Regulation of Nrf2/Keap1 signalling in human skeletal muscle during exercise to exhaustion in normoxia, severe acute hypoxia and post-exercise ischaemia: Influence of metabolite accumulation and oxygenation

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**Abstract**

The Nrf2 transcription factor is induced by reactive oxygen and nitrogen species and is necessary for the adaptive response to exercise in mice. It remains unknown whether Nrf2 signalling is activated by exercise in human skeletal muscle. Here we show that Nrf2 signalling is activated by exercise to exhaustion with similar responses in normoxia (P\textsubscript{O2}: 143 mmHg) and severe acute hypoxia (P\textsubscript{O2}: 73 mmHg). CaMKII and AMPK\textsubscript{α2} phosphorylation were similarly induced in both conditions. Enhanced Nrf2 phosphorylation was achieved by raising Nrf2 total protein and Ser\textsuperscript{40} Nrf2 phosphorylation, accompanied by a reduction of Keap1. Keap1 protein degradation is facilitated by the phosphorylation of p62/SQSTM1 at Ser\textsuperscript{404} by AMPK, which targets Keap1 for autophagic degradation. Consequently, the Nrf2-to-Keap1 ratio was markedly elevated and closely associated with a 2-3-fold increase in Catalase protein. No relationship was observed between Nrf2 signalling and SOD1 and SOD2 protein levels. Application of ischaemia immediately at the end of exercise maintained these changes, which were reverted within 1 min of recovery with free circulation. While SOD2 did not change significantly during either exercise or ischaemia, SOD1 protein expression was marginally downregulated and upregulated during exercise in normoxia and hypoxia, respectively. We conclude that Nrf2/Keap1/Catalase pathway is rapidly regulated during exercise and recovery in human skeletal muscle. Catalase emerges as an essential antioxidant enzyme acutely upregulated during exercise and ischaemia. Post-exercise ischaemia maintains Nrf2 signalling at the level reached at exhaustion and can be used to avoid early post-exercise recovery, which is O\textsubscript{2}-dependent.

**1. Introduction**

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

The RONS produced during exercise are accompanied by...
intramuscular changes in oxygen pressure (PO₂), metabolites and signalling molecules (Ca²⁺, Pi, Cr, PCr, H⁺, NADH.H⁺, etc.), which return to pre-exercise levels with different time courses [16,17]. Such changes in metabolite accumulation and RONS production are exacerbated when the exercise is performed in hypoxia [18,19], leading to specific adaptations [20–22]. Animal and cell culture experiments indicate that skeletal muscle Nrf2 signalling is upregulated by hypoxia [23,24]. Nevertheless, it remains unknown whether metabolite accumulation and muscle oxygenation influence Nrf2 signalling in response to acute exercise.

Nrf2 signalling is principally regulated by Kelch-like ECH-associated protein 1 (Keap1), which under basal conditions binds to Nrf2 promoting its ubiquitination and proteasomal degradation [25]. Keap1 is a cysteine-rich protein sensitive to modification by electrophiles and oxidants, which cause conformational changes of Keap1 that stabilize the Keap1-Nrf2 interaction, preventing Nrf2 proteasomal degradation. Under lower availability of free Keap1, the newly formed Nrf2 accumulates and translocates to the nucleus where it binds with antioxidant response elements (AREs) to regulate the transcription of more than 250 genes involved in the xenobiotic and antioxidant response, mitochondrial biogenesis, metabolism, detoxification, cytoprotection, inflammation, autophagy, and cell differentiation [25]. Although, it is well established that exercise increases the gene expression of some antioxidant enzymes [11,17,26], the acute effects of exercise on the protein levels of Nrf2/Keap1, and their downstream regulated antioxidant enzymes superoxide dismutase isoenzyme 1 (SOD1), SOD2, and Catalase in human skeletal muscle remain mostly unknown. Moreover, the process of activation/deactivation of Nrf2 signalling in skeletal muscle with contractile activity has not been investigated in humans.

