra en el principal tema de la tesis, que inspiró su título (**Estudio 5**).

La mayoría de las enfermedades crónicas, el cáncer, la inflamación, la mutagénesis, las enfermedades neurodegenerativas y los procesos de envejecimiento se caracterizan por niveles elevados de ROS, que causan estrés oxidativo. Varias situaciones, incluido el ejercicio, aumentan la producción de ROS. Si bien los niveles excesivos de ROS pueden causar daño celular, el ejercicio regular mejora la capacidad antioxidante del músculo esquelético, mitigando el daño inducido por ROS y contribuyendo a las respuestas adaptativas. Nrf2 se activa principalmente por los niveles elevados de ROS y

orquesta la respuesta adaptativa al estrés oxidativo durante el ejercicio, principalmente al mediar la transcripción de genes antioxidantes.

CaMKII es una proteína inducida por las ROS y los cambios rápidos de concentración de calcio (Ca^{2+}), que regula el fenotipo muscular, la biogénesis mitocondrial, la autofagia y la señalización antioxidante. La caracterización precisa de las isoformas de CaMKII (δ_D, γ/δ y β_M) en el músculo esquelético humano es fundamental para comprender sus funciones específicas y respuestas al ejercicio. Por lo tanto, en el **Estudio 2**, desarrollamos un análisis de inmunoblotting basado en la incubación de extractos musculares con varios anticuerpos dirigidos contra isoformas específicas de CaMKII para facilitar la identificación de aquellas que responden más al ejercicio y a Zynamite PX®. En un tercer estudio (**Estudio 3**), determinamos si la expresión basal de CaMKII depende del nivel de fatiga provocado durante el programa de entrenamiento de fuerza y si los cambios de CaMKII con el entrenamiento estaban asociados con las respuestas adaptativas de la masa muscular (hipertrofia), la composición muscular de las cadenas pesadas de miosina (MHC) y la expresión de proteínas mitocondriales (OXPHOS). El **Estudio 3** también evaluó si la sarcolipina (SLN) cambia con el entrenamiento de fuerza. La sarcolipina es una proteína que, cuando se sobreexpresa, disminuye el acoplamiento de Ca^{2+} a la Ca^{2+}-ATPasa del retículo sarcoplásmico/endoplásmico (SERCA), lo que resulta en un aumento del gasto energético, estrés oxidativo, cambio de tipo de fibra muscular de rápida a lenta y la biogénesis mitocondrial.

La producción excesiva de ROS puede causar estrés oxidativo y daño celular, lo que puede afectar la función celular. La autofagia es un proceso celular que degrada componentes dañados y proteínas mal plegadas. Es esencial para mantener la salud muscular y es necesario para una respuesta adaptativa fisiológica al ejercicio. En el músculo esquelético se han descrito dos tipos principales de autofagia: la macroautofagia y la autofagia mediada por chaperonas (CMA). La macroautofagia es un proceso en el que vesículas

de doble membrana denominadas autofagosomas, capturan y degradan orgánulos y proteínas dañados para mantener la homeostasis celular. La autofagia mediada por chaperonas es un proceso similar que implica la degradación selectiva de proteínas con una secuencia específica reconocidas por proteínas chaperonas, que luego son transportadas al lisosoma para su degradación. Se desconoce cómo se regulan las vías de señalización de la autofagia con el ejercicio agudo hasta el agotamiento y cómo diferentes niveles de oxigenación muscular y acumulación de metabolitos en el músculo esquelético humano influyen en la macroautofagia y la CMA (**Estudio 4**). Para este propósito, hombres sanos realizaron un ejercicio incremental hasta el agotamiento (IE) en normoxia e hipoxia, seguido de aplicación inmediata de isquemia. Se tomaron biopsias del vasto lateral. El objetivo principal fue determinar la señalización de autofagia en el músculo esquelético en respuesta al IE con diferentes niveles de presión parcial inspiratoria de oxígeno (P_IO_2) e isquemia. Además, se analizó la regulación de FOXO, Akt-mTOR y GSK3β bajo estrés metabólico elevado y su influencia en la señalización de autofagia muscular.

Los polifenoles naturales y las xantonas, como la mangiferina y la quercetina, poseen propiedades antioxidantes y antiinflamatorias que pueden mejorar el rendimiento físico al inducir la señalización mediada por Nrf2, neutralizar los radicales libres e inhibir la xantina oxidasa (XO) y la NADPH oxidasa (NOX), las principales enzimas productoras de ROS durante el ejercicio. Zynamite®, un extracto de hojas de mango rico en mangiferina, ha sido estudiado en combinación con diferentes formulaciones, demostrando su capacidad para mejorar el rendimiento físico y acelerar la recuperación tras el daño muscular inducido por el ejercicio (EIMD). Sin embargo, ningún estudio en humanos ha determinado si Zynamite PX®, un extracto polifenólico que combina el extracto de hojas de mango con quercetina, ejerce efectos sobre la señalización muscular en el músculo esquelético en reposo y en respuesta a ejercicios de alta intensidad, con o sin isquemia-reperfusión posterior al ejercicio (**Estudio 5**).

Materiales y métodos

En el marco de esta tesis, se realizaron cuatro estudios experimentales (**estudios 2-5**) en los que se utilizó Western Blot como técnica principal para evaluar la expresión de proteínas en biopsias de músculo esquelético de hombres jóvenes y sanos. Los extractos musculares se prepararon con ~10 mg de tejido triturado con bolas de acero inoxidable y homogeneizado en un tampón de lisis con urea y cócteles inhibidores de proteasas y fosfatasas.

Estudio 1: Este artículo es una contribución a la edición especial titulada "Desbloqueando el potencial atlético: explorando la fisiología del ejercicio desde los mecanismos hasta el rendimiento" de la revista Free Radical Biology & Medicine de 2024. Para realizar esta revisión, se buscaron todos los artículos que contenían los términos en "Nrf2", "Keap1" y "skeletal muscle" en Web of Science (WOS). Además, se realizó una búsqueda con los términos " glutathione " y "skeletal muscle". Se llevaron a cabo búsquedas adicionales basadas en las referencias citadas en estos artículos si no habían sido identificadas previamente.

Estudio 2: Este estudio fue diseñado para optimizar la detección de las diferentes isoformas de CaMKII mediante Western blot, *stripping* y reincubado utilizando ocho anticuerpos comerciales diferentes de CaMKII en músculo esquelético humano. Se utilizó músculo esquelético humano de un participante sano obtenido en condiciones de reposo para incubar con los siguientes anticuerpos: Aldrich oxidado-Met$^{281/282}$ (no. 07-1387, Sigma Aldrich), CST pThr^{287} (no. 12716, Cell Signaling Technology), BD Total (no. 611292, BD Biosciences), CST Total (no. 4436, Cell Signaling Technology), Abcam δ (no. 181052, Abcam), Badrilla δ (no. A010-55AP, Badrilla), Proteintech γ (no. 12666-2-AP, Proteintech), SCBT β (no. 376828, Santa Cruz Biotechnology). Como ensayo adicional para validar el patrón de bandas, se obtuvieron biopsias del músculo vasto lateral antes y después de una intervención de entrenamiento de fuerza de 3 sujetos [1] y antes y después de una sesión de ejercicio agudo de alta intensidad de 1 sujeto [2], las cuales se incubaron (pThr^{287}-CaMKII y ox-

$Met^{281/282}$-CaMKII, respectivamente), seguidas de *stripping* y reincubado con el anticuerpo para CaMKII total. Se utilizaron las herramientas de Análisis de bandas y peso molecular del software Image Lab© 6.0.1 (Bio-Rad) para determinar los pesos moleculares experimentales.

Estudio 3: Este estudio fue una extensión de un estudio previo [3]. El objetivo de este estudio fue determinar si CaMKII y SLN estaban involucrados en los cambios de fenotipo y rendimiento muscular provocados por el entrenamiento de fuerza. Para este propósito, 22 hombres jóvenes siguieron un programa de entrenamiento de fuerza basado en la velocidad durante 8 semanas utilizando el ejercicio de sentadilla completa mientras se monitorizaba la velocidad de ejecución de las repeticiones. Los sujetos fueron asignados aleatoriamente a dos programas de entrenamiento de fuerza que diferían en la pérdida de velocidad de repetición permitida en cada serie: 20% (VL20) vs 40% (VL40). Se tomaron biopsias del vasto lateral antes y después del entrenamiento. La expresión de proteínas de CaMKII, SLN y OXPHOS se determinó mediante Western blot. Los datos previamente publicados [3] sobre las mejoras en la fuerza máxima (1RM), el rendimiento en salto vertical, el número de repeticiones realizadas durante el programa de entrenamiento, el grado de hipertrofia muscular y el cambio en el porcentaje de cadena pesada de miosina IIx (MHC-IIx) se utilizaron para la interpretación de las respuestas de señalización muscular. Los análisis estadísticos se realizaron mediante un ANOVA de medidas repetidas 2 x 2 con el software SPSS versión v.18 para Windows (SPSS Inc). Se aplicó un análisis de correlación de Pearson para determinar las asociaciones entre variables.

Estudio 4: Esta investigación se realizó para determinar los cambios en la expresión proteica de las vías de señalización de macroautofagia y CMA después de ejercicio de alta intensidad y aplicación de isquemia post-esfuerzo incluidas las principales señales reguladas por Zynamite®. Se determinaron los marcadores de Forkhead box O (FOXO), los relacionados con la elongación proteica (GSK3β y EEF2), así como marcadores hipóxicos (HIF1α) y los

involucrados en la síntesis de proteínas (Akt y mTOR). Para lograr estos objetivos, 11 hombres realizaron un ejercicio incremental hasta el agotamiento en normoxia (Nx, P_IO_2:143 mmHg) e hipoxia (Hyp, P_IO_2:73 mmHg, AltiTrainer200), en orden aleatorio. Al agotamiento, la circulación de una pierna se ocluyó instantáneamente durante 60 s con un inflador rápido (300 mmHg, Hokanson) conectado a un manguito (SC10D Hokanson) mientras los sujetos eran trasladados cuidadosamente a una camilla. Se tomaron biopsias del vasto lateral antes (Pre), 10 s después (Post, solo pierna ocluida) y 60 s después del IE en las piernas ocluida (Oc1m) y no ocluida (FC1m). Las biopsias de FC1m solo se tomaron después de la prueba hipóxica, obtenidas de la pierna que se estaba recuperando con circulación libre en normoxia durante 60 s. El consumo de oxígeno se midió respiración a respiración utilizando un carro metabólico (Vmax N29; Sensormedics, Yorba Linda, CA, EE. UU.). Los metabolitos musculares se evaluaron y reportaron previamente [4]. Los análisis estadísticos se realizaron utilizando un ANOVA de medidas repetidas 3 x 2. Se compararon las piernas ocluidas y no ocluidas utilizando una prueba t pareada de dos colas. Se determinaron las asociaciones lineales entre proteínas aplicando un modelo mixto lineal. Se calculó la prueba de razón de verosimilitudes para los efectos aleatorios (LRT) junto con los valores de R-cuadrado marginal y condicional. Todos los análisis estadísticos se realizaron con el software IBM SPSS, v.29.0, para Mac (IBM, Nueva York, NY, EE. UU.) y Jamovi v2.4.8. (The jamovi project 2023).

Estudio 5: Esta investigación fue diseñada para determinar los efectos de 48 h de suplementación con Zynamite PX® sobre los niveles de Nrf2 en músculo esquelético y la señalización inducida por Nrf2 en condiciones basales y en respuesta al ejercicio de alta intensidad combinado con isquemia-reperfusión en humanos. También se determinaron las principales proteínas reguladoras de los niveles de Nrf2, denominadas, CaMKII, GSK3β, p38 MAPK y Keap1, así como la expresión proteica de las enzimas antioxidantes GR y catalasa. Para ello, 25 hombres jóvenes (17 grupo Control; 8 grupo Zynamite PX®) realizaron un ejercicio incremental (IE) hasta el agotamiento,

seguido de oclusión instantánea unilateral de la pierna durante 60 s con un inflador rápido (300 mmHg, Hokanson) conectado a un manguito (SC10D Hokanson). Al liberar el manguito, los sujetos realizaron un sprint máximo de 30 s (prueba de Wingate) con el ergómetro configurado en modo isocinético (80 RPMs), y posteriormente, la misma pierna fue ocluida instantáneamente durante 90 s. Inmediatamente después de la oclusión, los sujetos fueron trasladados cuidadosamente a una camilla, donde descansaron en posición supina mientras la circulación de la pierna biopsiada permanecía completamente ocluida durante los 90 s. La suplementación consistió en 140 mg de Zynamite® (estandarizado al 60% de mangiferina, un extracto acuoso de *Mangifera indica*) en combinación con 140 mg de quercetina (proporcionada como 280 mg de extracto de *Sophora japonica*, estandarizado al 50% de quercetina), comercializado como Zynamite PX®, cada 8 h para un total de seis dosis 48 h antes de la biopsia en reposo. Se ingirió una última dosis del suplemento ~60 min antes del inicio del ejercicio. Se tomaron biopsias del vasto lateral en reposo (antes de ingerir la última dosis de Zynamite PX®, Pre – B1), 20 s después del IE (Post, pierna ocluida – B2) y 10 s después de Wingate (OcW, pierna ocluida – B3), y de la pierna ocluida y no ocluida a los 90 s (Oc90 y nOc90 – B4) y 30 min post-Wingate (Oc30m y nOc30m – B5). El consumo de oxígeno se midió respiración a respiración utilizando un carro metabólico (Vyntus, Jaeger-CareFusion, Höchberg, Alemania). Se utilizó una prueba t de dos colas no apareada para estudiar los cambios en la expresión de proteínas en condiciones de reposo entre el grupo suplementado y el grupo de control. Una prueba t apareada evaluó las diferencias entre la piernas isquémica y de libre circulación a los 90 s y 30 min post-ejercicio. Debido a respuestas similares en ambas piernas en cada punto temporal, se promediaron las biopsias a los 90 s, al igual que las biopsias a los 30 min, y estos promedios se utilizaron para representar las respuestas post-ejercicio a los 90 s y 30 min, respectivamente. Los análisis estadísticos se realizaron utilizando un ANOVA de medidas repetidas 5 x 2 con el software IBM SPSS, v. 29.0, para Mac (IBM, Nueva York, NY, EE. UU.).

Resultados

El **Estudio 1** ofrece una visión general del rol de Nrf2 en la mejora del rendimiento y de las adaptaciones al entrenamiento en humanos. La señalización de Nrf2 se activa principalmente en respuesta al desequilibrio redox por ROS o electrófilos en el músculo esquelético [5, 6]. Estudios en ratones knockout para Nrf2 ($Nrf2^{-/-}$) reportaron menos fuerza y mayor fatigabilidad debido a una menor capacidad respiratoria mitocondrial basal y adaptabilidad mitocondrial al entrenamiento [7]. En humanos, la expresión basal de Nrf2 en músculo esquelético se ha asociado con el consumo máximo de oxígeno (VO_2máx) [8] y el rendimiento en ejercicios de alta intensidad [9, 10].

Durante el ejercicio, los ROS se producen principalmente por NOX2 y XO [11-13], así como por la cadena de transporte de electrones mitocondrial. La contribución de cada fuente de ROS varía con la intensidad del ejercicio, duración, oxidación de sustratos, estado de entrenamiento, oxigenación, nivel de fatiga y edad [11, 14-16]. Los ROS son contrarrestados por antioxidantes enzimáticos, como las superóxido dismutasas (SODs), glutatión reductasa (GR), catalasa, glutatión peroxidasa (GPx) y antioxidantes no enzimáticos. El radical superóxido (O_2^-) es reducido a peróxido de hidrógeno (H_2O_2) por las SODs. El peróxido de hidrógeno, que es un oxidante menos reactivo, es reducido por catalasa y GPx. La actividad enzimática de GPx consume glutatión reducido (GSH) y produce glutatión oxidado (GSSG), siendo el ratio GSH:GSSG un indicador teórico de la capacidad de tamponamiento redox [17]. No obstante, los estudios que evalúan el ratio GSH:GSSG en el músculo esquelético humano muestran alta variabilidad, lo que indica que podría no ser un biomarcador fiable de la capacidad de tamponamiento antioxidante de la célula a menos que se mida con precisión [18].

En condiciones basales, Nrf2 forma un complejo con Keap1 [19, 20], que actúa como proteína adaptadora facilitando la acción de un complejo E3 ubiquitina ligasa (CUL-E3), el cual ubiquitina a Nrf2 [20]. Esto provoca la disociación de Nrf2 y Keap1 y la posterior degradación proteasomal de Nrf2

ubiquitinado [21, 22]. Ante el estrés oxidativo, los ROS modifican a Keap1, deshabilitando su función adaptadora para CUL E3, impidiendo la ubiquitinación de Nrf2 [21, 23, 24] y estabilizando el complejo Keap1-Nrf2. El primer Nrf2 recién sintetizado es secuestrado por Keap1 libre restante, y solo cuando Keap1 libre se agota, Nrf2 transloca al núcleo [19, 25], donde se une a los elementos de respuesta antioxidante (AREs) para activar la expresión de enzimas antioxidantes [7, 26-32]. Además, la degradación de Keap1 también es mediada por sequestosoma 1 (p62/SQSTM1), que se une directamente a Keap1 a través de una secuencia específica, la región de interacción con Keap1 (KIR) [2, 33, 34], lo que lleva a la degradación lisosomal de Keap1 y promueve la señalización de Nrf2 [35]. La regulación de Nrf2 durante el estrés oxidativo también puede ser independiente de Keap1. La retención nuclear de Nrf2 está modulada por múltiples sitios de fosforilación de Nrf2, que son regulados por quinasas inducidas por el ejercicio, como p38 MAPK en $pSer^{40}$-Nrf2 [36, 37]. Adicionalmente, la regulación al alza de las quinasas inducidas por el ejercicio (AMPK, Akt, PKA y PKC) [38-40] puede producir una disminución en la actividad de GSK3β, lo que atenúa la fosforilación de Nrf2 en Ser^{338} y Ser^{342}, impidiendo la degradación de Nrf2 por el proteasoma. La inhibición de GSK3β también puede facilitar la retención nuclear de Nrf2 al impedir la fosforilación de Nrf2 en Ser^{344} y Ser^{347} [41-43] y en Ser^{550} [44]. Además, el peróxido de hidrógeno y el sulforafano pueden estimular la traducción de Nrf2 cap-independiente a través de un sitio interno de entrada del ribosoma ($IRES_{Nrf2}$) [45, 46], un proceso que requiere la fosforilación del factor de iniciación eucariota 2 alfa (eIF2α) por quinasas inducidas por estrés [47-49]. La acetilación de Nrf2 promueve la translocación nuclear [50]. La exportación nuclear y la posterior ubiquitinación y degradación son facilitadas por la quinasa Fyn al fosforilar a Nrf2 en Tyr^{568} [51].

Los polifenoles pueden reducir la disponibilidad de Keap1, mejorar la translocación nuclear de Nrf2, inducir la transcripción y traducción de Nrf2 [46, 52], y reducir la degradación proteasomal de Nrf2 [52]. Aunque se ha sugerido que la ingesta de antioxidantes antes del ejercicio puede atenuar algunas de las adaptaciones al ejercicio, esto sigue siendo controvertido en humanos sanos [53]

y solo se puede observar después de la ingesta de altas dosis de vitamina C y E [54, 55]. En cambio, se considera que la ingesta de polifenoles derivados de frutas es positiva para mejorar el rendimiento y la recuperación en atletas [56].

El **Estudio 2** determinó los pesos moleculares de las isoformas de CaMKII en músculo esquelético humano en función de la movilidad en SDS-PAGE, que corresponden a: δ_D, con 54.2 ± 2.1 kDa; γ/δ, con 59.0 ± 1.2 kDa y 61.6 ± 1.3 kDa; y la isoforma β_M, con 76.0 ± 1.8 kDa. También evaluamos la especificidad de los anticuerpos de CaMKII. El anticuerpo β CaMKII (no. 376828, Santa Cruz Biotechnology) reconoció una sola banda, alineada con el peso molecular (MW) correspondiente a una banda similar marcada por los anticuerpos dirigidos contra CaMKII total, fosfo- y oxidada. Además, el anticuerpo δ CaMKII (no. 181052, Abcam) mostró alta especificidad para δ_D, la isoforma más sensible a ROS y a gradientes de Ca^{2+} intracelular en el músculo esquelético humano. Por el contrario, el anticuerpo γ CaMKII (no. 12666-2-AP, Proteintech) mostró reactividad cruzada con todas las isoformas de CaMKII, lo que no permitió distinguir entre las isoformas γ/δ.

El **Estudio 3** mostró que la cantidad de fatiga permitida en cada serie de la intervención de entrenamiento de fuerza determinó los niveles de CaMKII basal, la expresión basal de SLN y los cambios en el fenotipo muscular. El entrenamiento de fuerza provocó hipertrofia muscular, mejoró el 1RM e incrementó CaMKII total, principalmente por un aumento de la CaMKII δ_D total. El grupo que entrenó con mayor fatiga intra-serie (VL40) experimentó mayor hipertrofia muscular y reducción del porcentaje de MHC-IIx (lo que podría limitar la mejora de la potencia muscular con el entrenamiento) y un aumento de la fosforilación de CaMKII δ_D en Thr^{287}. En contraste, el grupo que entrenó con menor fatiga intra-serie (VL20) incrementó la expresión de SLN y atenuó el cambio de MHC-IIx a IIa sin variaciones en la fosforilación de CaMKII. Los cambios en los niveles de fosforilación de CaMKII δ_D en Thr^{287} se asociaron positivamente con la hipertrofia muscular y el número de repeticiones durante el entrenamiento, y negativamente con los cambios en

MHC-IIx. La expresión de SLN no se asoció con cambios en la hipertrofia muscular, el porcentaje de MHC-IIx, el número de repeticiones ni las mejoras en el rendimiento del salto vertical y 1RM, mientras que se asoció negativamente con pThr^{287}-CaMKII δ_D, SDHB (Complejo II) y ATP5A (Complejo V). La mayoría de las proteínas de OXPHOS permanecieron sin cambios, excepto NDUFB8 (Complejo I), que se redujo después del entrenamiento en ambos grupos. No se observó ninguna asociación entre pThr^{287}-CaMKII y OXPHOS, lo que indica que una mayor activación de CaMKII no indujo la expresión de proteínas mitocondriales.

El **Estudio 4** mostró que un ejercicio incremental hasta el agotamiento seguido de la aplicación de isquemia durante 60 s indujo la macroautofagia y autofagia mediada por chaperonas de manera similar en normoxia (Nx) e hipoxia (Hyp). Bajos niveles del ratio AMP:ATP y el estrés metabólico activaron la señalización de la macroautofagia a través de AMPKα-UKL1. Esto fue seguido por la fosforilación en Ser^{15} de BECN1, crucial para la formación del fagóforo. Los niveles elevados de pSer^{349}-SQSTM1/p62, la reducción de SQSTM1/p62 total y una mayor proporción de la ratio LC3B-II:LC3B-I sugirieron un aumento de la degradación de autofagosomas. Esta señalización fue acompañada por el aumento de PHAF1/MYTHO, un nuevo biomarcador de macroautofagia, que se asoció positivamente con la ratio LC3B-II:LC3B–I. El aumento de LAMP2A indicó la activación de la autofagia mediada por chaperonas en respuesta al ejercicio agudo de alta intensidad, independientemente de la disminución post-ejercicio de HSPA8/HSC70. Además, pSer^{40}-Nrf2 y LAMP2A se asociaron positivamente, lo que sugiere una estimulación de CMA dependiente de ROS. Los FOXOs no parecieron regular la macroautofagia y CMA en músculo esquelético sano, ya que su señalización fue inhibida después del ejercicio de alta intensidad. La disminución de pThr^{56}-EEF2, que está inhibido por EEF2K, indicó una disminución de la señalización de elongación proteica después del ejercicio. EEF2K es activado por AMPKα activa y GSK3β. Además, la disminución de la activación de Akt podría indicar una disminución de la síntesis de proteínas

bajo un alto estrés metabólico. El aumento de hidroxi-Pro^{564} HIF1α en condiciones de ejercicio en normoxia no fue más estimulado por la hipoxia o la isquemia. La asociación positiva entre pSer^{571}-PGC1α y pThr^{172}-AMPKα sugirió un aumento de la biogénesis mitocondrial y de la transcripción de genes de macroautofagia. A pesar de una mayor activación del metabolismo glucolítico, la hipoxia severa no exacerbó la respuesta de señalización de la autofagia, lo que indica que los niveles de autofagia fueron casi máximos con el ejercicio en normoxia. La mayoría de los cambios de señalización revirtieron en un minuto de recuperación con circulación libre, mientras que la aplicación inmediata de isquemia post-ejercicio impidió la recuperación.

El **Estudio 5** demostró que, en comparación con los controles, el grupo suplementado con Zynamite PX® aumentó la expresión basal de pThr^{287}-CaMKII δ_D, que fosforila GSK3β en Ser^{9}, inhibiendo su actividad. El aumento de la inhibición de GSK3β puede disminuir la tasa de degradación proteasomal de Nrf2, y, en consecuencia, se observó un aumento no significativo de Nrf2 total ($p = 0.099$) y pSer^{40}-Nrf2 ($p = 0.061$). No se observaron cambios basales en las enzimas antioxidantes (catalasa y GR), lo que sugiere que se podrían necesitar dosis más altas o que el sistema enzimático antioxidante está estrictamente regulado. En el grupo de control, el ejercicio combinado con isquemia-reperfusión y recuperación condujo a la activación de quinasas de estrés (pThr^{287}-CaMKII δ_D y pThr^{180}/Tyr^{182}-p38 MAPK) y a la respuesta de señalización antioxidante (Nrf2 total, Nrf2/Keap1, catalasa y GR). Sin embargo, esta señalización fue parcialmente atenuada en el grupo Zynamite PX®, lo que sugiere que Zynamite PX® puede reducir el estrés metabólico y la señalización inducida por ROS aumentando la señalización basal de Nrf2 después de la ingesta de Zynamite PX®.

Conclusiones

La regulación del factor de transcripción Nrf2 en el músculo esquelético durante el ejercicio involucra una interacción entre el equilibrio redox, el estrés celular y las respuestas adaptativas. Nrf2 activa la expresión de varias enzimas y vías antioxidantes en respuesta al estrés oxidativo y se regula a través de Keap-1 y de varias modificaciones postraduccionales que controlan su localización nuclear, traducción proteica y degradación proteasomal, de manera independiente de la regulación mediada por Keap1. Además, los polifenoles dietéticos han emergido como moduladores potenciales de la actividad de Nrf2 (**Estudio 1**).

Los pesos moleculares de las isoformas de CaMKII caracterizadas por immunoblotting son: 54.2 ± 2.1 kDa (δ_D), 59.0 ± 1.2 kDa, 61.6 ± 1.3 kDa (γ/δ) y 76.0 ± 1.8 kDa (β_M), siendo la δ_D la más responsiva a ROS y gradientes de Ca^{2+} (**Estudio 2**).

CaMKII δ_D total aumenta en respuesta al entrenamiento de fuerza, y realizar repeticiones cerca del fallo dentro de cada serie se asocia con niveles basales elevados de $pThr^{287}$-CaMKII δ_D. El aumento de $pThr^{287}$-CaMKII δ_D se asocia positivamente con la hipertrofia muscular y negativamente con los cambios en la expresión de MHC-IIx. La expresión de sarcolipina aumenta en respuesta al entrenamiento de fuerza, pero este efecto solo se observa cuando el nivel de fatiga en la serie es bajo. Se ha demostrado una asociación inversa entre los cambios basales de $pThr^{287}$-CaMKII δ_D y los de sarcolipina, lo que es compatible con la existencia de un loop negativo que limita una expresión excesiva de SLN después de contracciones musculares fatigantes (**Estudio 3**).

Los niveles de macroautofagia y CMA son aumentados de manera similar durante el ejercicio hasta el agotamiento en normoxia e hipoxia severa. Los ROS producidos durante el ejercicio incremental y la isquemia pueden activar la macroautofagia y la CMA, pero no los FOXOs. Hay una mayor expresión del nuevo biomarcador de autofagia PHAF1/MYTHO en el músculo

esquelético humano en respuesta al ejercicio en normoxia, hipoxia e isquemia post-ejercicio. Hay una activación concurrente de la autofagia con la inhibición de la elongación y síntesis proteica (**Estudio 4**).

La ingesta oral de Zynamite PX® aumenta los niveles basales de pThr^{287}-CaMKII δ_D y de la fosforilación inhibidora de GSK3β en el músculo esquelético humano, lo que puede provocar adaptaciones musculares similares a las inducidas por el ejercicio. Consecuentemente, la respuesta de las quinasas de estrés inducidas por el ejercicio y la isquemia-reperfusión se atenuó parcialmente después de la suplementación con Zynamite PX®. Esto puede explicarse por 1) la mejora de la señalización de Nrf2 antes del ejercicio por la suplementación de Zynamite PX® durante 48 h y 2) la dosis de Zynamite PX® tomada 60 min antes del ejercicio, que puede haber contribuido a atenuar parte de la respuesta de señalización de Nrf2/ROS inducida por el ejercicio (**Estudio 5**).

En general, esta tesis proporciona valiosos conocimientos sobre la regulación redox y los efectos de Zynamite PX® en el músculo esquelético humano. Este conocimiento es de gran interés para identificar nuevas dianas terapéuticas para contrarrestar las enfermedades derivadas de ROS y las lesiones isquémicas. También ofrece conocimientos moleculares sobre cómo los polifenoles regulan la señalización del músculo esquelético, lo que puede resultar útil para desarrollar nuevas aplicaciones para Zynamite PX®.

III

INTRODUCTION

3. INTRODUCTION

This thesis aimed to investigate the molecular effects of oral Zynamite PX® ingestion, a polyphenolic extract rich in mangiferin and quercetin, on skeletal muscle signalling under both basal and during exercise. Given that Zynamite PX® formulation has potent antioxidants that modulate enzymes involved in ROS production, the research focused on understanding redox balance regulation in human muscle. The thesis included a comprehensive literature review on the key mechanisms regulating Nrf2 signalling in basal and in response to exercise and the impact of some supplements (**Study 1**). Following this, a methodological study was carried out to more precisely identify and determine CaMKII isoforms, an exercise-induced kinase responsive to ROS and Ca^{2+} transients, in human skeletal muscle (**Study 2**). Additionally, the role of CaMKII in response to strength training performed under different levels of metabolic stress (fatigue) was analysed (**Study 3**). **Study 4** explored how redox balance, muscle oxygenation and energy metabolism influence muscle signalling and autophagy. Finally, **study 5** focused on the main objective of this thesis, which was to determine the effects of Zynamite PX® in human skeletal muscle exercise-induced stress kinases (p38 MAPK, CaMKII, GSK3β), the Nrf2-Keap1 signalling system and the principal antioxidant enzymes at rest and in response to high-intensity exercise and ischaemia. The five studies included in this thesis investigate ROS-mediated mechanisms that could guide future research in the development of new applications for Zynamite®.

Since 1954, when reactive oxygen species (ROS) were reported for the first time in biological materials [57], a predominant concept prevailed considering free radicals and ROS as “dangerous agents” that should be avoided and counteracted [58]. Free radicals are atoms or molecules that contain one or more unpaired electrons and are capable of independent

existence. Free radicals can have a very short lifespan due to their high reactivity with other molecules. Reactive oxygen species (ROS) are compounds (or atoms) that contain oxygen, which can be free radicals or highly reactive non-radical oxygen derivatives, such as hydrogen peroxide (H_2O_2). Reactive nitrogen species (RNS) are nitrogen radicals and non-radical reactive nitrogen molecules [14]. The term reactive oxygen and nitrogen species (RONS) is often used to refer indistinctively to ROS and RNS.

RONS are involved in multiple mechanisms of disease, including inflammation, cancer [59], mutagenesis, radiation-induced injury [60], neurodegenerative diseases [61] and ageing [62-64]. It was not until the 1970s that some experimental evidence emerged indicating that ROS are necessary for the normal functioning of the cells [65]. In addition, it was discovered that free radicals or their derivatives are essential for regulating vascular tone, O_2 sensing, and for several signal transduction mechanisms and oxidative stress adaptive responses necessary for maintaining redox homeostasis and health [66, 67]. RONS are also required for normal protein folding and disulfide bond formation that regulate the structure and function of many cellular proteins [68].

ROS are produced during exercise depending on intensity, duration and training status. At rest, ROS are primarily generated as by-products of mitochondrial respiration [67]. However, during exercise ROS are mostly produced by xanthine oxidase (XO) and nicotinamide adenine dinucleotide phosphate-oxidase isoform 2 (NAD(P)H oxidase 2 or NOX2) [11, 12, 69]. The production of ROS is exacerbated during high-intensity and prolonged exercise, mainly if performed until exhaustion [11, 12, 15, 70]. Severe hypoxia [71] and ischaemic conditions [2] increase the involvement of glycolytic energy metabolism causing the accumulation of lactate, hydron (H^+), and inorganic phosphate (P_i) and exacerbating ROS production. Although uncontrolled

ROS production could cause oxidative damage and fatigue [72], exercise training increases skeletal muscle antioxidant capacity [36, 73, 74] and attenuates ROS induced signalling [11, 15] and damage [75]. Importantly, part of the signalling responses needed for the adaptive response to exercise are mediated by ROS [76].

Redox homeostasis is maintained by antioxidants and modulation of ROS production. Antioxidants can be divided into two categories: non-enzymatic and enzymatic antioxidants. The Nrf2-Keap1 signalling system plays a crucial role in regulating redox cellular homeostasis [77] and its disruption could be implicated in cellular senescence and sarcopenia [78]. In skeletal muscle, oxidative stress caused by high-intensity exercise, activates Nrf2 signalling [5, 6]. Under basal conditions, Nrf2 forms a complex with Keap1 [19, 20]. Keap1 acts as an adaptor protein facilitating the action of a ubiquitin E3 ligase complex (CUL-E3), which ubiquitinates Nrf2 [20]. The latter causes the dissociation of Nrf2 from Keap1 and subsequent proteasomal degradation of the ubiquitinated Nrf2 [21, 22]. Additionally, Nrf2 is also targeted for degradation through ubiquitination by β-transducin repeat-containing protein (β-TrCP). This process requires Nrf2 phosphorylation at Ser^{338} and Ser^{342} by glycogen synthase kinase-3 beta (GSK3β). GSK3β is constitutively active but is inhibited by phosphorylation at Ser^{9}, a process driven by kinases like Akt [79], PKA^{80}, p70S6K [81], and CaMKII [82], which are activated during high-intensity exercise or exercise to exhaustion [2, 38, 83-85]. Upon oxidative stress, Keap1 structure can be modified by ROS and electrophiles, rending inoperative its adaptor function for CUL-E3, what impedes the ubiquitination of Nrf2 [21, 23, 24] and disaggregation of the Keap1-Nrf2 complex. The first newly synthesized Nrf2 is sequestered by the remaining free Keap1, and only once the reservoir of free Keap1 has been exhausted will Nrf2 translocate to the nucleus [25, 86] , where it binds to antioxidant response elements (AREs) in

the promoter of most antioxidant enzymes [7, 26-28, 30]. The latter include the main antioxidant enzymes involved in redox balance in human skeletal, i.e., superoxide dismutase 1 and 2 (SOD1 and SOD2), catalase, GR, and GPx, thioredoxin isoforms (Trx1 and Trx2), among others [7, 26-31, 87-95]. The action of Nrf2 is facilitated by its nuclear retention which may be elicited by its phosphorylation at Ser^{40} by several exercise-stimulated kinases [36, 37].

Calcium (Ca^{2+})/calmodulin-dependent protein kinase II (CaMKII) is activated during exercise by ROS [96] and Ca^{2+} transients triggering muscle contraction [97]. CaMKII is a multimeric enzyme encoded by four homologous genes (α, β, γ, and δ) [98, 99]. The presence of β, γ, and δ CaMKII isoforms in skeletal muscle has been revealed mainly by Western blot with isoform-specific antibodies [100-105]. In human skeletal muscle, different isoforms characterisation [104-108] and specific regulation of some of these isoforms following exercise training [109, 110] has been reported. However, it remains unknown which of the CaMKII isoforms are more responsive to exercise. Given the importance of this kinase in human skeletal muscle, correctly identifying the CaMKII isoforms is crucial for better understanding their specific functions and regulation in response to exercise and other stimuli. Strength training to failure is accompanied by higher activation of the anaerobic metabolism [111] and possibly greater Ca^{2+} transients than training with considerably less fatigue in each set [112]. A higher level of fatigue (i.e., greater reduction in strength, velocity, and power) by performing repetitions closer to muscle failure has been associated with greater muscle hypertrophy, a IIx-to-IIa myosin heavy chain (MHC) transition [3]. CaMKII is a principal regulator of muscle phenotype [112, 113] and modulates the activity of the transcription factor coactivator peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), which is a critical activator of mitochondrial biogenesis [114]. Nevertheless, the molecular mechanisms explaining the regulation of

muscle phenotype are based on previous evidence coming from extreme experimental rodent models [112, 115-117] and information in humans is lacking. Moreover, CaMKII modulates the antioxidant signalling pathways mediated by Keap1 and Nrf2 [2, 83, 106] and is implicated in the regulation of autophagy [118], muscle contraction and relaxation [112] and glycolytic metabolism [119 82 120 121], among others.

Excessive ROS production can cause damage to organelles and protein structures. Autophagy is a cellular process by which a double-membrane vesicle, an autophagosome, sequesters old and damaged organelles and proteins that must be degraded in the autolysosome [122, 123]. Maintaining adequate levels of autophagy is crucial for health. Neurodegenerative diseases have been linked to disrupted autophagy, leading to the accumulation of damaged structures or proteins and impairing the ability for neurogenesis [124]. Autophagy has been shown to decrease in older cells, contributing to age-related diseases. For example, a decline in neurogenesis related to decreased autophagy has been observed in old mice brain [125, 126] and elderly humans [127], associated with the Parkinson's and Alzheimer's diseases. Moreover, excessive autophagy activation can lead to muscle mass loss, increasing the development of metabolic diseases, morbidity, and mortality [128]. In bed resting critically ill patients, lack of muscle activity triggers an overactivation of autophagy, contributing to atrophy [129]. In skeletal muscle, autophagy is essential for adaptive response to exercise to prevent mitochondrial damage [130], facilitate muscle regeneration [131] , and maintain glucose homeostasis [132]. ROS production and metabolite accumulation disrupt homeostasis during incremental exercise to exhaustion, hypoxia, and ischaemia [4]. Although ROS measurements in human skeletal muscle are challenging, cell and animal studies have reported higher ROS levels concomitantly with raised autophagy activation [133-135]. Two main types of autophagy have been

characterised in skeletal muscle: macroautophagy and chaperone-mediated autophagy (CMA).

Macroautophagy, which involves autophagosome and autolysosome formation and subsequent degradation processes, has been shown to be initiated in response to endurance exercise [85, 136-145] and training [138, 139, 143, 146] in human skeletal muscle. The increase of the AMP:ATP ratio activates adenosine monophosphate-activated protein kinase alpha (AMPKα) [141], which phosphorylates and activates unc-51 like kinase 1 (ULK1) at Ser^{556}, initiating macroautophagy [142]. The molecular evidence for markers of autophagosome content and catabolism of cargoreceptors is less conclusive, in particular regarding the protein expression of the microtubule-associated protein 1 light chain 3 beta (MAP1LC3B/LC3B) and sequestosome 1 (SQSTM1/p62) [85, 138-143, 145, 147-149]. Chaperone-mediated autophagy is a selective mechanism for the degradation of cytosolic proteins in lysosomes. Proteins and organelles containing the KFERQ-like motif are recognised by heat shock protein family A [Hsp70] member 8 (HSPA8/HSC70) [150-152]. The damaged proteins and HSPA8 bind to the lysosomal-associated membrane protein 2A (LAMP2A) receptor, eventually resulting in their digestion in the lysosomal lumen. This process is acutely activated in cells under oxidative stress [153] and hypoxia [154]. ROS may elicit CMA since the knockout Nrf2 reduces LAMP2A mRNA and protein levels in different cell types [155]. Nevertheless, the mechanisms leading to the activation of macroautophagy and chaperone-mediated autophagy in human skeletal muscle in response to high-intensity exercise remain poorly understood. Elucidating the signalling pathways involved in autophagy in skeletal muscle during exercise and ischaemia is essential to gain insights into the physiological adaptations to oxidative stress.

Natural polyphenols and xanthones are non-enzymatic antioxidants that possess antioxidant, anti-inflammatory, cardioprotective,

neuroprotective, anticancer, immunomodulatory, prebiotic, and antimicrobial properties [26, 56, 156], which may have an ergogenic effect (enhancement of exercise performance). Mangiferin is a xanthone with remarkable antioxidant power due to its free-radical scavenging properties and its ability to inhibit NOX and XO [157-160]. Quercetin is a polyphenol with potent antioxidant and anti-inflammatory actions [161], which may enhance aerobic exercise performance [162] and power output during sprint exercise when administered together with mangiferin [163, 164]. Although some antioxidants and XO inhibitors can partly block the acute signalling response to exercise [11, 76], cell and animal experiments indicate that polyphenols such as mangiferin and quercetin may overcome this limitation by inducing Nrf2 [157, 165, 166]. Zynamite®, an extract from mango leaves abundant in mangiferin, has been shown to enhance power output when combined with quercetin or luteolin [163, 164], accelerate recovery after exhausting exercise [156] and attenuate the adverse effects of ischaemia-reperfusion on muscle function [163, 164, 167]. Zynamite PX®, a polyphenolic extract combining the mango leaf extract with quercetin, has been demonstrated to enhance exercise performance after a single dose [167], as well as following 48 h [163, 164] and 15 days of supplementation [163, 164]. While the physiological effects of Zynamite® are well-studied, its impact on muscle signalling has not been studied. Thus, it remains to be determined if Zynamite PX® exerts Nrf2 signalling effects in resting skeletal muscle and whether short-term Zynamite PX® supplementation modifies the Nrf2 signalling response to high-intensity exercise. Understanding the dynamics of Nrf2 signalling in skeletal muscle during exercise provides valuable insights into the physiological adaptations to oxidative stress and the interaction between training and diet, offering avenues for optimising performance and mitigating oxidative stress-related muscle damage.

This knowledge may contribute to preventing ageing-related diseases, cancer, and other conditions characterised by either excessive ROS levels and/or deficient or excessive autophagy. Moreover, investigating ischaemia-reperfusion in human skeletal muscle can provide valuable information on how cardiac muscle might respond to ischaemic events, such as myocardial infarctions. Additionally, this research may facilitate the identification of therapeutic targets and the development of novel treatments to modulate these processes. This research may contribute to elucidating the role of polyphenols and dietary components in mitigating ROS signalling pathways in response to oxidative stress.

IV

AIMS AND HYPOTHESES

4. AIMS AND HYPOTHESES

In the scope of this thesis, five studies have been conducted, each with specific objectives and hypotheses.

The following section details the objectives and hypotheses for each study.

Study 1: Review article

- **Aims:**

  To describe the main mechanisms of ROS production and counteraction in the exercising skeletal muscle.

  To describe the main regulatory processes leading to Nrf2 signalling in response to acute and chronic exercise and the influence of Nrf2 on exercise performance.

  To describe the impact of the diet and some supplements on the basal expression of Nrf2 and Nrf2 signalling to acute and chronic exercise.

Study 2: Methodological article

- **Aim:**

  To develop a protocol to optimise the identification of the β_M, γ/δ and δ_D CaMKII isoforms and their respective activating phosphorylation (Thr^{287}) and oxidation ($Met^{281/282}$) using eight different commercial antibodies through immunoblotting, stripping and reprobing in human skeletal muscle.