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

Given the intrinsic difficulty in assessing RONS production in human skeletal muscle and the low specificity and sensitivity of the oxidative markers at use, we examined potential changes in RONS production by assessing phosphorylation changes known to be mediated by RONS. This is the case of the phosphorylation of Nrf2 at its serine 40 by protein kinase Cδ (PKCδ), a ROS-sensitive kinase [27]. Likewise, we determined the phosphorylation of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) at its threonine 287. CaMKII is activated by oxidation and autophosphorylation [28], and effect likely amplified by ROS-induced inhibition of phosphatases [29]. During high-intensity exercise, Thr³⁸⁷ CaMKII phosphorylation is blunted by the administration of antioxidant agents before exercise [5]. Likewise, overexpression of antioxidant enzymes prevents Thr³⁸⁷ CaMKII phosphorylation in other experimental models [30,31]. As downstream indicators of Nrf2 signalling, we determined the protein expression levels of Catalase, and SOD1 and SOD2. Animal data and cell culture experiments indicate that the gene expression of Catalase [25], SOD1 [32], and SOD2 [33] are stimulated by Nrf2, while the physiological ROS-induced expression of Catalase is blunted in Nrf2⁻/⁻ mice [6,34]. We also measured Thr³⁸² AMPKα phosphorylation as a marker of metabolic stress, since this enzyme is activated principally depending on the AMP/ATP ratio [35], and is necessary to enhance the expression of SOD2 in response to training [36]. Besides, due to the short half-life of RONS and the fast recovery of the energy metabolism upon cessation of exercise, a new experimental model was developed in humans to impede early recovery through the instantaneous application of complete post-exercise ischaemia with a pneumatic cuff in one leg only, using the contralateral leg as a control.

Since Nrf2/Keap1 signalling is expected to be activated by exercise models eliciting redox perturbations [6–8], we used an exercise protocol that allows the achievement of maximal oxygen uptake (VO₂max) in 10–15 min and elicits a marked activation of the glycolysis close to exhaustion [2,37], resulting in oxidative stress [38].

We hypothesised that Nrf2 protein amount and its downstream-regulated proteins Catalase, SOD1, and SOD2 would be increased in response to exhaustive exercise, and more markedly during exercise in hypoxia than normoxia. We also hypothesised that these changes would revert to pre-exercise levels within 1 min of the cessation of exercise in the leg recovering with free circulation. At the same time, Nrf2 depending signalling would increase further in the ischaemic leg due to the additional accumulation of metabolites and the reduction of PO₂.
to anoxic levels.

2. Methods

2.1. Subjects

Eleven physically active men (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%) agreed to participate in this investigation. Subjects were recruited among physical education students with a specific interest in exercise physiology. The inclusion criteria were as follows: a) age between 18 and 35 years, b) sex: male, c) body mass index: < 30 kg cm⁻², d) normal 12-lead electrocardiogram, and e) having a physically active lifestyle exercising regularly 2–4 times a week, but without following a specific training program at the time of enrolment. The exclusion criteria were: a) smoking, b) any disease or allergy, c) any medical contraindication to exercise, and d) being under medical treatment. All volunteers were informed of the purpose of the study, experimental procedures and potential risks in written and orally before providing their written consent. The study was carried out by the Declaration of Helsinki and was approved by the Ethical Committee of the University of Las Palmas de Gran Canaria.

2.2. Study design

The current investigation is part of a larger project initially designed to determine the mechanisms that limit performance during whole-body exercise in humans. The results focusing on exercise performance and muscle metabolism have been already published [39–42]. The present study contains unpublished material and new analyses to determine how Nrf2 signalling is regulated during exercise and recovery in human skeletal muscle. Subjects were requested to avoid physical activity and refrain from carbonated, caffeinated and alcohol-containing beverages during the 24-h period preceding all experimental days. They were also asked to record their dinner on the day preceding the first experimental day and thoroughly reproduce it before the subsequent experimental days, as well as maintain their usual diet along the experimental phases.

2.3. Pre-test and familiarization

On the first visit to the laboratory, anthropometric and body composition assessments were performed by dual-energy X-ray absorptiometry (DEXA) (Hologic QDR-1500, software version 7.10, Hologic Corp., Waltham, MA, USA) [42]. These were continued by familiarization with the exercise tests. The next two sessions were used to determine their peak oxygen consumption (VO2peak), maximal heart rate (HRmax) and maximal power output reached at exhaustion (Wmax) in normoxia (N; FIO2 = 0.21; PO2 = 143 mmHg) and severe acute hypoxia (Hyp; FIO2 = 0.104; PO2 = 73 mmHg). For these purposes, subjects performed ramp incremental exercise tests to exhaustion (IE) on a Lode Excalibur Sport 925900 (Groningen, The Netherlands), as previously reported [42]. In all exercise tests, VO2 was measured by indirect calorimetry with an open-circuit metabolic cart operated in breath-by-breath mode (Vmax N29; Sensormedics, Yorba Linda, CA, USA). The metabolic cart was calibrated before each test according to the manufacturer’s instructions employing high-grade calibration gases using a reference gas mixture made of 16% O2 and 4% CO2 (Carburco Metalicos, Las Palmas de Gran Canaria, Spain) [42]. The validity of the metabolic cart was established by a butane combustion test [43] and cross-checking end-tidal CO2 pressures with arterial blood CO2 partial pressures (ABL90, Radiometer, Copenhagen, Denmark) [44]. Respiratory variables were averaged every 20 s for VO2peak assessment [45].