- **Hypotheses:**

We hypothesised that CaMKII antibodies would specifically identify each CaMKII isoform and that combining eight different commercial antibodies would aid in their identification.

We also hypothesised that employing exercise-stimulated samples (strength training and acute exercise) could facilitate accurately identifying the CaMKII isoforms.

Study 3:

- **Aims:**

To determine whether basal CaMKII phosphorylation in skeletal muscle is modified depending on the level of fatigue elicited during a strength training program.

To ascertain whether the changes in muscle mass (hypertrophy), MHC composition, and mitochondrial protein expression (OXPHOS) are associated with the changes in CaMKII phosphorylation.

To assess whether SLN expression in skeletal muscle is increased by strength training and if it is associated with muscle hypertrophy.

- **Hypotheses:**

We hypothesised that CaMKII phosphorylation would increase more in subjects training with greater fatigue allowed during each set, stimulating a IIx-to-IIa shift in MHC expression, muscle hypertrophy, and the expression of mitochondrial proteins.

We hypothesised that training with more fatigue would be associated with a higher increase in SLN expression.

Study 4:

- **Aims:**

To determine whether macroautophagy and chaperone-mediated autophagy are activated by acute exercise to exhaustion in human skeletal muscle.

To ascertain the role played by muscle oxygenation and metabolite accumulation in autophagy signalling.

To describe the temporal course of these signalling responses during the early recovery after exercise and post-exercise ischaemia.

To determine the regulation of FOXO, Akt-mTOR and GSK3β under high metabolic stress and its influence on skeletal muscle autophagy signalling.

- **Hypotheses:**

We hypothesised that exercise would activate macroautophagy and chaperone-mediated autophagy.

We hypothesised that these effects would be more marked when exercise is performed in severe acute hypoxia and further enhanced by post-exercise ischaemia.

We hypothesised that these changes would be reverted to pre-exercise levels early after the cessation of exercise in the muscles recovering with free circulation.

Study 5:

- **Aims:**

To determine the effects of 48 h Zynamite PX® supplementation on skeletal muscle Nrf2 protein levels and Nrf2-induced signalling under basal conditions and in response to high-intensity exercise combined with ischaemia-reperfusion in humans.

To determine the effects of Zynamite PX® on CaMKII, GSK3β and Keap1 as main regulatory mechanism of Nrf2 levels.

- **Hypotheses:**

We hypothesised that Zynamite PX® supplementation would increase basal Nrf2 protein levels and attenuate the signalling responses induced by high-intensity exercise and ischaemia-reperfusion.

V

JUSTIFICATION

5. JUSTIFICATION

The proposed research for this thesis is justified by its potential to elucidate the molecular mechanisms underlying the human skeletal muscle regulation by exercise, ischaemia-reperfusion and polyphenol intake, providing insights into how these processes contribute to enhance health and physical performance by modulating ROS levels.

Age-related diseases, cancer, and chronic and muscular dystrophies are associated with high oxidative stress. High-intensity exercise protocols, particularly those involving ischaemia-reperfusion, are characterised by a temporary increase in ROS. ROS acute increase is necessary to trigger an adaptive response in skeletal muscle tissue, activating metabolic pathways that improve the capacity to counteract ROS and preserve muscle cell functionality. Additionally, the intake of natural polyphenols, i.e. a mango leaf extract combined with quercetin (Zynamite PX®), may enhance the antioxidant capacity of skeletal muscle, potentially mitigating ROS production during exercise, delaying muscle fatigue and accelerating recovery.

Despite existing evidence on ROS-mediated skeletal muscle signalling, human studies are limited. CaMKII is crucial for exercise physiology, as it regulates autophagic and antioxidant pathways. Proper characterisation of CaMKII isoforms could help to better understand the role played by each CaMKII isoform in human skeletal muscle. Furthermore, there is limited evidence regarding the regulation of autophagy in humans, particularly in models inducing high levels of ROS production by exercise and ischaemia. Moreover, the redox-regulated signalling in response to short-term Zynamite PX® ingestion remains unknown.

Regular exercise activates autophagic and antioxidant systems through the activation of regulatory proteins, further validating the methodology applied. Understanding the molecular action of Zynamite PX® could provide a comprehensive approach to how polyphenols, at appropriate concentrations, can help to counteract oxidative stress. This makes human skeletal muscle an ideal tissue for studying the molecular mechanisms triggered by ROS, potentially leading to the identification of novel therapeutic targets for counteracting ROS.

The general aims of the thesis are firstly, to provide a comprehensive approach of Nrf2 regulation during exercise. Secondly, to discern CaMKII isoforms and determine CaMKII and SLN protein expression following a strength training program and their association with hypertrophy, MHC composition, and mitochondrial protein expression. Thirdly, to assess autophagic signalling in human skeletal muscle in response to high-intensity exercise under both normoxic and hypoxic conditions, including ischaemia application at exhaustion. This includes assessing protein expression levels regulating macroautophagy and chaperone-mediated autophagy. Lastly, to determine the antioxidant signalling mediated by Zynamite PX® in human skeletal muscle under basal conditions and in response to high-intensity exercise and ischaemia-reperfusion. This involves assessing protein expression levels of the antioxidant system (Nrf2-Keap1) and its association with exercise-induced kinases and the key muscle antioxidant enzymes.

Therefore, this thesis has the potential to enhance understanding of the molecular adaptations caused by exercise-induced oxidative stress. Key contributions to skeletal muscle signalling include: 1) understanding the mechanisms regulating Nrf2 activity in human skeletal muscle, 2) providing insights into the characterisation and physiological functions of CaMKII, 3) elucidating the activation of exercise-induced kinases, 4)

determining the regulation of autophagy and antioxidant pathways in human skeletal muscle in response to high-intensity exercise, and 5) exploring the molecular effects of polyphenol intake on the skeletal muscle antioxidant system. Understanding these mechanisms may have clinical implications for treating chronic diseases and ischaemic injuries. Additionally, insights into the molecular mechanisms of Zynamite PX® could help develop new protocols for polyphenol intake to enhance performance and recovery, validate the nutraceutical product and provide a background for further studies and applications.

VI

CONCLUSIONS

6. CONCLUSIONS

Study 1:

- The intricate regulation of the Nrf2 transcription factor in skeletal muscle with exercise reveals a complex interplay between redox balance, cellular stress, and adaptive responses to regular exercise.
- Nrf2 orchestrates a comprehensive antioxidant defence system by activating the expression of various antioxidant enzymes and pathways in response to oxidative or electrophilic stress, as those elicited in skeletal muscle tissue by intense exercise and ischaemia-reperfusion.
- Nrf2 signalling is crucial for mitigating oxidative damage and maintaining redox homeostasis.
- Nrf2 is tightly regulated by several posttranslational modifications that control for nuclear retention, nuclear extrusion, increased protein translation and proteasome degradation. These mechanisms are independent of Keap1 regulation.
- The activation of Nrf2 in human skeletal muscle in response to exercise may involve Keap1 modifications, metabolites, non-coding RNAs, and autophagy.
- Some dietary polyphenols have emerged as potential modulators of Nrf2 activity, although their effects on skeletal muscle Nrf2 signalling remain to be fully elucidated.

Study 2:

- The corresponding molecular weight for the CaMKII isoforms in human skeletal muscle are as follows: δ_D, at 54.2 ± 2.1 kDa; γ/δ, at 59.0 ± 1.2 kDa and 61.6 ± 1.3 kDa; and β_M isoform, at 76.0 ± 1.8 kDa.
- Some commercially available antibodies have high specificity for the δ_D, the most responsive isoform to ROS and intracellular Ca^{2+} transients in human skeletal muscle, while others, despite the commercial claims, failed to show such specificity.
- Exercise induces posttranslational modifications, like phosphorylation and oxidation, allowing the identification of specific bands by multitargeting them with different antibodies after stripping and reprobing.

Study 3:

- CaMKII δ_D increases in response to strength training, and executing repetitions within each set close to failure is associated with elevated basal Thr^{287}-CaMKII δ_D phosphorylation levels, which seem dependent on the total number of repetitions performed.
- The increase in $pThr^{287}$-CaMKII δ_D phosphorylation is positively associated with muscle hypertrophy and negatively with changes in MHC-IIx expression.
- Sarcolipin protein expression increases in response to strength training, but this effect is only observed when the level of fatigue allowed in the set is low.

- An inverse association has been shown between the changes in basal pThr^{287}- CaMKII δ_D phosphorylation and those of sarcolipin, which is compatible with the existence of a negative loop limiting an excessive expression of SLN after fatiguing muscle contractions.
- Changes in basal SLN protein expression do not seem to play a role in muscle hypertrophy in human skeletal muscle, at least under physiological conditions.

Study 4:

- Macroautophagy and CMA are similarly upregulated during exercise to exhaustion in normoxia and severe acute hypoxia.
- ROS produced during incremental exercise and ischaemia may activate macroautophagy and CMA but not FOXOs.
- There is an increased expression of the novel autophagy PHAF1/MYTHO biomarker in human skeletal muscle in response to exercise in normoxia, hypoxia, and post-exercise ischaemia.
- There is a concurrent activation of autophagy and inhibition of protein elongation and synthesis.
- Exercise-induced autophagy activation is not enhanced by severe acute hypoxia and is reversed within 60 s of recovery as long as the circulation is intact.

Study 5:

- In resting muscle, Zynamite PX® intake induces molecular adaptations in part similar to those caused by exercise. The increased Thr^{287}-CaMKIIδ_D phosphorylates and inhibits GSK3β, preventing Nrf2 degradation and promoting the activation of antioxidant enzymes.

- During high-intensity exercise to exhaustion combined with ischaemia-reperfusion, the response of stress kinases is partly reduced after Zynamite PX® supplementation. This can be explained by 1) the enhanced Nrf2 signalling prior to exercise by 48 h Zynamite PX® supplementation and 2) the dose of Zynamite PX® taken 60 min before exercise, which may have contributed to attenuate part of the Nrf2/ROS-induced signalling response.

VII

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7. REFERENCES

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VIII

APPENDIX

STUDY 1

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Invited Review Article

Unlocking peak performance: The role of Nrf2 in enhancing exercise outcomes and training adaptation in humans☆

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ABSTRACT

Since the discovery of the nuclear factor erythroid-derived 2-like 2 (Nrf2) transcription factor thirty years ago, it has been shown that it regulates more than 250 genes involved in a multitude of biological processes, including redox balance, mitochondrial biogenesis, metabolism, detoxification, cytoprotection, inflammation, immunity, autophagy, cell differentiation, and xenobiotic metabolism. In skeletal muscle, Nrf2 signalling is primarily activated in response to perturbation of redox balance by reactive oxygen species or electrophiles. Initial investigations into human skeletal muscle Nrf2 responses to exercise, dating back roughly a decade, have consistently indicated that exercise-induced ROS production stimulates Nrf2 signalling. Notably, recent studies employing Nrf2 knockout mice have revealed impaired skeletal muscle contractile function characterised by reduced force output and increased fatigue susceptibility compared to wild-type counterparts. These deficiencies partially stem from diminished basal mitochondrial respiratory capacity and an impaired capacity to upregulate specific mitochondrial proteins in response to training, findings corroborated by inducible muscle-specific Nrf2 knockout models. In humans, baseline Nrf2 expression in skeletal muscle correlates with maximal oxygen uptake and high-intensity exercise performance. This manuscript delves into the mechanisms underpinning Nrf2 signalling in response to acute exercise in human skeletal muscle, highlighting the involvement of ROS, antioxidants and Keap1/Nrf2 signalling in exercise performance. Furthermore, it explores Nrf2's role in mediating adaptations to chronic exercise and its impact on overall exercise performance. Additionally, the influence of diet and certain supplements on basal Nrf2 expression and its role in modulating acute and chronic exercise responses are briefly addressed.

1. Introduction

Since Moi and co-workers discovered the nuclear factor erythroid-derived 2-like 2 (Nrf2) transcription factor thirty years ago [1], it has been shown that it regulates more than 250 genes [2] involved in a multitude of biological processes, including redox balance, mitochondrial biogenesis, metabolism, detoxification, cytoprotection, inflammation, immunity, autophagy, cell differentiation, and xenobiotic metabolism [3–5]. In skeletal muscle, Nrf2 signalling is primarily activated in response to the perturbation of redox balance by reactive oxygen species (ROS) or electrophiles [6,7].

About twenty years ago, it was reported that Nrf2 is involved in the response to exercise in kidney [8], and more recently, it was shown that Nrf2 intervenes in the exercise response in the myocardium [9], neurons [10], and skeletal muscle [11–14]. The first studies assessing skeletal muscle Nrf2 responses to exercise in humans were published about ten years ago [7,15,16]. These studies conclude that Nrf2 signalling in

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Abbreviations

Akt	Protein kinase B
ALDH2	Aldehyde dehydrogenase 2
AMPK	AMP-activated protein kinase
Akr1c14	Aldo-keto reductase family 1 member C14
AREs	Antioxidant response elements
β-TrCP	β-transducin repeat-containing protein
CK2	Casein kinase 2
CRM1	Chromosomal maintenance 1
eIF2α	Eukaryotic initiation factor 2 alpha
eNOS	Endothelial nitric oxide synthase
GCN2	General control nonderepressible 2
GCL	Glutamate-cysteine ligase
GLO1	Glyoxalase 1
GPx	Glutathione peroxidase
GR	Glutathione reductase
Grx	Glutaredoxin
GSK3β	Glycogen synthase kinase-3 beta
GST	Glutathione S-Transferase
GS	Glutathione synthase
H2O2	Hydrogen peroxide
HAT p300/CBP	Histone acetyltransferase CREB-binding protein
HO	Heme oxygenase
IRESs	Internal ribosomal entry sites
Keap1	Kelch-like ECH-associated protein 1
KIR	Keap1 interacting region
LLM	Lower extremities lean mass
MHC I	Myosin heavy chain type I
MHC IIa	Myosin heavy chain type IIa
MG-H1	Methylglyoxal-derived hydroimidazolone
NES_{TA}	Nuclear export signal in the transactivation domain
NOX2	Nicotinamide adenine dinucleotide phosphate oxidase (NAD(P)H oxidase isoform 2
NQO1	NAD(P)H oxidoreductase 1
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
O2	Superoxide radical
PERK	Protein Kinase R (PKR)-like Endoplasmic Reticulum Kinase
PKA	Protein kinase A
PKC	Protein Kinase C
PKCδ	Protein kinase C delta
PKR	Protein Kinase RNA-activated or Protein Kinase R
PLA2	Phospholipase A2
Prx	Peroxiredoxins
RBS	rRNA binding site
ROS	Reactive oxygen species
RONS	Reactive oxygen and nitrogen species
RNS	Reactive nitrogen species
SOD	Superoxide dismutase
Srx1	Sulfiredoxin
SnoRNAs	Small nucleolar RNAs
SR/ER	Sarcoplasmic/endoplasmic reticulum
Trx	Thioredoxin
TrxR	Thioredoxin reductase
VO2	Oxygen uptake
VO2max	Maximal oxygen uptake
XO	Xanthine oxidase

skeletal muscle is stimulated by the production of reactive oxygen species during exercise [7]. A few years ago, it was shown that skeletal muscle contractile function is impaired in Nrf2 knockout mice ($Nrf2^{-/-}$) [11]. $Nrf2^{-/-}$ mice have 35 % less force, being more fatigable than their wild-type controls [11], partly due to a reduced basal mitochondrial respiratory capacity and impaired ability to increase specific mitochondrial proteins in response to training [11]. These effects have been confirmed in inducible muscle-specific $Nrf2^{-/-}$ mice [6]. In humans, Nrf2 basal expression in skeletal muscle has been associated with maximal oxygen uptake (VO_2max) [17] and high-intensity exercise performance [18,19].

Since ROS are the main activators of Nrf2 signalling during exercise, this manuscript briefly reviews the main mechanisms of ROS production and counteraction in the exercising skeletal muscle. Then, the main regulatory processes leading to Nrf2 signalling in response to acute and chronic exercise and the influence of Nrf2 on exercise performance are addressed. Finally, the impact of diet and some supplements on the basal expression of Nrf2 and Nrf2 signalling to acute and chronic exercise are succinctly analysed.

1.1. Skeletal muscle ROS production and antioxidant mechanisms

Reactive oxygen species (ROS) are chemical compounds (or atoms) containing oxygen, which may be free radicals or non-radical highly reactive oxygen derivatives, like hydrogen peroxide. Reactive nitrogen species (RNS) are nitrogen radicals and non-radical nitrogen reactive molecules [20]. The term reactive oxygen and nitrogen species (RONS) is often used to refer indistinctively to ROS and RNS.

Since 1954, when ROS were reported for the first time in biological materials [21], a predominant concept prevailed considering free radicals and ROS as “dangerous agents” that should be avoided and counteracted [22]. It was not until the 1970s that some experimental evidence emerged indicating that ROS are necessary for the normal functioning of the cells. In that period, it was observed that rats in 100 % O_2 (1 atm) died within 3 days. However, if rats were exposed to 80 % oxygen for 5 days, they survived and adapted to the high O_2 environment, so if they were exposed to 100 % O_2, they could survive for a very long time [23]. Thus, it became clear that cells and living organisms could adapt to sublethal oxidative stress, increasing their tolerance to subsequent exposures to ROS. In addition, it was discovered that free radicals or their derivatives are essential for regulating vascular tone, O_2 sensing, and for several signal transduction mechanisms and oxidative stress adaptive responses necessary for maintaining redox homeostasis and health [24,25]. RONS are also required for normal protein folding and disulfide bond formation that regulate the structure and function of many cellular proteins [26].

At rest, ROS are primarily generated in the mitochondria [25]. However, during exercise, RONS are produced mostly by nicotinamide adenine dinucleotide phosphate oxidase (NAD(P)H oxidase, particularly in the isoform 2 (NOX2), xanthine oxidase (XO), phospholipase A2 (PLA2), lipoxygenases and cyclooxygenases, endothelial nitric oxide synthase (eNOS), sarcoplasmic/endoplasmic reticulum (SR/ER) stress, and myoglobin [20,25,27,28]. In the presence of excess superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), myoglobin can catalyse the formation of highly reactive hydroxyl radicals [27]. In contrast to resting conditions, during aerobic exercise mitochondrial ROS production amounts to 1-2 orders of magnitude lower than previously thought (see Calbet et al., for review [29]). Moreover, the main contribution of each ROS source varies with the exercise intensity, duration, substrate oxidation, training state, oxygenation, level of fatigue, and age [20,29–31]. However, the fractional or relative contribution of each of these sources of ROS remains to be elucidated regarding actual ROS generation during exercise.

Redox homeostasis is maintained by antioxidants and modulation of ROS production. Antioxidants can be divided into two categories: Non-enzymatic and enzymatic antioxidants (non-enzymatic antioxidants are beyond the scope of this manuscript; the interested reader is referred to

excellent published reviews on the topic [20,32,33]). The main enzymatic antioxidants in skeletal muscle are superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx, isoforms GPx-1, GPx-3 and GPx-4), and glutathione reductase (GR) [25]. Superoxide dismutase is the most abundant enzymatic antioxidant in skeletal muscle, which expresses the three isoforms of the enzyme: SOD1, SOD2 and SOD3 (extracellular SOD). SOD1, the cytosolic isoform (although there is some SOD1 in the mitochondrial intermembrane space), which requires Cu and Zn as cofactors, accounts for 65–85 % of the whole SOD activity. SOD2, the mitochondrial isoform, requires manganese as cofactor [20]. SOD2 accounts for 15–35 % of skeletal muscle SOD activity [20]. SOD3 localises in the extracellular matrix and extracellular fluids, including plasma [34]. Superoxide dismutases catalyse the dismutation of $O_2^{\cdot-}$ into the less powerful oxidant H_2O_2 (Fig. 1).

Hydrogen peroxide has a longer life than $O_2^{\cdot-}$, and unlike $O_2^{\cdot-}$, H_2O_2 can cross lipid membranes by diffusion or through channels such as aquaporins [35]. Theoretically, excessive H_2O_2 production can lead to the formation of the highly reactive and damaging hydroxyl radical in the presence of free Fe^{2+} (Fenton reaction) or heme groups from myoglobin [36], which may be facilitated by acidosis. Normally, excessive H_2O_2 production is prevented by glutathione peroxidases (GPxs, particularly GPx-1), catalase, and peroxiredoxins. Catalase and GPx-1 are ubiquitous in the cells [37]. GPx-1, unlike catalase, can also reduce lipid hydroperoxides, peroxynitrites [38], soluble hydroperoxides, and phospholipid-monoacylglycerol hydroperoxides [37], but not tri- or diacylglycerol hydroperoxides [39], which are reduced by GPx-4, a ubiquitous isoform of GPx, generally associated with membranes [37]. The enzymatic action of GPx-1 consumes GSH to generate GSSG, preventing the oxidation of reactive protein thiols. The pentose-phosphate pathway provides the reducing equivalents ($NADP^+$/NADPH redox couple) necessary to restore the normal GSH/GSSG ratio [37]. GSH plays a central role in maintaining redox homeostasis, and the GSH to oxidised glutathione ratio (GSH/GSSG) provides an estimate of cellular redox buffering capacity [40] (Fig. 1).

Other enzymatic antioxidants, such as thioredoxin (Trx), thioredoxin reductase (TrxR), glutaredoxin (Grx), and sulfiredoxin (Srx1), are also present in skeletal muscle; however, their relative contribution to redox homeostasis in exercising skeletal muscle is uncertain [25,32].

During low-intensity exercise, mitochondria are predominantly in state 3 respiration (ADP-stimulated) [41] and produce less ROS relative to the VO_2 than at rest. Most of the $O_2^{\cdot-}$ produced in mitochondria is swiftly converted to H_2O_2 by SOD2 and SOD1, located close to the production site. Hydrogen peroxide can also be eliminated rapidly by GPx-1, which is abundant in the mitochondria and cytosol, and by the ubiquitous catalase, which is more abundant in the peroxisomes. During high-intensity exercise, most superoxide originates from NOX2 and, to a lesser extent, from XO [20,30,42]. NOX and XO-produced superoxide is rapidly converted into H_2O_2 by SODs close to their site of production (Fig. 1).

1.2. *Glutathione and reduced-to-oxidised glutathione ratio (GSH/GSSG) in human skeletal muscle and Nrf2*

The tripeptide glutathione (γ-glutamyl-l-cysteinylglycine, GSH) is the most abundant non-enzymatic antioxidant in cells, reaching 1–10 mM [43] (Fig. 2). Under exposure to oxidants, the sulfhydryl group of the cysteine residue of one glutathione molecule reacts with the cysteine residue of another glutathione molecule to form glutathione disulphide (GSSG) in a process that transfers two electrons to an acceptor molecule which becomes reduced while glutathione is oxidised [44]. The GSH/GSSG molar ratio is considered to reflect the redox balance, such that a reduction of GSH/GSSG indicates oxidative stress and a low GSH/GSSG ratio has been associated with an increased risk of death in patients with coronary artery disease [45]. The GSH/GSSG ratio is tightly controlled in the cells, such that under oxidative stress conditions, GSSG is reduced back to GSH by GR while the synthesis of GSH and GR is stimulated by Nrf2 [46].

Thus, the GSH/GSSG ratio could be envisaged as a regulator of Nrf2

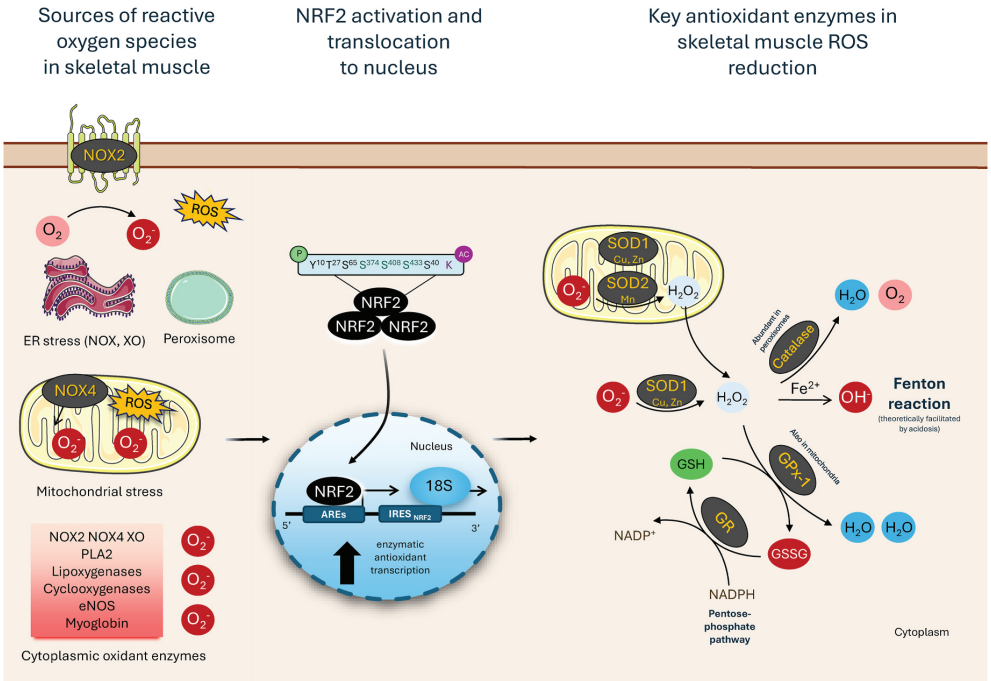

Fig. 1. ROS sources, Nrf2 activation and translocation to the nucleus, and key antioxidant enzymes in skeletal muscle.

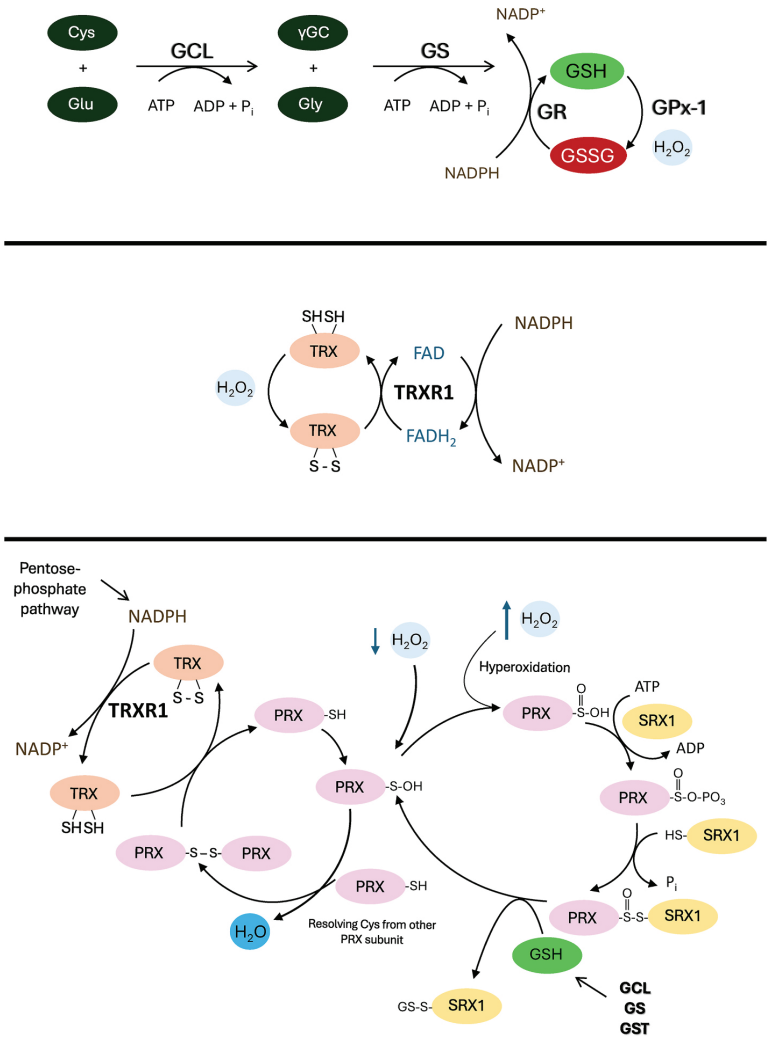

Fig. 2. Metabolism of glutathione and H_2O_2 (adapted from Tebay et al. [4]).

expression in human skeletal muscle since a high ratio will prevent Nrf2 signalling activation due to the increased antioxidant potential of the muscle. However, the GSH/GSSG measurement in skeletal muscle has high variability, likely due to the intrinsic difficulty of preventing GSH oxidation during sample manipulation [44], limiting its value to detect subtle changes in ROS production. The GSH/GSSG ratio was measured in the *vastus lateralis* of thirteen physically active men before and after a 6-week interval training program on the cycle ergometer [47]. The GSH/GSSG ratio for muscle homogenates was close to 3 to 4 and remained unchanged with training. The authors reported high variability in the assessment, which was performed with a fluorometric kit [47]. Also, using a colorimetric assay, a mean GSH/GSSG ratio close to 5 was reported at rest in 6 males and 1 female (6M/1F) (physically active), which remained unchanged by an N-acetylcysteine infusion at rest [48]. From the data reported by Morrison et al. [49] in eleven males, the calculated GSH/GSSG ratio of resting *vastus lateralis*, assessed spectrophotometrically, laid close to 55, a value 10-fold higher than observed in previous studies [49]. Neither GSSG nor the ratio GSH/GSSG were acutely changed by exercise nor four weeks of high-intensity interval training with or without four weeks of vitamin C (1 g/d) + E (400 IU/d) supplementation [48,49].

Additional studies reported GSH/GSSG values in muscle biopsies. For example, the GSH/GSSG ratio was measured at rest in the *vastus lateralis* of eight physically active males before (mean value close to 10, range 6–18) and after supplementation with beetroot juice (mean value close to 13, range: 6–23; differences were not statistically significant) in homogenates measured by HPLC [50]. In ten patients (5M/5F), 70 years old, who underwent surgery, the GSH/GSSG ratio measured with HPLC was 29 ± 13 (mean ± SD), also indicating high interindividual variability [51]. In eight physically active young subjects (7M/1F), the reported GSH/GSSG ratio measured at rest by HPLC was 19 in the *vastus lateralis* [52]. No significant changes in GSH nor GSSG were observed after 2 h (120 min at 60 % of VO_2max, which reduced muscle glycogen content by 88 %) [52]. In eight endurance-trained males, no changes in the GSH/GSSG ratio measured with HPLC were observed after 45 min pedalling at 70 % of VO_2max followed by exercise at 90 % of VO_2max

until exhaustion [53]. No change in GSSG (nor in ubiquinone, alpha-tocopherol, or malondialdehyde) was observed in the working muscle in seven subjects after repeated isometric contractions (static knee extension at 30 % MVC, 10 s on and 10 s off, repeated for 80 min or until fatigue) [54]. No significant changes in the resting GSH/GSSG ratio were observed in the *vastus lateralis* of 7 males who performed interval cycling training for six weeks, training one leg without blood flow restriction and the other with blood flow restriction [47]. In the latter study, the GSH/GSSG ratio was unchanged by N-acetylcysteine infusion at rest before training in the control leg and the leg training with blood flow restriction. After training, N-acetylcysteine infusion at rest increased the GSH/GSSG ratio in the control leg but not in the leg training with blood flow restriction [47]. Nevertheless, this significant effect was not supported by a significant interaction in the statistical analysis [47].

Overall, these studies show high variability in the reported GSH/GSSG ratios for human skeletal muscle, which may preclude detecting changes in redox balance with interventions known to increase ROS production or antioxidant capacity. Although the GSH/GSSG ratio conceptually reflects the redox balance in skeletal muscle, the reported values depart greatly from those expected, which should lay close to 1000 [44]. Thus, GSH/GSSG ratios reported in the exercise physiology literature should be interpreted cautiously [44]. Nonetheless, this GSH/GSSG ratio limitation for detecting increased ROS production can be circumvented by analysing the redox-sensitive signalling pathways [55–57].

1.3. *Nrf2 is the main transcription factor regulating cellular redox balance*

Nrf2 is a transcription factor that regulates the gene expression of the main enzymes involved in redox balance regulation, namely SOD1, SOD2, catalase, the eight characterised GPx isoforms, the two thioredoxin isoforms (Trx1 and Trx2), thioredoxin reductase (TrxR1 and TrxR2), at least three of the peroxiredoxins (Prx1, Prx2 and Prx6), sulfiredoxin (Srx1), GR, the different classes of Glutathione S-Transferase (GST), glutathione synthase (GS), the two isoforms of heme oxygenase expressed in skeletal muscle (HO-1 and HO-2), NAD(P)H:quinone oxidoreductase 1 (NQO1), and the two subunits of glutamate-cysteine ligase (GCL), which is the rate-limiting enzyme in the synthesis of glutathione [2,4,11,13,58–69] (Fig. 2).

The Nrf2 protein contains six functional domains, designated Neh1

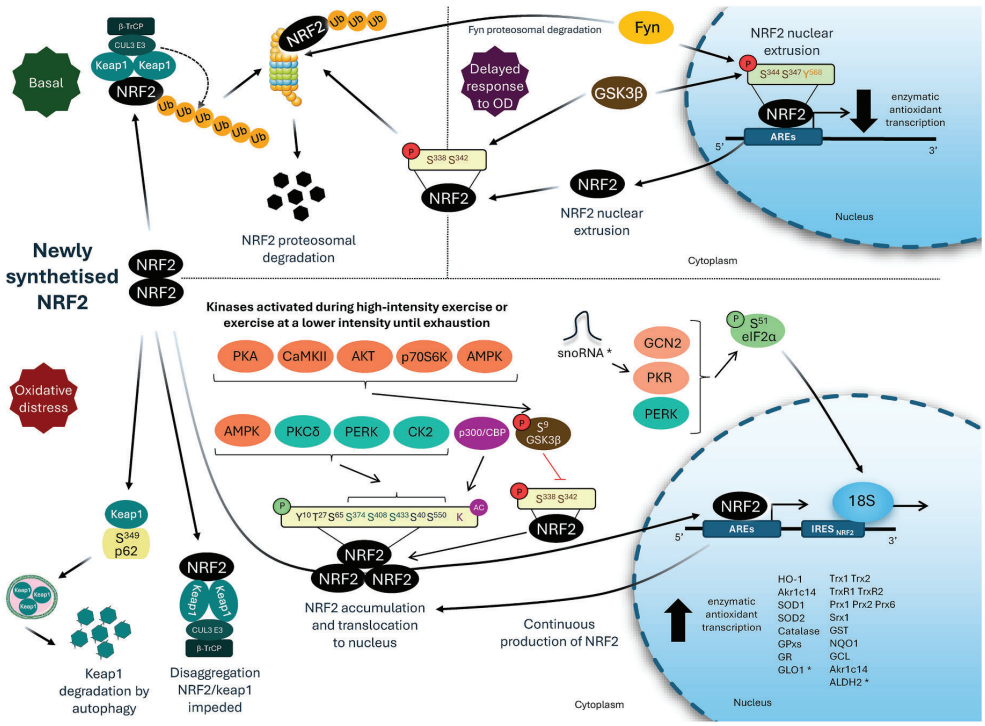

Fig. 3. Regulatory mechanisms of Nrf2. Under basal conditions, Nrf2 is ubiquitinated and degraded by the proteasome. The delayed response to oxidative stress is triggered by Fyn kinase, which promotes the nuclear export and subsequent ubiquitination and degradation of Nrf2 by phosphorylating Nrf2 at Tyr^{568}. GSK3β can directly phosphorylate Nrf2 Ser^{344} and Ser^{347} for nuclear extrusion. GSK3β phosphorylates Fyn kinase to elicit its exportation from the nucleus, and Nrf2 in Ser^{338} and Ser^{342} for subsequent ubiquitination and proteasomal degradation. In the presence of oxidative or electrophilic stress, several Keap1 cysteine residues undergo covalent modifications impeding the ubiquitination and proteasomal degradation of Nrf2. Since oxidative stress is not able to dissociate Nrf2 from Keap1, the newly translated Nrf2 accumulates in the cytoplasm. p62/SQSTM1 directly binds to Keap1 through a specific sequence, the Keap1 interacting region (KIR) leading to the autophagic degradation of Keap1, promoting Nrf2 signalling. The accumulated Nrf2 is activated by phosphorylation which may be elicited by AMPK, PKCδ, PERK, CK2. Nrf2 acetylation by HAT p300/CBP increases Nrf2 transcription rate and promotes nuclear localisation. Moreover, GSK3β is inhibited by Ser^{9} phosphorylation, which may be elicited by PKA, CaMKII, AKT, p70S6K and AMPK leading to Nrf2 accumulation. The accumulated Nrf2 translocates to nucleus where it binds to antioxidant response elements (AREs) in the promoter regions of most antioxidant enzymes. H_2O_2 and sulforaphane may trigger Nrf2 mRNA cap-independent translation via the $IRES_{Nrf2}$, a process that requires the phosphorylation of Eukaryotic Initiation Factor 2 alpha (eIF2α) in serine 51. Ser^{51}-eIF2α phosphorylation may be elicited by several stress-induced kinases in skeletal muscle, including PERK, GCN2, and PKR. PKR may be activated in response to metabolic stress by small nucleolar RNAs (snoRNAs). * Little is known during exercise conditions.

through Neh6 [4]. The Neh2 domain, located at the N-terminus, facilitates binding with Kelch-like ECH-associated protein 1 (Keap1) dimers [4]. Within Neh2, two conserved motifs, DLG and ETGE, which are separated by a sequence containing seven lysine residues, may be subject to ubiquitination [70]. The ETGE motif of Nrf2 can attach to the DC region of one monomeric subunit of the Keap1 dimer, while the DLG motif binds to the DC region of the other subunit of the same Keap1 dimer [71]. Keap1 binds with 100-fold higher affinity to the ETGE than DLG Neh2 motifs [72]. Thus, the formation of the Keap1-Nrf2 complex occurs in two phases: initially, the ETGE motif mediates an "open" Keap1-Nrf2 complex, followed by the DLG motif facilitating an intra-complex association to form a "closed" Keap1-Nrf2 complex [70,72]. It has been proposed that these two binding sites act as a hinge and latch mechanism, where the ETGE motif plays the role of the hinge [71].

When the Keap1 dimer is bound to both Neh2 motifs (i.e., Keap1-Nrf2 complex in the close state), Keap1 acts as an adaptor protein facilitating the action of a ubiquitin E3 ligase complex (CUL-E3), which ubiquitinates Nrf2 [70]. The latter causes the dissociation of Nrf2 from Keap1 and subsequent proteasomal degradation of the ubiquitinated Nrf2 [73,74] (Fig. 3). Keap1 contains multiple cysteines, whose thiol groups can be modified by ROS and electrophiles, which rends inoperative its adaptor function for CUL E3 and impedes the ubiquitination of Nrf2 [73,75,76]. Upon exposure to oxidative or electrophilic agents, the interaction between Keap1 and Nrf2 is consolidated, impeding the disaggregation of the Keap1-Nrf2 complex.

Consequently, under basal conditions, there is little Nrf2 in the cells because the proteasome continuously degrades it, with the estimated Nrf2 half-life being 6–20 min [72]. Moreover, Keap1 is in excess relative to Nrf2, and only a very small fraction of Nrf2 total abundance remains free [72]. Upon oxidative stress, the first newly synthetised Nrf2 is sequestered by the remaining free Keap1, and only once the reservoir of free Keap1 has been exhausted will Nrf2 translocate to the nucleus [77,78], where it binds to antioxidant response elements (AREs) in the promoter regions of most antioxidant enzymes [4,11,13,58–61,79]. Thus, the Nrf2-induced activation of the antioxidant gene program depends on the synthesis rate of Nrf2, the basal concentrations of Keap1 and the redox balance. However, under unstressed resting conditions, mRNA Nrf2 translation is inhibited [80], while ROS release this translation inhibition [80]. When the Keap1 reservoir is small, the antioxidant gene program can be activated faster in response to oxidative or reductive stress, as it occurs during exercise or ischaemia [55].

Nrf2 contains specific peptide motifs recognised by β-transducin repeat-containing protein (β-TrCP), an adaptor protein for E3 ubiquitin ligase. Once bound, β-TrCP facilitates the transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme to Nrf2, thereby tagging it for degradation. This process requires Nrf2 phosphorylation in Ser^{338} and Ser^{342} by glycogen synthase kinase-3β (GSK3β) [81,82]. GSK3β is a constitutively active enzyme regulated by inhibition through phosphorylation at Ser^{9}.

Nrf2 signalling may be amplified by increased nuclear retention (which may be facilitated by several posttranslational modifications) [2], enhanced ribosomal translation, and increased transcription rate of Nrf2 mRNA [78]. Interestingly, enhanced translation does not require increased mRNA or changes in the rate of Nrf2 degradation [83]. Protein translation is mainly regulated via cap-dependent ribosome scanning under normal physiological conditions. In contrast, under cellular stress, the cap-independent internal ribosome entry mediated by internal ribosomal entry sites (IRESs) is recruited [84]. The human Nrf2 mRNA hosts an IRES within the 5′ untranslated region, which contains an 18S rRNA binding site (RBS) necessary for internal initiation [80]. Hydrogen peroxide and sulforaphane may trigger Nrf2 mRNA cap-independent translation via the $IRES_{Nrf2}$, a process that requires the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α) in Ser^{51}. Ser^{51}-eIF2α phosphorylation may be elicited by several stress-induced kinases in skeletal muscle, including PERK (Protein kinase R(PKR)-like endoplasmic reticulum kinase, activated by endoplasmic reticulum stress) [85], GCN2 (General control nonderepressible 2, activated by amino acid deprivation) [86], and PKR (Protein kinase RNA-activated or protein kinase R) [87]. Besides, PKR may be activated in response to metabolic stress by small nucleolar RNAs (snoRNAs) [88] (Fig. 3).

Nrf2 protein abundance may be enhanced by a positive feedback loop in which Nrf2 acts as a transcriptional activator of its own gene promoter [89]. This is possible because the Nrf2 gene promoter contains two ARE-like sites to which the Nrf2 protein can bind [89]. The Nrf2 binding to the target genes (ARE-containing promoters) is facilitated by the histone acetyltransferase (HAT) p300/CBP (CREB-binding protein), which acetylates Nrf2 [90]. Acetylation increases Nrf2 transcription rate and promotes nuclear localisation, and vice versa; deacetylation reduces the transcription rate and promotes relocalisation to the cytoplasmic compartment [91].

Apart from acetylation, nuclear retention of Nrf2 is also regulated through phosphorylation by mechanisms that have not been fully elucidated due to the high complexity of Nrf2 that hold numerous potential phosphorylation sites (Ser^{65}, Thr^{27}, and Tyr^{10}) [92]. Some evidence supports Ser^{40}-Nrf2 phosphorylation as a mechanism promoting nuclear retention. Several kinases may elicit Ser^{40} phosphorylation, including PERK [93], casein kinase 2 (CK2) [94], and PKC, particularly the delta isoform (PKCδ) [95]. AMPK can also phosphorylate several serine residues of Nrf2 (Ser^{374}, Ser^{408} and Ser^{433} in human Nrf2), facilitating the transactivation of selected target genes [HO-1 and aldo-keto reductase family 1, member C14 or (Akr1c14)], but without affecting the half-life of Nrf2 [96] (Fig. 3).