2.4. Main experiments and biopsy sampling

The muscle biopsies analysed here were obtained in two main experimental sessions administered in random order and separate days (Fig. 1). Each session included one incremental exercise to exhaustion on an isokinetic ergometer (Lode Excalibur Sport 925900, Groningen, The Netherlands), one day in normoxia (N; FIO2 = 0.21; barometric pressure 735–745 mmHg) and the other in hypoxia (Hyp; FIO2 = 0.104; barometric pressure 735–745 mmHg). Exhaustion was defined by the subject stopping pedalling or dropping pedalling rate below 50 rpm despite strong verbal encouragement for 5 s.

On each experimental day, subjects reported to the laboratory at 08.00 h, after overnight fasting from 22.00 h. In the Nx session, after 10 min of rest in the supine position, the skin of the lateral aspect of both thighs (middle third) was anaesthetized (lidocaine (lignocaine) 2%, 2 ml, without adrenaline), avoiding infiltrating the muscle belly with lidocaine. Fifteen minutes later, a 5-mm incision of the skin and superficial fascia was performed, and the Bergstrom-type biopsy needle inserted to obtain a muscle biopsy from the m. vastus lateralis of one of the two thighs, which were randomly assigned (Bergstrom technique with suction). This biopsy was labelled as Pre Nx. For this first biopsy, the needle was directed distally with 45° of inclination. An additional incision was similarly performed in the contralateral leg before the start of the exercise. Both incisions were covered with temporary plasters. Then, a cuff (SCD10, Hokanson, Bellevue, WA, USA) connected to a rapid cuff inflator (Hokanson, E20 AG101) was placed unilaterally around the leg biopsied first and taped as close as possible to the inguinal crease. Subsequently, the volunteers seated on the cycle ergometer, which was pre-configured with saddle height and handlebar position used in familiarization tests. Then, after verification of electrocardiograph and metabolic cart connections, the resting measurements started, and after 2-min of data collection, the incremental exercise in normoxia begun with an initial load of 80 W, which was increased by 30 W every 2 min until exhaustion. At exhaustion, the cuff was inflated instantaneously at 300 mmHg, and a countdown started to obtain a second biopsy (labelled as Post Nx) exactly 10 s after exhaustion, i.e. after 10 s of ischaemia. For the second biopsy (Post Nx), the Bergstrom-type needle was introduced perpendicularly to the thigh. Subsequently, while maintaining the cuff inflated the subject rested quietly on the cycle ergometer and the third biopsy was taken exactly 60 s after exhaustion directing the biopsy needle proximally (45° inclination) (labelled as Oc1m Nx) [46]. This novel experimental approach permitted the assessment of muscle signalling changes during ischaemia, while the energy metabolism relied only on anaerobic sources, i.e., the energy supplied by phosphagens (ATP and phosphocreatine (PCr)) and the glycolysis [42].

In the hypoxia session, similar procedures were applied for subject preparation and biopsy sampling at rest, the latter obtained while the volunteers were breathing normoxic air (labelled as Pre Hyp). The hypoxic test started with 2 min collection at rest (Pinr ~73 mmHg, Altitrainer200, SMTEC, Nyon, Switzerland), followed by 2 min at 60 W, and after that, increments of 20 W every 2 min until exhaustion. At exhaustion, the cuff was inflated instantaneously, and the second biopsy was taken at the 10th s (labelled as Post Hyp). Then, while maintaining the cuff inflated the subjects were carefully moved to a stretcher prepared beside the cycle ergometer where the third biopsy was obtained exactly after 60 s of ischaemia (labelled as Oc1m Hyp). Sixty seconds after exhaustion, a fourth biopsy was obtained from the contralateral leg, which had been recovering in normoxia and without occlusion (labelled as FC1m). This means that the 3rd and 4th biopsies were taken simultaneously. Upon collection, all muscle samples (80–160 mg) were immediately dried on a sterile gauze, carefully freed from visible blood, dissected of any debris and fat tissue and immediately frozen in liquid nitrogen and stored at −80 °C until further analysis.
Switzerland). Almost equal final concentration in all muscle protein extracts was obtained (4.01 μg/μL) by following an individual adjustment of the extract volume using a volume calibration curve. Then, the supernatant was diluted with electrophoresis loading buffer (160 mM Tris-HCl, pH 6.8, 5.9% SDS, 25.5% glycerol, 15% β-mercaptoethanol–bromophenol blue).