In contrast, Fyn kinase promotes the nuclear export and subsequent ubiquitination and degradation of Nrf2 by phosphorylating Nrf2 at Tyr^{568} [97]. This action of Fyn kinase should be preceded by the accumulation of Fyn kinase in the nucleus, a process that requires several hours and constitutes a part of the delayed response to oxidative stress [97,98]. In turn, GSK3β phosphorylates Fyn kinase to elicit its exportation from the nucleus and subsequent ubiquitination and proteasomal degradation [97,99]. AMPK facilitates Nrf2 nuclear retention by phosphorylating Nrf2 at the Ser^{550} residue [100]. GSK3β can directly phosphorylate Nrf2 Ser^{344} and Ser^{347} [81,101], facilitating nuclear extrusion [102] and the formation of a phosphodegron that is recognised by the ubiquitin ligase adapter β-TrCP, tagging Nrf2 for proteasomal degradation [92]. Thus, the inhibition of GSK3β through its Ser^{9} phosphorylation leads to increased Nrf2 signalling by redundant mechanisms (Fig. 3).

The transactivation (TA) domain of Nrf2 harbours a chromosomal maintenance 1 (CRM1 or exportin 1)-dependent nuclear export signal (NES_{TA}), which contains a redox-sensitive cysteine residue at position 138 [103]. This cysteine residue is modified upon electrophilic or oxidative stress, inhibiting nuclear exportation [103]. This process is facilitated by S-nitrosylation of CRM1 [104].

2. Regulation of Nrf2 during exercise

In addition to H_2O_2, several metabolites produced during exercise can react with Keap1 cysteines to trigger Nrf2 signalling [105–108] (Fig. 3). High-intensity exercise requires elevated glycolytic rates, causing accumulation of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate [109–112], which, in part, can be converted into methylglyoxal, a highly reactive dicarbonyl electrophile [113,114]. Methylglyoxal can bind to arginine residues, generating hydroimidazolone adducts (MG-H1), causing covalent modifications of protein structure [115]. Methylglyoxal can target Keap1, eliciting cross-linking and dimerisation of Keap1 molecules, facilitating Nrf2 signalling [107]. Besides, glyceraldehyde 3-phosphate can stimulate S-lactoylation of several cysteines in Keap1 [108]. These covalent modifications of Keap1 promote Nrf2 signalling [108]. Furthermore, Nrf2 upregulates the expression of the main enzymes capable of eliminating methylglyoxal: glyoxalase-1 (GLO1) and aldehyde dehydrogenase 2 (ALDH2), which convert methylglyoxal into lactate and pyruvate,

respectively [116,117]. Interestingly, animal studies have demonstrated that both enzymes (GLO1 and ALDH2) are upregulated with exercise training [118]. In situations of insufficient expression of GLO1, methylglyoxal-modified proteins accumulate, leading to a condition known as a "dicarbonyl stress" [119], a form of glycative stress observed in insulin resistance, obesity, diabetes, and ageing [120,121]. Methylglyoxal accumulation may damage mitochondria and increase ROS production [120]. Nevertheless, it remains unknown how the human skeletal muscle protein expression of GLO1 and ALDH2 is regulated by exercise [118].

Low Keap1 levels may enhance the basal expression of antioxidant and pentose phosphate pathway enzymes through Nrf2 signalling [108]. In fact, the gene expression of several of the pentose phosphate pathway enzymes is regulated by Nrf2 [2,122]. Consequently, trained subjects are expected to display lower Keap1 levels at rest, increased resting Nrf2/Keap1 ratios, and reduced activation of Nrf2 in response to exercise due to: 1) more efficient quenching of the ROS produced during exercise due to the upregulation of antioxidants enzymes with training; 2) lower ROS production in the trained state; and 3) reduced production of metabolites capable of interacting with Keap1 to activate Nrf2 signalling.

Exercise with high energy deficit and high-intensity exercise may induce autophagy activation [55,123,124,131]. Prolonged exercise with severe energy restriction elicits p62/SQSTM1 accumulation [124]. p62/SQSTM1 directly binds to Keap1 through a specific sequence, the Keap1 interacting region (KIR), leading to the autophagic degradation of Keap1, promoting Nrf2 signalling [125]. The latter is facilitated by Ser^{351}-p62 phosphorylation [126], which is observed during high-intensity exercise in human skeletal muscle [55]. Besides, p62 is a competitive inhibitor of Nrf2 binding to Keap1 [77]. Thus, sustained oxidative stress during prolonged submaximal exercise or repeated high-intensity exercise may cause increased degradation of Keap1 and reduce Keap1 half-life in skeletal muscle [127] (Fig. 3).

2.1. Regulation of Nrf2 signalling during exercise may occur independently of Keap1

High-intensity or low-intensity exercise, the latter when performed to near exhaustion, may activate AMPK, Akt, PKA, and PKC [128–130], all of which can lead to Ser^{9}-GSK3β phosphorylation. A drop in Ser^{9}-GSK3β phosphorylation has been reported during prolonged exercise with severe energy deficit [145] (Fig. 3). The exercise-induced inhibition of GSK3β blunts Nrf2 Ser^{338} and Ser^{342} phosphorylations, hindering Nrf2 ubiquitination by β-TrCP, and thereby promoting an increased abundance of Nrf2 with exercise.

Nrf2 signalling during exercise may be facilitated by increased nuclear retention [2], ribosomal translation, and transcription rate of Nrf2 mRNA [78]. The exercise-induced inhibition of GSK3β may facilitate the nuclear retention of Nrf2 by impeding Nrf2 phosphorylation at Ser^{344} and Ser^{347} [81,101], which should result in reduced nuclear exportation of Nrf2 [102]. Likewise, AMPK activation with high-intensity [131] or prolonged exercise [124] may facilitate Nrf2 nuclear retention by phosphorylating Nrf2 at Ser^{550} [100]. Nuclear retention during exercise may also be promoted by Ser^{40}-Nrf2 phosphorylation, which may be elicited by several kinases activated during exercise, such as PERK [85] and PKC [95,132,133]. However, the impact that exercise might have on CRM1 regulation of Nrf2 nuclear retention remains unknown.

Exercise may stimulate Nrf2 cap-independent translation via the $IRES_{Nrf2}$ through Ser^{51}-eIF2α phosphorylation by PERK and PKR, which may be activated by exercise-induced endoplasmic reticulum stress [85,134], commonly observed during high-intensity or prolonged submaximal exercise [134]. In theory, PKR may be activated during exercise by small nucleolar RNAs (snoRNAs) [88], but little is known about snoRNAs response to exercise or PKR activation during exercise [135] (Fig. 3). The Nrf2 produced during exercise may also stimulate its own transcription [89], contributing to explain why Nrf2 mRNA is increased in muscle biopsies taken immediately after exercise [136].

Besides, exercise may facilitate Nrf2 acetylation by p300/CBP HAT, which has been shown to promote Nrf2 nuclear localisation and transcription [91]. Exercise-induced Nrf2 could enhance Nrf2 binding to the ARE of its own promoter to increase Nrf2 transcription. Nrf2 acetylation with exercise could also promote the expression of other Nrf2-regulated genes [89,90]. Nevertheless, whether Nrf2 acetylation occurs during exercise in skeletal muscle remains unknown [137,138].

The main kinases eliciting Ser^{9}-GSK3β phosphorylation, i.e., Akt [139], PKA [140], p70S6K [141], and CaMKII [142], are activated during exercise, particularly during high-intensity exercise or exercise to exhaustion [55,57,128,143–145]. Thus, either of these exercise-activated kinases could stimulate Nrf2 signalling via the inhibition of GSK3β through its Ser^{9} phosphorylation (Fig. 3).

2.2. Impact of polyphenols and sulforaphane in Nrf2 regulation in human skeletal muscle

Several dietary polyphenols, such as resveratrol, piceatannol, curcumin, mangiferin, quercetin, isoquercetin, naringenin, hesperidin, kaempferol, hydroxytyrosol, apigenin, anthocyanins, catechins, and pterostilbene, as well as the isothiocyanate sulforaphane, have been shown to activate Nrf2 signalling in cells and animal models [146–157] (Fig. 4). It is likely that some marketed juice products rich in polyphenols [158,159] as well as dietary polyphenols [160] can also activate skeletal muscle Nrf2 signalling; however experimental evidence in humans is lacking.

Polyphenols may reduce Keap1 availability, enhance Nrf2 nuclear translocation, induce Nrf2 transcription and translation, and reduce proteasomal Nrf2 degradation. For example, sulforaphane may facilitate Ser^{9}-GSK3β phosphorylation [161] and IRES activation of ribosomal translation of Nrf2 mRNA [80]. Apigenin, resveratrol and the resveratrol metabolite piceatannol may release the inhibitory mechanisms by interfering with the processes repressing Nrf2 mRNA translation, which are operative under unstressed conditions [157,162].

However, the scientific evidence linking oral intake of polyphenols to skeletal muscle Nrf2 signalling is scarce [57,163–167]. In rodents, sulforaphane intraperitoneal injection (four times for 3 days, i.e., 72, 48, 24, and 3 h before exhaustive treadmill tests) was associated with increased Nrf2 gene expression [165] and reduced oxidative stress after high-intensity exercise [168]. In humans, consumption of glucosinolate-rich broccoli sprouts twice daily during 7 days of high-intensity interval training elicited increased Nrf2 protein expression in skeletal muscle [167], which was associated with reduced lactate accumulation in blood during submaximal exercise an enhanced maximal power output during incremental exercise to exhaustion. The increase in maximal power output was not associated with increased VO_2max nor changes in mitochondrial respiration, which was unaffected by the intervention [167] (Fig. 4)

Supplementation three times a day for two days with Zynamite PX® (a mango leaf extract rich in mangiferin combined with quercetin) was associated with increased basal protein amount of Thr^{287}-$CaMKII\delta_D$ and Ser^{9}-GSK3β and a non-significant increase of total Nrf2 (1.7-fold, $p = 0.099$) and Ser^{40}-Nrf2 (1.2-fold, $p = 0.061$) in skeletal muscle of 8 male young physically active volunteers [57]. After high-intensity exercise with intercalated episodes of ischaemia-reperfusion, Zynamite PX® supplementation blunted the expected redox-signalling response in skeletal muscle observed in the control group [57] (Fig. 4). In other words, the mangiferin-quercetin combination elicited muscle signalling changes in resting skeletal muscle resembling those described for exercise training and partly abrogated the stress kinases response to exercise, likely due to an increased muscle antioxidant capacity before the exercise. This type of response is also observed in trained muscles. Despite these encouraging findings with some polyphenols [169], it has been shown that antioxidants may also blunt some of the adaptive responses to exercise when ingested chronically, although these effects have been

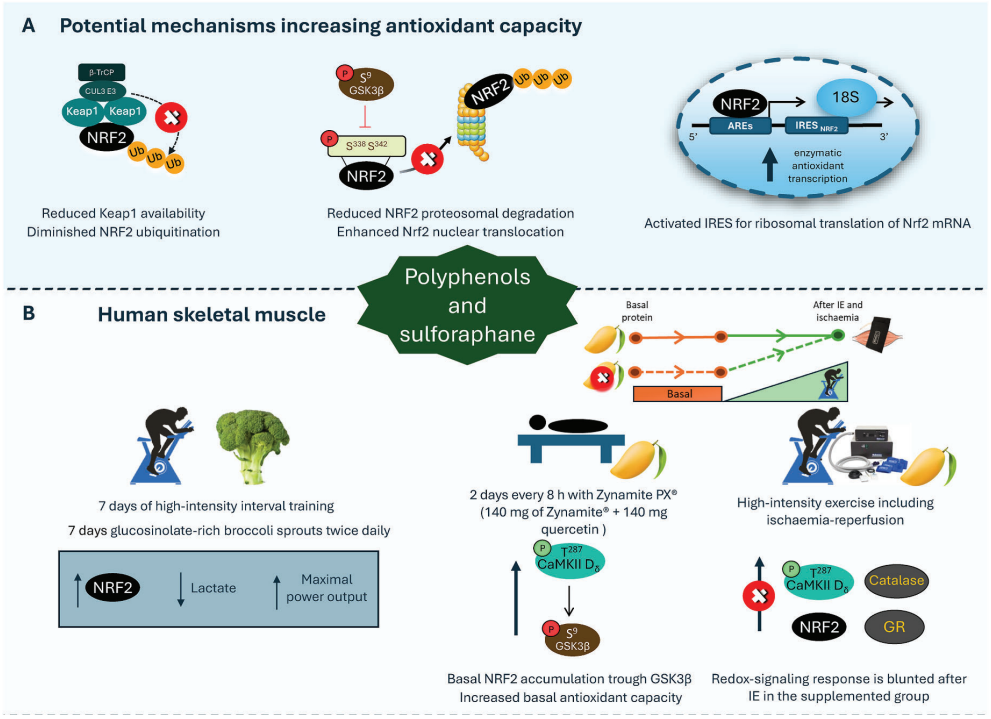

Fig. 4. Potential mechanisms through which polyphenols and sulforaphane may increase antioxidant capacity in human skeletal muscle.

reported mainly with the intake of high doses of vitamin C or the combination of vitamins C and E [49,170,171].

3. Nrf2 and exercise performance

Skeletal muscle basal levels of Nrf2, Keap1 and antioxidant enzymes have been associated with exercise performance. Some studies have reported that regular exercise training may increase enzymatic antioxidant capacity in skeletal muscle [32,172]. Cross-sectional studies have reported increased SOD2 expression in endurance-trained athletes [173,174]. Most endurance training research in humans has reported increases in SOD2 in skeletal muscle [49,175–177]. However, other studies have reported no significant changes in SOD, catalase, GPx or glutathione status in skeletal muscle with endurance [178–180] or sprint interval training [181] despite improvements in VO_2max. The effects of exercise training on Trx, TrxR, Grx, and Prx remain uncertain [32].

SOD2 seems critical for exercise performance since heterozygous SOD2-knockout mice, which have 30–80 % lower expression of SOD2, have reduced exercise capacity [182]. This concurs with the pioneer observation by Jenkins et al. [183], who found a positive correlation between VO_2max in mL.kg^{-1}.min^{-1} and the skeletal muscle enzymatic activities of SOD and catalase in 12 males aged between 17 and 19 years [183]. The latter agrees with a recent study carried out in our laboratory. Specifically, SOD1, SOD2, catalase and GR and their regulatory factors Nrf2 and Keap1 basal protein expression levels were determined in skeletal muscle biopsies obtained from 189 volunteers (120 males and 69 females) [17], in which VO_2max and body composition (dual energy x-ray absorptiometry) was also assessed. No significant sex differences were observed between males and females in Ser^{40}-Nrf2, Total Nrf2, Ser^{40}-Nrf2/Total Nrf2 ratio, and Nrf2/Keap1 ratio, catalase, GR and SOD1, after accounting for VO_2max expressed as mL.kg LLM^{-1}.min^{-1} (where LLM indicates lower extremities lean mass) (Fig. 5). In this study, SOD2 was the antioxidant enzyme more closely associated with VO_2max [17] (Fig. 6). A positive linear association was also observed between the myosin heavy chain (MHC) percentage of MHC I + MHC IIa and the protein expression levels of GR, catalase, and SOD2 [17]. The latter concurs with several studies in rodents reporting higher antioxidant expression in aerobic muscle fibres [32].

These findings indicate enhanced capacity to counteract ROS generated by mitochondrial respiration is likely a critical factor for a higher VO_2max, hence endurance performance. Not surprisingly, animals with exceptional VO_2max possess remarkably increased levels of SOD in their skeletal muscles [184]. What remains to be determined is whether increasing antioxidant enzymes in skeletal muscle without concomitant training could enhance VO_2max or endurance performance in humans. Animal studies indicate that unchecked ROS production in skeletal muscle increases fatigue, suggesting that increasing antioxidant capacity may improve muscle performance [32,185]. However, most studies in humans indicate that antioxidant supplementation fails to improve exercise performance or enhance the adaptations to exercise training [186,187].

Animal studies have shown that genetic deletion of Nrf2 reduces muscle performance [11], while the opposite is observed in the Nrf2-overexpressing mice [58,188]. From these observations, it was postulated that Nrf2 overexpression may improve endurance by increasing the antioxidant capacity [188], promoting mitochondrial biogenesis [189,190] and protecting mitochondria against oxidative damage [191].

Mice lacking Keap1 in their skeletal muscles have enhanced endurance performance, although this effect was restricted to female rodents [188,192]. Genetic deletion of Keap1 has been associated with constitutively elevated Nrf2, likely explaining the observed more aerobic phenotype of skeletal muscle fibres in these mice [192]. It has been

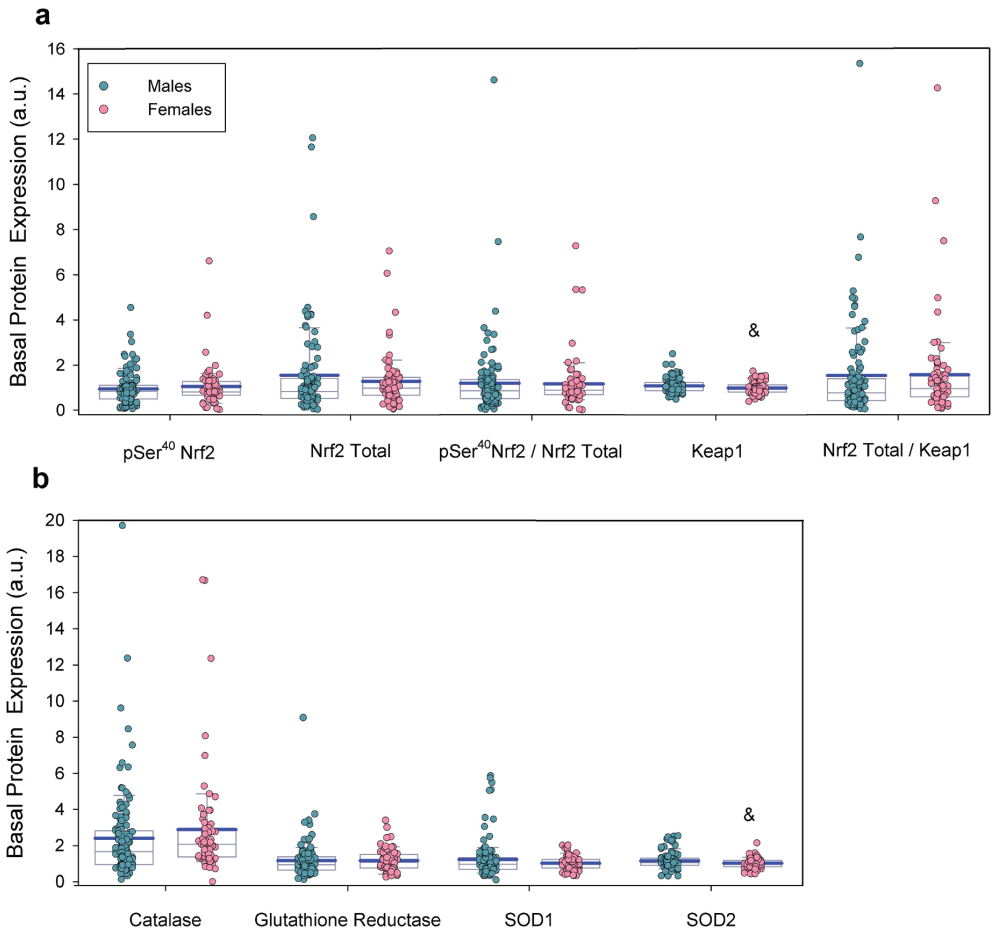

Fig. 5. Sex-related differences in protein expression levels of: Ser^{40}-Nrf2, total Nrf2, Ser^{40}-Nrf2/Nrf2 ratio, Keap1, and Nrf2/Keap1 ratio (a); catalase, glutathione reductase, superoxide dismutase 1 (SOD1), and superoxide dismutase 2 (SOD2) (b). n = 189 (120 males and 69 females) expressed in arbitrary units (a.u.). Box and whisker plots: the extremes of the whiskers represent the limits of the 5th and 95th percentiles, respectively; the thick and thin horizontal lines inside the boxes correspond to the mean and median values, respectively; and the lower and upper limits of the box delimit the 1st and 3rd quartiles, respectively. Male data are green, while female data are pink. & $p < 0.05$ males compared to females. Statistical differences were not significant after accounting for differences in age and VO_2max in mL.kg lower extremities lean $mass^{-1}.min^{-1}$. (From Galvan-Alvarez et al. [17]).

recently reported in humans that sprint performance and mean power output during repeated high-intensity exercise bouts interspaced with brief resting periods of ischaemia are associated with lower expression levels of Keap1 in skeletal muscle [18,19]. This lower basal expression of Keap1 may facilitate a fast upregulation of antioxidant enzymes [55], attenuating the potential detrimental effects of excessive ROS production during the extremely high metabolic rates reached during sprint exercise [19].

In summary, the intricate regulation of the Nrf2 transcription factor in skeletal muscle with exercise reveals a complex interplay between redox balance, cellular stress, and adaptive responses to regular exercise. Nrf2 orchestrates a comprehensive antioxidant defence system by activating the expression of various antioxidant enzymes and pathways in response to oxidative or electrophilic stress. It is well-established that Nrf2 signalling is crucial for mitigating oxidative damage and maintaining redox homeostasis. New research reveals the roles of Nrf2 in human skeletal muscle exercise responses, which may involve Keap1 modifications, metabolites, non-coding RNAs, and autophagy. Moreover, some dietary polyphenols have emerged as potential modulators of Nrf2 activity, although their effects on skeletal muscle Nrf2 signalling remain to be fully elucidated. Understanding the dynamics of Nrf2 signalling in skeletal muscle during exercise provides valuable insights into the physiological adaptations to oxidative stress and the interaction between training and diet, offering avenues for optimising performance and mitigating oxidative stress-related muscle damage.

4. Disclosure summary

The authors have nothing to disclose.

CRediT authorship contribution statement

Miriam Martinez-Canton: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Victor Galvan-Alvarez:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Marcos Martin-Rincon:** Writing – review & editing, Methodology, Investigation, Data curation,

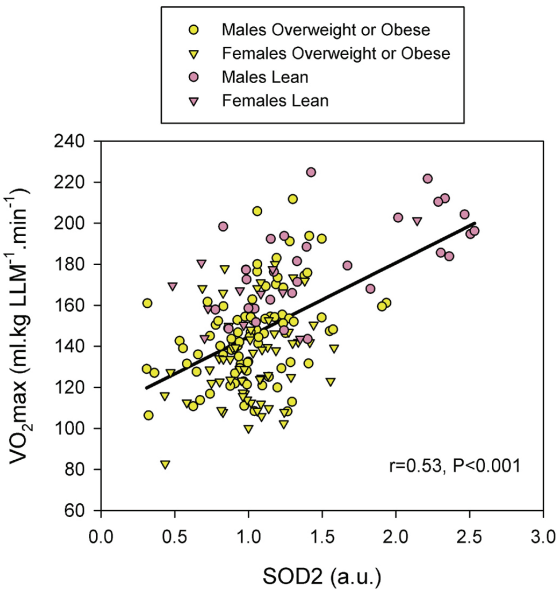

Fig. 6. Relationship between VO_2max in mL.kg of lower extremities lean mass^{-1}.min^{-1} and protein expression of superoxide dismutase 2 (SOD2). n = 188 (41 lean and 147 with overweight or obesity). Data from lean participants are presented in magenta, while data for overweight or obese participants are presented in yellow, including a circle shape for males and a triangle shape for men. The values shown are means ± standard errors and expressed in mL.kg LLM^{-1}.min^{-1} and arbitrary units (a.u.). Statistical significance was set at $p < 0.05$. (From Galvan-Alvarez et al. [17]).

Conceptualization. **Jose A.L. Calbet:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Angel Gallego-Selles:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.Jose A L. Calbet is scientific advisor of Nektium Pharma S.L. and co-inventor of the patent for Zynamite PX.

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STUDY 2

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CaMKII protein expression and phosphorylation in human skeletal muscle by immunoblotting: Isoform specificity

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ABSTRACT

Calcium (Ca^{2+})/calmodulin-dependent protein kinase II (CaMKII) is activated during exercise by reactive oxygen species (ROS) and Ca^{2+} transients initiating muscle contraction. CaMKII modulates antioxidant, inflammatory, metabolic and autophagy signalling pathways. CaMKII is coded by four homologous genes (α, β, γ, and δ). In rat skeletal muscle, δ_D, δ_A, γ_D, γ_B and β_M have been described while different characterisations of human skeletal muscle CaMKII isoforms have been documented. Precisely discerning between the various isoforms is pivotal for understanding their distinctive functions and regulatory mechanisms in response to exercise and other stimuli. This study aimed to optimize the detection of the different CaMKII isoforms by western blotting using eight different CaMKII commercial antibodies in human skeletal muscle. Exercise-induced posttranslational modifications, i.e. phosphorylation and oxidations, allowed the identification of specific bands by multitargeting them with different antibodies after stripping and reprobing. The methodology proposed has confirmed the molecular weight of β_M CaMKII and allows distinguishing between γ/δ and δ_D CaMKII isoforms. The corresponding molecular weight for the CaMKII isoforms resolved were: δ_D, at 54.2 ± 2.1 kDa; γ/δ, at 59.0 ± 1.2 kDa and 61.6 ± 1.3 kDa; and β_M isoform, at 76.0 ± 1.8 kDa. Some tested antibodies showed high specificity for the δ_D, the most responsive isoform to ROS and intracellular Ca^{2+} transients in human skeletal muscle, while others, despite the commercial claims, failed to show such specificity.

1. Introduction

Calcium (Ca^{2+})/calmodulin-dependent protein kinase II (CaMKII) is activated during exercise by reactive oxygen species (ROS) [1] and the Ca^{2+} transients triggering muscle contraction [2]. CaMKII modulates the antioxidant signalling pathways mediated by Keap1, NRF2 and NF-κB [3–5] and is implicated in the regulation of glycolytic enzymes [6], glycogen synthesis [7], glucose transport [8,9], protein synthesis and ion concentrations [10,11], muscle contraction and relaxation [10], nitric oxide synthase [12], and autophagy [13], among other functions. Besides, CaMKII is instrumental in the muscle adaptive response to regular exercise and is a chief determinant of muscle phenotype [14,15]. For these reasons, studying CaMKII is fundamental for a mechanistic approach in skeletal muscle physiology research, explaining the growing

Abbreviations: CaMKII, Calcium (Ca^{2+})/calmodulin-dependent protein kinase II; HDAC4, Histone deacetylase 4; Keap1, Kelch-like ECH-associated protein 1; MHC-IIx, Myosin heavy chain isoform IIX; MW, molecular weight; NF-κB, Nuclear factor kappa B; NRF2, Nuclear factor erythroid 2-related factor 2; ROS, Reactive oxygen species; RT, Room temperature; TBS-T, Tris-buffered saline containing 0.1 % Tween 20; VL, *Vastus lateralis*.

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interest in better characterising this enzyme.

CaMKII is a multimeric enzyme encoded by four homologous genes (α, β, γ, and δ) [16,17]. Each isoform comprises a catalytic domain, a variable domain, and an oligomerisation domain. The N-terminal catalytic domain features a CaM-binding site housing the stimulatory autophosphorylation site (Thr^{287}) and the inhibitory phosphorylation site (Thr^{306}). The central variable domain, susceptible to alternative splicing, contributes to the notable diversity within this kinase family [18]. Despite high sequence homology among the four genes [19], indicating conservation of essential functions, the holoisoforms exhibit differential tissue expression [20,21] and exist as several alternative spliced variants. The β isoform is neuron-specific, and its spliced variant β_M is present in skeletal muscle [22]. The δ and γ isoforms are ubiquitous [22], the δ being predominant in cardiac myocytes [23–25] and the γ prevalent in smooth muscle [26–28]. The α isoform (54.0 kDa) is expressed primarily in the brain [29].

The presence of β, γ, and δ CaMKII isoforms in skeletal muscle has been revealed mainly by Western blot with isoform-specific antibodies [30–35]. In rodent skeletal muscle, the isoforms β_M, $\gamma_{B,}$ δ_D, and δ_A have been described [30,36]. In human skeletal muscle, some researchers did not differentiate between γ and δ, reporting the two variants of the δ and the γ isoforms conjointly while identifying the β_M isoform separately [4,34,35,37,38]. Interestingly, a specific regulation of some of these isoforms has been reported after exercise training. For example, Thomassen et al. [39] reported upregulation of $pThr^{287}$- γ/δ CaMKII and $pThr^{287}$-β_M CaMKII, as well as an increased amount of the total protein expression of γ/δ CaMKII after a 7-week high-intensity training in cyclists. Likewise, Popov et al. [40] reported increased basal $pThr^{287}$-CaMKII protein expression without distinguishing between isoforms after aerobic training in untrained men. The latter may be due to the difficulty of separating and distinguishing between the different isoform bands in Western blot analyses from human skeletal muscle [3–5,14,34,35,37–42]. Correctly identifying the CaMKII isoforms in human skeletal muscle, particularly the δ isoform [14], is crucial for better understanding their specific functions and regulation in response to exercise and other stimuli.

Therefore, the purpose of this study was to develop a protocol to optimize the identification of the β_M, γ/δ and δ_D CaMKII variants and their respective activating phosphorylation (Thr^{287}) and oxidation ($Met^{281/282}$) using eight different commercial antibodies through immunoblotting in human skeletal muscle.

2. Materials and methods

2.1. General overview

This study is based on the analysis of resting and post-exercise skeletal muscle biopsies obtained in research projects from our laboratory to determine mechanisms of fatigue after a strength-training intervention [14,43] and high-intensity exercise [3,4,44]. In the main experiments 1 and 2, resting muscle biopsies were obtained from the *vastus lateralis* (VL) muscle of a healthy, physically active human. Experiment 3 included twenty-two physically active young male participants from whom VL muscle biopsies were taken before and after a strength-training program [14]. Biopsies corresponding to three participants were randomly chosen for the current determinations. In experiment 4, eleven young active males had VL muscle biopsies taken at rest and immediately after a bout of intense exercise [3,4]. The biopsies pertaining to one of the participants were included in the present analyses. These experiments showed that CaMKII positively correlates with muscle hypertrophy and the total number of repetitions executed and negatively with the changes in MHC-IIx induced by the training program [14], and support a ROS-mediated CaMKII Thr^{287} phosphorylation during high-intensity exercise [3].

2.2. Muscle biopsies

Resting muscle biopsies were obtained after a 10-min rest in the supine position. Post-exercise muscle biopsies were obtained immediately after the exercise, with the subject seated on a cycle ergometer and leaning backwards, supported by a helper. The skin over the middle portion of the VL muscle was anaesthetised with 2 % lidocaine (2 mL) without adrenaline and injected above the superficial fascia, i.e., avoiding infiltrating the muscle belly. Following a 15-min interval, a 5-mm incision of the skin and superficial fascia was performed. Subsequently, a Bergstrom's biopsy needle was introduced through the incisions, and a muscle biopsy was obtained 2 cm below the fascia. The same medical doctor performed all biopsy procedures, ensuring standardisation. Complete information regarding participants' physical characteristics can be found in previous publications [3,4,14].

2.3. Muscle homogenates

Human skeletal muscle whole tissue lysates were prepared with ~10 mg of tissue ground by stainless steel balls for 1 min at 22 Hz in a Mikro-Dismembrator S (Sartorius, Goettingen, Germany) and immediately homogenised in urea lysis buffer (6 M urea, 1 % SDS, supplemented with 50X cOmplete protease and 10X PhosSTOP phosphatase inhibitor cocktails). Then, the lysates were centrifuged for 12 min at 25,200 g at 16 °C. The resulting supernatants were quantified in triplicate using the bicinchoninic acid assay [45]. The homogenised volumes were adjusted to obtain a concentration ranging from 5 to 6.5 μg/μL in experiments 1 and 4, 0.4 μg/μL and 5.5 μg/μL in experiment 2, and ~2.8 μg/μL in experiment 3. For experiments 1 and 2, 0.5–22.5 μg of the same lysate was loaded and electrophoresed. To ensure the reproducibility of the different antibodies and to provide sufficient area for proper incubation, the same lysate was loaded in duplicate for each antibody tested. After trying different protein amounts, the protein quantities were chosen to allow the visualisation of most CaMKII isoforms within a singular chemiluminescent exposure employing the antibody conditions defined in the following lines. For experiment 2, the anti-CaMKII total (no. 611292, BD Biosciences) was used as a reference antibody to be compared with the isoform-specific antibodies.

For experiments 3 and 4, 10–22.5 μg were loaded for electrophoresis. The amount of protein loaded was optimised by determining the range of protein loading for which the antigen-antibody response was linear, generally ranging from 5 to 30 μg, considering potential changes in protein expression levels [3,4,14]. Experiments 3 and 4 were conducted as additional assays to validate the banding patterns observed in experiments 1 and 2. Given the staining consistency and relatively low variability observed between the duplicates in experiments 1 and 2, running experiments 3 and 4 in duplicate was not deemed necessary.

2.4. Western blot

The samples were diluted one-third (v/v) in Laemmli reducing buffer (160 mM Tris-HCl, pH 6.8, 5.9 % SDS, 25.5 % glycerol, 15 % β-mercaptoethanol-bromophenol blue) and electrophoresed at 4 °C for 50 min at 80V to stack the samples and 90 min at 170V in 10 % hand-cast gels. After SDS-PAGE electrophoresis, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (0.45 μm pores, Bio-Rad Laboratories, Hercules, CA, USA) with a Trans-Blot Cell (Bio-Rad) chamber for 90 min at 4 °C and 0.4 Å. For the accurate assessment of assay variability and to ensure optimal loading, transfer efficiency, and band separation, all membranes underwent staining using Reactive Brown 10 (Sigma Aldrich, St. Louis, MO, USA and Santa Cruz Biotechnology, Dallas, TX, USA) or Ponceau S (Sigma Aldrich, St. Louis, MO, USA). The membranes were blocked for 1 h at RT in 4 % bovine serum albumin (BSA) or 5 % skimmed milk-blocking buffer diluted in Tris-buffered saline containing 0.1 % Tween 20 (TBS-T) and incubated overnight for 12–15 h at 4 °C with primary antibodies. The primary

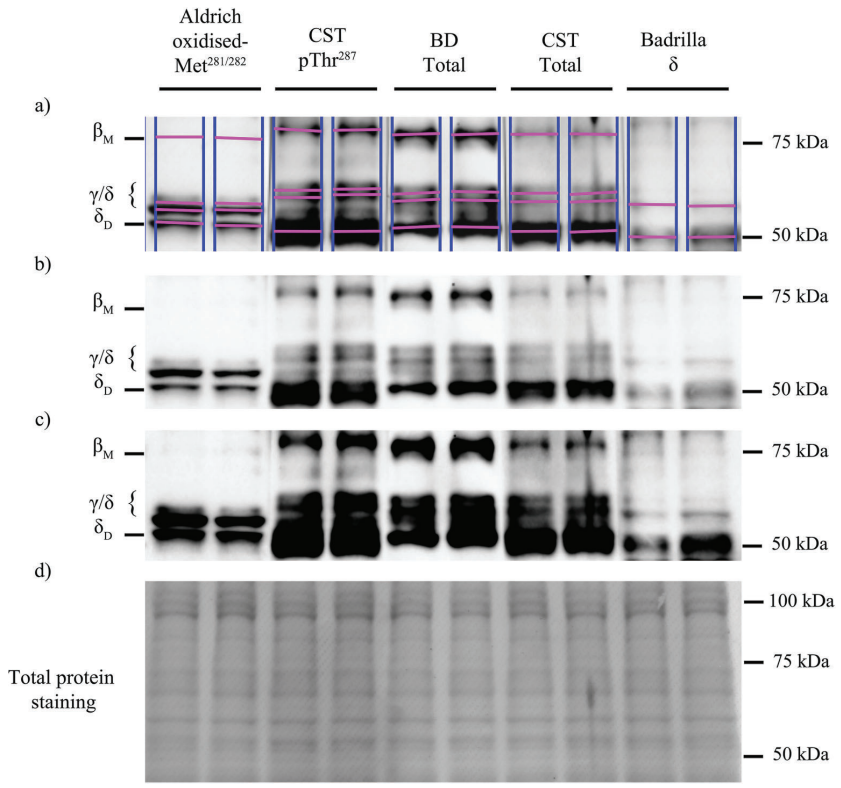


Fig. 1. CaMKII isoforms identification through immunoblotting in human skeletal muscle from a healthy participant obtained under resting conditions. **a) Representative image** obtained using Image Lab© software 6.0.1 (Bio-Rad) employing Lanes and Bands and MW Analysis Tools. The continuous purple line denotes the selected band used to calculate the molecular weight. Blue vertical lines delimit each lane. **b) Representative immunoblot** employing five commercial antibodies: Aldrich oxidised-Met$^{281/282}$, CST pThr^{287}, BD Total, CST Total, Badrilla δ. **c) Over-exposed immunoblot** targeting β_M with the Aldrich oxidised-Met$^{281/282}$ antibody. **d) Total protein staining** using Ponceau S. Isoforms (β_M, γ/δ and δ_D) are depicted on the left side of the panel. The band migration markers indicate the molecular weight in kDa.
Aldrich oxidised-Met$^{281/282}$: anti-oxidised-Met$^{281/282}$-CaMKII (no. 07–1387, Sigma Aldrich); CST pThr^{287}: anti-phospho-CaMKII-Thr^{287} (no. 12716, Cell Signaling Technology), BD Total: anti-CaMKII total (no. 611292, BD Biosciences), CST Total: anti-CaMKII total (no. 4436, Cell Signaling Technology), Badrilla δ: anti-CaMKII delta isoform (no. A010-55AP, Badrilla).

antibodies were anti-oxidised-Met$^{281/282}$-CaMKII diluted 1:5,000 in 5 % skimmed milk (no. 07–1387, Sigma Aldrich), anti-phospho-CaMKII-Thr^{287} diluted 1:2,000 in 4 % BSA (no. 12716, Cell Signaling Technology), anti-CaMKII total diluted 1:3,000 in 5 % skimmed milk (no. 611292, BD Biosciences), anti-CaMKII total diluted 1:2,000 in 4 % BSA (no. 4436, Cell Signaling Technology), anti-CaMKII delta isoform diluted 1:2,000 in 4 % BSA (no. 181052, Abcam), anti-CaMKII delta isoform diluted 1:2,500 in 4 % BSA (no. A010-55AP, Badrilla), anti-CaMKII gamma isoform diluted 1:2,000 in 4 % BSA (no. 12666-2-AP, Proteintech), and anti-CaMKII beta isoform diluted 1:2,000 in 4 % BSA (no. 376828, Santa Cruz Biotechnology). After the primary antibody incubation, the membranes were washed thrice for 10 min each with TBS-T. Subsequently, incubation with HRP-conjugated anti-rabbit (no. 111-035-144, Jackson ImmunoResearch; SC2004, Santa Cruz Biotechnology) or anti-mouse antibody (no. 115-035-003, Jackson ImmunoResearch; SC2031, Santa Cruz Biotechnology) was performed, with a dilution range of 1:5,000–1:50,000 in 5 and 2.5 % skimmed milk blocking buffers for all instances. Following incubation, chemiluminescent visualisation was conducted using Clarity™ Western ECL Substrate (Bio-Rad) and subsequent visualisation using a ChemiDoc™ Touch Imaging System (Bio-Rad). Pre-stained protein standards obtained from Bio-Rad (catalogue reference: 1610373, Bio-Rad) were captured under white light immediately after chemiluminescent imaging, with the membranes maintained in the same position.

2.5. Identification of CaMKII isoforms: Image Lab© software 6.0.1

The identification of CaMKII isoforms was based on their predicted electrophoretic size mobility (β_M: 72.7 kDa; δ_A: 60.0 kDa; γ_C: 56.0; γ_B:58.4 kDa; and δ_D: 58.4 kDa) [19] and the information provided in the antibody datasheet. Experimental molecular weights (MW) of each band resulting from the reactivity with antibodies targeting all total CaMKII and all phosphorylated isoforms, the specific antibodies for the β, γ, δ isoforms, and an antibody specific for oxidised methionine 281/282 were determined using the Lanes and Bands and MW Analysis Tools within the Image Lab© software 6.0.1 (Bio-Rad).

2.6. Identification of CaMKII isoforms: membrane stripping

To accurately identify CaMKII isoforms, the membrane in experiment 3, which contained the before and after strength training intervention samples from 3 subjects, and the membrane in experiment 4, which contained the resting and after acute exercise samples from 1 subject, underwent a stripping procedure and subsequent reprobing

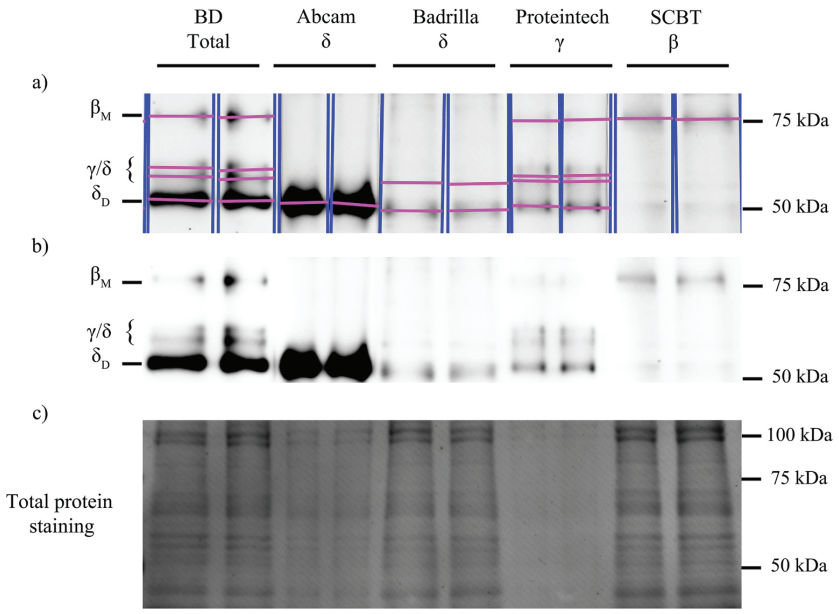


Fig. 2. CaMKII isoforms identification through immunoblotting in human skeletal muscle from a healthy participant obtained under resting conditions. **a) Representative image** obtained using Image Lab© software 6.0.1 (Bio-Rad) employing Lanes and Bands and MW Analysis Tools. The continuous purple line denotes the selected band used to calculate the molecular weight. Blue vertical lines delimit each lane. **b) Representative immunoblot** employing five commercial antibodies: BD Total, Abcam δ, Badrilla δ, Proteintech γ and SCBT β. **c) Total protein staining** using Reactive Brown 10. Isoforms (β_M, γ/δ and δ_D) are depicted on the left side of the panel. The band migration markers indicate the molecular weight in kDa.
BD Total: anti-CaMKII total (no. 611292, BD Biosciences); Abcam δ anti-CaMKII delta (no. 181052, Abcam); Badrilla δ anti-CaMKII delta (no. A010-55AP, Badrilla); Proteintech γ: anti-CaMKII Gamma (no. 12666-2-AP, Proteintech); SCBT β: anti-CaMKII Beta (no. 376828, Santa Cruz Biotechnology). Note that the antibody targeting the γ isoform displays high cross-reactivity with β and δ CaMKII, being unable to specifically resolve the γ isoform.

with the antibody for the total CaMKII. This was done as an additional assay to validate the bands obtained using the same PVDF membranes. After a 30-min incubation at room temperature (RT) in 2 % (v/v) sodium dodecyl sulfate containing 100 mM β-mercaptoethanol [46], the membranes were washed with TBS-T and stained with Reactive Brown 10 or Ponceau S for total protein presence confirmation. Following a 1-h blocking with 5 % skimmed milk, membranes were incubated with an HRP-conjugated anti-rabbit diluted 1:5,000–1:20,000 in 5 % skimmed milk blocking buffer for 1 h (no. 111-035-144, Jackson ImmunoResearch; SC2004, Santa Cruz Biotechnology). Afterwards, Clarity™ Western ECL Substrate (Bio-Rad) was added for subsequent visualisation (ChemiDoc™ Touch Imaging System, Bio-Rad), aiming to verify the effectiveness of antibody removal by assessing the absence of signal from the previously probed antibodies. Then, membranes were blocked again in 4 % BSA and reprobed for 12–15 h at 4 °C with anti-CaMKII total diluted 1:2,000 in 4 % BSA (no. 4436, Cell Signaling Technology). The HRP-conjugated anti-rabbit diluted 1:5,000 in 5 % skimmed milk (no. 111-035-144, Jackson ImmunoResearch) was incubated for 1 h, and the visualisation of the second protein was executed following the same procedural steps. See Tables S1 and S2, as well as the Appendix, for a detailed description of the materials and their composition.

3. Results

3.1. Identification and molecular weight determination of CaMKII isoforms from experiments 1, 2, 3 and 4

We examined the CaMKII isoform composition of human skeletal muscle by immunoblot analysis to determine the MW of each isoform using the 8 commercial antibodies (Figs. 1 and 2). Due to the inherent lack of exact measurements, the data shown has been rounded to the nearest unit separately for each isoform across experiments 1, 2, 3 and 4. Based on SDS–PAGE mobility [30,32–35,37], we identified the band with the lowest MW as δ_D (54.2 ± 2.1 kDa, n = 51). The group of intermediate bands above δ_D, exhibiting a slightly higher MW, was designated as γ/δ (59.0 ± 1.2 kDa, n = 45 and 61.6 ± 1.3 kDa, n = 45). The band with the highest MW corresponded to the β_M isoform (76.0 ± 1.8 kDa, n = 47).

3.2. Additional human experiments to verify CaMKII isoforms

In experiment 3, strength training induced a shift from MHC-IIx to MHC-IIa, driven by changes in sarcoplasmic [Ca^{2+}] sensed by CaMKII [14]. This intervention enabled the detection of changes in $pThr^{287}$-δ_D CaMKII, which is positively associated with muscle hypertrophy and the number of repetitions performed during training and negatively with the changes in MHC-IIx. In experiment 4, ROS produced during exercise until exhaustion triggered ROS-mediated signalling, leading to the upregulation of NRF2 and Thr^{287} CaMKII phosphorylation [3]. The close positive association between NRF2 total/Keap1 and $pThr^{287}$ CaMKII further validates this model for studying CaMKII isoforms.

3.2.1. Experiment 3: Identification of phosphorylated Thr^{287} CaMKII incubation, membrane stripping, and Total-CaMKII reprobing in human skeletal muscle

In the biopsies obtained before and after strength training (experiment 3, Fig. 3) [14], the membranes were first incubated with $pThr^{287}$-CaMKII (exposure time 110 s, binning 4 × 4) (Fig. 3a, upper panel). After a successful 30-min stripping (Fig. 3b, upper panel) of the PVDF membrane (exposure time 371 s, binning 4 × 4), the presence of

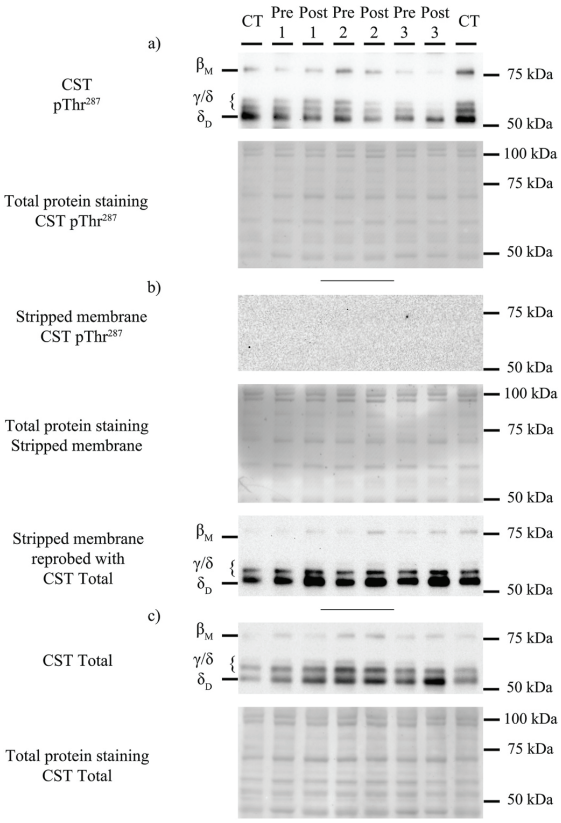


Fig. 3. Immunoblotting steps for experiment 3, including three representative young male participants assessed before and after a strength training programme. a) Membrane 1. Upper panel: Incubation with pThr^{287}-CaMKII (exposure time 110 s, binning 4 × 4); Lower panel: Total protein staining using Reactive Brown 10. **b) Stripped membrane 1**. Upper panel: Chemiluminescent signal (exposure time 371 s, binning 4 × 4) of pThr^{287}-CaMKII after 30 min of stripping with custom-made stripping buffer; middle panel: Total protein staining using Reactive Brown 10 after 30 min of stripping; lower panel: Reprobing with total-CaMKII (exposure time 720 s, binning 2 × 2) of the stripped membrane. **c) Membrane 2**. Upper panel: Incubation with total-CaMKII (exposure time 37 s, binning 4 × 4); lower panel: Total protein staining using Reactive Brown 10. Isoforms (β_M, γ/δ and δ_D) are depicted on the left side of the panel. The band migration markers indicate the molecular weight in kDa. CST pThr^{287}: anti-phospho-CaMKII-Thr^{287} (no. 12716, CST), CST Total: anti-CaMKII total (no. 4436, CST), CST: Cell Signaling Technology, CT: human control sample (non-experimental, Pre: biopsy sample taken before a strength-training programme, Post: biopsy sample taken after a strength-training programme.

proteins was confirmed by staining with Reactive Brown 10 (Fig. 3b, middle panel). Next, the membrane was reprobed with the total-CaMKII antibody for band validation (Fig. 3b, lower panel). The resulting bands from the pThr^{287}-CaMKII were positioned in the lower region, encompassing δ_D, followed by γ/δ bands, while the upper region featured the β_M isoform. This observed pattern was consistent when employing total-CaMKII in both the stripped membrane (exposure time 720 s, binning 2 × 2 (Fig. 3b, lower panel) and the non-stripped membrane (exposure time 37 s, binning 4 × 4) (Fig. 3c, middle panel).

3.2.2. Experiment 4: Identification of isoforms using an antibody specific for oxidised-Met$^{281/282}$ -CaMKII: incubation, membrane stripping, and Total-CaMKII reprobing in human skeletal muscle

For the acute exercise biopsies (experiment 4, Fig. 4) [3,4], membranes were initially subjected to incubation with an antibody directed against oxidised-Met$^{281/282}$-CaMKII (exposure time 72 s, binning 2 × 2) (Fig. 4a, upper panel). After a successful 30-min stripping (Fig. 4b, upper panel) of the PVDF membrane (exposure time 720 s, binning 4 × 4), the presence of proteins using Reactive Brown 10 was confirmed (Fig. 4b, middle panel). Next, the membrane was reprobed with the antibody against total-CaMKII for verification (Fig. 4b, lower panel). The band stained with anti-oxidised-Met$^{281/282}$-CaMKII with lower MW comprised δ_D, followed by γ/δ bands, while the β_M isoform laid at a higher MW. This distinctive pattern was consistently manifested when employing the antibody against total-CaMKII in both the non-stripped membrane (exposure time 37 s, binning 4 × 4) (Fig. 4b, lower panel) and the non-stripped membrane (exposure time 30s, binning 2 × 2) (Fig. 4c, upper panel).

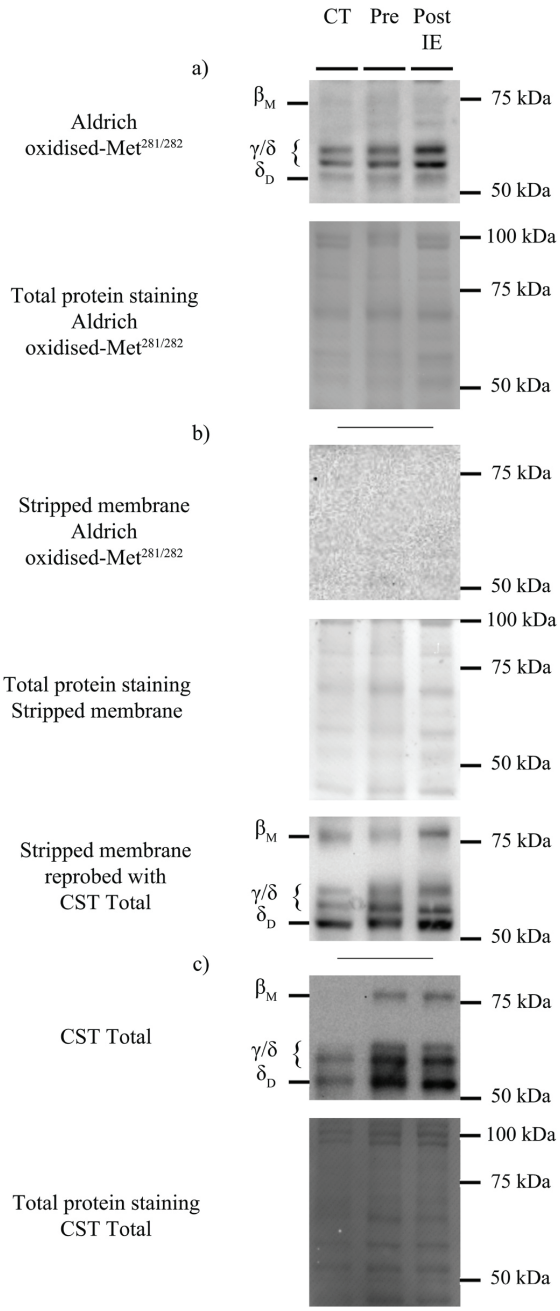
4. Discussion

Most studies in human skeletal muscle have distinguished only two main CaMKII isoforms: γ/δ and β_M. γ/δ (γ and δ, analysed conjointly) represented the sum of the electrophoretic bands with MW ranging between 50 and 65 kDa, while β_M corresponded to a single upper band with an MW close to 75 kDa [34,37–39]. Notably, alternative characterisations have defined CaMKII as a singular 50 kDa band [47], a band range spanning 50–60 kDa [40] omitting β_M isoform, or a band assembly without specifying MWs [5,41,42,48]. In this context, we present an easy and cost-effective approach to distinguish different CaMKII β_M, γ/δ and δ_D isoforms, suitable for implementation in most laboratories. The immunoblotting assessment of phosphorylated and oxidised CaMKII with specific antibodies, followed by stripping and subsequent positive incubation of the specific band using an antibody that binds with all CaMKII, verified the band identification. Despite the technical limitations of immunoblotting, the usage of 8 different commercial antibodies conferred robustness to the assays. The parallel findings in the banding pattern across membranes and the reproducibility of findings across experiments and conditions underscore the overall validity of the experimental procedures.

The β_M CaMKII is abundantly expressed in mouse skeletal muscle [19,22], which Tombes et al. [19] identified as a 72.7 kDa band, i.e., slightly less than the 76.0 ± 1.8 kDa observed in our experiments. However, our results align with the studies in human skeletal muscle, which consistently report β_M CaMKII band above the molecular marker of 70 kDa [3,4,14,34,35,37–39]. Studying β_M in human skeletal muscle is of significant interest, as it forms a complex with the glycolytic machinery at the sarcoplasmic reticulum, regulating glycolytic metabolism, calcium transport, and muscle excitation-relaxation [6].

The δ_D CaMKII isoform is exclusive of skeletal muscle, theoretically weighing 58.4 kDa [19], while our data indicate that the corresponding MW for human skeletal muscle is 54.2 ± 2.1 kDa. We tested two different δ-specific CaMKII antibodies. Our assays indicated that the δ CaMKII Badrilla and δ CaMKII Abcam identified a band in 50.9 ± 0.31 kDa and 51.7 ± 0.08 kDa, respectively. In humans, the basal protein expression level of the δ_D isoform increases following a strength training program in parallel with the degree of muscle hypertrophy elicited by the training program [14]. Likewise, the specific δ CaMKII expressed in rodent myocardiocytes, the δ_B, plays an essential role in hypertrophy after myocardial ischemia/reperfusion injury [49,50]. Our human studies support the emerging role of the δ_D CaMKII isoform as the most exercise-responsive CaMKII isoform in skeletal muscle [3,4], which appears to contribute to the NF-κB [4] and NRF2/Keap1 [3] signalling activation.

The γ CaMKII isoform displays widespread expression with diverse spliced variants across neural and nonneural tissues [26,28,30,51]. γ_B



(caption on next column)

Fig. 4. Immunoblotting steps for experiment 4, including one representative young male participant who underwent an incremental exercise until exhaustion. a) Membrane 1. Upper panel: Incubation with oxidised-Met$^{281/282}$-CaMKII (exposure time 72 s, binning 2 × 2); Lower panel: Total protein staining using Reactive Brown 10. **b) Stripped membrane 1**. Upper panel: Chemiluminescent signal (exposure time 720 s, binning 4 × 4) of oxidised-Met$^{281/282}$-CaMKII after 30 min of stripping with custom-made stripping buffer; middle panel: Total protein staining using Reactive Brown 10 after 30 min of stripping; lower panel: Reprobing with total-CaMKII (exposure time 139 s, binning 2 × 2) of the stripped membrane. **c) Membrane 2**. Upper panel: Incubation with total-CaMKII (exposure time 30 s, binning 2 × 2); lower panel: Total protein staining using Ponceau S. Isoforms (β_M, γ/δ and δ_D) are depicted on the left side of the panel. The band migration markers indicate the molecular weight in kDa
Aldrich oxidised-Met$^{281/282}$: anti-oxidised-Met$^{281/282}$-CaMKII (no. 07–1387, Sigma Aldrich), CST Total: anti-CaMKII total (no. 4436, CST), CST: Cell Signaling Technology, CT: human control sample (non-experimental, Pre: biopsy sample taken at rest, Post IE: biopsy sample taken after incremental exercise until exhaustion.

CAMKII protein [30] and γ_C mRNA have been reported in the skeletal muscle of rats [26,28] and different human tissues [51]. As Tombes et al. [19] outlined, the γ_C variant is ubiquitously present in tissues with a 56 kDa MW. The γ_B is present in T-cells, smooth muscle, astrocytes and islets with a 58.4 kDa MW. The δ_A CaMKII isoform has been reported as a band positioned below β_M CaMKII in rodent skeletal muscle [36] being involved in the modulation of muscle fibre type-specific gene expression, primarily via the phosphorylation of histone deacetylase 4 (HDAC4), enabling the transition from a fast-to-slow muscle phenotype [52]. The MW for this band has been postulated to lay close to 60 kDa [19]. In the present investigation, we identified γ/δ as the two upper bands below β_M CaMKII, with MWs of 59.0 ± 1.2 kDa and 61.6 ± 1.3 kDa, respectively. The γ CaMKII antibody used here was not isoform-specific due to its cross-reactivity with all the CaMKII isoforms, even if a minimal amount of protein was loaded (0.5 μg of human skeletal muscle lysate). The δ CaMKII purchased from Badrilla targeted a band at 57.9 ± 0.42 kDa (n = 4), which was not detected by δ CaMKII purchased from Abcam. Discerning between δ_A, γ_C, and γ_B splicing variants was not achievable with Western blot, despite using antibodies that claimed specificity. Further research is needed to distinguish between these CaMKII isoforms, targeted by total and phosphorylated CaMKII antibodies.

4.1. Specificity of CaMKII isoform antibodies

High homology between isoforms results in cross-reactivity with isoform-specific antibodies. Most studies using the four specific isoform antibodies report bands without specifying the MW at which these bands were observed.

The β CaMKII antibody has been tested in pig skeletal muscle without reporting MW [53]. In Fan et al. [54], β CaMKII is reported at 57 kDa in PC12 cells (neural tissue). Our study revealed that the specific antibody against β CaMKII marked a single band, which precisely aligned at MW corresponding to a similar band marked by the antibodies directed against the total, phospho- and oxidised CaMKII. The MW (76.0 ± 1.8 kDa) concured with that reported for β_M CaMKII in human skeletal muscle by Christiansen et al. [38] and Thomassen et al. [39] using pThr^{287}-CaMKII (no. 12716, Cell Signaling Technology) and total-CaMKII (no. 4436, Cell Signaling Technology) and total-CaMKII (no. 611293, same as 611292, BD Biosciences) and pThr^{287}-CaMKII (no. 3361, Cell Signaling Technology, discontinued).

In rodents, the δ_D CaMKII has been described in skeletal muscle [19] and cardiac myocytes [49,50]. Previous evidence using the antibody purchased from Abcam reports a 56 kDa band in HL-1 myocytes [55], while non-specific MW is reported in other studies [56,57]. In the present investigation, δ CaMKII from Abcam only detected a clear single band at 51.7 ± 0.08 kDa, which concurred with the Total and pThr^{287} CaMKII antibodies at this MW. The δ CaMKII from Badrilla detects a group of bands in the atrial tissue of rats [58] without specifying the MW. Our study detected two bands, the lowest at 50.9 ± 0.3 kDa, which coincided with the Abcam δ CaMKII band, and the highest at 57.9 ± 0.42 kDa. Although no MW is defined, the γ CaMKII antibody has been knock-down validated in multiple myeloma cells [59]. Other studies

report a single band at 59 kDa [60,61] or without MW [62]. The γ CaMKII antibody has also been used to target total CaMKII [63–65]. Even when loading a low amount of protein (0.5 μg of human skeletal muscle in our study), the γ CaMKII antibody had high cross-reactivity with β and δ CaMKII. Despite the characterisation of β_M, γ_D-δ_D and δ_A [36] and β_M, γ_B, δ_A and δ_B [30] in rat skeletal muscle, discerning between γ and the upper δ CaMKII band was not achievable using current commercial antibodies. Therefore, γ/δ have been analysed conjointly in the present investigation.

4.2. Limitations

This study has several limitations. Although none of the bands was immunoprecipitated and submitted to mass spectrometry, most commercial antibodies have been shown to be specific and have been used in numerous previous studies. Besides, the experiments executed in the present investigation took advantage of protein modifications (phosphorylation and oxidation) that are specific for CaMKII and can be tracked with the antibodies used. Greater knowledge could be gained regarding the regulation of the total protein changes by adding measurements of gene expression. However, the protein levels may change independently from changes in mRNA expression due to changes in translation efficiency or protein degradation rate, as reported for Keap1 and Nrf2 [66]. Additional information regarding gene expression can be obtained using the MetaMex tool, which allows the generation of meta-analysis of multiple experiments using published transcriptomic data from skeletal muscle biopsies obtained in human experiments [67]. In response to acute and chronic exercise, no significant changes have been observed in mRNA levels of CAMK2B (beta isoform) or CAMK2G (gamma isoform) [67]. In agreement with our previous publication [14], an increased CAMK2D (delta isoform) mRNA expression has been observed for resting muscle biopsies obtained 72 h after the end of strength training programmes lasting between 12 and 26 weeks, compared to the values observed before training [67].

In summary, this study shows a practical immunoblotting approach for discerning the CaMKII isoforms β_M, γ/δ and δ_D in human skeletal muscle with commercial antibodies. Remarkably, our findings confirm the MW of β_M CaMKII and allows distinguishing between γ/δ and δ_D CaMKII, the latest being the most responsive isoform to ROS stimulation. It has been confirmed that, as indicated by the manufacturer, the γ CaMKII antibody displays cross-reactivity with all CaMKII isoforms, not allowing distinguishing between γ/δ isoforms. Correctly identifying CaMKII isoforms is crucial for elucidating the functional role of each isoform. Here we have demonstrated that exercise can be used to elicit specific posttranslational modifications, such as phosphorylations and oxidations, which allow the identification of specific bands by multi-targeting them with different antibodies after stripping and reprobing.

Disclosure summary

The authors have nothing to disclose.

Data availability statement

The data that support the findings of this study are available on request from the corresponding authors.

CRediT authorship contribution statement

Miriam Martinez-Canton: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Angel Gallego-Selles:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Victor Galvan-Alvarez:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Eduardo Garcia-Gonzalez:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Giovanni Garcia-Perez:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Alfredo Santana:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Marcos Martin-Rincon:** Writing – review & editing, Supervision, Software, Resources, Methodology, Formal analysis, Data curation, Conceptualization. **Jose A.L. Calbet:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary materials and methods

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2024.08.030.

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STUDY 3

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ORIGINAL ARTICLE

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Role of CaMKII and sarcolipin in muscle adaptations to strength training with different levels of fatigue in the set

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Abstract

Strength training promotes a IIX-to-IIA shift in myosin heavy chain (MHC) composition, likely due to changes in sarcoplasmic [Ca^{2+}] which are sensed by CaMKII. Sarcoplasmic [Ca^{2+}] is in part regulated by sarcolipin (SLN), a small protein that when overexpressed in rodents stimulates mitochondrial biogenesis and a fast-to-slow fiber type shift. The purpose of this study was to determine whether CaMKII and SLN are involved in muscle phenotype and performance changes elicited by strength training. Twenty-two men followed an 8-week velocity-based resistance training program using the full squat exercise while monitoring repetition velocity. Subjects were randomly assigned to two resistance training programs differing in the repetition velocity loss allowed in each set: 20% (VL20) vs 40% (VL40). Strength training caused muscle hypertrophy, improved 1RM and increased total CaMKII protein expression, particularly of the δ_D isoform. Phospho-Thr^{287}-CaMKII δ_D expression increased only in VL40 (+89%), which experienced greater muscle hypertrophy, and a reduction in MHC-IIX percentage. SLN expression was increased in VL20 (+33%) remaining unaltered in VL40. The changes in phospho-Thr^{287}-CaMKII δ_D were positively associated with muscle hypertrophy and the number of repetitions during training, and negatively with the changes in MHC-IIX and SLN. Most OXPHOS proteins remained unchanged, except for NDUFB8 (Complex I), which was reduced after training (−22%) in both groups. The amount of fatigue allowed in each set critically influences muscle CaMKII and SLN responses and determines muscle phenotype changes. With lower intra-set fatigue, the IIX-to-IIA MHC shift is attenuated.

KEYWORDS

exercise, fatigue, human, myosin heavy chain, skeletal muscle, velocity-based training

1 | INTRODUCTION

The amount of fatigue allowed during resistance training determines the structural and phenotypic changes elicited by a strength training program.[1] In this study, a higher level of fatigue (ie greater reduction in strength, velocity, and power) by performing repetitions closer to muscle failure was associated with greater muscle hypertrophy, a IIX-to-IIA myosin heavy chain (MHC) switch,[1] and a lesser improvement in jumping performance despite each repetition being executed at the maximal intended velocity. However, we did not address the molecular mechanisms explaining these differences in muscle adaptation.

Experiments involving genetic manipulation, denervation, and immobilization indicate that Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a principal regulator of muscle phenotype.[2,3] CaMKII regulates the expression of the transcription factor coactivator peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), which is a critical regulator of mitochondrial biogenesis.[4] Besides this, CaMKII has been implicated in the regulation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB),[5,6] which is stimulated by high-intensity exercise[7] and muscle metabolism.[8-10] Nevertheless, previous evidence on the role of CaMKII in the regulation of muscle phenotype comes from extreme experimental models and information using physiological models in humans is lacking.[2] CaMKII has been implicated in muscle hypertrophy in animal models.[11-13] CaMKII is encoded by four genes (α, β, γ, and δ) and more than 40 splice variants have been reported[14] of which, the isoforms β_M, γ, δ_A, and δ_D are expressed in skeletal muscle.[15-19]

Although increases of resting CaMKII γ/δ expression and phosphorylation status have been reported after sprint training,[20] no study has determined the effect of strength training on basal total expression and phosphorylation state of CaMKII isoforms in human skeletal muscle. Moreover, it remains unknown whether differences in CaMKII phosphorylation specific to the level of fatigue could be implicated in the IIX-to-IIA MHC changes elicited by most strength training programs.[21] The latter could be the case, since it has been shown that CaMKII is activated by Ca^{2+}-calmodulin binding and autophosphorylation,[2] particularly in response to Ca^{2+} transients elicited by prolonged contractions[22] or repeated briefs contractions.[23] In turn, CaMKII activation may stimulate PGC-1α expression and mitochondrial biogenesis.[22]

Another mechanism that could explain muscle phenotype changes is a reduction in expression of the protein sarcolipin (SLN).[24] Sarcolipin is a small protein expressed only in skeletal and cardiac muscle,[25] which regulates the maximal activity and thermodynamic efficiency of the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA).[26] Animal experiments indicate that SLN could have a role in exercise performance and muscle phenotype.[27,28] Mice overexpressing SLN (Sln^{OE}) have superior resistance to fatigue during electrically induced contractions than their wild-type (WT) counterparts.[27] Likewise, compensatory muscle hypertrophy elicited by overloading of the *plantaris* muscle by bilateral surgical excision of the *soleus* and *gastrocnemius* muscles (synergist ablation) is associated with increased SLN mRNA[29] and protein[24] expression in mice. However, the effects of strength training on SLN protein expression remain unknown. Therefore, the principal aim of the present investigation was to determine whether basal CaMKII phosphorylation in skeletal muscle is modified depending on the level of fatigue elicited during a strength training program. A secondary aim was to ascertain whether the changes in muscle mass (hypertrophy),[1] MHC composition,[1] and mitochondrial protein expression are associated with the changes in CaMKII phosphorylation. Finally, we sought to identify whether SLN expression in skeletal muscle is increased by strength training and if it associates with muscle hypertrophy, as previously reported in rodents.

We hypothesized that CaMKII phosphorylation would increase more in subjects training with greater fatigue (reduction in power) allowed during each set, stimulating a IIX-to-IIA shift in MHC expression, muscle hypertrophy, and the expression of mitochondrial proteins. We also hypothesized that training with more fatigue would be associated with a higher increase in SLN expression.

2 | MATERIALS AND METHODS

2.1 | Participants

This study is an extension of our previous study (see Pareja-Blanco et al[1]). The characteristics of the two groups analyzed, experimental design and general procedures have been reported previously.[1] Twenty-four young and healthy men volunteered to participate in this study. Their initial one-repetition maximum (1RM) strength for the full (deep) squat (SQ) exercise was 1.41 ± 0.19 kg body mass^{-1}. All subjects were physically active sports science students with a resistance training experience ranging from 1.5 to 4 years (1-3 sessions/wk) and were accustomed to performing the SQ with a correct technique. Subjects were randomly assigned to one of two groups only differing in the magnitude of repetition velocity loss allowed within each set during training: 20% (VL20; n = 12) or 40% (VL40; n = 12). Consequently, training volume was larger for VL40 than VL20 (310.5 ± 42.0 vs 185.9 ± 22.2 total repetitions, respectively).

Two subjects from the VL40 dropped out, one of them due to an injury not related to the training intervention. All subjects were informed about the experimental procedures and potential risks before they provided their written informed consent. The study was approved by the institutional review committee of

Pablo de Olavide University and was performed in accordance with the Declaration of Helsinki. Subjects refrained from taking drugs, medications, or dietary supplements during the study and had not been on medical treatments or taken supplements for at least two months before the start of the study.

2.2 | Study design

Subjects trained twice a week (48-72 hours apart) during 8 weeks for a total of 16 sessions. A progressive resistance training program which comprised only the SQ exercise was used (see Pareja-Blanco et al[1] for a thorough description of the training program). Briefly, the two groups trained at the same relative intensity (%1RM), which was increased gradually from 70% to 85% of 1RM between the 1st and 16th training sessions) but differed in the maximum percentage velocity loss allowed in each exercise set (20% vs 40%, of the mean propulsive velocity [MPV] attained in the fastest repetition of the set). The 40% velocity loss limit for the SQ exercise allowed performing repetitions to, or close to, failure, while the 20% velocity loss limit is reached when the athlete has performed ~50% of the possible repetitions per set.[30] When the corresponding target velocity loss limit was exceeded, the set was finished. Consequently, the VL40 groups performed almost twice as many repetitions as the VL20 group (310.5 ± 42.0 vs 185.9 ± 22.2 total repetitions, respectively). All training sessions were supervised and carried out in the laboratory at the same time of the day (±1 hour) for each subject and under controlled environmental conditions (20°C and 60% humidity). Subjects were asked not to engage in any other type of strenuous physical activity, exercise training, or sports competition for the duration of the investigation. The use of any putative recovery treatments, nutritional, and anti-inflammatory supplements was prohibited during the study. Training compliance was 100% of all sessions for the subjects that completed the intervention. The two groups were assessed on two occasions: 48 hours before (Pre) and 48 hours after (Post) the 8-week training intervention. Both pre- and post-training testing took place in one session in the following order: a 20-m running sprint (data not reported here), countermovement vertical jump (CMJ), a progressive loading test in the SQ exercise and the fatigue test.[1] Five minutes after the SQ progressive loading test, the subjects performed the fatigue test described below.

2.3 | Fatigue test

This test was performed with the same absolute load (kg) at pre- and post-training measurements. This load corresponded to 60% of the 1RM attained at pre-training. During each repetition, volunteers were required to execute the eccentric phase in a controlled manner and performing the concentric phase at maximal intended velocity. Subjects were required to complete as many repetitions as possible until the MPV felt below 0.5 m s^{-1}. The following variables derived from this test were used for analysis: (a) the maximal number of repetitions and (b) the average MPV attained against the same number of repetitions to pre- and post-training. The MPV was defined as the average bar vertical velocity during the propulsive phase, which is defined as the portion of the concentric phase where acceleration is greater than −9.8 m s^{-2}.[31] Sprint, CMJ, and SQ progressive loading tests were performed as described previously.[1]

2.4 | Muscle biopsies, protein extraction, Western blotting, and MHC composition

Subjects reported to the laboratory after a 12 hours overnight fast, two days after the strength tests. The dinner preceding the biopsy day was standardized for pre- and post-training. After 10 minutes of rest in the supine position, the skin over the middle portion of the *vastus lateralis* (*VL*) muscle was anaesthetized with 2% lidocaine (2 mL). After that, muscle biopsy samples (80-160 mg) were obtained from the superficial region (2-3 cm depth) using the Bergstrom technique with suction. The leg to be biopsied was assigned randomly. The same leg was biopsied after training, 2 cm more proximal than the pre-training biopsy.

The same medical doctor performed all muscle biopsies, and great care was taken to standardize the site and depth of the sample. Upon collection, muscle samples were dried on a sterile gauze, carefully freed from visible blood, connective tissue and fat, and fractionated into two pieces. The first half was mounted on cork blocks with the use of Tissue-Tek OCT™ embedding medium and orientated so that myofibers could be cut transversely. Specimens were frozen by 10-15 seconds immersion in isopentane pre-cooled in liquid nitrogen. The other piece of the muscle biopsy was immediately frozen in liquid nitrogen. Both biopsy pieces were stored at −80°C until analyzed.

Whole skeletal muscle lysates were prepared as described previously[32] and total protein content quantified using the bicinchoninic acid assay.[33] In brief, ~10 mg of muscle was grinded by stainless steel balls during one minute in a Mikro-Dismembrator S (Sartorius) and immediately homogenized in urea lysis buffer (6 mol/L urea, 1% SDS) and 50× Complete protease inhibitor and 10× PhosSTOP phosphatase inhibitor cocktails (Roche). The concentration of protein was adjusted to 2.8 μg/μL in all muscle extracts. 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 mmol/L Tris-HCl, pH 6.8, 5.9% SDS, 25.5% glycerol, 15% β-mercaptoethanol-bromophenol blue).

For Western blotting, the optimal amounts of protein loaded and antibodies were determined using a gradient of protein extracts ranging 2.5-30 μg and the linear relationship between the total protein loaded and the optical band density. Following assurance of linearity within this range, equal amounts of protein (5-15 μg) of each sample were electrophoresed with SDS-PAGE using the system of Laemmli[34] and transferred to Immun-Blot PVDF Membranes for Protein Blotting (Bio-Rad Laboratories). For SLN protein expression determination, two samples (pre- and post-training) from each subject (independently of the group) were run in duplicate together with two control samples (prepared from healthy human skeletal muscle). In the case of phospho-Thr^{287}-CaMKII, total CaMKII, and oxidative phosphorylation (OXPHOS) proteins, two samples (pre- and post-training) were run together with three control samples. All gels included two protein ladders (prestained protein standards All Blue; Bio-Rad Laboratories). Samples from the same subject were loaded onto the same gel with two or three control samples for quality check. The membranes were blocked for one hour in 4% bovine serum albumin or 5% nonfat dry milk diluted in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) (BSA or Blotto blocking buffers). Antibodies were diluted in BSA (phosphorylated and total CaMKII and SLN) or Blotto blocking buffer (OXPHOS) at a 1:2000 concentration and incubated overnight at 4°C. After incubation with primary antibodies, the membranes were incubated with an HRP-conjugated antibody (diluted 1:5000 in Blotto blocking buffer in all instances) for 1 hour at room temperature and subsequent chemiluminescent visualization with Clarity™ Western ECL Substrate (Bio-Rad Laboratories) using a ChemiDoc™ Touch Imaging System (Bio-Rad Laboratories). Protein ladders were captured under white light immediately following chemiluminescent imaging with the membranes positioned in the same position. Finally, densitometry band quantification was performed with the Image Lab© software 5.2.1 (Bio-Rad Laboratories). All membranes were stained with Reactive Brown 10 (Sigma-Aldrich) to control for differences in loading and transfer efficiency,[35] and the bands captured with the ChemiDoc™ Touch Imaging System and quantified with the Image Lab 5.2.1 software. Since loading was homogeneous in all membranes, no further corrections were performed. MHC composition was assessed as previously reported in the same extracts used for Western blotting.[1] Identification of CaMKII isoforms was based on their electrophoretic mobility (β_M: 72.7 kDa; δ_A: 60.0 kDa; γ: 56.0-62.2 kDa; and δ_D: 58.4 kDa), their predicted molecular weights (MW) and their reactivity with the antibodies for the total and phosphorylated forms of the enzyme, and an antibody specific for the δ isoforms.[15,19] The band showing the lowest MW in the membranes was considered as δ_D, the band appearing immediately above δ_D, which had a somewhat higher MW, was considered as γ (which was not marked by the antibody specific for the δ isoforms), the small thin band with a slightly higher MW just above the γ band was considered as δ_A band, and the band with the highest MW was considered as the β_M isoform. The results are reported and analyzed for each CaMKII isoform band separately or for combinations of bands, as done by others.[20,36-38]

The corresponding catalogue numbers of primary antibodies were as follows: anti-phospho-CaMKII (Thr^{287}), no. 12716 and anti-CaMKII, no. 4436, were purchased from Cell Signaling Technology. Anti-sarcolipin, no. ABT13 was purchased from EMD-Millipore. The anti-OXPHOS antibody was an optimized premixed cocktail antibody (total OXPHOS human antibody cocktail, no. ab110411) purchased from Abcam which targets 5 oxidative phosphorylation proteins: Complex I subunit NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8 (NDUFB8) (no. ab110242), Complex II subunit succinate dehydrogenase [ubiquinone] iron-sulfur subunit (SDHB) (no. ab14714), Complex III subunit cytochrome b-c1 complex subunit 2 (UQCRC2) (no. ab14745), Complex IV subunit cytochrome c oxidase subunit 2 (COX-II) (no. ab110258), and ATP synthase F1 subunit alpha (ATP5A) (no. ab14748). A CaMKII δ isoform-specific antibody (anti- CaMKII delta isoform no. A010-55AP; Badrilla) was used to distinguish between the γ and δ isoforms. The secondary HRP-conjugated goat anti-rabbit (no. 111-035-144) and goat anti-mouse (no. 115-035-003) antibodies were purchased from Jackson ImmunoResearch.

2.5 | Muscle volume and cross-sectional area determination

Magnetic resonance imaging 1.5-T scanner (General Electric, Milwaukee, Wisconsin, USA) was used to determine the total volume of the quadriceps muscle of both extremities as previously described.[1] The muscle cross-sectional area was determined using adenosine triphosphatase (ATPase) histochemical analysis, microscope visualization, and image analysis of fiber boundaries as described in detail elsewhere.[1]

2.6 | Statistical analyses

Descriptive values are presented as means ± standard deviations (SD). The normality of distribution of the variables was assessed with the Shapiro-Wilk test and, when necessary, data were transformed logarithmically before analysis. Homogeneity of variance was checked with the Levene's test. Data were analyzed using a repeated-measures ANOVA including one within-subjects factor (time, with two levels: pre- and post-training) and one between-subjects factor (training program, with two levels: VL20 and VL40). Associations between variables were examined by Pearson's correlation

analysis. Statistical significance was set at $P < .05$. All statistical analyses were performed using SPSS software version v.18 for Windows (SPSS Inc).

3 | RESULTS

The effects of the strength training program have been reported previously.[1] Briefly, both groups had similar improvements in 1RM, but the enhancement in jumping height performance was higher when training with less fatigue in the set (VL20) than when training close to failure (VL40). Training close to failure elicited greater hypertrophy of *vastus lateralis* and *intermedius* (+9%) than training with less fatigue (+3.4%). MHC-IIX percentage was reduced in VL40 but not in VL20.[1] Likewise, training close to failure elicited greater increase of *vastus lateralis* CSA at the level where the muscle biopsies were taken (+11.0%, from 29.3 ± 4.4 to 32.5 ± 5.1 cm^2, pre- and post-training, respectively, $P < .001$) than training with less fatigue (+0.7%, from 26.6 ± 2.9 to 26.8 ± 3.6 cm^2, pre- and post-training, respectively, $P = .67$) (time effect $P < .001$, group × time interaction $P < .001$).[1]

After training, the total expression of CaMKII protein, which includes all the isoforms detected, was increased by 20% (time effect $P < .001$, group × time interaction $P = .70$) (Figure 1A, B). This effect was due to a 40% higher expression of the CaMKII δ_D isoform after training (time effect $P < .001$, group × time interaction $P = .45$) (Figure 2D), while no statistically significant changes were observed in the expression of the other isoforms (Figure 2A-C).

After training, phospho-Thr^{287}-CaMKII protein expression was 20% higher than before training when all isoforms were analyzed conjointly (time effect $P = .055$, group × time interaction $P = .039$) (Figure 1C,D). Nevertheless, the Thr^{287}-CaMKII δ_D was increased by 89% in VL40 while it remained unchanged in VL20 (time effect $P = .01$, group × time interaction $P = .024$) (Figure 2H). No statistically significant changes were observed in protein expression of the Thr^{287}-CaMKII β, δ_A, and γ isoforms (Figure 2E-G), nor in the corresponding fractional phosphorylation ratios. The fractional phosphorylation of δ_D isoform was reduced by 27% in VL20, while it increased not significantly in VL40 (time effect $P = .74$, group × time interaction $P = .048$) (Figure 3). No significant changes

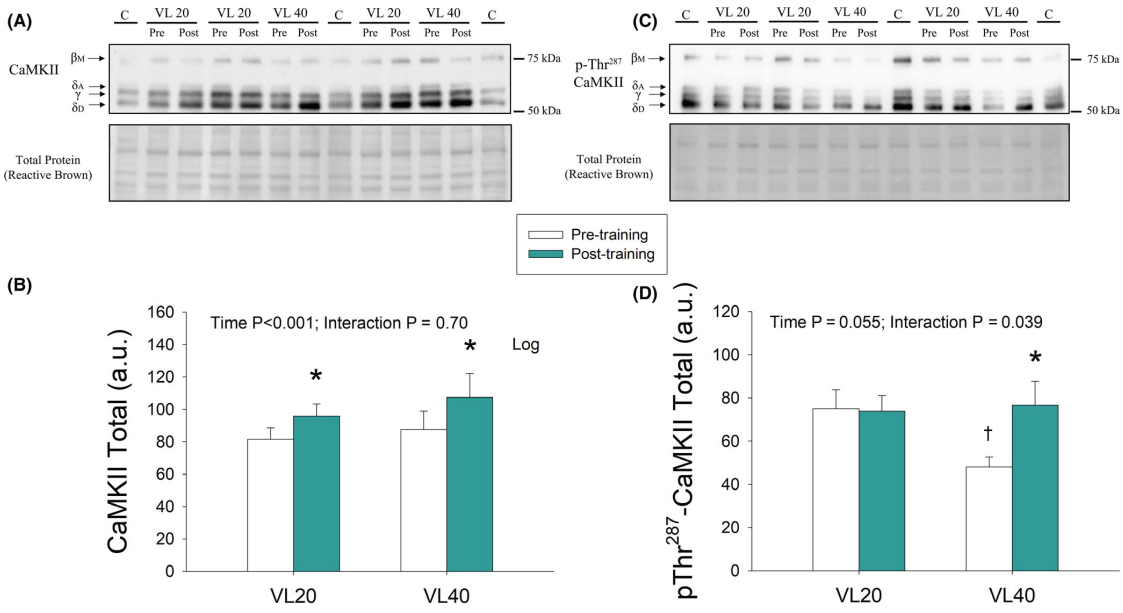

FIGURE 1 Skeletal muscle protein expression levels of total and phosphorylated CaMKII including all isoforms before (pre-training) and after (post-training) an 8-week velocity-based resistance training program using the full squat exercise in two groups of subjects allowing an intra-set repetition velocity loss of 20% (VL20, n = 12) or 40% (VL40, n = 10). (A) and (C): representative immunoblot images and total amount of protein loaded (Reactive Brown staining) of total CaMKII and phospho-Thr^{287}-CaMKII, respectively, from five participants in the study belonging to both training groups at pre- and post-training. A control human sample was run on triplicate on each gel as quality control. Estimated molecular weights are indicated on the right side of the blot. (B) and (D): protein expression levels of total CaMKII and phospho-Thr^{287}-CaMKII, respectively, with all isoforms analyzed conjointly. The values shown are means ± standard errors and expressed in arbitrary units (a.u.). *Significantly different from pre-training, $P < .05$; †Significantly different from VL20 at the same time point, $P < .05$

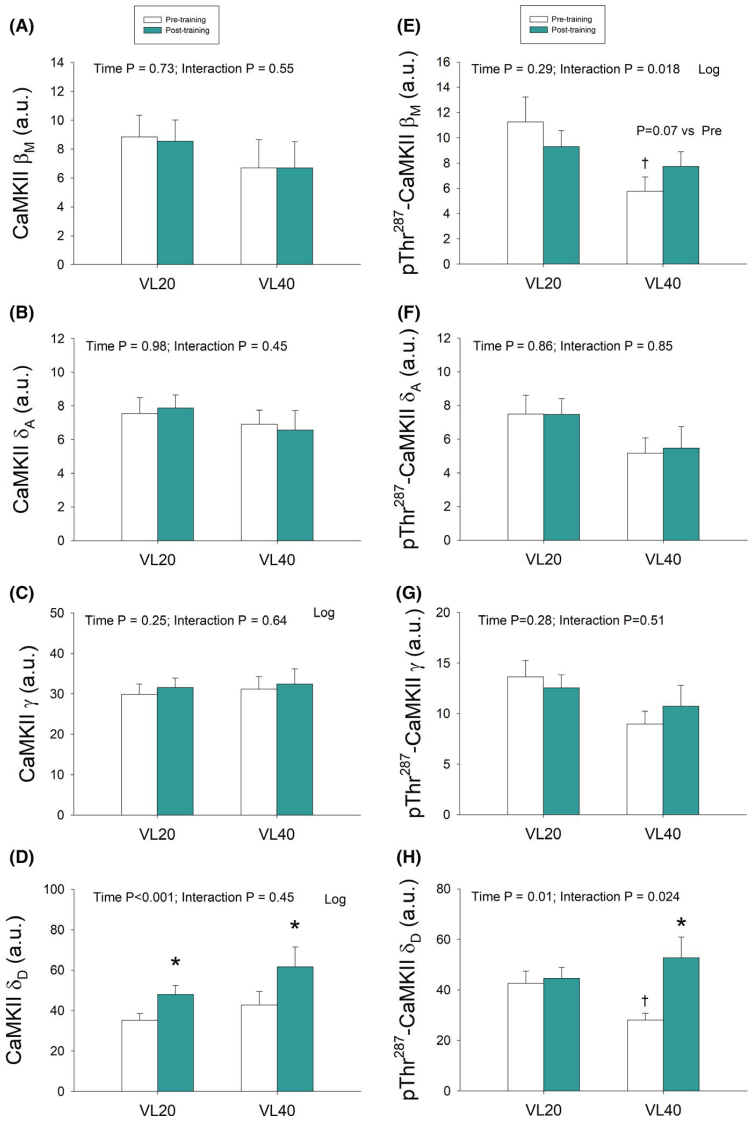

FIGURE 2 Skeletal muscle protein expression levels of total and phosphorylated CaMKII isoforms β_M, δ_A, γ, and δ_D analyzed separately before (pre-training) and after (post-training) an 8-week velocity-based resistance training program using the full squat exercise in two groups of subjects allowing an intra-set repetition velocity loss of 20% (VL20, n = 12) or 40% (VL40, n = 10). Left panel, total CaMKII expression: (A) CaMKII β_M, (B) CaMKII δ_A, (C) CaMKII γ, and (D) CaMKII δ_D. Right panel, phosphorylated form: (E) Thr^{287}-CaMKII β_M, (F) Thr^{287}-CaMKII δ_A, (G) Thr^{287}-CaMKII γ, (H) Thr^{287}-CaMKII δ_D. See the top of Figure 1 for representative immunoblots. The values shown are means ± standard errors and expressed in arbitrary units (a.u.). *Significantly different from pre-training, $P < .05$. † Significantly different from VL20 at the same time point, $P < .05$. Log: statistical analysis performed with logarithmically transformed data

were observed in the fractional phosphorylation of the other isoforms.