For Western Blotting, the optimal amount of total protein to be loaded and the antibody concentration for each assay was determined by loading protein extracts in different amounts ranging from 2 to 45 μg. After verification of linearity within this range, equal amounts of protein of each sample (10–25 μg) were electrophoresed on SDS-PAGE gels using the system of Laemmli and transferred to Immun-Blot polyvinylidene fluoride (PVDF) membranes for protein blotting (Bio-Rad Laboratories, Hercules, CA, USA) as arbitrary units (a.u.). Since loading was homogeneous in all membranes, no further corrections were performed. In the case of Thr172 AMPKα phosphorylation, the antibody was stripped off using a buffer containing Tris-HCL 1 M pH 6.7, SDS 20%, β-mercaptoethanol 14.3 M and H2O. Subsequently, membranes were stained with Reactive Brown, re-blocked and re-incubated with HRP-conjugated anti-rabbit or anti-mouse antibody (diluted 1:5000 in 5% Blotto blocking buffer) and secondary antibodies employed in this investigation were obtained from different manufacturers.

2.5. Muscle metabolites, protein extraction and western blotting

Determination of muscle metabolites was performed as previously described [42]. Extracts of muscle protein (whole skeletal muscle lysates) were prepared as reported elsewhere [5], and total protein content quantified using the bicinchoninic acid assay [47]. In brief, ~10 mg of muscle were ground by stainless steel balls during 1 min in a Mikro-Dismembrator S (Sartorius, Goettingen, Germany) and immediately homogenised in urea lysis buffer (6 M urea, 1% SDS) and 50X Complete protease inhibitor (Cat. #4906837001) cocktails (Roche, Basel, Switzerland) were prepared as reported elsewhere [5], and total protein content quantified using the bicinchoninic acid assay [47]. In brief, ~10 mg of muscle were ground by stainless steel balls during 1 min in a Mikro-Dismembrator S (Sartorius, Goettingen, Germany) and immediately homogenised in urea lysis buffer (6 M urea, 1% SDS) and 50X Complete protease inhibitor (Cat. #4906837001) cocktails (Roche, Basel, Switzerland).

After verification of linearity within this range, equal amounts of protein of each sample (10–25 μg) were electrophoresed on SDS-PAGE gels using the system of Laemmli and transferred to Immun-Blot polyvinylidene fluoride (PVDF) membranes for protein blotting (Bio-Rad Laboratories, Hercules, CA, USA) as arbitrary units (a.u.). Since loading was homogeneous in all membranes, no further corrections were performed. In the case of Thr172 AMPKα phosphorylation, the antibody was stripped off using a buffer containing Tris-HCL 1 M pH 6.7, SDS 20%, β-mercaptoethanol 14.3 M and H2O. Subsequently, membranes were stained with Reactive Brown, re-blocked and re-incubated with HRP-conjugated antibody to check for stripping efficiency and subsequently re-blocked and re-incubated with the corresponding antibody for total AMPKα.

2.6. Materials

The Protein Plus Precision All Blue Standards were acquired from Bio-Rad Laboratories (Hemel Hempstead Hertfordshire, UK). The antibodies employed in this investigation were obtained from different manufacturers. The corresponding catalogue numbers from Abcam (Cambridge, USA) were as follows: pSer40 Nrf2 (no. ab76026), Nrf2 (no.
ab62352), Keap1 (no. ab119403), SOD1 (no. ab56416) and SQSTM1/p62 (pSer394) (no. ab211324). The antibodies purchased from Cell Signaling Technology (Danvers, MA, USA) were: Thr172 CaMKII (no. 12716), AMPKα (no. 2532), Thr172 AMPKα (no. 2535), Catalase (no.14097) and SOD2 (no. 13141). Some secondary HRP-conjugated goat anti-rabbit (no. 111-035-144) and the HRP-conjugated goat anti-mouse (no.115-035-003) antibodies were acquired from Jackson ImmunoResearch (West Grove, PA, USA). Other secondary HRP-conjugated goat antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA): anti-rabbit (no. sc2004) and anti-mouse (no. sc2031). See Supplementary Table 1 for a more detailed description of the antibodies and procedures.