There was a positive association between the change in the expression of phospho-Thr^{287}-CaMKII δ_D and the total number of repetitions performed during training ($r = 0.59$, $P = .004$, n = 22) (Figure 4A), and the degree of *vastus lateralis* and *intermedius* hypertrophy ($r = 0.48$, $P = .03$, n = 22) (Figure 4B) as well as with the degree of *quadriceps* muscle hypertrophy ($r = 0.43$, $P = .048$, n = 22); while the association was negative with the change in MHC-IIX ($r = -0.72$, $P < .001$, n = 22) (Figure 4C). These associations depended specially on the changes observed in the VL40 group ($r = 0.59$, $P = .07$; $r = 0.72$, $P = .02$, and $r = -0.86$, $P = .002$, n = 10, for repetitions performed during training, degree of *vastus lateralis* and *intermedius* hypertrophy, and change in MHC-IIX, respectively).

Sarcolipin expression was increased by 33% in VL20 ($P = .048$) while the 11% reduction observed in VL40 did not reach statistical significance ($P = .38$, group × time interaction $P = .04$) (Figure 5A,B). No association was observed between the changes in muscle volume and those of SLN expression when all subjects were analyzed together ($r = -0.07$, $P = .76$, n = 22) or each group separately ($r = 0.30$, $P = .35$, n = 12; and $r = -0.28$, $P = .44$, n = 10, for the VL20 and VL40, respectively). Similar results were obtained between the changes in muscle CSA from the

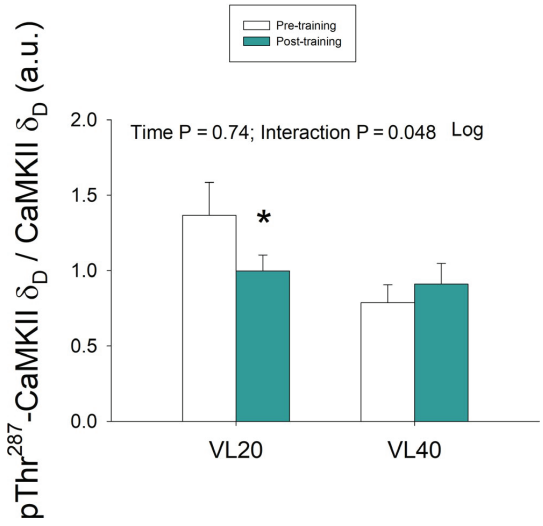

FIGURE 3 Phosphorylation fraction of the CaMKII δ_D isoform (phospho-Thr^{287}-CaMKII δ_D/CaMKII δ_D) before (pre-training) and after (post-training) an 8-week velocity-based resistance training program using the full squat exercise in two groups of subjects allowing an intra-set repetition velocity loss of 20% (VL20, n = 12) or 40% (VL40, n = 10). *Significantly different from pre-training, $P < .05$. Log: statistical analysis performed with logarithmically transformed data

muscle biopsies (previously reported[1]) and SLN expression when all subjects were analyzed together ($r = -0.20$, $P = .37$, n = 22) or each group separately ($r = 0.02$, $P = .95$, n = 12; and $r = -0.55$, $P = .07$, n = 10, for the VL20 and VL40, respectively).

There was a negative association between the changes in SLN and the changes in phospho-Thr^{287}-CaMKII δ_D ($r = -0.50$, $P = .018$, n = 22) and a positive association between the improvements in MPV during the fatigue test and the increase in SLN with strength training ($r = 0.42$, $P = .05$, n = 22). No associations were observed between the change in SLN expression on one side and the improvement in CMJ height ($r = 0.29$, $P = .18$, n = 22) and 1RM ($r = 0.35$, $P = .11$, n = 22). Before training, no association was observed between the relative cross-sectional area of type I fibers and SLN expression ($r = -0.37$, $P = .09$, n = 22) nor between the MHC-I percentage and SLN expression ($r = -0.28$, $P = .21$, n = 22).

In general, no significant changes were observed in OXPHOS proteins, except NDUFB8 which was reduced after training by 22% (time effect $P = .03$, group × time interaction $P = .26$) (Figure 6A-F). No association was observed between the changes in OXPHOS proteins and phospho-Thr^{287}-CaMKII isoforms.

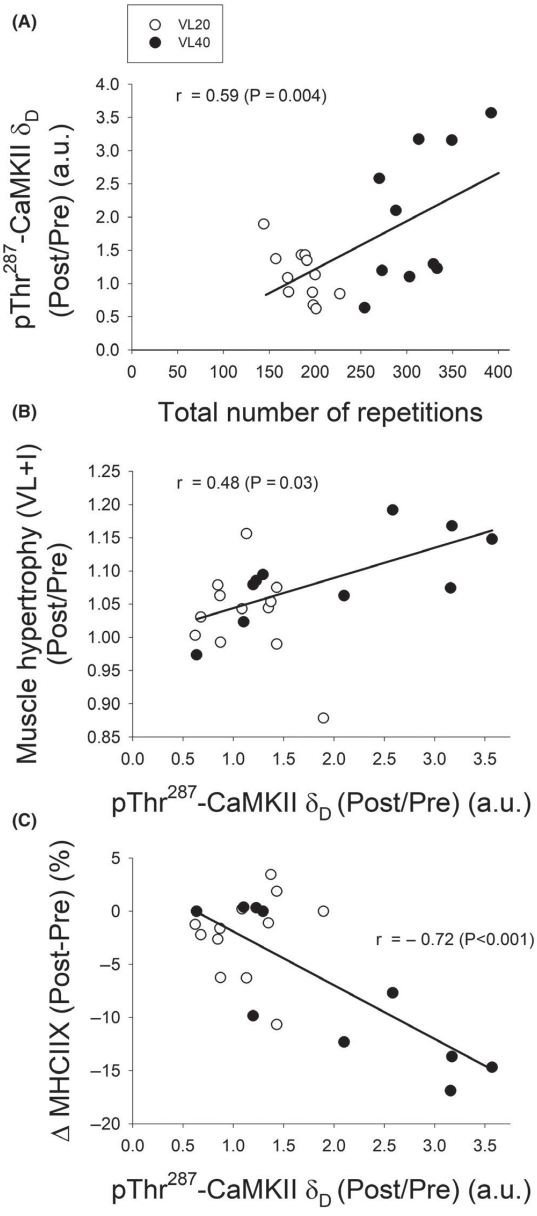

FIGURE 4 Relationship between the change in phospho-Thr^{287}-CaMKII δ_D and (A) the number of repetitions carried out during the training program, (B) the degree of muscle hypertrophy of m. *vastus lateralis* and *intermedius* (sum of both extremities) and (C) the delta change in myosin heavy chain IIX percentage (MHC-IIX) after 8 weeks of strength training allowing a 20% loss in mean velocity of the propulsive phase in the set (group VL20, n = 12, plain circles) or 40% (group VL40, n = 10, black circles). Note that in panel (B) there is an outlier, after removing this outlier the correlation improved to $r = 0.65$ ($P < 001$). Log: statistical analysis performed with logarithmically transformed data

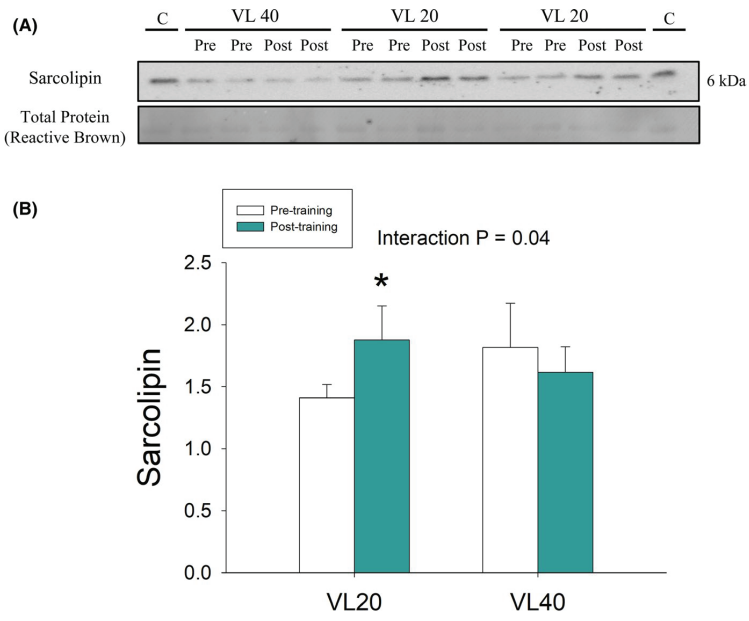

FIGURE 5 Skeletal muscle protein expression levels of sarcolipin before (pre-training) and after (post-training) an 8-week velocity-based resistance training program using the full squat exercise in two groups of subjects allowing an intra-set repetition velocity loss of 20% (VL20, n = 12) or 40% (VL40, n = 10). (A) Protein expression levels of sarcolipin, (B) Representative immunoblot image and total amount of protein loaded (Reactive Brown staining) in three subjects pre- and post-training. Duplicate assays pre- and post-training were included for all samples. A control human sample was run on duplicate on each gel as a loading control. Estimated molecular weights are indicated on the right side of the blot. The values shown are means ± standard errors and expressed in arbitrary units (a.u.). *Significantly different from pre-training, $P < .05$

4 | DISCUSSION

This study shows that strength training induces specific adaptations in basal levels of CaMKII and SLN protein depending on the level of fatigue allowed in the sets. Both types of training resulted in muscle hypertrophy and increased expression of CaMKII, particularly of the isoform δ_D. However, basal phospho-Thr^{287}-CaMKII phosphorylation levels responded in an isoform- and training-specific way, such that Thr^{287}-CaMKII δ_D was increased only when training with more fatigue, and a similar trend was observed for Thr^{287}-CaMKII β_M isoform. The fact that there was a positive association between the total number of repetitions carried out and the change in basal Thr^{287}-CaMKII δ_D phosphorylation is compatible with a cause-and-effect relationship. The latter is also supported by the observed association between the increase in Thr^{287}-CaMKII δ_D phosphorylation and the reduction in MHC-IIX expression, and the increase in muscle mass elicited by the training program. In contrast with our hypothesis, the changes in SLN expression were not associated with the changes in muscle mass, MHC-IIX percentage, or strength, while a negative association was observed between SLN expression and the mitochondrial proteins ATP5A and SDHB, and with the changes in phospho-Thr^{287}-CaMKII δ_D.

4.1 | Muscle phenotype changes with strength training and CaMKII

Although little is known regarding the specific role played by each isoform in human skeletal muscle, it has been reported that the protein expression of the CaMKII δ/γ isoforms is increased after sprint training[20] and endurance training[39,40] in humans, as observed in the present study. The increase in basal CaMKII δ/γ expression has been strongly associated with the increase in maximal CaMKII enzymatic activity and basal Thr^{287}-CaMKII protein expression.[40]

Also in agreement with our results, a significant increase in the total amount of CaMKII has been reported a few days after a blood flow-restricted strength training program,[41] although the phosphorylated form of CaMKII was not assessed in that study.[41] Likewise, rodent experiments have shown that a higher CaMKII activity promotes muscle hypertrophy[11-13] and a fast-to-slow MHC shift.[2] Collectively, the present investigation and these studies show that the basal protein expression levels of CaMKII are increased in response to diverse training stimuli. However, in these studies, the δ and γ isoform were analyzed conjointly[20,39,40] precluding any conclusion regarding the specific CaMKII isoform accounting for the reported effects.

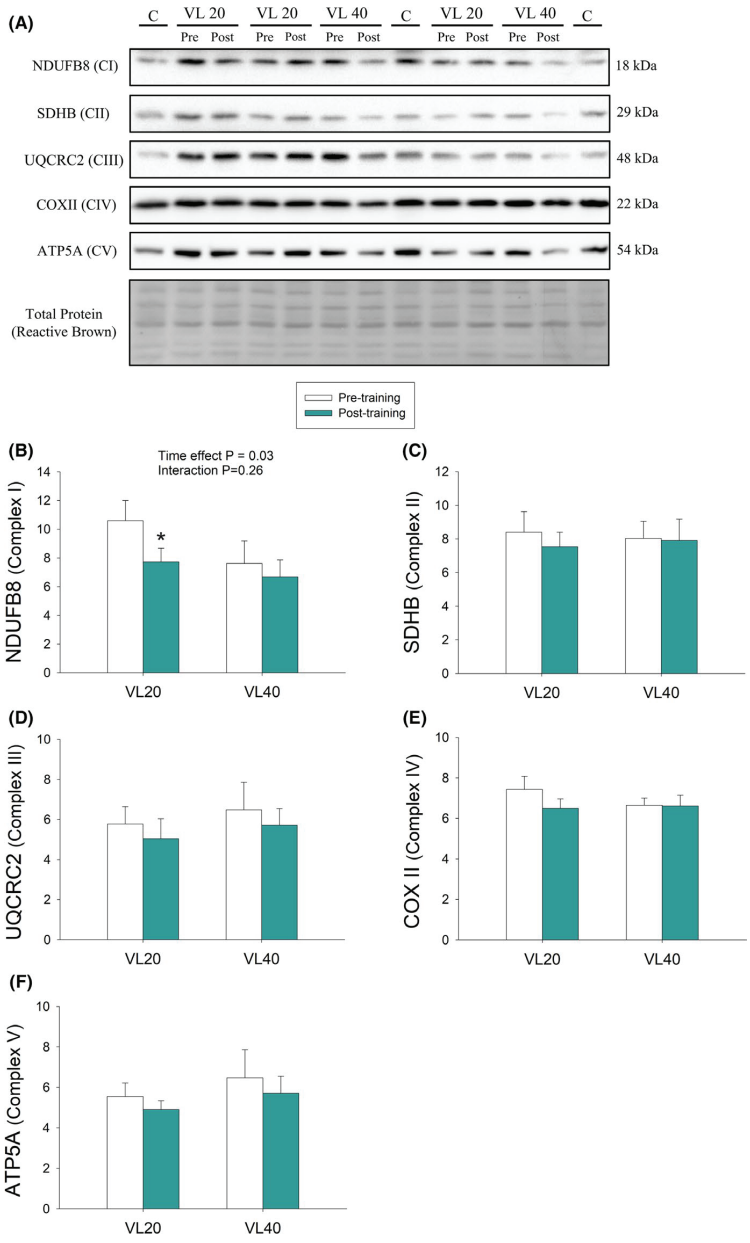

FIGURE 6 Skeletal muscle protein expression of different subunits of OXPHOS complexes: (A) Representative immunoblot images and total amount of protein loaded (Reactive Brown staining) from five participants in the study belonging to both training groups at pre- and post-training. A control human sample was run on triplicate on each gel as a loading control. Estimated molecular weights are indicated on the right side of the blot, (B) NDUFB8 (Complex I), (C) SDHB (Complex II), (D) UQCRC2 (Complex III), (E) COX-II (Complex IV), and (F) ATP5A (Complex V) before (pre-training) and after (post-training) an 8-week velocity-based resistance training program using the full squat exercise in two groups of subjects allowing an intra-set repetition velocity loss of 20% (VL20, n = 12) or 40% (VL40, n = 10). The values shown are means ± standard errors and expressed in arbitrary units (a.u.). *Significantly different from pre-training, $P < .05$

Thr^{287}-CaMKII phosphorylation is more pronounced right after sprint exercise,[42] prolonged-moderate intensity exercise,[43] and workouts eliciting more metabolite accumulation,[44] than with less severe exercise.[43,44] Strength training to failure is accompanied by higher activation of the anaerobic metabolism[45] and possibly greater Ca^{2+} transients than training with considerably less fatigue in each set.[2] The latter, combined with the higher oxidative stress associated with fatiguing contractions, should have facilitated the activation of CaMKII.[46,47] These facts could explain the observed associations between the change in basal Thr^{287}-CaMKII δ_D phosphorylation and the total number of repetitions completed during the training intervention, the reduction in MHC-IIX expression, and the increase in muscle volume elicited by the training program. Likewise, since CaMKII promotes recovery of muscle pH via activation of the Na^+/H^+ exchanger,[48] increased phospho-Thr^{287}-CaMKII could indirectly promote adaptive changes that could accelerate the recovery between sets.

An isoform and muscle type-specific Thr^{287}-CaMKII phosphorylation pattern have been reported in rodent skeletal muscle depending on stimulation frequency with special responsiveness of the δ_A isoform,[38] which has been implicated in the regulation of gene expression[49] via histone deacetylase 4 (HDAC4) phosphorylation to promote a fast-to-slow transition.[3] However, no association was observed here between the changes in the basal level of CaMKII phosphorylation and the expression of OXPHOS proteins, indicating that just a moderate rise in basal Thr^{287}-CaMKII phosphorylation may not suffice to induce an increase of mitochondrial protein.

4.2 | Sarcolipin protein expression is increased by strength training with less fatigue and is not associated with muscle hypertrophy

Animal studies have suggested that SLN may also determine the muscle phenotype by modulating the resting sarcoplasmic [Ca^{2+}].[24,29] In the present research, no significant changes in SLN protein expression were observed in the group experiencing more muscle hypertrophy, and no relationship was found between muscle hypertrophy and SLN expression. This is at odds with some previous studies in rodents.[24,29] Riedl et al.[29] overloaded the *plantaris* muscle of mice by synergist ablation of the *soleus* and *gastrocnemius*. Consequently, the *plantaris* muscle doubled its mass in 14 days, likely because this model of overloading may cause muscle hyperplasia in mice[24] and also due to potential inaccuracies in the dissection.[24] This level of hypertrophy was associated with a ~20- to 40-fold increase in *Sln* mRNA, in part explained by the fact that murine adult *plantaris* muscles do not typically express SLN protein.[24] Using the same experimental model, Fajardo et al.[24] determined the changes in SLN protein expression in the overloaded *plantaris* and the unloaded/tenotomized *soleus* muscle of *Sln-null* (*Sln*$^{-/-}$) and WT mice. In Fajardo et al.[24] experiments, the compensatory hypertrophy of the *plantaris* was more modest and similar in WT and *Sln*$^{-/-}$ mice (~20%-25%). SLN protein expression increased 14-fold in the unloaded *soleus* of the WT and was accompanied by a slow-to-fast fiber type shift and atrophy, being these effects more pronounced in the *Sln*$^{-/-}$ mice. Thus, the two studies published in mice indicate that SLN protein expression occurs with overloading and unloading. This apparent counterintuitive adaptation could be due to the fact that immobilization and disuse cause inflammation, increase oxidative stress and elevate resting sarcoplasmic [Ca^{2+}].[50-53]

In the present investigation, the highest level of *vastus lateralis* muscle hypertrophy was 19%, but SLN protein expression was reduced by 16% in the same subject. The apparent discrepancy between our findings and those obtained in mice may be due to the extreme nature of the intervention used in animal studies (gene manipulation and compensatory hypertrophy after surgery), as well as differences between species. In fact, nucleotide identity between human and mice SLN cDNAs is no more than 44%; thus, some functional between-species differences are expectable.[54]

The changes in SLN expression were not associated with the improvements in either maximal strength (1RM) nor vertical jump performance. Nonetheless, the improvement in jumping performance was greater in the group that experienced an increase in SLN protein expression. A weak positive association was observed between the changes in SLN and the improvement in the fatigue test. Rodent experiments have shown that SLN overexpression stimulates mitochondrial biogenesis and oxidative metabolism,[28] resulting in improved endurance capacity.[27] Even though SLN expression was increased in VL20, this was not accompanied by an increase in OXPHOS proteins. This indicates that the small changes elicited by strength training in SLN proteins levels in human skeletal muscle do not seem to play a direct or indirect role in the regulation of mitochondrial biogenesis. However, we cannot rule out a transient effect that may have escaped detection given the fact that we only did single-point measurements under basal conditions. Likely, a higher level or more sustained activation of CaMKII is required to induce mitochondrial biogenesis with strength training.

Although overexpression of SLN induces a fast-to-slow shift in muscle fiber type in rodents,[24,28] no such effect was observed in the present investigation. The reduction in MHC-IIX was statistically significant and larger in the VL40 group, which trained with more fatigue and did not experience significant changes in SLN expression. In agreement with Odermatt et al.,[54] SLN expression was enhanced after training in the VL20 group, which maintained a faster muscle fiber phenotype. Since SLN is more abundantly expressed in type II fibers, a muscle fiber type shift from type II to I could, in theory, explain a reduction in SLN in VL40 (if we assume a type II error in VL40 SLN results). However, no significant changes were observed in MHC-I % with training in either group, that is, such a type II to I shift did not occur.[1] Moreover, in VL40 there was significant hypertrophy of type II fibers,[1] which should have been associated with increased SLN rather than the observed reduction. This indicates that the changes in SLN expression observed herein cannot be explained by a training-induced shift in muscle fiber types. We also examined whether SLN changes were associated with the changes in muscle volume (MRI) and CSA (histology) in the VL20 group, to avoid the potential confounding effects of the changes in MHC composition observed in the VL40 group. This analysis also yielded negative results. Thus, SLN may change in response to certain types of exercise, but its physiological role in humans remains uncertain.

In summary, the level of fatigue permitted during the sets is a crucial variable determining the adaptations to strength training, as recently confirmed[55,56] when all repetitions are performed with the maximal intended velocity during the concentric phase. CaMKII δ_D increases in response to strength training and executing repetitions within each set close to failure is associated with elevated resting Thr^{287}-CaMKII δ_D phosphorylation levels, which seem dependent on the total number of repetitions performed during the training program. The increase in Thr^{287}-CaMKII δ_D phosphorylation is positively associated with muscle hypertrophy but also with the reduction in MHC-IIX expression. The latter could limit the enhancement of muscle power with training. Sarcolipin protein expression increases in response to strength training, but this effect is only observed when the level of fatigue allowed in the set is low. An inverse association has been revealed between the changes in basal Thr^{287}-CaMKII δ_D phosphorylation and those of sarcolipin, which is compatible with the existence of a negative loop limiting an excessive expression of SLN after fatiguing muscle contractions. Lastly, changes in basal SLN protein expression do not seem to play a role in muscle hypertrophy in human skeletal muscle, at least under physiological conditions.

5 | PERSPECTIVE

The amount of fatigue allowed during strength training emerges as a critical variable influencing skeletal muscle adaptations and performance improvements through specific signaling events. Future studies should examine whether similar effects are observed in other populations of different sex, age, and fitness. It will also be interesting to obtain additional data during the training program and the detraining phase. Since this is the first time that the effects of two different strength training programs on CaMKII isoforms and SLN protein expression in human skeletal muscle have been tested, our findings will need independent confirmation by other researchers. We induced only modest changes in SLN, which were similar to those observed after sprint training[57]; therefore, it remains unknown whether larger changes in SLN expression could produce adaptations similar to those described in mice overexpressing SLN. In the present investigation, there was a negative association between the changes phospho-Thr^{287}-CaMKII δ_D and those of SLN suggesting a potential negative feedback loop of phospho-Thr^{287}-CaMKII δ_D on SLN expression that requires testing in animal and cellular models.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

JJGB conceived and designed the training study with assistance by FPB, DRR, JSM, CD, and JAC. JJGB, FPB, and JAC collected data. JJGB, FPB, DRR, MGR, MMC, MMR, AGS, DMA, JSM, and CD analyzed and interpreted data. MMC, MGR, MMR, and JAC drafted the manuscript. MMR prepared all figures. All authors read and approved the final version of the manuscript.

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Activation of macroautophagy and chaperone-mediated autophagy in human skeletal muscle by high-intensity exercise in normoxia and hypoxia and after recovery with or without post-exercise ischemia

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ABSTRACT

Autophagy is essential for the adaptive response to exercise and physiological skeletal muscle functionality. However, the mechanisms leading to the activation of macroautophagy and chaperone-mediated autophagy in human skeletal muscle in response to high-intensity exercise remain elusive. Our findings demonstrate that macroautophagy and chaperone-mediated autophagy are stimulated by high-intensity exercise in normoxia (P_IO_2: 143 mmHg) and severe acute hypoxia (P_IO_2: 73 mmHg) in healthy humans. High-intensity exercise induces macroautophagy initiation through AMPKα phosphorylation, which phosphorylates and activates ULK1. ULK1 phosphorylates BECN1 at Ser^{15}, eliciting the dissociation of BECN1-BCL2 crucial for phagophore formation. Besides, high-intensity exercise elevates the LC3B-II:LC3B–I ratio, reduces total SQSTM1/p62 levels, and induces *p*-Ser^{349} SQSTM1/p62 phosphorylation, suggesting heightened autophagosome degradation. PHAF1/MYTHO, a novel macroautophagy biomarker, is highly upregulated in response to high-intensity exercise. The latter is accompanied by elevated LAMP2A expression, indicating chaperone-mediated autophagy activation regardless of post-exercise HSPA8/HSC70 downregulation. Despite increased glycolytic metabolism, severe acute hypoxia does not exacerbate the autophagy signaling response. Signaling changes revert within 1 min of recovery with free circulation, while the application of immediate post-exercise ischemia impedes recovery. Our study concludes that macroautophagy and chaperone-mediated autophagy pathways are strongly activated by high-intensity exercise, regardless of PO_2, and that oxygenation is necessary to revert these signals to pre-exercise values. PHAF1/MYTHO emerges as a pivotal exercise-responsive autophagy marker positively associated with the LC3B-II:LC3B–I ratio.

1. Introduction

Human skeletal muscle (hSKM), which accounts for approximately 40 % of body mass in healthy humans [1], transforms chemical energy into mechanical tension, enabling movement. During incremental exercise to exhaustion, the energy demand can rise above 100-fold resting levels, significantly disrupting homeostasis [2,3]. The latter is accompanied by a marked reduction of muscle oxygen pressure (PO_2) and high activation of substrate-level phosphorylation that complements oxidative energy production to match ATP resynthesis with demand. The involvement of substrate-level phosphorylation is intensified when the exercise is performed in hypoxic or ischemic conditions, causing accumulation of lactate, hydron (H^+), and inorganic phosphate (P_i), as well as exacerbating the production of reactive oxygen species (ROS) [2,3], which may lead to oxidative stress and damage. However, skeletal muscle has remarkable plasticity, which relies on tissue remodeling, enabling structural and functional adaptations to cope more efficiently

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Abbreviations:

AMPKα AMP-activated protein kinase alpha
ATP Adenosine triphosphate
AKT AKT serine/threonine kinase
BECN1 Beclin-1
BSA Bovine serum albumin
CMA Chaperone-mediated autophagy
EEF2K Eukaryotic elongation factor 2 (EEF2) kinase
EEF2 Eukaryotic elongation factor 2
FC1m 60 s after exercise cessation during recovery with free circulation
F_IO_2 Inspired oxygen fraction
FOXO Forkhead box O
GSK3B Glycogen synthase kinase 3 beta
H^+ Hydron
HIF1A Hypoxia inducible factor 1 subunit alpha
HSPA8/HSC70 Heat shock protein family A [Hsp70] member 8
Hyp Hypoxia
hSKM Human skeletal muscle
H_2O_2 Hydrogen peroxide
KEAP1 Kelch Like ECH Associated Protein 1
LAMP2A Lysosomal-associated membrane protein 2A
MAP1LC3B/LC3B Microtubule associated protein 1 light chain 3 beta
MTOR Mammalian target of rapamycin
NFE2L2/NRF2 Nuclear factor erythroid-derived 2-like 2
NF-κB Nuclear Factor-kappa Beta
Nx Normoxia
Oc1m 60 s after exercise cessation during ischemic recovery
PCr Phosphocreatine
PHAF1/MYTHO Phagophore assembly factor 1/macro-autophagy and youth optimizer
PHDs Prolyl hydroxylases
PPARGC1A/PGC1A Peroxisome proliferator gamma coactivator 1-alpha
P_i Inorganic phosphate
P_IO_2 Partial pressure of inspired O_2
PO_2 Oxygen pressure
Post 10 s after exercise cessation during ischemic recovery
Pre before exercise
ROS Reactive oxygen species
SQSTM1/p62 Sequestosome 1
SDS Sodium dodecyl sulphate
ULK1 Unc-51 like autophagy activating kinase 1
UPS Ubiquitin-proteasome system
$\dot{V}O_2$peak Peak oxygen consumption
Wmax Maximal power output at exhaustion

with homeostatic challenges. This process allows the synthesis of new proteins but also requires the renewal of damaged proteins and organelles by autophagy [4–7]. Two main types of autophagy have been characterized in skeletal muscle: macroautophagy and chaperone-mediated autophagy (CMA).

Macroautophagy is particularly active in exercising hSKM. At high intensity, the increase of the AMP:ATP ratio activates adenosine monophosphate-activated protein kinase alpha (AMPKα), which positively upregulates the expression of autophagy genes [8] and phosphorylates and activates unc-51 like autophagy activating kinase 1 (ULK1) at Ser^{556}, initiating macroautophagy [9]. Some studies indicate macroautophagy stimulation following endurance exercise [9–19] and training [12,13,16,20]. Nevertheless, the evidence for markers of autophagosome content and catabolism of cargoreceptors is less conclusive, in particular regarding the protein expression of the microtubule-associated protein 1 light chain 3 beta (MAP1LC3B/LC3B) and sequestosome 1 (SQSTM1/p62) [9,12–16,18,19,21–23]. Little is known regarding phagophore assembly factor 1/macro-autophagy and youth optimizer (PHAF1/MYTHO), a novel regulator of muscle autophagy [24,25], which remains uncharacterized in hSKM.

Chaperone-mediated autophagy is a selective mechanism to degrade soluble damaged proteins and organelles containing the KFERQ-like motif, which are recognized by heat shock protein family A [Hsp70] member 8 (HSPA8/HSC70) [26–28]. The damaged protein and HSPA8 bind to the lysosomal-associated membrane protein 2A (LAMP2A) receptor, eventually resulting in its digestion in the lysosomal lumen. This process is acutely activated in cells under oxidative stress [29] and hypoxia [30]. Reactive oxygen and nitrogen species may elicit CMA since the knockout of nuclear factor erythroid-derived 2-like 2 (NFE2L2/NRF2) reduces LAMP2A mRNA and protein levels in different cell types [31]. Nevertheless, the role and contribution of CMA in exercise-induced skeletal muscle autophagy remains unknown in humans.

The present investigation aimed to determine whether macroautophagy and chaperone-mediated autophagy are activated by acute exercise to exhaustion in hSKM, and ascertain the role played by muscle oxygenation and metabolite accumulation in autophagy signaling. We also aimed at determining the regulation of FOXO, AKT-MTOR and GSK3B under high metabolic stress and its influence on skeletal muscle autophagy signaling. Furthermore, we wanted to describe the temporal course of these signaling responses during the early recovery after exercise and post-exercise ischemia. We hypothesized that exercise would activate macroautophagy and chaperone-mediated autophagy. We also hypothesized that these effects would be more marked when exercise is performed in severe acute hypoxia and further enhanced by post-exercise ischemia. Lastly, we hypothesized that these changes would be reverted to pre-exercise levels early after the cessation of exercise in the muscles recovering with free circulation.

2. Materials and methods

A more detailed description of the methods is provided in the Supplementary Materials and Methods document.

2.1. Subjects

Eleven physically active healthy men were recruited among physical education students (means ± SD; age: 21.5 ± 2.0 years, height: 174 ± 8 cm, body mass: 72.3 ± 9.3 kg, body fat: 16.1 ± 4.9 %, VO_2max in normoxia: 50.7 ± 4.0 mL $kg^{-1}.min^{-1}$). All volunteers were briefed about the aims of the study and the potential risks before providing their written consent. The study was authorized by the Ethical Committee of the University of Las Palmas de Gran Canaria and executed by the Declaration of Helsinki.

For 24 h before the experiments, subjects were asked to avoid ingesting alcohol, caffeine, and taurine-containing drinks and exercise. Likewise, subjects were also requested to maintain a similar dinner the day before the experimental sessions.

2.2. Study design

Even though the current investigation was initially planned to elucidate the mechanisms that limit performance during whole-body exercise in humans [32–35], the study also aimed to determine the

signaling pathways regulated by autophagy and redox stress during exercise and post-exercise ischemia. The NRF2 and NF-κB mechanisms of activation/deactivation during exercise and recovery have been recently described [31,36]. The current article encompasses singular outcomes determining how autophagy signaling is regulated in hSKM during acute exercise in normoxia and severe acute hypoxia, as well as during recovery with either free circulation or ischemia.

2.3. Pre-test and familiarization

Preliminary visits to the laboratory were dedicated to assessing the body composition (dual-energy X-ray absorptiometry [Lunar iDXA] {GE Healthcare, Milwaukee, WI, USA}) [35] and peak oxygen consumption ($\dot{V}O_2$peak) in normoxia (Nx; $F_IO_2 = 0.21$; partial pressure of inspired [P_IO_2] ~143 mmHg) and hypoxia (Hyp; $F_IO_2 = 0.104$; P_IO_2 ~73 mmHg) using a ramp incremental exercise test to exhaustion on a isokinetic ergometer (Lode Excalibur Sport 925900 [Groningen, The Netherlands]) [35]. The respiratory variables were averaged every 20 s to determine the $\dot{V}O_2$peak [37].

2.4. Main experiments and biopsy sampling

A schematic representation of the experimental protocol is presented in Fig. 1. Subjects reported to the laboratory at 8.00 h after fasting from 22:00 h. Upon arrival, they rested supine for 90 min. Then, a baseline muscle biopsy (Pre Nx and Pre Hyp) was taken from one of the m. *vastus lateralis* (assigned randomly) and a cuff (SC10D Hokanson, Bellevue, Washington, USA) connected to a fast cuff inflator (Hokanson, E20 AG101) was placed unilaterally around the thigh biopsied just below the inguinal crease, as previously reported [35]. After a 2-min verification of proper readings and connections, subjects performed an incremental exercise starting with 80 W in normoxia (increasing 30 W every 2 min) and 60 W in hypoxia (increasing 20 W every 2 min) until exhaustion. For the experiment in hypoxia, the air was diluted with medical grade nitrogen, setting the P_IO_2 at ~73 mmHg (equivalent to an altitude of 5300 m above sea level) using an AltiTrainer200 (SMTEC, Nyon, Switzerland in Hyp). In the Nx trial, immediately at exhaustion, the cuff was inflated instantaneously at 300 mmHg to occlude the circulation completely. Exactly 10 s after exhaustion and ischemia, the second biopsy was taken (Post Nx), and 50 s later, while maintaining the cuff inflated, the third biopsy was performed (occluded 1 min, Oc1m Nx, Bergstrom needle introduced with 45° inclination to the head) [38]. The Oc1m biopsy permitted the evaluation of muscle signaling changes after 60-s ischemia when the energy sources depend almost exclusively on substrate-level phosphorylation (ATP, PCr, and glycolysis) while PO_2 decreased immediately to zero [35]. The incremental exercise in hypoxia was performed after similar preparations, obtaining the first baseline biopsy while the subjects breathed room air (normoxia). Then, the volunteers sat on the cycle ergometer and, after 2 min breathing hypoxic gas, the incremental exercise was started. At exhaustion, the cuff was inflated instantaneously while the volunteers were switched to breathe normoxic room air. Ten seconds later, the second biopsy was taken from the cuffed leg (Post Hyp). Immediately, the subjects were carefully moved to a stretcher while maintaining the cuff inflated to obtain the third biopsy exactly after 60 s of ischemia from the cuffed leg (Oc1m Hyp). At the same time, a fourth biopsy was obtained from the contralateral leg (FC1m), which had been recovering with free circulation in normoxia for 60 s. Thus, one leg recovered for 60 s in ischemia and the other with unrestrained free circulation. All muscle samples were immediately frozen in liquid nitrogen and stored at −80 °C.

2.5. Muscle metabolites, protein extraction, and western blotting

The procedures for analyzing the muscle samples are detailed in the Supplementary Materials and Methods document. In short, ~10 mg of muscle were ground and homogenized in urea lysis buffer, supplemented with protease and phosphatase inhibitors. After centrifugation, the total protein content was quantified, and the extract volume was adjusted to a ~5.7 μg/μL final concentration in all muscle protein extracts. Optimal amounts of protein were loaded and tested with antibodies and electrophoresed on SDS-PAGE gels. The proteins were transferred to Immun-Blot polyvinylidene fluoride (PVDF) membranes. Four control samples were processed and run together onto the same gel with the samples from each volunteer. Reactive Brown 10 (R0385, Sigma Aldrich) or Ponceau S (P3504, Sigma Aldrich) were used as total protein staining methods to control for optimal loading and transfer

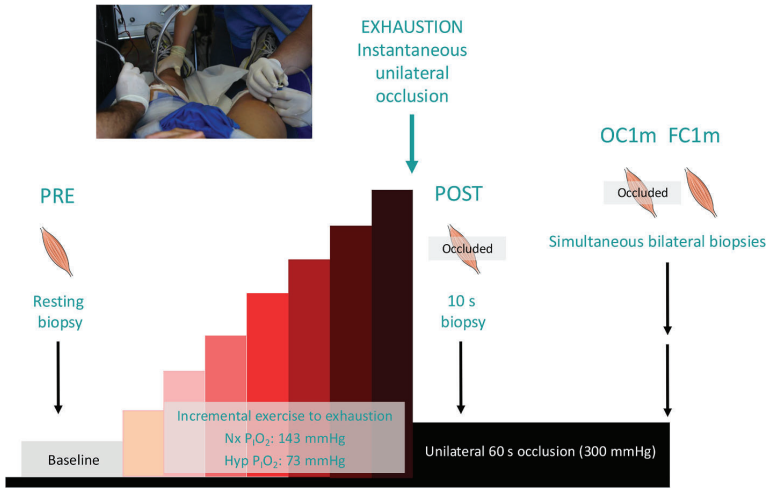

Fig. 1. Schematic representation of the experimental protocol. Eleven volunteers performed an incremental exercise until exhaustion in normoxia ($P_IO_2 = 143$ mmHg) or severe normobaric hypoxia ($P_IO_2 = 73$ mmHg), in random order. A resting skeletal muscle biopsy was obtained from the m. vastus lateralis before warm-up, followed by an incremental exercise test until exhaustion. Upon exhaustion, one leg was occluded with a cuff inflated at 300 mmHg for 60 s. Muscle biopsies were taken from the occluded leg at 10 s and 60 s in both trials (Nx and Hyp). In the test performed in hypoxia, an extra biopsy was obtained at 60 s from the leg recovering with free circulation. During recovery, the subjects breathed normoxic room air.

efficiency. Membranes were blocked and incubated with primary and secondary HRP-conjugated antibodies (see Tables S1 and S2 for details).

2.6. Statistical analysis

Variables were checked for Gaussian distribution using the Shapiro–Wilks test, and when appropriate, data were transformed logarithmically before further analysis. Signaling responses were examined with 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. The Mauchly's test of sphericity was run 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 observed, pairwise comparisons were adjusted by applying the Holm-Bonferroni procedure. When differences between the post-exercise conditions were not significant, the two pre-exercise conditions were averaged and compared with the four post-exercise points using ischemia (two in normoxia and two in hypoxia). The latter was done by a contrast analysis based on a two-way repeated measures analysis (R Foundation for Statistical Computing, Vienna, Austria, Version: 2023.12.0 Build 369). The occluded and non-occluded legs were compared using a paired two-tailed *t*-test. Linear relationships between variables were determined by applying a linear mixed model, and the Likelihood Ratio Test for the random effects (LRT) was calculated and reported with the marginal and conditional r-squared values. If the residuals of the linear mixed model were not normally distributed, the outcome variable was logarithmically transformed, and the model recalculated. 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.29 for Mac (SPSS Inc., Chicago, IL, USA) and jamovi v2.4.8. (The jamovi project 2023, retrieved from https://www.jamovi.org).