2.7. Statistical analysis

Variables were checked for Gaussian distribution using the Shapiro-Wilk test, and when appropriate, data were transformed logarithmically before further analysis. A two-way 3 x 2 repeated-measures ANOVA with time (Pre, Post, and Oc1m) and F2,7. Statistical analysis

The protein expression of pSer349/p62 showed a tendency to increase with time (ANOVA time effect p = 0.051). When the mean of the two Pre conditions was compared to the mean of Post and Oc1m conditions, pSer349/p62 was 26% higher after exercise (p = 0.04) (Fig. 2E). Following the IE performed in Hyp, pSer493/p62 protein was 41% lower in the leg recovering without occlusion compared with the occluded leg (p = 0.026), and similar to that observed at Pre (Fig. 2E). No significant changes in p62 were detected using raw data (ANOVA F2,7 effect p = 0.84, time effect p = 0.07, F2,7 by time interaction p = 0.50). A secondary analysis was carried out to compare the mean of the two Pre conditions with the mean of the two post-exercise conditions (Post and Oc1m), which showed that p62 was reduced by ~20% after exercise (p = 0.02) (Fig. 2F). The ratio pSer349/p62/p62 total was increased by 1.8 and 1.5-fold at Post and Oc1m (Post vs Oc1m p = 0.17; ANOVA time effect p = 0.029), with a similar response in Nx and Hyp (F2,7 effect p = 0.34, ANOVA F2,7 by time p = 0.19, Fig. 2G). Following the IE performed in Hyp, the ratio pSer349/p62/p62 total was 43% lower in the leg recovering without occlusion (p = 0.017), and similar to that observed before the exercise (Fig. 2G). After the IE performed in Hyp, the pSer493/p62/p62 total ratio increased by 1.5-fold during the period of ischaemia (p = 0.026). There was an association between the ratio pThr172 AMPKα/AMPKα total and the ratio pSer349/p62/p62 total across conditions (r = 0.42, p < 0.001, n = 73) (Fig. 2H).

4. Results

4.1. Muscle metabolites

The responses regarding the metabolite accumulation in both conditions have been reported previously [42] and will only be summarized here. Briefly, following IE (i.e. at Post), muscle lactate, phosphocreatine (PCr) and ATP changed similarly in Nx and Hyp. From Post to the subsequent biopsy (ischaemic or free circulation period), muscle lactate increased only at Oc1m (25%; p < 0.05). PCr was reduced by 94 and 48%, compared to Pre levels, in Oc1m and FC1m, respectively (p < 0.005), regardless of exercise. F2O2 indicating recovery to 47.7% of the Pre-exercise PCr levels in the leg recovering with free circulation. Femoral vein F2O2 was 21.1 ± 2.0 and 10.6 ± 2.8 mmHg at Wmax, in Nx and Hyp, respectively (p < 0.001).

4.2. Muscle signalling

4.2.1. CaMKII, AMPK and p62

pThr172 CaMKII expression was increased by 1.7-fold after IE, remaining at this level after 1 min of occlusion (1.9-fold above Pre), with a similar response in Nx and Hyp (ANOVA F2O2 effect p = 0.83, time effect p = 0.001, F2O2 by time interaction p = 0.9) (Fig. 2A). Solely in the leg recovering with free circulation, pThr172 CaMKII levels returned to pre-exercise values 1 min after the end of the IE.

Compared to Pre, pThr172 AMPKα expression was increased by 2.4 and 3.0-fold at Post and Oc1m (Post vs Oc1m p = 0.07; ANOVA time effect p < 0.001), with a similar response in Nx and Hyp (ANOVA F2O2 effect p = 0.71, F2O2 by time interaction p = 0.68, Fig. 2B). After the IE performed in Hyp, no significant differences were observed between the occluded and non-occluded leg 1 min after exercise (p = 0.19) (Fig. 2B).

Compared to Pre, AMPKα total expression was reduced by 25 and 27% at Post and Oc1m (ANOVA time effect p = 0.007), with a similar response in Nx and Hyp (ANOVA F2O2 effect p = 0.71, F2O2 by time interaction p = 0.72, Fig. 2C). One minute after the end of the IE, AMPKα total expression returned to the pre-exercise levels only in the non-occluded leg (Fig. 2C).

The ratio pThr172 AMPKα/AMPKα total was increased by 4.5 and 4.0-fold at Post and Oc1m (Post vs Oc1m p = 0.38; ANOVA time effect p < 0.004), with a similar response in Nx and Hyp (ANOVA F2O2 effect p = 0.71, F2O2 by time interaction p = 0.42, Fig. 2D). After the IE performed in Hyp, the pThr172 AMPKα/AMPKα total ratio tended to be 52% lower in the leg recovering without occlusion (p = 0.057) (Fig. 2D).

The protein expression of pSer349/p62 showed a tendency to increase with time (ANOVA time effect p = 0.051). When the mean of the two Pre conditions was compared to the mean of Post and Oc1m conditions, pSer349/p62 p62 protein was 26% higher after exercise (p = 0.04) (Fig. 2E). Following the IE performed in Hyp, pSer493/p62 protein was 41% lower in the leg recovering without occlusion compared with the occluded leg (p = 0.026), and similar to that observed at Pre (Fig. 2E). No significant changes in p62 were detected using raw data (ANOVA F2,7 effect p = 0.84, time effect p = 0.07, F2,7 by time interaction p = 0.50). A secondary analysis was carried out to compare the mean of the two Pre conditions with the mean of the two post-exercise conditions (Post and Oc1m), which showed that p62 was reduced by ~20% after exercise (p = 0.02) (Fig. 2F). The ratio pSer349/p62/p62 total was increased by 1.8 and 1.5-fold at Post and Oc1m (Post vs Oc1m p = 0.17; ANOVA time effect p = 0.029), with a similar response in Nx and Hyp (F2,7 effect p = 0.34, ANOVA F2,7 by time p = 0.19, Fig. 2G). Following the IE performed in Hyp, the ratio pSer349/p62/p62 total was 43% lower in the leg recovering without occlusion (p = 0.017), and similar to that observed before the exercise (Fig. 2G). After the IE performed in Hyp, the pSer493/p62/p62 total ratio increased by 1.5-fold during the period of ischaemia (p = 0.026). There was an association between the ratio pThr172 AMPKα/AMPKα total and the ratio pSer349/p62/p62 total across conditions (r = 0.42, p < 0.001, n = 73) (Fig. 2H).

4.2.2. Antioxidant enzymes: Catalase, SOD1 and SOD2

Catalase protein expression was increased by 2.3 and 2.8-fold immediately after IE (Post) and after 1 min of ischaemic recovery (Oc1m), respectively, with a similar response in Nx and Hyp (ANOVA time effect p = 0.001, F2O2 by time interaction p = 0.12). Since the level of Catalase tended to be lower in the Pre value obtained the day of the experiment in Hyp (p = 0.10), we additionally analysed the experiment performed in Hyp separately. During the IE in Hyp, compared to Pre, Catalase expression levels were elevated 1.9-fold and 3.3-fold immediately after the IE (Post) and after 1 min of occlusion (Oc1m), respectively. From the end of the IE in Hyp, the level of Catalase doubled during the occlusion (Post vs Oc1m, p = 0.018) (Fig. 3A). One minute after the IE in Hyp, Catalase protein levels were reduced by 56% in the non-occluded leg (FC1m), remaining 1.5-fold above Pre levels (p = 0.048) (Fig. 3A).

Although no significant changes were observed in SOD1 protein levels with exercise nor ischaemia, SOD1 tended to decrease and increase after the IE in Nx and Hyp, respectively (ANOVA F2O2 effect p = 0.58; time effect p = 0.86, F2O2 by time interaction p = 0.053). To reduce variability between starting values on the testing day, we also repeated the analysis for fold changes regarding the pre-exercise value of each day. This analysis showed that SOD1 was reduced and increased after the IE in Nx and Hyp, respectively (ANOVA F2O2 effect p = 0.002, time effect p = 0.90, F2O2 by time interaction p = 0.044). The mean values of the Post and Oc1m after the IE in Nx and Hyp were compared using a paired Student’s t-test. This analysis found a 30% higher SOD1
Fig. 2. Skeletal muscle intracellular CaMKII, AMPKα and p62 signalling in response to incremental exercise to exhaustion in normoxia and severe hypoxia and the application of immediate ischaemic or non-ischaemic recovery. Protein expression levels of pThr^{287} CaMKII (A), pThr^{172} AMPKα (B), AMPKα total (C), pThr^{172} AMPKα/AMPKα total ratio (D), pSer^{349} p62 (E), p62 total (F), pSer^{349} p62/p62 total ratio (G), and association between the ratio of pThr^{172} AMPKα/AMPKα total and the ratio of pSer^{349} p62/p62 total (H). Nx; test performed in normoxia (FIO$_2$ = 0.21, PPO$_2$: 143 mmHg), Hyp; test performed in severe acute normobaric hypoxia (FIO$_2$ = 0.104, PPO$_2$: 73 mmHg); Pre, before exercise; Post, 10 s after the end of exercise with ischaemic recovery; Oc1m, 60 s after the end of exercise with ischaemic recovery; FC1m, 60 s after the end of exercise in the leg recovering without occlusion (free circulation); p62/SQSTM1 (shortened to p62), n = 11 in all conditions except for Oc1m Nx (n = 9), Post Hyp (n = 10), and Oc1m Hyp (n = 10). In (H), large symbols represent the mean of the subjects studied in each condition. The correlation coefficient and regression line have been calculated using the individual values (small white circles, n = 73). A detailed description of the experimental phases is explained in Fig. 1. The statistical analysis was performed with logarithmically transformed data for the pThr^{172} AMPKα/AMPKα total ratio and p62 total. The values shown are means ± standard errors and expressed in arbitrary units (a.u.). *p < 0.05 vs Pre Nx; †p < 0.05 vs Pre Hyp; #p < 0.05 vs Post Hyp; §p < 0.05 vs Oc1m Hyp.
protein content after exercise + occlusion in Hyp than in Nx (p = 0.001). In both experiments, SOD1 remained unchanged from 10 to 60 s of ischaemia. One min after IE in Hyp, no significant differences were observed in SOD1 expression between the occluded and the non-occluded leg (p = 0.11) (Fig. 3A). No significant changes were observed in SOD2 protein levels with exercise or ischaemia (ANOVA F = 40, p = 0.7; ANOVA time effects, p = 0.01, Fig. 3A). The exercise-elicited increase of pSer40 Nrf2 was maintained at the same level after 1 min of ischaemia, while it recovered to pre-exercise values in the non-occluded leg. Similar changes were observed in the expression of Nrf2 total protein (Fig. 4B). Although no statistically significant changes were observed in the pSer40 Nrf2/Nrf2 total ratio between Nx and Hyp, 1 min after IE in Hyp, this ratio was higher in the occluded compared to the non-occluded leg (p = 0.02) (Fig. 4C).