3. Results

3.1. Muscle metabolites

The changes in metabolites following exercise have been reported previously [35] and will only be summarized here. In brief, 10 s after the end of the incremental exercise (i.e., at Post), muscle lactate was increased and PCr and ATP decreased. These responses were similar in normoxia (Nx) and hypoxia (Hyp). At the first minute following

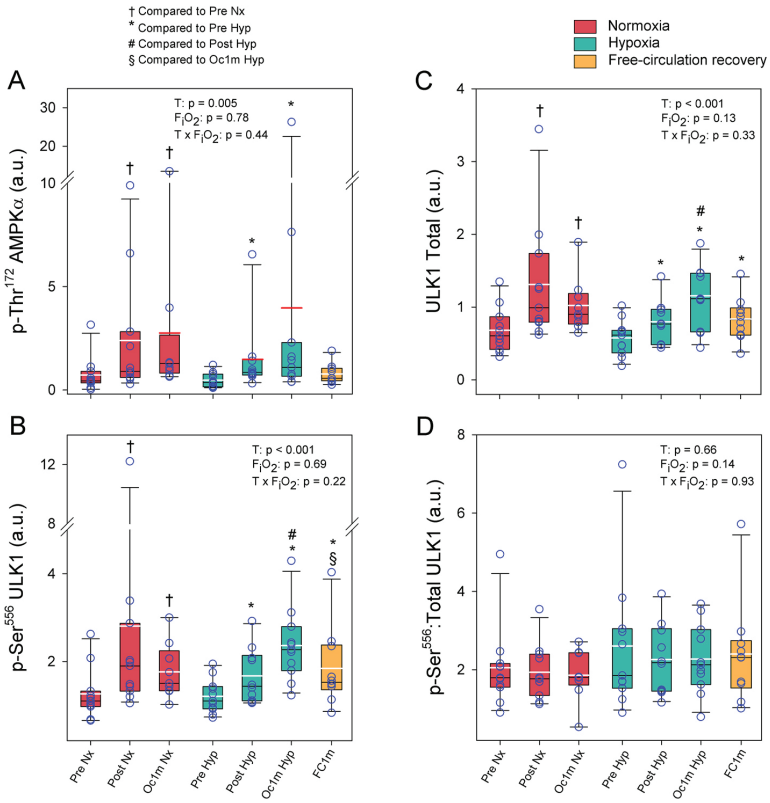

Fig. 2. Protein expression levels of AMPKα and ULK1 signaling in response to incremental exercise to exhaustion in normoxia, severe acute hypoxia, and post-exercise ischemia. Values for (**A**) *p*-Thr^{172} AMPKα, (**B**) *p*-Ser^{556} ULK1, (**C**) ULK1 Total, and (**D**) *p*-Ser^{556}:total ULK1 in arbitrary units (a.u.). The whiskers delimit the 5th and 95th percentiles; the thin and thick horizontal lines correspond to the median and the mean values, respectively; the red horizontal lines correspond to the mean when it lies outside the limits of the box; and the upper and lower ends of the boxes define the 1st and 3rd quartiles, respectively. Nx: Normoxia ($P_IO_2 = 143$ mmHg); Hyp: severe normobaric hypoxia ($P_IO_2 = 73$ mmHg); Pre: before exercise; Post: 10 s after exercise cessation during ischemic recovery; Oc1m: 60 s after exercise cessation during ischemic recovery; FC1m: 60 s after exercise cessation during recovery with free circulation. Individual values are represented with a circle. For panels (A–D), n = 11 for all conditions except for Oc1m Nx (n = 9), Post Hyp 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 all proteins. †$p < 0.05$ vs. Pre Nx; *$p < 0.05$ vs. Pre Hyp; #$p < 0.05$ vs. Post Hyp; §$p < 0.05$ vs. Oc1m Hyp.

exercise, muscle lactate concentration was further increased by 25 % in the leg recovering with ischemia ($p < 0.05$), while it remained unchanged in the leg recovering with free circulation. PCr was reduced by 94 and 48 %, in the occluded (Oc1m) and free-circulation (FC1m) legs, respectively ($p < 0.005$), regardless of inspired oxygen fraction (F_IO_2). Thus, PCr levels were partly restored in the leg recovering with free circulation. Femoral vein PO_2 was 21.1 ± 2.0 and 10.6 ± 2.8 mmHg at maximal power output at exhaustion (Wmax), in Nx and Hyp, respectively ($p < 0.001$).

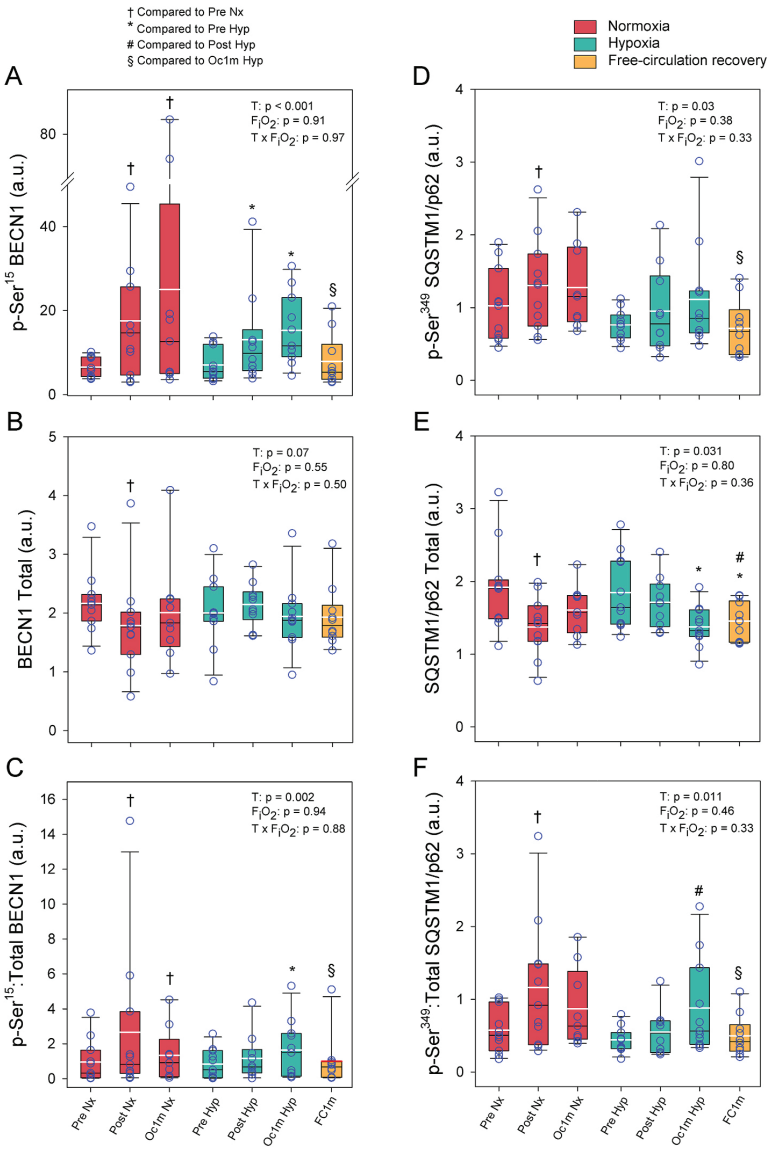

Fig. 3. Protein expression levels of BECN1 and SQSTM1/p62 signaling in response to incremental exercise to exhaustion in normoxia, severe acute hypoxia, and post-exercise ischemia. Values for (**A**) *p*-Ser^{15} BECN1, (**B**) BECN1 Total, (**C**) *p*-Ser^{15}:Total BECN1, (**D**) *p*-Ser^{349} SQSTM1/p62, (**E**) SQSTM1/p62 Total, and (**F**) *p*-Ser^{349}:total SQSTM1/p62 in arbitrary units (a.u.). The whiskers delimit the 5th and 95th percentiles; the thin and thick horizontal lines correspond to the median and the mean values, respectively; the red horizontal lines correspond to the mean when it lies outside the limits of the box; and the upper and lower ends of the boxes define the 1st and 3rd quartiles, respectively. Nx: Normoxia ($P_IO_2 = 143$ mmHg); Hyp: severe normobaric hypoxia ($P_IO_2 = 73$ mmHg); Pre: before exercise; Post: 10 s after exercise cessation during ischemic recovery; Oc1m: 60 s after exercise cessation during ischemic recovery; FC1m: 60 s after exercise cessation during recovery with free circulation. Individual values are represented with a circle. For panels (A–F), n = 11 for all conditions except for Oc1m Nx (n = 9), Post Hyp 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 all proteins, except for *p*-Ser^{349}:total SQSTM1/p62. †$p < 0.05$ vs. Pre Nx; *$p < 0.05$ vs. Pre Hyp; #$p < 0.05$ vs. Post Hyp; §$p < 0.05$ vs. Oc1m Hyp.

3.2. Macroautophagy signaling

Compared to pre-exercise resting values, *p*-Thr^{172} AMPKα (Fig. 2A), *p*-Ser^{556} ULK1 (Fig. 2B), ULK1 total (Fig. 2C), *p*-Ser^{15} BECN1 (Fig. 3A), *p*-Ser^{15} :total BECN1 ratio (Fig. 3C), *p*-Ser^{349} p62 (Fig. 3D), *p*-Ser^{349}:total p62 ratio (Fig. 3F) and MYTHO (Fig. 3E) were significantly increased at Post (1.3 to 4-fold) and Oc1m (1.3 to 8-fold). In contrast, p62 total (Fig. 3E) protein expression was reduced by 19 and 21 % at Post and Oc1m, respectively. Responses in Nx and Hyp were similar.

After 1 min of recovery with free circulation, *p*-Thr^{172} AMPKα, *p*-Ser^{556} ULK1, *p*-Ser^{15} BECN1, *p*-Ser^{15} :total BECN1 ratio, *p*-Ser^{349} p62, *p*-Ser^{349}:total p62 ratio expression returned to pre-exercise levels in the leg

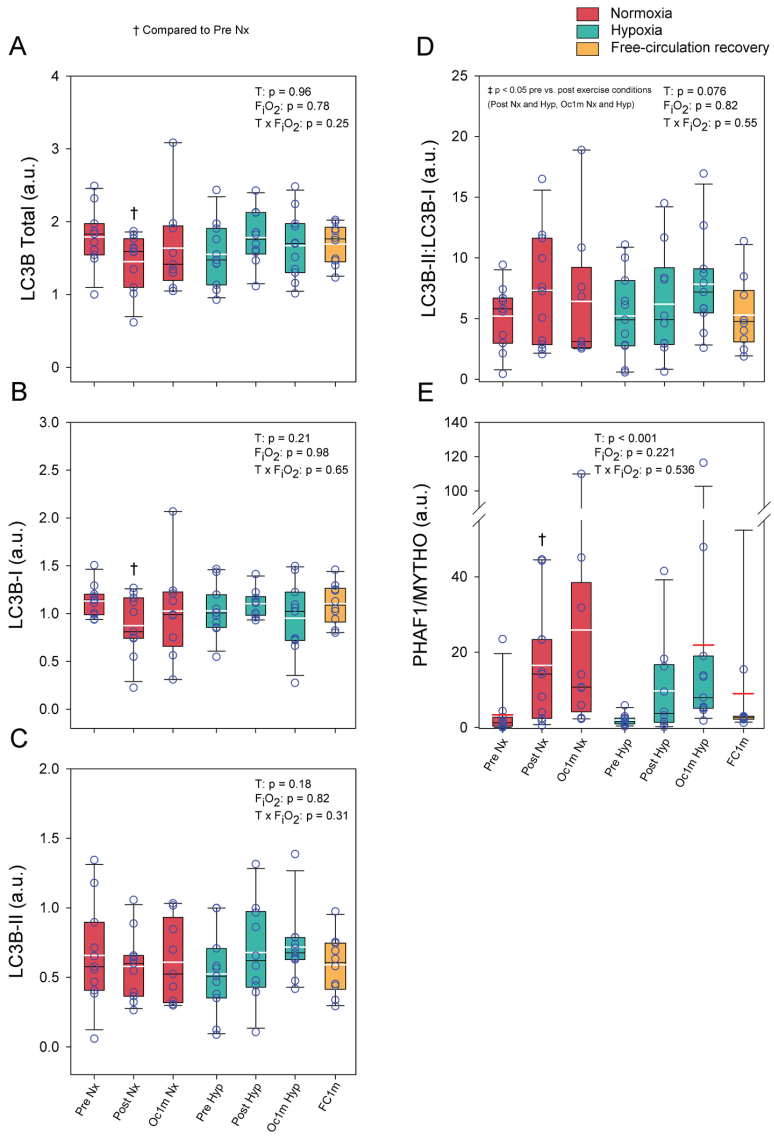

Fig. 4. Protein expression levels of LC3B and MYTHO signaling in response to incremental exercise to exhaustion in normoxia, severe acute hypoxia, and post-exercise ischemia. Values for (**A**) LC3B Total, (**B**) LC3B–I, (**C**) LC3B-II, (**D**) LC3B-II:LC3B–I, and (**E**) MYTHO in arbitrary units (a.u.). The whiskers delimit the 5th and 95th percentiles; the thin and thick horizontal lines correspond to the median and the mean values, respectively; the red horizontal lines correspond to the mean when it lies outside the limits of the box; and the upper and lower ends of the boxes define the 1st and 3rd quartiles, respectively. Nx: Normoxia (P_IO_2 = 143 mmHg); Hyp: severe normobaric hypoxia (P_IO_2 = 73 mmHg); Pre: before exercise; Post: 10 s after exercise cessation during ischemic recovery; Oc1m: 60 s after exercise cessation during ischemic recovery; FC1m: 60 s after exercise cessation during recovery with free circulation. Individual values are represented with a circle. For panels (A–D), n = 11 for all conditions except for Oc1m Nx (n = 9), Post Hyp and FC1m (n = 10). For panels (E), n = 11 for all conditions except for Oc1m Nx (n = 9) and Post Hyp (n = 10). See Fig. 1 for a detailed description of the experimental phases. The statistical analysis was performed with logarithmically transformed data for LC3B Total, LC3B-II and MYTHO. A contrast analysis comparing the two pre with the post exercise (Post Nx and Hyp, Oc1m Nx and Hyp) conditions was performed for LC3B-II:LC3B–I ratio. †p < 0.05 vs. Pre Nx; ‡p < 0.05 two pre vs. the post exercise conditions (Post Nx and Hyp, Oc1m Nx and Hyp).

recovering with free circulation. However, this recovery was incomplete for *p*-Ser^{556} ULK1 and MYTHO, who remained 46 % and 4.5-fold above the resting value after 1 min of recovery with free circulation. One minute after the end of exercise p62 remained similarly reduced in the leg recovering with free circulation and in the leg recovering with ischemia.

Compared to the occluded leg, *p*-Ser^{556} ULK1, ULK1 total, *p*-Ser^{15} BECN1, *p*-Ser^{15} :total BECN1 ratio, *p*-Ser^{349} p62, *p*-Ser^{349}:total p62 ratio and MYTHO were 24–59 % lower in the leg recovering with free circulation. No significant differences were observed between the occluded and non-occluded leg 1 min after exercise in *p*-Thr^{172} AMPKα and p62 total.

The *p*-Ser^{556}:total ULK1 ratio (Fig. 2D), BECN1 total (Fig. 3B), LC3B total (Fig. 4A), LC3B–I (Fig. 4B), LC3B-II (Fig. 4C), and LC3B-II:LC3B–I ratio (Fig. 4D) did not change significantly with ANOVA. However, a contrast analysis comparing the two pre-exercise conditions with those following exercise with ischemia application (Post Nx and Hyp, Oc1m Nx and Hyp) was performed to increase the statistical power and determine whether there was an exercise effect. This analysis revealed that LC3B-II:LC3B–I ratio was significantly increased by exercise 1.4-fold.

3.3. Chaperone-mediated autophagy

Compared to pre-exercise, LAMP2A (Fig. 5A) was increased 1.2 and 1.3-fold at Post and Oc1m, respectively, while HSPA8 (Fig. 5B) was reduced by 25 % at Oc1m, with similar responses in Nx and Hyp. After 1 min of recovery with free circulation, LAMP2A and HSPA8 expression returned to pre-exercise levels. Compared to the occluded leg, HSPA8 was 1.4-fold higher in the leg recovering with free circulation.

3.4. FOXOs signaling

Compared to pre-exercise, *p*-Ser^{256} FOXO1 (Fig. 6A) and *p*-Ser^{256}:total FOXO1 ratio (Fig. 6C) were increased at Post (2.2 and 2.4-fold, respectively) and Oc1m (2.6 and 3.2-fold, respectively). Compared to pre-exercise, *p*-Ser^{253} FOXO3 (Fig. 6D) and FOXO3 total (Fig. 6E) were increased by 1.8 and 1.3-fold, respectively, at Oc1m, while FOXO1 total (Fig. 6B) protein expression was significantly reduced by 13 % at Oc1m. Responses in Nx and Hyp were similar.

After 1 min of recovery with free circulation, *p*-Ser^{256} FOXO1, FOXO1 total, *p*-Ser^{256}:total FOXO1 ratio, *p*-Ser^{253} FOXO3, FOXO3 total expression returned to pre-exercise levels in the leg recovering with free circulation, being 30–65 % lower in the leg recovering with free-circulation compared with the occluded leg.

The ratio *p*-Ser^{253}:total FOXO3 (Fig. 6F) did not change significantly with ANOVA. However, a contrast analysis comparing the two pre with the post-exercise (Post Nx and Hyp, Oc1m Nx and Hyp) conditions indicated that the ratio *p*-Ser^{253}:total FOXO3 increased significantly by 27 % after exercise.

3.5. Protein synthesis and protein elongation signaling

Compared to pre-exercise, *p*-Ser^{9} GSK3B (Fig. 7A) and *p*-Ser^{473} AKT (Fig. 7B) were reduced at Post (20 and 54 %, respectively) and Oc1m (23 and 65 %, respectively). Phospho-Thr^{56} EEF2 (Fig. 7C) and *p*-Thr^{56}:total EEF2 ratio (Fig. 7E) were increased at Post (3.5 and 3.6-fold, respectively) and Oc1m (3.4 and 4.2-fold, respectively). Responses in Nx and Hyp were similar.

After 1 min of recovery with free circulation, *p*-Ser^{473} AKT remained 73 % below the resting value in the leg recovering with free circulation. In contrast, *p*-Thr^{56} EEF2 and *p*-Thr^{56}:total EEF2 ratio remained 4.3 and 6-fold above the resting value, while *p*-Ser^{9} GSK3B returned to Pre-exercise levels. No significant differences were observed between the occluded and non-occluded leg in *p*-Ser^{473} AKT, *p*-Thr^{56} EEF2, *p*-Thr^{56}: total EEF2 ratio and *p*-Ser^{9} GSK3B. Phospho-Ser^{2448} MTOR (Fig. 7F) and EEF2 total (Fig. 7D) did not change significantly.

3.6. Hypoxia signaling

Compared to pre-exercise, hydroxy-Pro^{564} HIF1A (Fig. 8A) and hydroxy-Pro^{564}:total HIF1A ratio (Fig. 8C) were increased at Post (1.9 and 1.7-fold, respectively) and Oc1m (1.8 and 1.7-fold, respectively), with a similar response in Nx and Hyp.

After 1 min of recovery with free circulation, hydroxy-Pro^{564} HIF1A expression returned to resting values, while hydroxy-Pro^{564}:total HIF1A

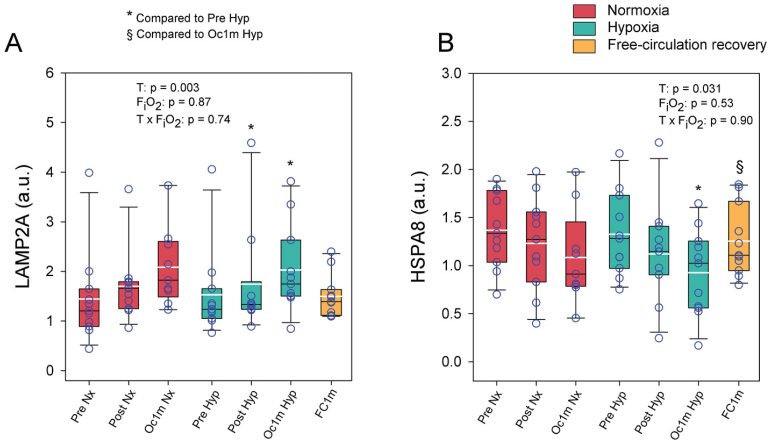

Fig. 5. Protein expression levels of LAMP2A and HSPA8 signaling in response to incremental exercise to exhaustion in normoxia, severe acute hypoxia, and post-exercise ischemia. Values for (**A**) LAMP2A and (**B**) HSPA8 in arbitrary units (a.u.). The whiskers delimit the 5th and 95th percentiles; the thin and thick horizontal lines correspond to the median and the mean values, respectively; and the upper and lower ends of the boxes define the 1st and 3rd quartiles, respectively. Nx: Normoxia (P_IO_2 = 143 mmHg); Hyp: severe normobaric hypoxia (P_IO_2 = 73 mmHg); Pre: before exercise; Post: 10 s after exercise cessation during ischemic recovery; Oc1m: 60 s after exercise cessation during ischemic recovery; FC1m: 60 s after exercise cessation during recovery with free circulation. Individual values are represented with a circle. For panel (A), n = 11 for all conditions except for Oc1m Nx (n = 9) and Post Hyp (n = 10). 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. *p < 0.05 vs. Pre Hyp; §p < 0.05 vs. Oc1m Hyp.

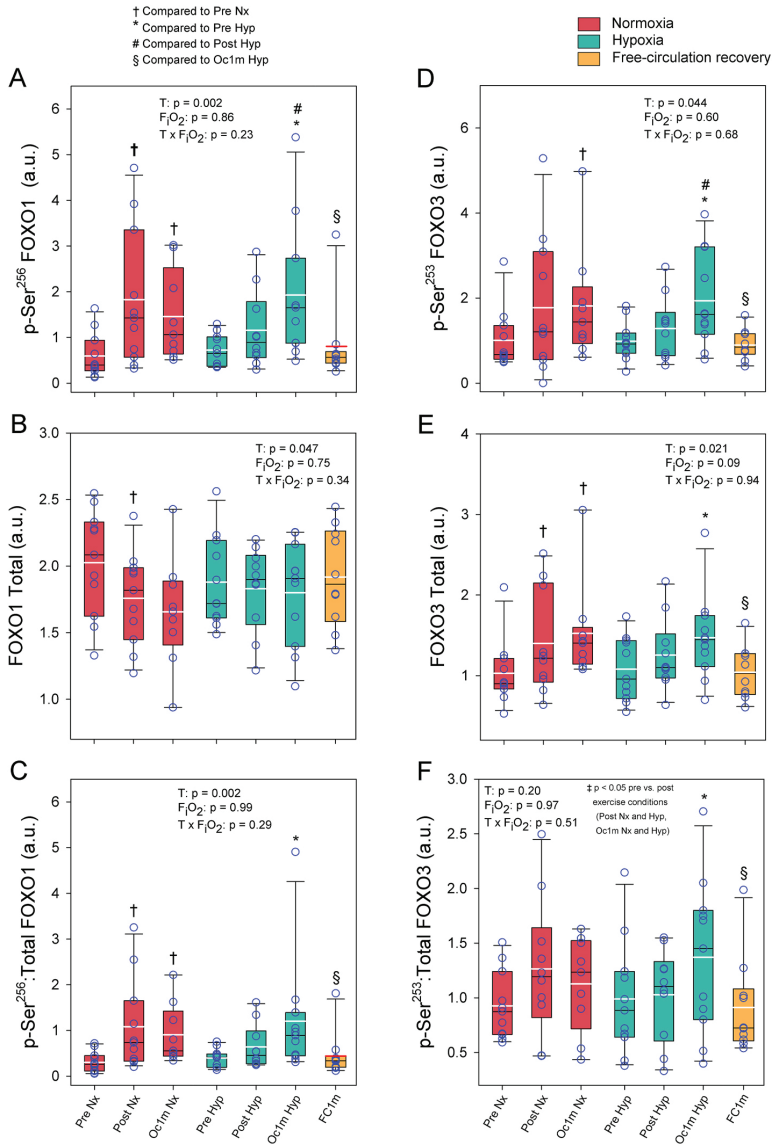

Fig. 6. Protein expression levels of FOXOs signaling in response to incremental exercise to exhaustion in normoxia, severe acute hypoxia, and post-exercise ischemia. Values for (**A**) *p*-Ser^{256} FOXO1, (**B**) FOXO1 total, (**C**) *p*-Ser^{256}:total FOXO1, (**D**) *p*-Ser^{253} FOXO3, (**E**) FOXO3 total, and (**F**) *p*-Ser^{253}:total FOXO3 in arbitrary units (a.u.). The whiskers delimit the 5th and 95th percentiles; the thin and thick horizontal lines correspond to the median and the mean values, respectively; the red horizontal lines correspond to the mean when it lies outside the limits of the box; and the upper and lower ends of the boxes define the 1st and 3rd quartiles, respectively. Nx: Normoxia (P_IO_2 = 143 mmHg); Hyp: severe normobaric hypoxia (P_IO_2 = 73 mmHg); Pre: before exercise; Post: 10 s after exercise cessation during ischemic recovery; Oc1m: 60 s after exercise cessation during ischemic recovery; FC1m: 60 s after exercise cessation during recovery with free circulation. Individual values are represented with a circle. For panels (A–D), n = 11 for all conditions except for Oc1m Nx (n = 9), Post Hyp and FC1m (n = 10). For panels (E) and (F), n = 11 except for Oc1m Nx (n = 9), Post Nx, Post Hyp 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 *p*-Ser^{256} FOXO1, *p*-Ser^{256}: total FOXO1 and FOXO3 total. A contrast analysis comparing the two pre with the post exercise (Post Nx and Hyp, Oc1m Nx and Hyp) conditions was performed for *p*-Ser^{253}:total FOXO3. †p < 0.05 vs. Pre Nx; *p < 0.05 vs. Pre Hyp; #p < 0.05 vs. Post Hyp; §p < 0.05 vs. Oc1m Hyp; ‡p < 0.05 two pre vs. the post-exercise conditions (Post Nx and Hyp, Oc1m Nx and Hyp).

ratio was reduced by 28 % compared to Pre-exercise. Compared to the occluded leg, hydroxy-Pro^{564}:total HIF1A ratio and hydroxy-Pro^{564} HIF1A were 66 and 77 % lower in the leg recovering with free circulation, respectively.

No significant changes were observed in HIF1A total (Fig. 8B) protein content with the ANOVA. A contrast analysis comparing the two pre- and the post-exercise (Post Nx and Hyp, Oc1m Nx and Hyp) conditions showed that exercise increased significantly HIF1A total by 1.2-fold.

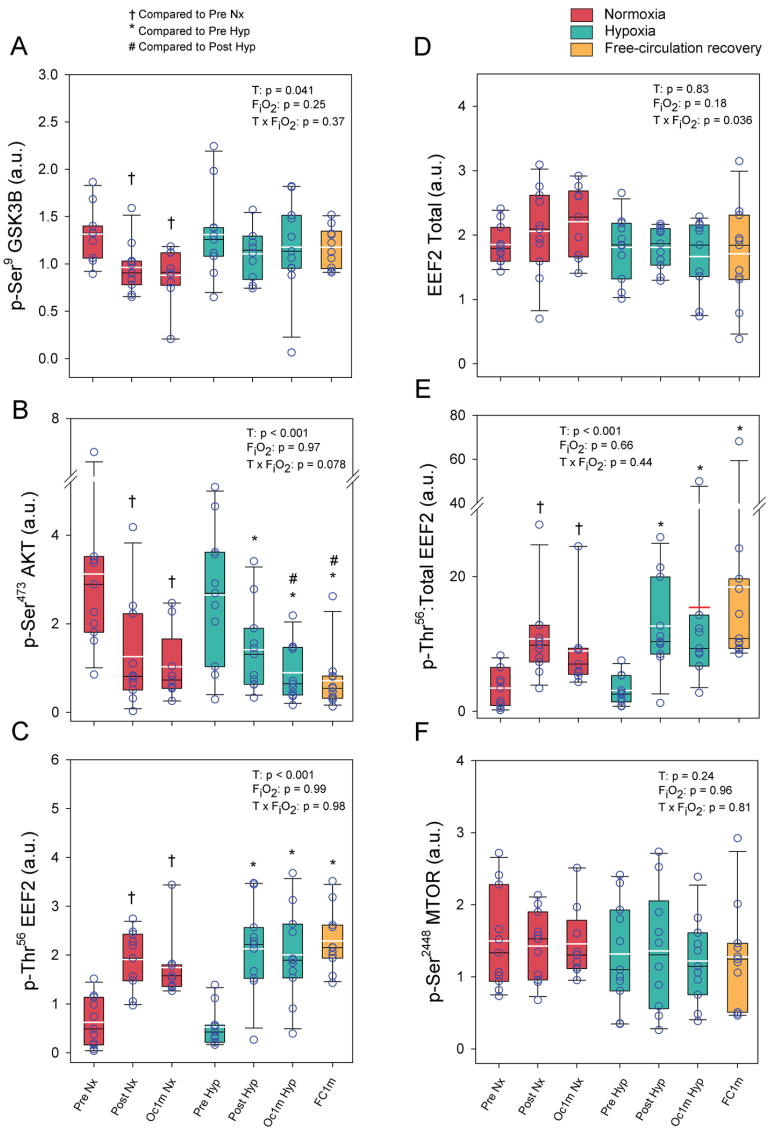

Fig. 7. Protein expression levels of the GSK3B, AKT, EEF2, and MTOR signaling in response to incremental exercise to exhaustion in normoxia, severe acute hypoxia, and post-exercise ischemia. Values for (**A**) *p*-Ser^{9} GSK3B, (**B**) *p*-Ser^{473} AKT, (**C**) *p*-Thr^{56} EEF2, (**D**) EEF2 Total, (**E**) *p*-Thr^{56}:Total EEF2 and (**F**) *p*-Ser^{2448} MTOR in arbitrary units (a.u.). The whiskers delimit the 5th and 95th percentiles; the thin and thick horizontal lines correspond to the median and the mean values, respectively; the red horizontal lines correspond to the mean when it lies outside the limits of the box; and the upper and lower ends of the boxes define the 1st and 3rd quartiles, respectively. Nx: Normoxia ($P_IO_2 = 143$ mmHg); Hyp: severe normobaric hypoxia ($P_IO_2 = 73$ mmHg); Pre: before exercise; Post: 10 s after exercise cessation during ischemic recovery; Oc1m: 60 s after exercise cessation during ischemic recovery; FC1m: 60 s after exercise cessation during recovery with free circulation. Individual values are represented with a circle. For panel (A), n = 11 for all conditions except for Oc1m Nx (n = 9), Post Hyp and FC1m (n = 10). For panels (B–E), n = 11 for all conditions except for Oc1m Nx (n = 9). For panel (F), n = 11 for all conditions except for Oc1m Nx (n = 9) and Post Hyp (n = 10). See Fig. 1 for a detailed description of the experimental phases. The statistical analysis was performed with logarithmically transformed data for *p*-Ser^{473} AKT, *p*-Thr^{56} EEF2, and *p*-Thr^{56}:Total EEF2. †p < 0.05 vs. Pre Nx; *p < 0.05 vs. Pre Hyp; #p < 0.05 vs. Post Hyp.

3.7. PGC1A expression

Compared to Pre, *p*-Ser^{571} PGC1A (Fig. 8D) was increased by 1.9 and 2.5-fold at Post and Oc1m, respectively, with similar responses in Nx and Hyp. After 1 min of recovery with free circulation *p*-Ser^{571} PGC1A returned to Pre-exercise values and was 51 % lower than in the occluded leg.

3.8. Linear associations

Positive linear associations were observed between *p*-Thr^{172} AMPKα and ULK1 phosphorylation at Ser^{556} (R^2 marginal = 0.48, R^2 conditional = 0.98, intercept and slope random effect LRT p < 0.001) (Fig. S1A) and PGC1A phosphorylation at Ser^{571} (R^2 marginal = 0.67, R^2

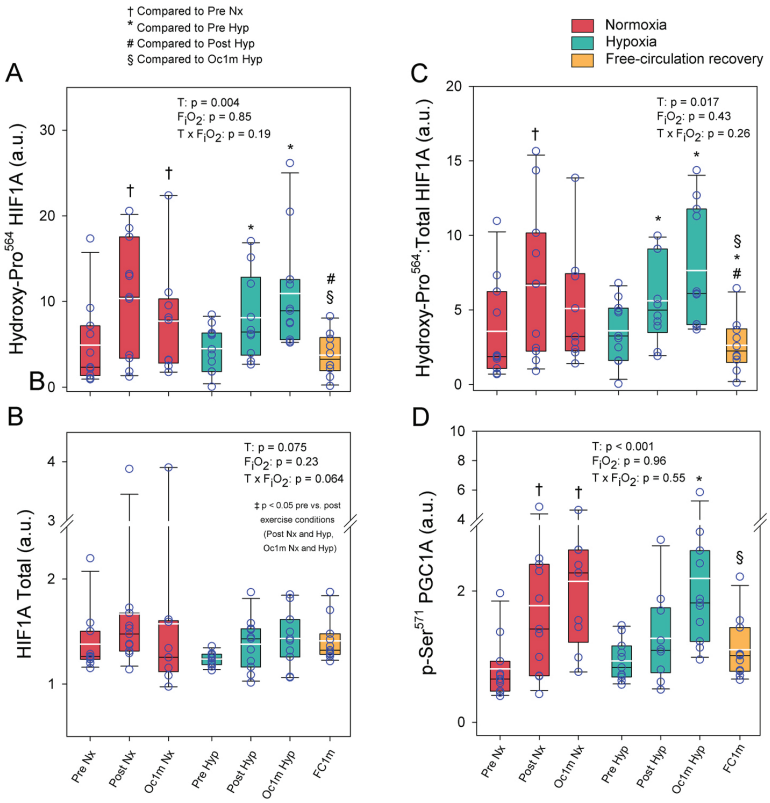

Fig. 8. Protein expression levels of the HIF1A and PGC1A signaling in response to incremental exercise to exhaustion in normoxia, severe acute hypoxia, and post-exercise ischemia. Values for (**A**) Hydroxy-Pro^{564} HIF1A, (**B**) HIF1A Total, (**C**) Hydroxy-Pro^{564}:Total HIF1A, (**D**) *p*-Ser^{571} PGC1A in arbitrary units (a.u.). The whiskers delimit the 5th and 95th percentiles; the thin and thick horizontal lines correspond to the median and the mean values, respectively; and the upper and lower ends of the boxes define the 1st and 3rd quartiles, respectively. Nx: Normoxia (P_IO_2 = 143 mmHg); Hyp: severe normobaric hypoxia (P_IO_2 = 73 mmHg); Pre: before exercise; Post: 10 s after exercise cessation during ischemic recovery; Oc1m: 60 s after exercise cessation during ischemic recovery; FC1m: 60 s after exercise cessation during recovery with free circulation. Individual values are represented with a circle. For panels (A) and (C), n = 11 for all conditions except for Oc1m Nx (n = 9), Post Hyp and FC1m (n = 10). For panel (B), n = 11 for all conditions except for Oc1m Nx (n = 9). For panel (D), n = 11 for all conditions except for Oc1m Nx (n = 9) and Post Hyp (n = 10). See Fig. 1 for a detailed description of the experimental phases. The statistical analysis was performed with logarithmically transformed data for HIF1A Total and *p*-Ser^{571} PGC1A. A contrast analysis comparing the two pre with the post exercise (Post Nx and Hyp, Oc1m Nx and Hyp) conditions was performed for HIF1A total. †p < 0.05 vs. Pre Nx; *p < 0.05 vs. Pre Hyp; #p < 0.05 vs. Post Hyp; §p < 0.05 vs. Oc1m Hyp; ‡p < 0.05 two pre vs. the post exercise conditions (Post Nx and Hyp, Oc1m Nx and Hyp).

conditional = 0.91, intercept and slope random effect LRT p = 0.046) (Fig. S1B). ULK1 phosphorylation at Ser^{556} was linearly associated to *p*-Ser^{15} BECN1 (R^2 marginal = 0.43, R^2 conditional = 0.84, intercept and slope random effect LRT p = 0.003) (Fig. S1C). MYTHO was linearly associated with the LC3B-II:LC3B–I ratio (R^2 marginal = 0.09, R^2 conditional = 0.46, intercept effect LRT p < 0.001) (Fig. S1D). Likewise, a linear association was observed between *p*-Ser^{40} NRF2 and LAMP2A (R^2 marginal = 0.05, R^2 conditional = 0.67, intercept effect LRT p < 0.001) (Fig. S1E).

Representative immunoblots of all proteins studied are presented in Fig. 9.

4. Discussion

This study showed that macroautophagy and chaperone-mediated autophagy (CMA) signaling pathways are activated by acute exercise to exhaustion in human skeletal muscle (hSKM) with a similar response in normoxia and severe acute hypoxia, even though femoral vein PO_2 at exhaustion was halved at exhaustion in hypoxia (Nx: 21.1 ± 2.0 Vs. Hyp: 10.6 ± 2.8 mmHg). It is worth noting that the level of hypoxia elicited is close to the limit of tolerance for non-altitude acclimatized humans. Macroautophagy remained activated during the 1 min post-exercise occlusion while it was restored to almost pre-exercise conditions in the leg recovering with free circulation just 1 min after the end of exercise, demonstrating that reversion of the exercise-mediated activation of macroautophagy requires O_2. The present findings indicated that macroautophagy signaling might be activated through upregulation of the AMPKα-ULK1, lowered phosphorylation levels of AKT and GSK3B, and unchanged *p*-Ser^{2448} MTOR. The latter was accompanied by increased signaling promoting autophagosome formation and cargodegradation, as indicated by the rise of the LC3B-II:LC3B–I ratio and MYTHO, and the decrease of SQSTM1/p62 total protein content. Chaperone-mediated autophagy was facilitated, as indicated by the observed increase in LAMP2A (a critical step for CMA) with exercise and ischemia. Moreover, the observed upregulation of the inhibitory phosphorylations of FOXO1 and FOXO3 indicates that there was no activation of FOXO signaling with exhaustive exercise, ischemia or systemic hypoxia, indicating that FOXOs are not necessary for macroautophagy

nor CMA activation in hSKM. Concomitantly with the activation of protein degradation signaling through macroautophagy and CMA, protein synthesis was blunted, as shown by the inhibition of protein elongation (see Fig. 10 for a graphical summary).

The signaling responses in this study were similar in normoxia and hypoxia; however, within 1 min after exercise cessation, most of the signaling changes were reverted to pre-exercise levels in the leg recovering with free circulation. This observation has important methodological implications for future studies since to capture the exercise-induced signaling response, muscle biopsies should be performed immediately at exhaustion or delayed a few seconds, but in the latter case, ischemia should be applied to maintain the exercise-induced signaling.

4.1. AMPKα-ULK1-triggered macroautophagy signaling initiation during high-intensity exercise and ischemia is unchanged under severe acute hypoxia in human skeletal muscle

The present investigation showed that an acute bout of exercise until exhaustion followed by 60s of total immediate ischemia increased

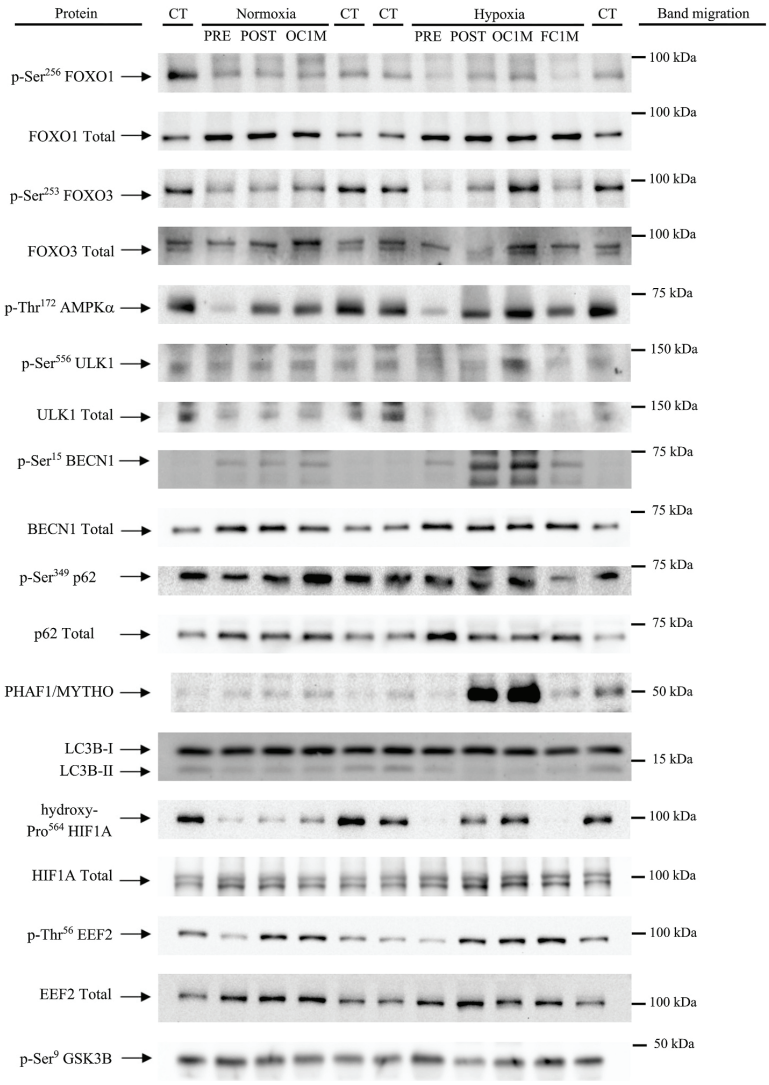

Fig. 9. Immunoblots and total amount of protein loaded (Reactive Brown Staining) from a representative subject of the study. Images from top to bottom: *p*-Ser^{256} FOXO1, FOXO1 total, *p*-Ser ^{253} FOXO3, FOXO3 total, *p*-Thr^{172} AMPKα, *p*-Ser^{556} ULK1, ULK1 total, *p*-Ser^{15} BECN1, BECN1 total, *p*-Ser^{349} SQSTM1/p62, SQSTM1/p62 total, PHAF1/MYTHO, LC3B–I, LC3B-II, hydroxy-Pro^{564} HIF1A, HIF1A total, *p*-Thr^{56} EEF2, EEF2 total, *p*-Ser^{9} GSK3B, LAMP2A, HSPA8, *p*-Ser^{473} AKT, *p*-Ser^{2448} MTOR, *p*-Ser^{571} PGC1A, and Reactive Brown (as total protein loading control). A detailed description of experimental phases is included in Fig. 1. CT, non-intervention healthy human sample included in quadruplicate onto each gel as a loading control. Normoxia: test performed with P_IO_2 = 143 mmHg; Hypoxia: test performed with P_IO_2 = 73 mmHg; Pre: before exercise; Post: 10 s after the end of exercise with ischemic recovery; Oc1m: 60 s after the end of exercise with ischemic recovery; FC1m: 60 s after exercise cessation during recovery with free circulation. The molecular weight standard markers closest to the migration of the band are indicated on the right side of the panel.

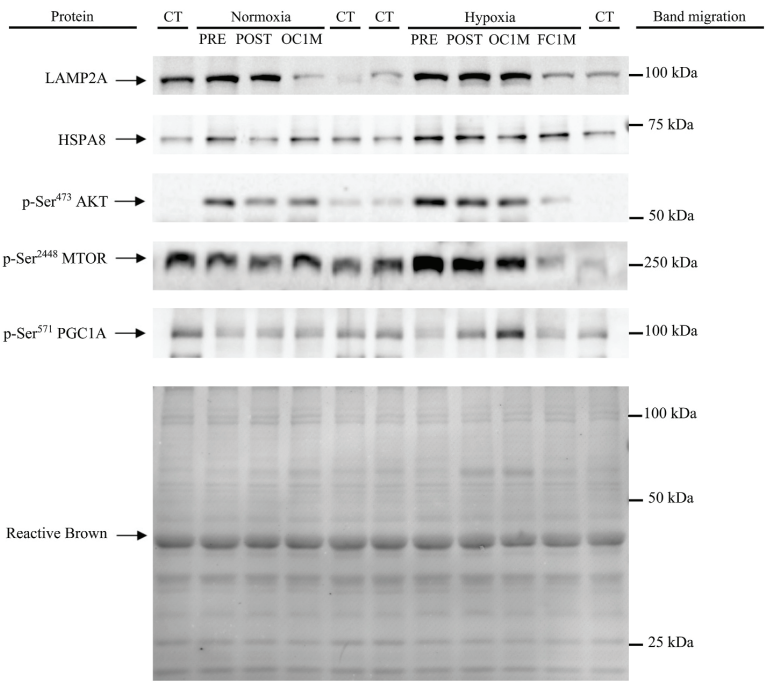

Fig. 9. (*continued*).

macroautophagy initiation signaling. Our results concur with the reported upregulation of *p*-Thr^{172} AMPKα and the subsequent phosphorylation of ULK1 at residues Ser^{317} or Ser^{556} seen after cycling for 60 min at 50–70 % peak oxygen consumption ($\dot{V}O_2$peak) [11, 13, 15, 17] and ultra-endurance exercise [14]. The observed positive linear association between *p*-Thr^{172} AMPKα and *p*-Ser^{556} ULK1 further supports the activation of ULK1 by AMPKα. No additional macroautophagy signaling activation was observed in hypoxia compared to normoxia, despite the very low femoral vein PO_2 values attained during exercise in hypoxia in the present investigation. This agrees with previous studies in moderate hypoxia (12 % O_2) [17]. As a novelty, we have shown that the application of leg ischemia at exhaustion maintained upregulated these signals similarly in Nx and Hyp. This is consistent with the activation of NRF2-KEAP1 [31] and nuclear factor-kappa Beta (NF-κB)-depending signaling in this experiment [36], which were neither upregulated with ischemia nor hypoxia. Thus, the levels of ROS and metabolite accumulation reached at the end of incremental exercise in normoxia are sufficient to stimulate macroautophagy signaling in human skeletal muscle maximally.