Compared to Pre, Keap1 expression was diminished by 23 and 29% at Post and Oc1m, respectively (ANOVA time effects, p = 0.015; ANOVA F = 40, p = 0.7; ANOVA time effects, p = 0.52). One minute after exercise, Keap1 recovered pre-exercise values in the non-occluded leg (Fig. 4D).

The Nrf2 total protein/Keap1 ratio was augmented by 3.3-fold (p = 0.001) across conditions. One minute after the end of the IE, Nrf2 total protein/Keap1 ratio returned to the pre-exercise levels in the non-occluded leg (p = 0.02) (Fig. 4E).

4.2.4. Phosphorylated CaMKII at Thr287 is closely associated with Nrf2 and Keap1 proteins

As illustrated in Fig. 5, pThr287 CaMKII protein levels were positively associated with those of pSer40 Nrf2 expression (r = 0.66, p < 0.001, n = 73), Nrf2 total protein (r = 0.63, p < 0.001, n = 73), and the Nrf2 total protein/Keap1 ratio (r = 0.51, p < 0.005, n = 73) and negatively with Keap1 (r = -0.23, p = 0.04) across conditions.

4.2.5. Nrf2 signalling during exercise is closely associated with Catalase protein expression but not with SOD1 or SOD2 protein levels

Catalase protein expression was closely associated with Nrf2 total protein expression (r = 0.74, p < 0.001, n = 73), pSer40 Nrf2 (r = 0.60, p < 0.001, n = 73), pThr287 CaMKII (r = 0.40, p < 0.001, n = 73), Keap1 (r = 0.37, p = 0.001, n = 73), and the Nrf2 total protein/Keap1 ratio (r = 0.77, p < 0.001, n = 73) (Fig. 6). No associations were observed between Nrf2 total protein and SOD1 (r = 0.01, p = 0.99, n = 73); pSer40 Nrf2 protein and SOD1 (r = -0.15, p
Fig. 4. Skeletal muscle intracellular Nrf2 and Keap1 signalling in response to incremental exercise to exhaustion in normoxia and severe hypoxia and the application of immediate ischaemic or non-ischaemic recovery. Protein expression levels of pSer⁴⁰ Nrf2 (A), Nrf2 total (B), pSer⁴⁰ Nrf2/Nrf2 total ratio (C), Keap1 (D), Nrf2 Total/Keap1 ratio (E). Nx; test performed in normoxia (F<sub>O₂</sub> = 0.21, P<sub>O₂</sub>: 143 mmHg), Hyp; test performed in severe acute normobaric hypoxia (F<sub>O₂</sub> = 0.104, P<sub>O₂</sub>: 73 mmHg); Pre, before exercise; Post, 10 s after the end of exercise with ischaemic recovery; Oc1m, 60 s after the end of exercise in the leg recovering without occlusion (free circulation). A detailed description of the experimental phases is explained in Fig. 1. The statistical analysis was performed with logarithmically transformed data for all proteins except for Keap1. The values shown are means ± standard errors and expressed in arbitrary units (a.u.). n = 11 in all conditions except for Oc1m Nx (n = 9), Post Hyp (n = 10), and Oc1m Hyp (n = 10). †p < 0.05 vs Pre Nx; *p < 0.05 vs Pre Hyp; ‡p < 0.05 vs Post Hyp; §p < 0.05 vs Oc1m Hyp.
5. Discussion