4.2. BECN1, LC3B, and SQSTM1/p62 signaling in response to acute exercise to exhaustion and ischemia

The complex BECN1/PIK3C3 is considered a marker of membrane nucleation, mediating the phagophore formation [39]. The activation of BECN1 Ser^{15} phosphorylation by ULK1 is crucial for disassembling the BECN1-BCL2 complex [40] and phagophore formation [7,18]. In agreement, a linear positive association was observed in the present investigation between *p*-Ser^{556} ULK1 and BECN1 Ser^{15} phosphorylation. As a novelty, we have seen a notable increase in BECN1 Ser^{15} phosphorylation after exercise and ischemia. In the present study, no changes were observed in the levels of total BECN1 after incremental exercise to exhaustion and recovery. This outcome concurs with the unchanged BECN1 protein expression reported after 60 min cycling at 50–60 % $\dot{V}O_2$peak [9,13] and ultra-endurance exercise [14,21,22].

LC3B has been widely considered an indirect marker of macroautophagy activity and autophagosome formation in rodent and human experimental models; however, changes in LC3B should be interpreted with caution [41]. In rodents, higher LC3B-II and LC3B-II:LC3B–I ratio values indicate increased macroautophagy and autophagosome content [16]. In human studies involving exercising for 30–120 min at 50–70 % of $\dot{V}O_2$peak, an elevated macroautophagy signaling is typically associated with a reduced LC3B-II:LC3B–I ratio, primarily driven by increased LC3B-II degradation [9,11,12,15,16]. In contrast, in the present investigation, the LC3B-II:LC3B–I ratio was increased after exercise due to reduced free LC3B–I but unchanged LC3B-II levels, which is compatible with increased lipidation activity to LC3B-II and autophagosome formation. SQSTM1/p62 harbors an LC3-interacting region domain involved in the specific transport of SQSTM1/p62-tagged substrates within the autophagosome [42]. Most studies on human skeletal muscle have reported no changes in SQSTM1/p62 total protein levels following prolonged exercise at 50–70 % of $\dot{V}O_2$peak [9,13,15,16] or ultra-endurance exercise [14]. In agreement with a potential increase of autophagic flux, SQSTM1/p62 was reduced after exercise, likely due to p62 autophagosome co-degradation [43]. The observed increased p62 Ser^{349} phosphory-lation further supports an increased macroautophagy flux, which facilitates p62 co-degradation with KEAP1 [31,42,44]. Our results combined with previous studies, indicate that a significant reduction of p62 after exercise is only seen at intensities above 80 % of $\dot{V}O_2$peak.

4.3. PHFA1/MYTHO, the novel macroautophagy biomarker, is upregulated in response to acute exercise and ischemia in human skeletal muscle

Our results have shown that MYTHO, a novel FOXO-dependent gene barely detectable at rest, experienced a marked upregulation after

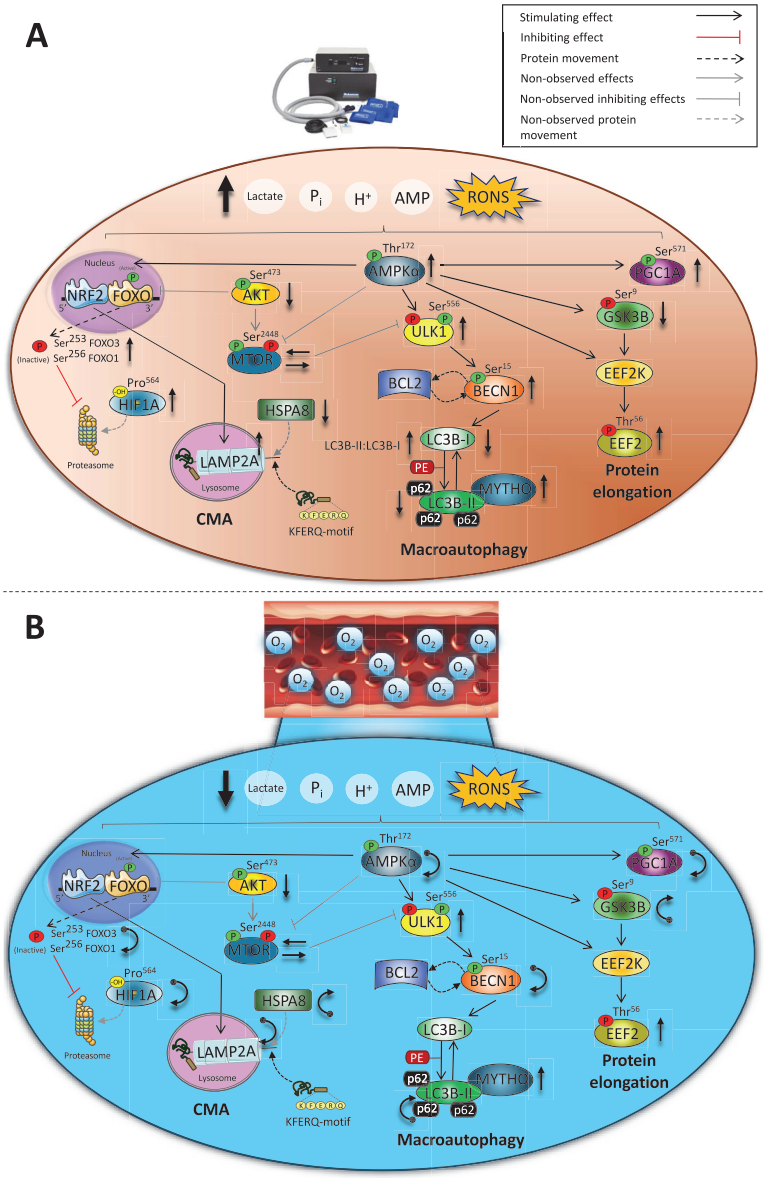

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Fig. 10. Schematic representation of the signaling pathways regulating autophagy activation in skeletal muscle in response to exercise to exhaustion in normoxia and severe normobaric hypoxia followed by ischemia-reperfusion. (A) Low levels of AMP:ATP ratio and ROS evoked by incremental exercise to exhaustion activate AMPKα. Phosphorylated AMPKα induces autophagy initiation by directly phosphorylating ULK1 at Ser^{556}. ULK1 stimulates phagophore formation through BECN1 Ser^{15} phosphorylation, which is crucial for disassembling the BECN1-BCL2 complex. Expansion of the autophagosome requires LC3B–I lipidation to LC3B-II. Ubiquitinated tagged substrates are recognized by SQSTM1/p62, which targets the material to the growing autophagosome by binding to LC3B-II. MYTHO/PHAF1 harbors an LC3 interacting region, which may regulate the elongation step of autophagosome formation. Chaperone-mediated autophagy degrades proteins containing the KFERQ motif, which are recognized by HSPA8. The damaged protein and HSPA8 are identified by the LAMP2A receptor, being digested in the lysosomal lumen. Protein synthesis is inhibited after exercise and ischemia as seen by a declined AKT phosphorylation at Ser^{473} and unchanged MTOR. MTOR, when active, phosphorylates and inhibits ULK1. EEF2K, a kinase that regulates protein elongation, is activated by active GSK3B, leading to increased phosphorylation of EEF2 at Thr^{56} and inhibition of protein elongation. Inhibitory phosphorylation of FOXO leads to its nuclear exclusion and inactivation. Insufficient energy availability could compromise proteasomal degradation, leading to the accumulation of hydroxylated HIF1A. AMPKα also positively regulates the activity of PGC1A, which increases the transcriptional activity of autophagy-related genes. **(B)** Most of the upregulated biomarkers rapidly reverted to pre-exercise levels within 60 s of free-circulation recovery, indicating a fast regulation of autophagy following the cessation of exercise, which depends on muscle reoxygenation. All proteins assessed are similarly regulated after exercise in normoxia and severe acute hypoxia. Stimulating/inhibiting effects are represented by black/red connecting lines. Protein movements are depicted with black dashed lines. Actions known but not observed/assessed in this investigation are depicted in grey (with dashed lines indicating a protein movement). The black arrows shown beside each biomarker illustrate the magnitude of the protein expression changes (increased/decreased/maintained/returned to pre-exercise levels) in this investigation.

exercise in normoxic and hypoxic conditions. In the present study, exercise resulted in FOXO inhibition, suggesting that the exercise induction of MYTHO does not require FOXO activation. In agreement, it has been reported that FOXO1 is not required for MYTHO induction with fasting in rodents [25]. In the present study, other molecular mechanisms should explain the increased MYTHO expression after high-intensity exercise. In our investigation, the observed increase in the LC3B-II:LC3B–I ratio and upregulation of MYTHO after acute exercise and ischemia may be linked. Studies in knockout MYTHO mice's skeletal muscles showed reduced autophagosome formation accompanied by lowered RNA levels of BECN1 and reduced LC3B-II:LC3B–I ratio [24,25]. Rodent experiments have shown that the interaction between MYTHO and LC3B is crucial for the elongation step of autophagosome formation, emphasizing the pivotal role of MYTHO in the autophagy machinery within skeletal muscle [45]. This is further supported by the positive association between the LC3B-II:LC3B–I ratio and MYTHO observed in the present investigation. As a novelty, our study demonstrates that muscle oxygenation is required to downregulate the exercise-induced elevation of MYTHO, which occurs quickly upon cessation of muscle contractions. This downregulation is necessary to prevent hyperactivation of autophagy [24,25]. We did not observe changes in MTOR protein levels, and there was a decline in AKT phosphorylation at Ser^{473}, indicating inhibition of protein synthesis.

4.4. LAMP2A and HSPA8 in response to high-intensity exercise, ischemia, and hypoxia induces chaperone-mediated autophagy signaling

The limiting step of CMA is the availability of the LAMP2A receptor at the lysosomal membrane, which is regulated by NRF2 [28,46], linking oxidative damage with CMA induction [47]. In mouse hepatocytes, knockout of NRF2 reduces LAMP2A mRNA and protein levels [47]. The present study provides for the first time evidence for upregulation of both NRF2 [31] and LAMP2A protein expression in response to high-intensity exercise and post-exercise ischemia in human skeletal muscle, underscored by the linear positive association observed here between *p*-Ser^{40} NRF2 and LAMP2A. HSPA8 selectively recognizes substrates with KFERQ-like motifs for lysosomal degradation [48,49] and is active under oxidative stress conditions [29]. We have previously shown that this exercise model elicits oxidative stress and increased protein carbonylation [50]. Thus, the results are compatible with increased degradation of muscle proteins with KFERQ-like motifs, facilitated by oxidative stress and the reduced cellular PO_2 observed at near maximal exercise in normoxia and hypoxia. The marked increase in LAMP2A observed here with exercise should have facilitated this process. The induction of macroautophagy in our exercise model should have elicited the fusion of autophagosomes and lysosomes, contributing to increased intracellular lysosomal pH, a known mechanism stimulating HSPA8 degradation [26]. Besides, the present study demonstrates that reduced HSPA8 and increased LAMP2A protein levels with exercise are rapidly reverted during recovery with free circulation.

4.5. Greater levels of muscle hypoxia do not enhance exercise-induced autophagy signaling or HIF1A levels

Production of ROS in skeletal muscle is exacerbated by exercise in hypoxia, likely due to a higher activation of the anaerobic metabolism and metabolic acidosis [50]. In contrast to our hypothesis, autophagy signaling markers were similar at exhaustion in normoxia and severe acute hypoxia, indicating that the activation was already maximal in normoxia or that the additional reduction in cellular PO_2 during exercise in hypoxia was not enough to stimulate autophagy further. These findings are consistent with the outcomes presented by Moberg et al. during submaximal exercise at a lower level of hypoxia than used in the present investigation [17].

Proline hydroxylation is enzymatically catalyzed by prolyl hydroxylases (PHDs), where oxygen acts as a co-substrate [51]. Under normoxic PO_2 levels, the PHDs catalyze the hydroxylation of HIF1A at residue Pro^{564}, targeting it for the E3 ubiquitin ligase and the subsequent proteasome-mediated degradation [52]. Here, we have observed a substantial increase in HIF1A hydroxylation at exhaustion, which remained elevated during ischemia. This change is compatible with the reported reduction of Von Hippel-Lindau tumor suppressor protein with exercise [33,53]. Other conceivable mechanisms accounting for the accumulation of hydroxylated HIF1A following incremental exercise could include a decrease in its proteasomal degradation rate due to insufficient energy availability [54]. This concurs with the observed inactivation of FOXO. Moreover, in the present investigation, the increase in the hydroxy-Pro^{564}: total HIF1A ratio was not attenuated in hypoxia despite the very low partial pressure of oxygen in arterial blood (P_aO_2) values (~33 mmHg near exhaustion) [33]. This could be because skeletal muscle is designed to work under the very low PO_2 levels reached during high-intensity exercise and forceful isometric contractions (which cause ischemia) [52].

4.6. Protein synthesis and elongation downregulation accompany the activation of autophagy

At low energy levels, AMPKα inhibits MTOR [55], suppressing protein synthesis. The latter impedes MTOR phosphorylation of ULK1 at Ser^{757}, which would inhibit ULK1 [56]. MTOR phosphorylation levels remained unchanged following exercise and ischemia in the present investigation, which concurs with the observed lack of activation of its upstream kinase AKT. Notably, our study participants had undergone a fasting period exceeding 12 h. These findings are consistent with Schwalm et al. [15], highlighting AKT activation's dependency on the fed state. They reported robust AKT stimulation in the fed state but

observed no such stimulation in AKT after exercise in a fasted state. Additionally, previous investigations in mouse skeletal muscle have documented a decline in AKT activity during fasting conditions [57,58]. The unchanged *p*-Ser^{2448} MTOR levels after post-exercise and ischemia in the present investigation concurs with Brandt et al. [13], which report unchanged *p*-Ser^{2448} MTOR after moderate cycling for 60 min at 60 % $\dot{V}O_2$peak. Furthermore, high-volume exercises, such as ultra-endurance activities, led to unchanged [14] and decreased [14,22] protein expression levels of MTOR and AKT.

Protein elongation is controlled by EEF2 [59], which EEF2K inhibits through Thr^{56} phosphorylation of EEF2 [54]. EEF2K, a calcium/calmodulin-dependent kinase (or CaMKIII), is activated by AMPKα [60,61] and GSK3B [62] and inhibited through proline hydroxylation [54]. In the present investigation, the inhibitory phosphorylation of EEF2 was markedly increased after exercise to a similar extent in normoxia and hypoxia. This concurs with the reported similar activation of AMPKα in both conditions [17] and the similar inhibition of GSK3B reported here. Therefore, it indicates downregulation of protein synthesis and elongation, which is expected due to the reduced energy charge during high-intensity exercise until exhaustion [35].

4.7. Upregulation of PGC1A by AMPKα and CaMKII following exercise and ischemia: another potential mechanism to stimulate exercise-induced autophagy

The peroxisome proliferator gamma coactivator 1-alpha (PPARGC1A/PGC1A) is the master regulator of mitochondrial biogenesis [63–66], preserving mitochondrial quality and promoting the transcription of macroautophagy genes [64,67–71]. Calmodulin-dependent protein kinase II and AMPKα are upstream regulators of PGC1A, which the current investigation has found upregulated after exercise and ischemia, both in normoxia and hypoxia [31,36]. Notably, these changes are reverted to their pre-exercise levels within just 1 min of muscle oxygenation [31,36]. The higher levels of intracellular Ca^{2+} and the heightened energy demand, along with the accumulation of metabolites during acute exercise, may lead to the phosphorylation of PGC1A by AMPKα [72], increasing its transcriptional activity [72], which may manifest a few hours after exercise [73].

4.8. Rapid reversal of exercise-induced autophagy activation by immediate post-exercise tissue reoxygenation

Another original feature of this study was the application of post-exercise total ischemia (cuff pressure: 300 mmHg) just immediately after the end of the incremental exercise to exhaustion in both tests. This approach allowed us to capture fast signaling events that may be reversed by cessation of muscle contractions combined with post-exercise hyperemia. During the first 3–5 s of the occlusion, the O_2 stores (O_2 trapped in capillary blood and bound to myoglobin) undergo depletion due to the heightened stimulation of oxidative phosphorylation [35,74]. This was evidenced by the fast reduction and stable plateau of muscle oxygenation measured by near-infrared spectroscopy, as previously reported [35]. Importantly, the initial post-exercise muscle biopsy was performed 10 s following exercise cessation, when the muscle conditions closely resembled those experienced at exhaustion while the cuff was inflated. The same leg was re-biopsied after 60 s of occlusion, while simultaneously an additional biopsy was obtained from the contralateral extremity, which recovered with a free circulation. This approach enabled a direct comparison between the occluded and non-occluded leg. During the 60 s occlusion, the energy metabolism continued to be active in the occluded leg, primarily relying on the energy derived from glycolysis. The latter resulted in lactate, H^+, P_i, and free creatine accumulation, while the concentration of ATP remained at the same level reached at exhaustion, i.e., ~-80 % of the concentration observed before exercise [35]. Despite the increased cytosolic acidification during the 60 s occlusion, no significant changes in signaling were observed between the 10th and the 60th s of post-exercise ischemia in the occluded leg. This suggests that autophagy signaling reached its maximum level at exhaustion and remained at that level during the ischemic period. The production of ROS during the 60 s of ischemia is also improbable, given the absence of oxygen supply from the 10th to the 60th second in the occluded leg [75]. In agreement, Gallego et al., using data from the same experiment, revealed that the redox-sensitive NRF2-KEAP1 pathway is not further activated from the 10th to the 60th s of post-exercise ischemia [31]. Moreover, most of the protein signaling changes elicited by exercise returned to the pre-exercise status just 60 s after the end of exercise in the leg recovering with free circulation, even though muscle lactate and H^+ remained almost at the same level reached at exhaustion [35]. In contrast, P_i and Cr were reduced due to PCr resynthesis during the 60 s recovery with free circulation but without reaching pre-exercise values [35]. This suggests that muscle lactate or acidification is not crucial in initiating or sustaining signaling activation. Instead, the large increase of P_i, accompanied by almost depletion of PCr during ischemia, may have inhibited the phosphatases [76], preserving during ischemia the phosphorylation levels of the proteins assessed here. The rapid restoration of autophagy signaling to pre-exercise levels requires oxygen and is essential to avoid the hazards associated with excessive and uncontrolled proteolytic stimulation [18,77,78].

4.9. Oxygenation and signaling in resting human skeletal muscle

The effect of oxygenation in resting skeletal muscle signaling has barely been researched in humans. A few studies have explored the impact of prolonged ischemia on muscle signaling, particularly in patients undergoing surgical procedures such as total knee arthroplasty (TKA) [79,80]. Leurcharusmee et al. [79] applied three cycles of 5-min ischemia followed by 5-min reperfusion just before performing a TKA in patients. They obtained the muscle biopsies from the *vastus medialis* 30 min after the last ischemia and upon release of the surgical tourniquet, which was inflated at 100 mmHg above systolic arterial pressure. In Leurcharusmee et al. [79], mitochondrial fusion proteins (MFN2 and OPA1) were increased at the onset of reperfusion. At the same time, no significant changes were observed in 4-HNE, SOD2, TNF, IL6, *p*-Ser^{616} DRP1:DRP1, MFN1, PGC1A, ETC complex I–V, CYCS, and cleaved CASP3/CASP3 expression between the end of ischemia and reperfusion [79]. Nevertheless, the baseline signaling was not measured, impeding the comparison between normal resting conditions and ischemia. Bailey et al. [80] obtained *vastus lateralis* muscle biopsies before, after ~43 min of ischemia (tourniquet pressurized above 300 mmHg), and after ~16 min of reperfusion in 70-year-old osteoarthritis patients (8 females and 4 males) during TKA. Ischemia was associated with the upregulation of the MAFbx and MURF1 [80]. Shadgan et al. [81] reported increased protein oxidation in *peroneus tertius* muscle biopsies obtained before and immediately after ~43 min of surgical ischemia (300 mmHg pressure). There is no data on the human skeletal muscle signaling responses to ischemia in healthy humans since previous research has been carried out in patients with osteoarthritis-related muscle atrophy [82] or in the context of other kinds of orthopedic surgery [81]. Direct comparisons between these studies and the present findings are challenging due to different baseline physiological conditions and a lack of information regarding biopsy timing in previous studies. Moreover, no other studies have analyzed the skeletal muscle signaling responses to post-exercise ischemia.

4.10. The role of ROS in autophagy signaling in skeletal muscle

During exercise, ROS are primarily produced by nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX2) and mitochondria [83,84], yet their direct involvement in skeletal muscle autophagy remains unclear. In our study, the application of immediate and complete

post-exercise ischemia trapped ROS and metabolites while muscle oxygen was depleted within 3–5 s, preventing early recovery, as previously reported [35]. In the present investigation, muscle lactate and H^+ were increased with exercise and ischemia [35]. Hydrogen ions can interact with superoxide radicals to form hydrogen peroxide (H_2O_2), which, under acidic conditions, can react with Fe^{2+} via the Fenton reaction, producing hydroxyl radicals (OH^-) [85]. In C2C12 cells, high doses of H_2O_2 activate AMPKα, increasing autophagy and PGC1A transcriptional activation [86,87]. After incremental exercise and ischemia, the highly acidic intra-cellular environment may have facilitated ROS production, AMPK Thr^{172} phosphorylation, autophagy, and PGC1A phosphorylation at Ser^{571} in our experimental conditions. Nutrient deprivation in Chinese Hamster Ovary (CHO) cells triggers mitochondrial H_2O_2 production, inhibiting Atg4 and prolonging autophagosome elongation by preventing LC3B-II delipidation [88]. We observed higher levels of LC3B-II:LC3B–I due to decreased LC3B–I, which may indicate increased LC3B-II lipidation activity. Talbert et al. [89] showed that increased mitochondrial ROS levels in a rat model of disuse muscle atrophy inhibit AKT/MTOR signaling, activating key proteolytic systems [89]. In our study, AKT activation decreased, and MTOR remained unchanged after incremental exercise, likely due to increased post-exercise and ischemia ROS production, prompting autophagy activation. Moreover, ROS are known to facilitate the translocation of FOXO to the nucleus [90]. However, the ROS levels in our study may not have been sufficient to activate FOXOs, as their activation typically occurs under pathological conditions associated with muscle atrophy [91]. ROS also upregulate NRF2 [31], a key promoter of p62 expression [92]. Despite upregulated transcription, low p62 levels indicated degradation by lysosomal proteases [93]. NRF2 also upregulates LAMP2A receptor [94], the primary regulator of CMA [28,46]. Thus, by stimulating autophagy and elimination of damaged organelles, ROS downregulate their own production.

In conclusion, our findings indicate that macroautophagy and CMA were similarly upregulated during exercise to exhaustion in normoxia and severe acute hypoxia. Furthermore, ROS produced during incremental exercise and ischemia may activate macroautophagy and CMA but not FOXOs. We have also shown increased expression of the novel autophagy PHAF1/MYTHO biomarker in human skeletal muscle in response to exercise in normoxia, hypoxia, and post-exercise ischemia. Our study does not support a critical involvement of FOXOs in exercise-induced autophagy. We have also provided evidence for a concurrent activation of autophagy with inhibition of protein synthesis. Additionally, we have demonstrated that exercise-induced autophagy activation was not enhanced by severe acute hypoxia and was reversed within 60 s of recovery as long as the circulation was intact.

4.11. Strengths and limitations

The main strength of the present investigation relies on the uniqueness of its experimental design, which allows it to address early signaling events elicited by exercise in humans. Besides, we have assessed these responses during exercise in normoxia and severe acute hypoxia close to the tolerable limit for humans. Our biopsies were obtained just 10 s after the end of incremental exercise and immediately after occlusion, with accurate timing. Other studies report the biopsy at rest and time 0 h (after exercise), but no accurate time details are provided. Although the principal limitation of this study is the absence of a direct assessment of autophagic flux, which cannot be performed in humans, we provide the combination of several indirect markers of autophagy, which are compatible with an increased autophagic flux with high-intensity exercise. Another limitation of the present study is that due to the small amount of tissue available, biomarkers of oxidative stress were not assessed. However, we have provided extensive evidence for ROS-induced signaling [31,36]. Our findings are based on Western blot analyses, which show high variability. Nevertheless, the latter was accounted for by the statistical analysis and proper normalization. It should be highlighted that recent research has shown that the autophagy response to exercise in humans is different from that observed in rodents, further emphasizing the relevance of this contribution. A final strength of this study is the comprehensive assessment of skeletal muscle signaling through the combination of experimental conditions and the thorough assessment of physiological and biochemical variables.

Disclosure statement

No potential conflict of interest was reported by the authors.

Data availability statement

The data that support the findings of this study are available on request from the co-senior author, MMR. The data are not publicly available due to ethical restrictions.

CRediT authorship contribution statement

Miriam Martinez-Canton: Writing – original draft, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Victor Galvan-Alvarez:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Angel Gallego-Selles:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Miriam Gelabert-Rebato:** Writing – review & editing, Investigation, Formal analysis. **Eduardo Garcia-Gonzalez:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Juan Jose Gonzalez-Henriquez:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Marcos Martin-Rincon:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jose A.L. Calbet:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

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STUDY 5

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A Mango Leaf Extract (Zynamite®) Combined with Quercetin Has Exercise-Mimetic Properties in Human Skeletal Muscle

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Abstract: Zynamite PX®, a mango leaf extract combined with quercetin, enhances exercise performance by unknown molecular mechanisms. Twenty-five volunteers were assigned to a control (17 males) or supplementation group (8 males, receiving 140 mg of Zynamite® + 140 mg quercetin/8 h for 2 days). Then, they performed incremental exercise to exhaustion (IE) followed by occlusion of the circulation in one leg for 60 s. Afterwards, the cuff was released, and a 30 s sprint was performed, followed by 90 s circulatory occlusion (same leg). *Vastus lateralis* muscle biopsies were obtained at baseline, 20 s after IE (occluded leg) and 10 s after Wingate (occluded leg), and bilaterally at 90 s and 30 min post exercise. Compared to the controls, the Zynamite PX® group showed increased basal protein expression of Thr^{287}-CaMKIIδ_D (2-fold, $p = 0.007$) and Ser^{9}-GSK3β (1.3-fold, $p = 0.005$) and a non-significant increase of total NRF2 (1.7-fold, $p = 0.099$) and Ser^{40}-NRF2 (1.2-fold, $p = 0.061$). In the controls, there was upregulation with exercise and recovery of total NRF2, catalase, glutathione reductase, and Thr^{287}-CaMKIIδ_D (1.2–2.9-fold, all $p < 0.05$), which was not observed in the Zynamite PX® group. In conclusion, Zynamite PX® elicits muscle signaling changes in resting skeletal muscle resembling those described for exercise training and partly abrogates the stress kinases responses to exercise as observed in trained muscles.

Keywords: ergogenic aids; signaling; antioxidant supplementation; high-intensity exercise; ischaemia-reperfusion; sports nutrition; polyphenols; muscle function; human

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1. Introduction

Natural polyphenols combine aromatic rings with a variety of functional groups, which entail a great diversity of physiological effects conferring antioxidant, cardioprotective, neuroprotective, anticancer, immunomodulatory, prebiotic, ergogenic (enhancement of exercise performance), and antimicrobial properties [1–3]. Zynamite®, an extract from mango leaves abundant in mangiferin, has been shown to enhance power output when combined with quercetin or luteolin [4,5]. Moreover, Zynamite® accelerates recovery after exhausting exercise [3] and attenuates the negative effects of ischemia-reperfusion on muscle function [4–6]. Zynamite PX®, a polyphenolic extract combining the mango leaf extract with quercetin, has been shown to enhance exercise performance after a single dose [6],

as well as after 48 h [4,5] and 15 days of supplementation [4,5]. Zynamite® has also been shown to improve reaction time in humans and long-term potentiation in the hippocampus in rodents [7]. However, no previous study has determined whether Zynamite PX® may trigger molecular changes in skeletal muscle analogous to the ones elicited by physical exercise, thus functioning as an exercise mimetic.

Mangiferin has iron-chelating capacity and remarkable antioxidant power due to its free-radical scavenging properties and its ability to inhibit nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase or NOX) and xanthine oxidase (XO), which are prominent producers of reactive oxygen species (ROS) in exercise [8] and inflammation [9–14]. Quercetin, which also has potent antioxidant and anti-inflammatory actions [15], may enhance aerobic exercise performance [16] and power output during sprint exercise when administered together with Zynamite® [4,5]. Animal and cell culture experiments have shown that both mangiferin [13,17–19] and quercetin exert protection against damage caused by ischemia-reperfusion [20–23], which could, in part, be explained by their inhibitory effects on XO and NOX [24,25].

ROS production during exercise is more prominent during high-intensity and prolonged exercise, mainly if performed until exhaustion [8,26–28] or in severe hypoxia [29]. Although uncontrolled ROS production could cause oxidative damage and fatigue [30], exercise training increases skeletal muscle antioxidant capacity [31–33] and reduces ROS-induced signaling [8,27] and damage [34]. Part of the signaling responses needed for the adaptive response to exercise is mediated by ROS [26,35]. Although several enzymes and transcription factors are ROS-sensitive, the nuclear factor erythroid-derived 2-like 2 (NRF2) is the primary ROS sensor in most cells, including skeletal muscle [36–39]. Upon stimulation, NRF2 translocates to the nucleus, where it can interact with more than 250 genes possessing specific deoxyribonucleic acid (DNA) sequences called antioxidant response elements (AREs) involved in inflammation, autophagy, metabolism, mitochondrial biogenesis, detoxification, cytoprotection, cell differentiation, and the xenobiotic and antioxidant response [2]. Free NRF2 levels are regulated by Kelch-like ECH-associated protein 1 (Keap1), which under normal unstressed conditions, binds to NRF2, prevents its translocation to the nucleus and facilitates NRF2 ubiquitination and proteasomal degradation [2]. However, oxidants and electrophiles can interact with the numerous cystines in Keap1, causing conformational changes that disrupt the Keap1-NRF2 union releasing free NRF2. NRF2 may be phosphorylated by several exercise-stimulated kinases, such as extracellular signal-regulated kinases (ERK), protein kinase C (PKC), c-Jun N-terminal kinases (JNK), and p38 mitogen-activated protein kinases (p38 MAPK) [32,40,41]. NRF2 phosphorylation by these kinases prevents NRF2 degradation and facilitates its translocation to the nucleus and gene transcription [2]. The exercise-induced activation of the NRF2-regulated gene program plays a vital role in the adaptation to exercise training [39,42,43]. In contrast, serine phosphorylation of NRF2 by glycogen synthase kinase 3 beta (GSK3β) promotes its proteasomal degradation [44,45].

Although some antioxidants and XO inhibitors can partly block the acute signaling response to exercise [8,35], cell and animal experiments indicate that polyphenols such as mangiferin and quercetin may circumvent this drawback by inducing NRF2 [9,46,47]. However, no human study has determined whether dietary polyphenols may increase NRF2 levels and signaling in resting skeletal muscle. There is no information regarding polyphenols' effects on the muscle signaling responses to exercise [8,48,49]. It also remains unknown whether Zynamite PX® exerts signaling effects on resting skeletal muscle and whether short-term Zynamite PX® supplementation modifies the signaling response to high-intensity exercise.

Therefore, this investigation aimed to determine the effects of 48 h Zynamite PX® supplementation on skeletal muscle NRF2 protein levels and NRF2-induced signaling under basal conditions and in response to high-intensity exercise in humans. To achieve these aims, we have also determined the effects of Zynamite PX® on Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and GSK3β and Keap1 as main regulatory mecha-

nisms of NRF2 levels [2,50,51]. Thus, we hypothesized that Zynamite PX® supplementation would increase basal NRF2 protein levels and attenuate the signaling responses induced by high-intensity exercise.

2. Materials and Methods

2.1. Subjects

Twenty-five young males agreed to take part in this research (means ± SD; age: 22.2 ± 2.1 years, body mass: 72.4 ± 7.4 kg, height: 177 ± 8 cm, body fat: 18.7 ± 5.1%, and maximal oxygen consumption (VO_2max): 47.0 ± 6.4 mL kg^{-1} min^{-1}) (Table 1). The criteria for eligibility included the following: (a) individuals aged between 18 and 35 years with a body mass index under 30 kg/m^2, (b) gender: male, (c) a normal resting 12-lead electrocardiogram, and (d) an active lifestyle with regular exercise 2–4 times a week, though not necessarily adhering to a particular training regimen. The disqualifying criteria encompassed: (a) the presence of any illness or allergy, (b) any medical condition contraindicating physical activity, (c) habits such as smoking, and (d) undergoing any form of medical treatment. The study was carried out following approval by the Ethical Committee of the University of Las Palmas de Gran Canaria (CEIH-2015-03 and CEIH-2017-02). All volunteers were informed about the study's aims and potential risks associated with the exercise and the invasive procedures and signed a written consent before starting the experiments. Participants were advised to avoid strenuous or unusual physical activities two days before all lab examinations and to abstain from beverages containing caffeine and alcohol the day preceding the preliminary tests and two days before the principal invasive experiment. In addition, they were instructed to continue with their usual dietary habits until the conclusion of the study. All the participants were physically fit and engaged in regular exercise.

Table 1. Physical characteristics, body composition, and VO_2max.

	Control Group (*n* = 17)	**Zynamite PX® Group (*n* = 8)**	***p***
Age (years)	22.5 ± 2.4	21.6 ± 1.2	0.350
Height (cm)	178 ± 8	177 ± 9	0.786
Weight (kg)	72.7 ± 7.6	71.6 ± 7.4	0.730
Body fat (%)	18.6 ± 5.8	18.8 ± 3.6	0.930
Fat body mass (kg)	13.7 ± 5.3	13.6 ± 3.7	0.930
Lean body mass (kg)	55.8 ± 5.1	54.9 ± 5.1	0.698
VO_2max (mL min^{-1})	3432 ± 489	3281 ± 367	0.448
VO_2max (mL kg^{-1} min^{-1})	47.5 ± 7.1	46.0 ± 4.7	0.594

2.2. General Overview

The research protocol was designed to identify the primary signaling routes triggered by cell stress during physical activity and post-exercise ischemia, utilizing a new experimental paradigm [36,52,53]. In this study, it was decided to allocate one subject to the Zynamite PX® supplementation group per each 2 subjects included in the control group. It was determined that to identify a 40% difference in the basal NRF2 protein expression between the control group and the group that received supplements, a sample size of 7 and 15 participants was required, assuming $\alpha = 0.05$ and $\beta = 0.80$, as calculated using G*Power version 3.1.9.6. Supplementation consisted of 140 mg of Zynamite® (standardized to 60% mangiferin, an aqueous extract from *Mangifera indica*) [4] in combination with 140 mg of quercetin (provided as 280 mg *Sophora japonica* extract, standardized to 50% quercetin) every 8 h for two days (six doses in total). The control subjects did not receive any supplement. Supplemented subjects were informed that the study's main aim was to examine the supplement's effect on muscle signaling responses.

2.3. Pre-Tests and Familiarization

The first visit to the laboratory was dedicated to anthropometric measurements and assessing the body composition (dual-energy X-ray absorptiometry, Lunar iDXA, GE Healthcare, Milwaukee, WI, USA) [53]. Next, the participants reported to the laboratory on three other days for familiarization with experimental procedures, including an incremental exercise to exhaustion and sprint exercise (Wingate tests, a 30 s all-out sprint). This was continued by a session devoted to determining their VO_2max using an incremental exercise to exhaustion [52] and another two sessions to measure their maximal functional reserve [52] using repeated supramaximal exercise bouts at 120% of VO_2max until exhaustion, interspaced with 20 s recovery periods, one day recovering with a free circulation and the other with total occlusion of the circulation [52]. The exercise tests were carried out on a Lode ergometer (Groningen, The Netherlands), while subjects were requested to keep a pedaling frequency close to 80 revolutions per minute (RPMs) [52,53]. Exhaustion was defined by the subject abruptly ceasing to pedal or by a decrease in the pedaling cadence to less than 50 RPMs for 5 s despite intense verbal encouragement. The highest 20-s averaged VO_2 value recorded during the incremental exercise to exhaustion or repeated supramaximal exercise bouts was taken as the VO_2max [54]. Oxygen uptake was measured breath-by-breath using a metabolic cart (Vyntus, Jaeger-CareFusion, Höchberg, Germany) calibrated immediately before each test using high-grade certified gases provided by the manufacturer and validated by a butane combustion test [55]. The flowmeter was calibrated before each test at low (0.2 L/s) and high (2 L/s) flows.

2.4. Main Experiments and Supplement Administration

A schematic representation of the experimental protocol is presented in Figure 1. One week after the VO_2max assessment, volunteers reported to the laboratory at 07:00 h, following a 12-h overnight fast. After resting supine for 90 min on a laboratory stretcher, the skin and subcutaneous tissue of the lateral aspect of the thigh was infiltrated with 1 mL of 2% lidocaine, and five min later, a baseline muscle biopsy was taken from one of the m. *vastus lateralis* (assigned randomly) using a Bergstrom's biopsy needle with suction, as previously reported [53]. For the initial biopsy, the needle was angled distally at a 45° tilt [56], and the skin incision was covered with a temporary plaster that was easy to remove at exhaustion for fast biopsy collection.

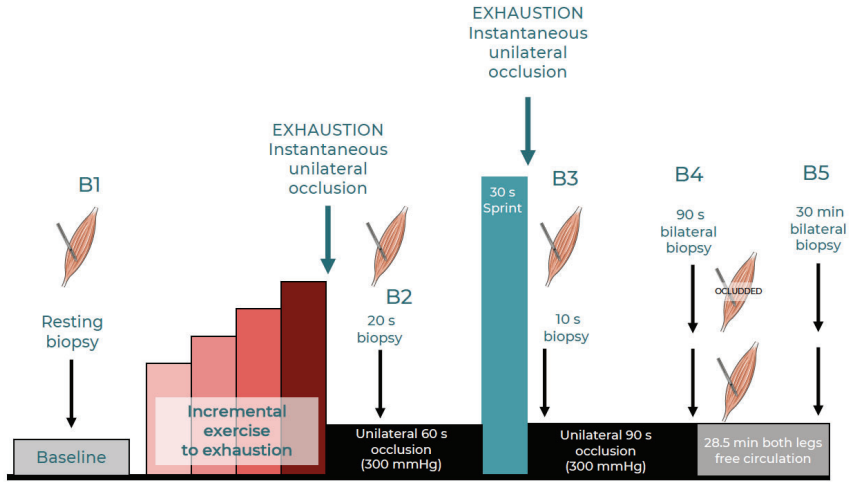

Figure 1. Schematic representation of the experimental protocol.

Then, the contralateral thigh was also anesthetized in the same area by applying similar procedures, and a 5 mm incision was performed and covered with a temporary plaster.

This was followed by the ingestion of the last dose of the supplement (140 mg of Zynamite® with 140 mg of quercetin, marketed as Zynamite PX®). After that, an SC10D Hokanson cuff was wrapped around one of the thighs (chosen randomly, which was the thigh having an incision ready but not yet biopsied), as close as possible to the inguinal crease. The SC10D cuff was connected via a plastic tube to a fast cuff inflator (Hokanson, E20 AG101). Then, the subjects sat on the cycle ergometer. After verification of proper readings, the incremental exercise test started (~60 min after the ingestion of the supplement), with three minutes at 20 W, followed by 20 W increases every three minutes until the respiratory exchange ratio (RER) was $\geq$1.00. After that, the ergometer was unloaded while the subjects kept pedaling at 30–40 RPMs for two minutes. Then, the exercise intensity was raised to the same load attained at the end of the initial phase and incremented by 15 W every minute until exhaustion. Upon exhaustion, the Hokanson was triggered to apply a 300-mmHg pressure around the thigh to completely occlude the circulation in less than 2 s. Twenty seconds after exhaustion, the second muscle biopsy was taken from the occluded leg. This second biopsy was obtained by introducing the needle with a 45° inclination and pointing distally. Then, the volunteer remained quietly seated on the cycle ergometer and prepared to sprint maximally for 30 s (Wingate test) with the ergometer set in isokinetic mode (80 RPMs) exactly 60 s after exhaustion. During these 60 s, the cuff remained inflated, and the circulation occluded. At the beginning of the 30 s sprint, the cuff was instantaneously released and re-inflated at 300 mmHg at the end of the 30 s sprint. Exactly 10 s after the sprint, a third muscle biopsy was obtained from the occluded leg. During this third biopsy, the needle was inserted at a right angle to the thigh [56]. Immediately after the biopsy, the incision was covered, and the subject was moved carefully to a stretcher, where he rested in the supine position with the circulation of the leg biopsied fully occluded. Ninety seconds after the end of the exercise, a bilateral biopsy was simultaneously obtained from the occluded and non-occluded leg, immediately after which the cuff was deflated. In the occluded leg, the biopsy needle was introduced with a 45° inclination and pointing proximally, while in the contralateral leg, the needle was introduced perpendicular to the thigh. Thirty minutes after the end of the exercise, a last bilateral muscle biopsy was obtained. The needle was introduced with a 30° inclination and pointing proximally in the leg that had already undergone three biopsies. Thus, one leg had an occluded circulation of 60 s after the incremental exercise and another lasting 90 s after the sprint exercise, whilst the contralateral leg always recovered with free circulation. All biopsies were immediately frozen in liquid nitrogen and stored at −80 °C.

2.5. Muscle Protein Extraction and Western Blotting

Skeletal muscle lysates were obtained by grinding 10 mg of muscle sample for one minute using a Mikro-Dismembrator S (Sartorius, Goettingen, Germany) equipped with stainless steel balls. The ground muscle was immediately homogenized in urea lysis buffer containing 6 M urea and 1% SDS, along with 10X PhosSTOP phosphatase inhibitor (Cat. #4906837001) and 50X Complete protease inhibitor (Cat. #11697498001) obtained from Roche (Basel, Switzerland). Subsequently, the muscle lysate was centrifuged (25,200× *g* for 12 min at 16 °C). Total protein content was quantified using the bicinchoninic acid assay [57]. The extract volume was adjusted to the muscle weighted individually to achieve a ~6.8 µg/µL final concentration in all muscle protein extracts. Afterwards, the supernatant was combined with an electrophoresis loading buffer. This buffer consisted of 160 mM Tris-HCl at 6.8 pH, 5.9% SDS, 25.5% glycerol, along with 15% β-mercaptoethanol-bromophenol blue.

The amount of protein to be loaded and the antibody concentration for each assay was determined empirically by loading different amounts of control protein (2 to 30 µg). The control protein was prepared using human skeletal muscle processed with the same procedures applied to the experimental samples. The range of protein amount for which there was a linear relationship between the amount of control protein and optical band density was determined for each antibody. Later, an amount of sample protein within

the linear range of the protein/optical density response was loaded for each kinase or signaling protein tested (7.5 to 15 μg), followed by electrophoresis on SDS-PAGE gels by the Laemmli system. The proteins were then transferred onto Immun-Blot polyvinylidene fluoride (PVDF) membranes for immunoblotting (Bio-Rad Laboratories, Hercules, CA, USA) (Supplementary Table S1). Loading and transfer efficiency was assessed by staining the membranes with Reactive Brown 10 (Sigma Aldrich, St. Louis, MO, USA). All samples corresponding to a given participant were run on the same gel intercalated with four control samples.

The membranes underwent a blocking process for a span of one hour using either 5% non-fat dried milk powder diluted in Tris-buffered saline containing 0.1% Tween 20 (Blotto) or 4% bovine serum albumin (BSA). This was followed by an overnight incubation period of 12–15 h at a temperature of 4 °C in the presence of primary antibodies. These primary antibodies were diluted in 5% Blotto or 4% BSA blocking buffers. Right after, the membranes were exposed to an HRP-linked anti-rabbit or anti-mouse antibody, the dilution of which ranged from 1:5000 to 1:20,000 in 5% Blotto-blocking buffer in every case. The membranes were then subjected to chemiluminescent visualization using the Clarity™ Western ECL Substrate procured from Bio-Rad Laboratories (Hemel Hempstead, UK) through a ChemiDocTM Touch Imaging System also from Bio-Rad Laboratories (Hercules, CA, USA). Lastly, the densitometric band data were quantified employing the Image Lab© software, version 6.0.1 from Bio-Rad Laboratories. The densitometric measurements were expressed in arbitrary units (a.u). No further corrections were carried out because the loading was consistent across membranes. Each sample was assessed with a single measurement taken per sample. The supplemented group had three missing values: the biopsy corresponding to the post-sprint exercise in one subject and the two corresponding to the 90 min post exercise in another subject.

The antibodies employed in the current study were acquired from different suppliers. From Abcam (Cambridge, MA, USA): Ser^{40}-NRF2 (no. ab76026) and total NRF2 (no. ab62352). From Cell Signaling Technology (Danvers, MA, USA): Thr^{287}-CaMKII (no. 12716), Thr^{180}/Tyr^{182}-p38 MAPK (no. 9211), Ser^{9}-GSK3β (no. 5558) and Catalase (no. 14097). From Proteintech (Rosemont, IL, USA): Glutathione reductase (GR) (no. 18257-1-AP) and Keap1 (no. 10503-2-AP). The horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (product no. 111-035-144), and the horseradish peroxidase-conjugated goat anti-mouse antibody (product no. 115-035-003) were both ordered from Jackson ImmunoResearch (West Grove, PA, USA). The CaMKIIδ_D isoform was identified by using an isoform-specific antibody for CaMKIIδ_D (anti-CaMKII delta isoform no. A010-55AP; Badrilla, Leeds, UK) [58,59]. See Supplementary Table S1 for more details regarding the antibodies and specific procedures. The Protein Plus Precision All Blue Standards were supplied by Bio-Rad Laboratories (Hercules, CA, USA).

2.6. Statistical Analysis

The values are reported as means ± standard deviations. The hypothesis of normality for each variable was assessed via the Shapiro–Wilks test. A logarithmic transformation was applied for variables demonstrating a significant departure from the Gaussian distribution. Unpaired *t*-tests were used to compare the mean expression values under basal conditions between the supplemented and the control group. A paired *t*-test was used to determine whether there was a difference between the leg that recovered with ischemia and the leg recovering with free circulation at 90 s and 30 min. Since both legs had similar responses at the 90 s biopsy, these two biopsies were averaged, and the resulting value was taken as representative of the 90 s post-exercise response. A similar approach was used for the 30 min responses since non-significant differences were observed between legs at this time point. Then, a repeated-measures ANOVA was run with one within-subjects factor: exercise (with five levels: resting (T1), post-VO_2max (T2), 10 s post-Wingate test (T3), 90 s post-Wingate test (T4), and 30 min post-Wingate test (T5)) and supplementation as between-subjects factors. The pre-requisite assumption of sphericity was verified using

Mauchly's test of sphericity prior to the execution of the ANOVA. When the assumption of sphericity was violated, the degrees of freedom (*df*) were corrected using the Huynh–Feldt epsilon procedure. The alpha level of statistical significance was established at $p \leq 0.05$. The statistical analyses were undertaken with IBM SPSS software, version 29.0, designed specifically for Apple Computers (IBM, New York, NY, USA).

3. Results

3.1. Effects of Zynamite PX® Supplementation on Basal Signalling

The protein expression levels of the enzymes and transcription factors measured under basal conditions are depicted in Figures 2 and 3. Basal total NRF2 and Ser^{40}-NRF2 protein expression were 1.7- and 1.2-fold higher in the Zynamite PX®-supplemented than in the control group ($p = 0.099$ and $p = 0.061$, respectively) (Figure 2A). Both groups had similar basal levels of Keap1 ($p = 0.975$) and total NRF2/Keap1 ratio ($p = 0.247$) (Figure 2A). The basal protein expression levels of catalase and glutathione reductase were similar in both groups ($p = 0.596$ and $p = 0.481$, respectively) (Figure 2B). Thr^{180}/Tyr^{182}-p38 MAPK protein expression was similar at pre in both groups ($p = 0.252$) (Figure 2B). Thr^{287}-CaMKIIδ_D basal expression was 2.1-fold higher in the Zynamite PX® supplemented than in the control group ($p = 0.007$) (Figure 2B). This was accompanied by 1.3-fold higher basal Ser^9-GSK3β phosphorylation in the Zynamite PX® supplemented group than in the control group ($p = 0.005$) (Figure 2B).

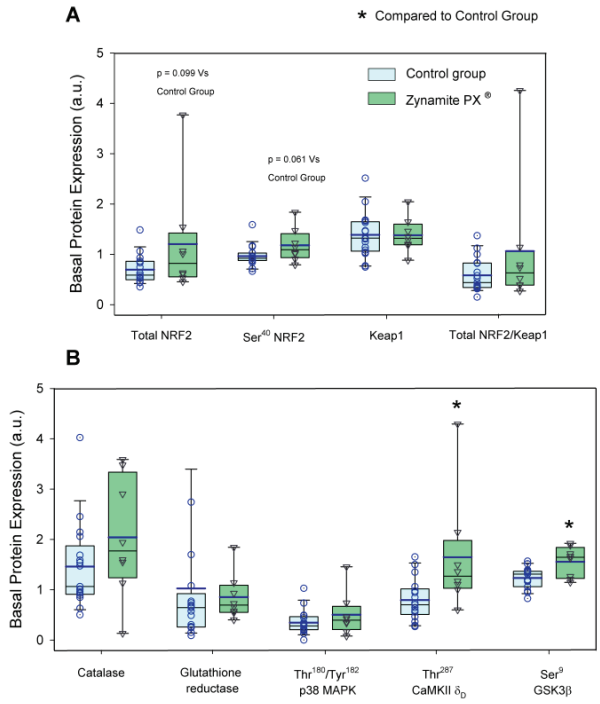

Figure 2. Protein expression levels of the enzymes and transcription factors measured under basal conditions. (**A**) Values for basal total NRF2, Ser^{40}-NRF2, Keap1, and total NRF2/Keap1 ratio (a.u.) and (**B**) values for catalase, glutathione reductase, Thr^{180}/Tyr^{182}-p38 MAPK, Thr^{287}-CaMKIIδ_D, and Ser^9-GSK3β (a.u.). The whiskers delimit the 5th and 95th percentiles; the thin and thick horizontal lines correspond to the median and the mean values, respectively; and the upper and lower ends of the boxes define the 1st and 3rd quartiles, respectively. Control group: circles ($n = 17$) and Zynamite PX® group triangles ($n = 8$). * $p < 0.05$ compared to the control group.

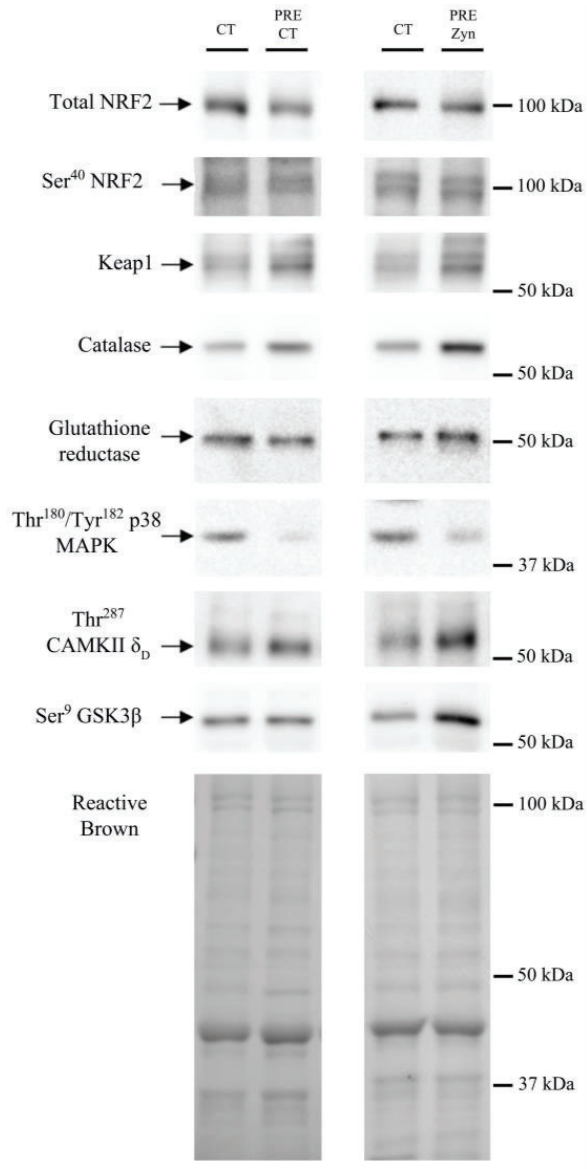

Figure 3. Immunoblots and total amount of protein loaded (Reactive Brown Staining) from a representative subject of the control group (Pre CT) and the Zynamite PX® supplemented group (Pre Zyn). CT corresponds to a human control sample (non-experimental) loaded onto each gel to allow for normalization and loading control. The markers indicate the closest molecular weight in kDa.

3.2. Effects of Zynamite PX® Supplementation Signalling Response to Exercise

Total NRF2 expression was increased by exercise only in the control group (1.3–1.7-fold, ANOVA exercise effect $p < 0.001$, F = 8.09, $df = 4$; exercise ×supplementation interaction $p = 0.032$, F = 2.79, $df = 4$) (Figure 4A). Ser^{40}-NRF2 protein expression remained unchanged with exercise in both groups (ANOVA exercise effect $p = 0.632$; F = 0.60, $df = 3.3$; exercise × supplementation interaction $p = 0.597$, F = 0.66, $df = 3.3$) (Figure 4B). Although non-significant changes were observed in Keap1 with exercise in either group (ANOVA exercise

effect $p = 0.925$, F = 0.22, $df = 4$; exercise × supplementation interaction $p = 0.921$, F = 0.23, $df = 4$) (Figure 5A), the total NRF2/Keap1 ratio was increased by exercise only in the control group (1.2–1.8-fold, ANOVA exercise effect $p < 0.001$, F = 6.94, $df = 4$; exercise × supplementation interaction $p = 0.061$, F = 2.35, $df = 4$) (Figure 5B). Catalase protein expression was increased in response to exercise only in the control group (1.5–1.8-fold, ANOVA exercise effect $p = 0.003$, F = 4.32, $df = 4$; exercise × supplementation interaction $p = 0.033$, F = 2.75, $df = 4$) (Figure 6A). Glutathione reductase protein expression was increased in response to exercise in the control group (1.9–2.9-fold, ANOVA exercise effect $p = 0.004$, F = 4.12, $df = 4$; exercise × supplementation interaction $p = 0.130$, F = 1.84, $df = 4$) (Figure 6B).

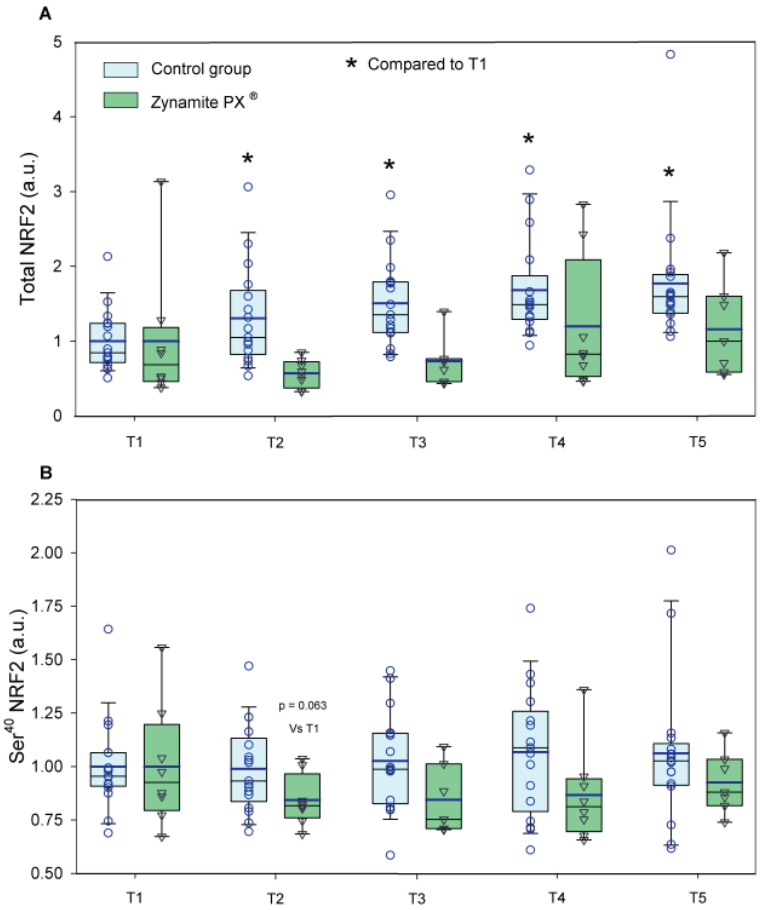

Figure 4. Protein expression levels of the enzymes and transcription factors during the exercise conditions and recovery. (**A**) Values for total NRF2 and (**B**) Ser^{40}-NRF2 (a.u.). Baseline (T1), post-VO_2max (T2), 10 s post-Wingate test (T3), 90 s post-Wingate test (T4), and 30 min post-Wingate test (T5). The whiskers delimit the 5th and 95th percentiles; the thin and thick horizontal lines correspond to the median and the mean values, respectively; and the upper and lower ends of the boxes define the 1st and 3rd quartiles, respectively. Control group: circles ($n = 17$) and Zynamite PX® group triangles ($n = 8$). * $p < 0.05$ compared to T1. Total NRF2: ANOVA exercise effect $p < 0.001$; exercise × supplementation interaction $p = 0.032$; Ser^{40}-NRF2: ANOVA exercise effect $p = 0.632$; exercise × supplementation interaction $p = 0.597$.

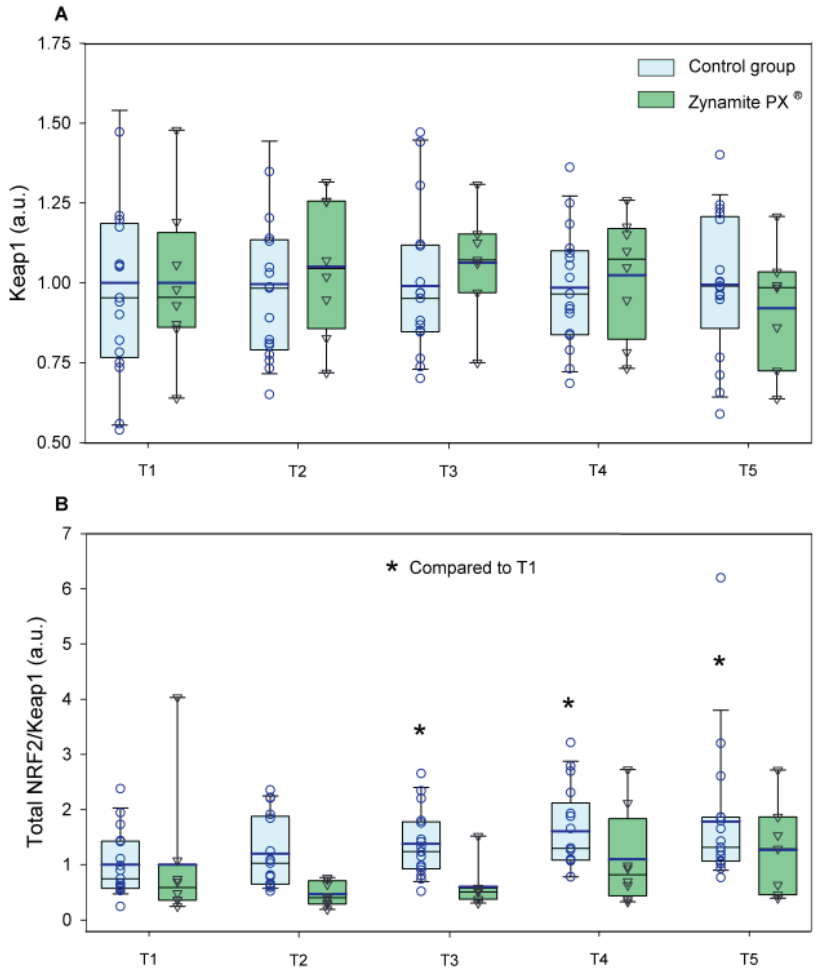

Figure 5. Protein expression levels of the enzymes and transcription factors during the exercise conditions and recovery. (**A**) Values for Keap1, and (**B**) total NRF2/Keap1 ratio (a.u.). Baseline (T1), post-VO_2max (T2), 10 s post-Wingate test (T3), 90 s post-Wingate test (T4), and 30 min post-Wingate test (T5). The whiskers delimit the 5th and 95th percentiles; the thin and thick horizontal lines correspond to the median and the mean values, respectively; and the upper and lower ends of the boxes define the 1st and 3rd quartiles, respectively. Control group: circles (n = 17) and Zynamite PX® group triangles (n = 8). * $p < 0.05$ compared to T1. Keap1: ANOVA exercise effect $p = 0.925$; exercise × supplementation interaction $p = 0.921$; NRF2/Keap1: ANOVA exercise effect $p < 0.001$; exercise × supplementation interaction $p = 0.061$.

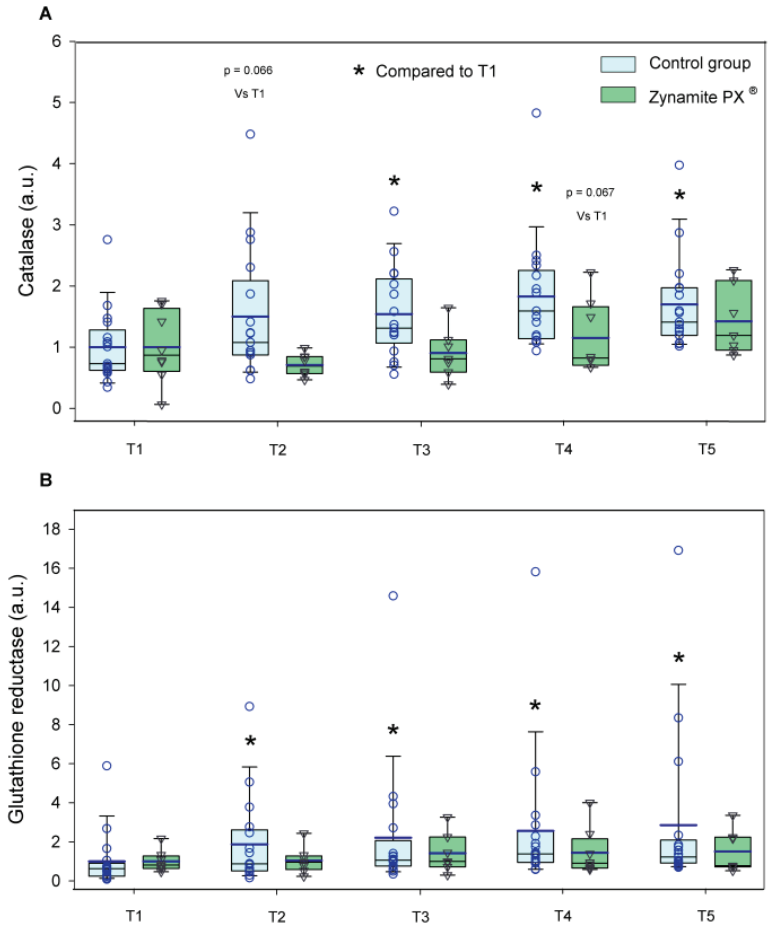

Figure 6. Protein expression levels of the enzymes and transcription factors during the exercise conditions and recovery. (**A**) Values for catalase, and (**B**) glutathione reductase (a.u.). Baseline (T1), post-VO_2max (T2), 10 s post-Wingate test (T3), 90 s post-Wingate test (T4), and 30 min post-Wingate test (T5). The whiskers delimit the 5th and 95th percentiles; the thin and thick horizontal lines correspond to the median and the mean values, respectively; and the upper and lower ends of the boxes define the 1st and 3rd quartiles, respectively. Control group: circles (n = 17) and Zynamite PX® group triangles (n = 8). * $p < 0.05$ compared to T1. Catalase: ANOVA exercise effect $p = 0.003$; exercise × supplementation interaction $p = 0.033$; Glutathione reductase: ANOVA exercise effect $p = 0.004$; exercise × supplementation interaction $p = 0.130$.

Thr^{180}/Tyr^{182}-p38 MAPK protein expression was similarly elevated in response to exercise in both groups (1.8–5.7-fold, ANOVA exercise effect $p < 0.001$, F = 35.3, df = 2.51; exercise × supplementation interaction $p = 0.080$, F = 2.49, df = 2.51) (Figure 7A). Thr^{180}/Tyr^{182}-p38 MAPK protein expression was slightly higher in the leg recovering with ischemia ($p = 0.01$, F = 7.89, df = 1); however, there was no significant interaction for the comparison of the responses observed in the leg with ischemia and the leg with free circulation recovery regardless of supplementation (recovery time × ischemia interaction $p = 0.845$, F = 0.03, df = 1; recovery time × ischemia × supplementation interaction $p = 0.830$, F = 0.05, df = 1). Thr^{287}-CaMKIIδ_D protein expression was increased in response to exercise in the control group (1.5–2.0-fold, ANOVA exercise effect $p = 0.008$, F = 3.72, df = 4; exercise × supplementation interaction $p = 0.064$, F = 2.31, df = 4) (Figure 7B). Thr^{287}-CaMKIIδ_D protein

expression was slightly higher in the leg recovering with ischemia (p = 0.033, F = 5.19, df = 1); nevertheless, there was no significant interaction for the comparison of the responses observed in the leg with ischemia and the leg with free circulation recovery regardless of supplementation (recovery time × ischemia interaction p = 0.403, F = 0.73, df = 1; recovery time × ischemia × supplementation interaction p = 0.628, F = 2.41, df = 1). Ser^{9}-GSK3β phosphorylation was reduced by 13% after the incremental exercise to exhaustion and then recovered pre-exercise values in both groups (ANOVA exercise effect $p < 0.001$, F = 5.79, df = 4; exercise × supplementation interaction p = 0.256, F = 1.36, df = 1) (Figure 8). Representative immunoblots of the enzymes and protein assessed during exercise can be seen in Figures 9 and 10.

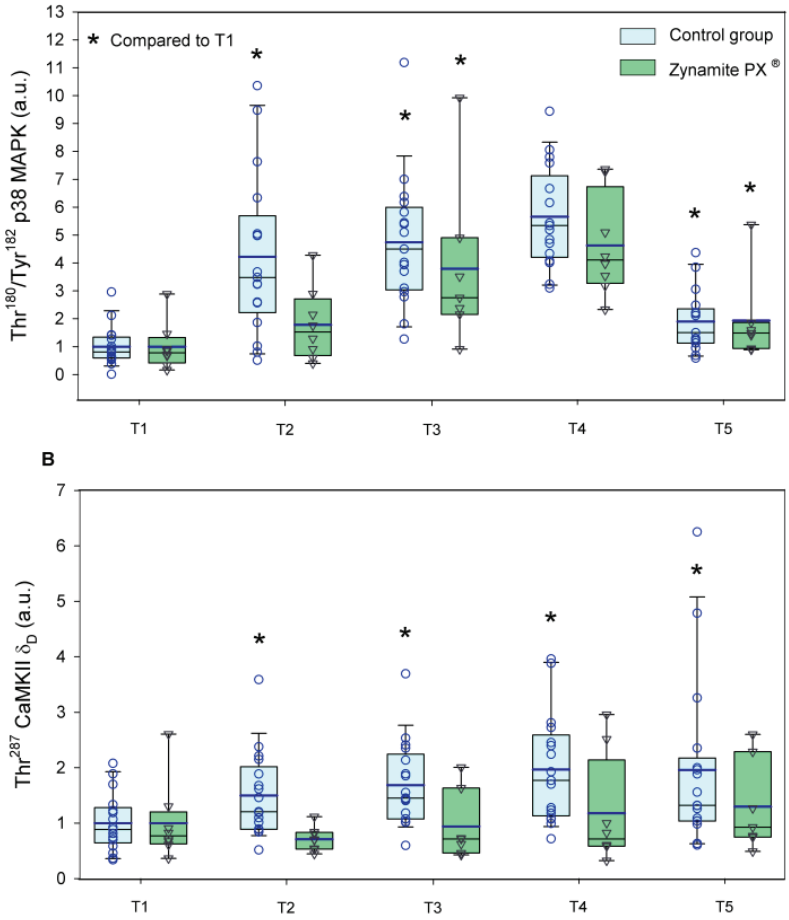

Figure 7. Protein expression levels of the enzymes and transcription factors during the exercise conditions and recovery. (**A**) Values for Thr^{180}/Tyr^{182}-p38 MAPK, and (**B**) Thr^{287}-CaMKIIδ_D (a.u.). Baseline (T1), post-VO_2max (T2), 10 s post-Wingate test (T3), 90 s post-Wingate test (T4), and 30 min post-Wingate test (T5). The whiskers delimit the 5th and 95th percentiles; the thin and thick horizontal lines correspond to the median and the mean values, respectively; and the upper and lower ends of the boxes define the 1st and 3rd quartiles, respectively. Control group: circles (n = 17) and Zynamite PX® group triangles (n = 8). * $p < 0.05$ compared to T1. Thr^{180}/Tyr^{182}-p38 MAPK: ANOVA exercise effect $p < 0.001$; exercise × supplementation interaction p = 0.080; Thr^{287}-CaMKIIδ_D: ANOVA exercise effect p = 0.008; exercise × supplementation interaction p = 0.064.

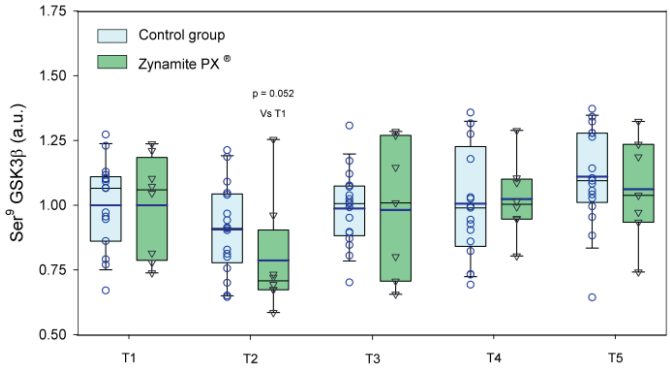

Figure 8. Protein expression levels of Ser^{9}-GSK3β (a.u.) during the exercise conditions and recovery. Baseline (T1), post-VO_2max (T2), 10 s post-Wingate test (T3), 90 s post-Wingate test (T4), and 30 min post-Wingate test (T5). The whiskers delimit the 5th and 95th percentiles; the thin and thick horizontal lines correspond to the median and the mean values, respectively; and the upper and lower ends of the boxes define the 1st and 3rd quartiles, respectively. Control group: circles (n = 17) and Zynamite PX® group triangles (n = 8). ANOVA exercise effect $p < 0.001$; exercise × supplementation interaction $p = 0.256$.

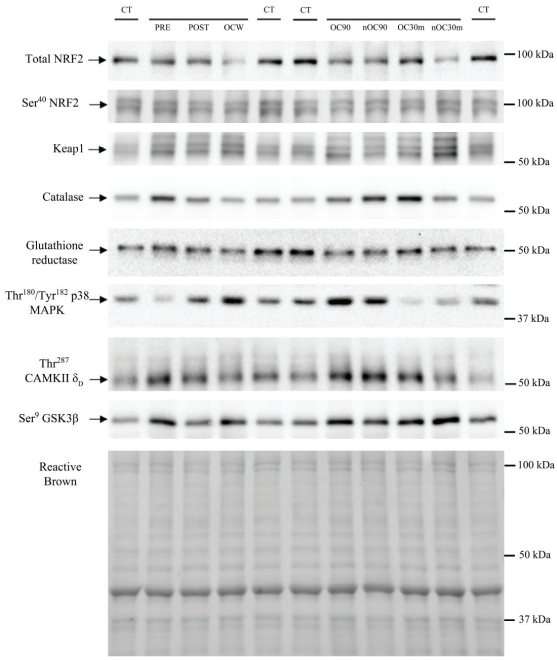

Figure 9. Immunoblots and total amount of protein loaded (Reactive Brown Staining) from a representative subject of the Zynamite PX® supplemented group. Images from top to bottom: total NRF2, Ser^{40}-NRF2, Keap1, catalase, glutathione reductase, Thr^{180}/Tyr^{182}-p38 MAPK, Thr^{287}-CaMKIIδ_D, and Ser^{9}-GSK3β. CT corresponds to a human control sample (non-experimental) loaded onto each gel in quadruplicate as a loading control. An illustration of experimental phases is depicted in Figure 1. PRE, before exercise. POST, 20 s after the end of the incremental exercise with ischemic recovery from the occluded leg. OCW, OC90, and OC30m, 10 s, 90 s, and 30 min after the end of the sprint, respectively, all from the occluded leg. nOC90 and nOC30m, 90 s and 30 min, respectively, from the leg recovering with free circulation. The markers indicate the closest molecular weight in kDa.

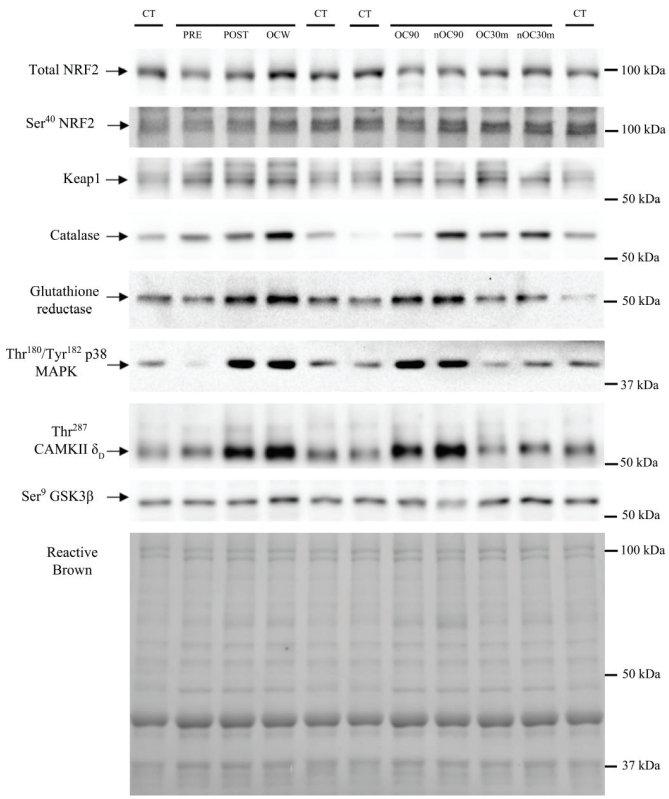

Figure 10. Immunoblots and total amount of protein loaded (Reactive Brown Staining) from a representative subject of the control group. Images from top to bottom: total NRF2, Ser^{40}-NRF2, Keap1, catalase, glutathione reductase, Thr^{180}/Tyr^{182}-p38 MAPK, Thr^{287}-CaMKIIδD, and Ser^{9}-GSK3β. CT corresponds to a human control sample (non-experimental) loaded onto each gel in quadruplicate as a loading control. An illustration of experimental phases is depicted in Figure 1. PRE, before exercise. POST, 20 s after the end of the incremental exercise with ischemic recovery from the occluded leg. OCW, OC90, and OC30m, 10 s, 90 s, and 30 min after the end of the sprint, respectively, all from the occluded leg. nOC90 and nOC30m, 90 s, and 30 min, respectively, from the leg recovering with free circulation. The markers indicate the closest molecular weight in kDa.

4. Discussion

The present investigation has shown that the oral administration of Zynamite PX®, a blend of natural polyphenols combining an extract from mango leaves abundant in mangiferin with a small amount of quercetin, elicits significant changes in resting skeletal muscle signaling molecules and modifies the signaling responses to high-intensity exercise (Figure 11). Our volunteers ingested 140 mg of mango leaf extract combined with 140 mg of quercetin every eight hours for a total of six doses before the resting biopsy. This was associated with increased CaMKII phosphorylation (Thr^{287}-CaMKIIδ_D), resulting in a stimulation of the activity of the enzyme, which phosphorylates and inhibits GSK3β [51] in the muscle biopsies obtained at rest. In unstressed conditions, GSK3β is constitutively active and phosphorylates NRF2 in C-terminal residues (different from Ser^{40}). This phosphorylation facilitates the interaction of NRF2 with β-transducin repeat-containing E3 ubiquitin-protein ligase (β-TrCP), leading to NRF2 ubiquitination and subsequent proteasomal degradation [50]. Thus, our results indicate that Zynamite PX® elicits the phosphorylation and inhibition of GSK3β, facilitating an increase in resting levels of NRF2 in human skeletal muscle [50,60]. This interpretation is further supported by the observed 1.7-fold higher

levels of NRF2 in the resting biopsies of the supplemented participants, which despite not achieving statistical significance, should not be ignored. Moreover, the level of Ser^{40}-NRF2 phosphorylation showed a similar trend, i.e., it was slightly increased in the supplemented participants. Ser^{40}-NRF2 phosphorylation prevents NRF2 degradation and facilitates its translocation to the nucleus and gene transcription [2]. However, no significant changes were observed in the basal protein expression levels of catalase and glutathione reductase, whose genes are regulated by NRF2. Thus, a more prolonged intake of Zynamite PX® may be required to upregulate the expression of these two genes. Nevertheless, at least in the case of catalase, this enzyme is tightly regulated, being acutely increased under conditions of increased ROS production, for example, high-intensity exercise, but immediately downregulated at the end of exercise [36].

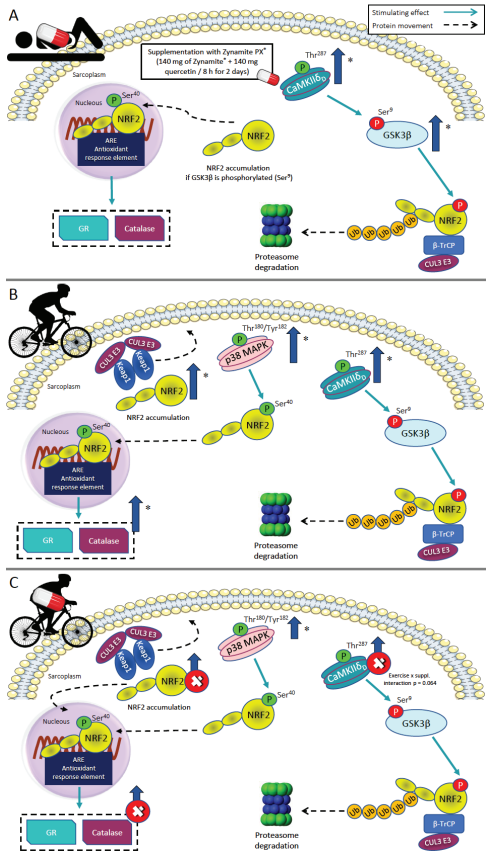

Figure 11. Schematic models of the NRF2 signaling pathway and its regulatory mechanisms in human skeletal muscle. (**A**) Basal signaling after 48 h of Zynamite PX® supplementation. * $p < 0.05$ compared to the control group. (**B**) Signaling elicited by high-intensity exercise without supplementation. * $p < 0.05$ compared to T1. (**C**) Signaling elicited by high-intensity exercise after supplementation with Zynamite PX®. * $p < 0.05$ compared to T1. Stimulating effects are represented by thin blue connecting lines. Changes in cellular localizations are presented with black dashed lines. The thick arrows in darker blue color located close to the specific markers illustrate this study's overall protein expression changes. Red crosses indicate suppression of the expected signaling response to high-intensity exercise following supplementation with Zynamite PX®. Activatory/inhibitory phosphorylations are depicted in green/red circles.

4.1. Potential Benefits Associated with Increased Inhibition of GSK3β

The present investigation shows that Zynamite PX® oral intake increases Ser^{9}-GSK3β phosphorylation, a mechanism that reduces its enzymatic activity [61,62]. Exercise also elicits Ser^{9}-GSK3β phosphorylation [63,64]. Increased Ser^{9}-GSK3β phosphorylation facilitates the preservation of muscle mass, protecting against muscle wasting due to disuse, chronic diseases, or caloric restriction [65–68], and facilitates myogenic differentiation and myoblast fusion [69,70]. Inactivation of GSK3β is necessary for the activation of muscle glycogen synthase [71,72], which is responsible for muscle glycogen resynthesis after exercise [73].

4.2. Zynamite PX® Increases Basal Levels of CaMKII Phosphorylation (Thr^{287}-CaMKIIδ_D)

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) plays a critical role in the regulation of muscle metabolism [74–76], muscle intracellular pH [77], calcium homeostasis [78], mitochondrial function [79] and biogenesis [80,81], redox balance [82], insulin sensitivity [83,84], and muscle growth [85], being strongly activated in response to exercise [86]. Increased CaMKIIδ_D expression and phosphorylation have been reported in skeletal muscle under basal conditions after sprint training [87] and strength training [59]. Interestingly, an association has been reported between the increase in Thr^{287}-CaMKIIδ_D phosphorylation and the magnitude of muscle hypertrophy elicited by a strength training program in humans [59].

4.3. Zynamite PX® Supplementation Attenuates the Activation of Stress Kinases and Redox Signalling in Response to High-Intensity Exercise to Exhaustion

This investigation shows that after brief supplementation with Zynamite PX®, the unstressed skeletal muscle displays some molecular adaptations like those elicited by exercise. Usually, exercise elicits Thr^{287}-CaMKII and Thr^{180}/Tyr^{182}-p38 MAPK phosphorylation [32,40,88], as observed in the present investigation in the control group. Thr^{287}-CaMKII phosphorylation is more prominent after sprint exercise [35] and workouts eliciting more metabolite accumulation [32], but it is also observed after prolonged exercise [89]. Nevertheless, when the exercise intensity is lower or elicits lesser metabolic stress, as observed in the trained state [90], the Thr^{287}-CaMKII phosphorylation response to exercise is attenuated or blunted [32,89]. The present investigation demonstrates that Zynamite PX® supplementation attenuates the expected Thr^{287}-CaMKII and Thr^{180}/Tyr^{182}-p38 MAPK phosphorylation observed in the control group. At least three mechanisms could explain this effect. Firstly, the exercise-mimetic action elicited by the 48 h Zynamite PX® supplementation may have attenuated the signaling response to exercise, as usually observed after training in humans [90]. Secondly, the marked increased basal phosphorylation of GSK3β after Zynamite PX® supplementation might have facilitated some NRF2 activity prior to the exercise resulting in lower ROS-induced signaling due to either lower ROS production or enhanced quenching during the exercise. In agreement, total NRF2 expression and the NRF2/Keap1 ratio were increased by exercise only in the control group, probably because NRF2 was already elevated before the start of exercise in the Zynamite PX® supplemented group. This explanation is supported by the fact that catalase and glutathione reductase, the antioxidant enzymes whose gene expression is stimulated by NRF2, were increased in response to exercise only in the control group. Thirdly, the dose of Zynamite PX® administered 60 min before the start of exercise may have contributed to abrogating part of the ROS signaling response in a similar way as observed after the ingestion of antioxidant cocktails [35] or inhibitors of XO [91]. Although it has been suggested that antioxidant ingestion before exercise may blunt part of the adaptations to exercise, this does not seem to be the case in healthy humans [92] and may be observed only after the intake of high doses of vitamin C and E [93,94]. In turn, the intake of fruit-derived polyphenols is considered favorable to enhance performance and recovery in athletes [1].

4.4. Limitations

This investigation has several limitations. First, some of the reported effects had "*p*" values between 0.05 and 0.1 for some of these effects; therefore, we cannot exclude the possibility of a type II error for some comparisons. Second, the changes reported in basal protein expression levels were observed ten hours after administering the last of the six Zynamite PX® administered. Whether more marked changes would be seen after a more prolonged supplementation remains unknown. Third, it is yet to be determined which of the polyphenols included in Zynamite PX® contributes the most to the reported effects or if they are partly due to specific metabolites generated by the gut microbiota entering the circulation. Fourth, only males were tested in the present investigation; nevertheless, we have previously shown that Zynamite PX® improves performance and enhances recovery in males and females [4,6,95].

5. Conclusions

The oral intake of Zynamite PX® increases basal Thr^{287}-CaMKIIδ_D and GSK3β phosphorylation in human skeletal muscle, which may elicit muscle adaptations to some extent, like those elicited by exercise. Consequently, the stress kinases' responses to exercise are partly blunted after Zynamite PX® supplementation. The increase in GSK3β phosphorylation may be associated with additional benefits which have not been assessed in the present investigation.

Future studies should determine whether a more prolonged supplementation may be associated with additional beneficial outcomes in males and females. GSK3β inhibits glycogen synthase; therefore, future studies should determine whether Zynamite PX® could accelerate muscle glycogen synthesis by inhibiting GSK3β and facilitate post-exercise recovery. Another aspect worth studying is the potential effects of Zynamite PX® on muscle protein synthesis, which the inhibition of GSK3β may facilitate [67].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu15132848/s1, Table S1: Detailed description of Western blotting antibodies and procedures.

Author Contributions: Conceptualization: M.M.-C., M.M.-R., L.L.-R., J.A.L.C. and T.V.-M.; methodology: A.S., J.A.L.C., L.L.-R., M.M.-C., M.M.-R. and T.V.-M.; formal analysis: A.S., A.G.-S., E.G.-G., G.G.-P., J.A.L.C., M.G.-R., M.M.-C., M.M.-R. and V.G.-A.; investigation: A.G.-S., J.A.L.C., E.G.-G., G.G.-P., M.G.-R., M.M.-C., M.M.-R. and V.G.-A.; resources: L.L.-R. and T.V.-M.; data curation: J.A.L.C., M.M.-C., M.M.-R., M.G.-R. and V.G.-A.; writing—original draft preparation: J.A.L.C. and M.M.-C.; writing—review and editing, all co-authors; validation, J.A.L.C., M.M.-C. and M.M.-R.; visualization, M.M.-C., M.M.-R. and J.A.L.C.; supervision, J.A.L.C., M.M.-C. and M.M.-R.; project administration: J.A.L.C. and M.M.-R.; funding acquisition: J.A.L.C. and L.L-R. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was carried out following approval by the Ethical Committee of the University of Las Palmas de Gran Canaria (CEIH-2015-03).

Informed Consent Statement: Informed written consent was obtained from all subjects involved in the study.

Data Availability Statement: Deidentified participant data are available from the senior author on reasonable request for research purposes.

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Conflicts of Interest: The present investigation was partially financed by Nektium Pharma S.L, who provided the polyphenolic compounds and participated in the experimental design, although they were fully excluded from participation in experimental data collection, data analysis and interpretation of results. The remaining funding agencies had no role in study design, data collection, management, data analysis, interpretation of data, writing of the report, and the decision to submit the report for publication.

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