This study shows that Nrf2 signalling is activated by exercise to exhaustion in human skeletal muscle. To our knowledge, this is the first study examining the response of the Nrf2 signalling to intense acute exercise in human skeletal muscle and its relationship with metabolite accumulation, O₂ delivery and tissue O₂ pressures. In contrast to our hypothesis, the degree of activation of Nrf2 signalling was essentially similar in normoxia and hypoxia, despite a 50% lower femoral vein PO₂ during the exercise in severe acute hypoxia. Increased Nrf2 signalling was achieved by enhancing the total Nrf2 protein content while reducing that of Keap1, the main inhibitor of Nrf2 signalling. The combination of the increase and reduction of Nrf2 and Keap1, respectively, resulted in a substantial elevation of the Nrf2-to-Keap1 ratio, facilitating the nuclear translocation of Nrf2 and subsequent upregulation of the antioxidant enzyme Catalase, whose expression was associated to that of Nrf2. No association was observed between Nrf2 signalling and SOD1 and SOD2 protein expressions. While SOD2 did not change significantly during either exercise or ischaemia, SOD1 protein expression was downregulated and upregulated during exercise in normoxia and hypoxia, respectively (see Fig. 8 for a graphical summary).

Contrary to our hypothesis, the application of immediate ischaemia at exhaustion did not amplify the changes elicited by the bout of intense exercise until exhaustion in pThr²⁸⁷ CaMKII, pThr²⁸⁷ Nrf2 and Keap1. Nonetheless, Catalase protein expression was further increased after the application of ischaemia at exhaustion in hypoxia. By using a novel experimental approach in humans, we have demonstrated that Nrf2, Keap1 and Catalase have a fast turnover in exercised human skeletal muscle, recovering pre-exercise levels within 1 min after the end of a bout of intense exercise by an O₂-dependent mechanism. Interestingly, during recovery with free circulation after exhausting exercise in hypoxia, SOD1 increased while Catalase was reduced, revealing a different regulation of these two critical antioxidant enzymes in response to exercise.

Fig. 5. Associations between the protein expression levels of phosphorylated CaMKII, Nrf2 and Keap1 across experimental phases. pThr²⁸⁷ CaMKII and pSer⁴⁰ Nrf2 (A) pThr²⁸⁷ CaMKII and Nrf2 total (B), pThr²⁸⁷ CaMKII and Nrf2/Keap1 ratio (C), pThr²⁸⁷ CaMKII and Keap1 (D). A description of the experimental phases is explained in Fig. 1. Nx; test performed in normoxia (FiO₂ = 0.21, P/O₂: 143 mmHg), Hyp; test performed in severe acute normobaric hypoxia (FiO₂ = 0.104, P/O₂: 73 mmHg); Pre, before exercise; Post, 10 s after the end of exercise with ischaemic recovery; Oc1m, 60 s after the end of exercise with ischaemic recovery; FC1m, 60 s after the end of exercise in the leg recovering without occlusion (free circulation). n = 11 in all conditions except for Oc1m Nx (n = 9), Post Hyp (n = 10), and Oc1m Hyp (n = 10). Large symbols: each point is representing the mean of the subjects studied in each condition. Correlation coefficients and regression lines have been calculated using the individual values (small white circles, n = 73). The values shown are means ± standard errors and expressed in arbitrary units (a.u.). Statistical significance was set at p < 0.05.
5.1. Skeletal muscle Keap1 protein levels are reduced by intense exercise

The present investigation demonstrates for the first time that exhaustive exercise reduces the amount of Keap1 protein in human skeletal muscle. Keap1 is a substrate adaptor protein for the Cul3 RING-box 1 (RBX1) E3 ubiquitin ligase which ubiquitinates Nrf2 for proteasomal degradation in response to oxidants and electrophiles [48–50]. Heavy metals and several oxidative and electrophilic agents may induce thiol modifications in Keap1 which impair Nrf2 ubiquitination, resulting in Nrf2 protein accumulation [25]. These type of Keap1 modifications have been observed in cells treated with oxidized lipids [51], H₂O₂ [52], and nitric oxide [52, 53].

The drop in Keap1 levels could be due to reduced synthesis of the protein, increased degradation, or both. Our results provide evidence for increased proteasomal and autophagic degradation of Keap1 during exercise, likely triggered by its oxidative modification by RONS [1–3]. Although oxidative stress markers were not directly assessed in this investigation, the increased Thr²⁸⁷ CaMKII phosphorylation supports this explanation [5]. Likewise, the observed augmented levels of Ser⁴⁰ Nrf2 phosphorylation combined with the reduction of Keap1 levels and the negative association between Thr²⁸⁷ CaMKII phosphorylation and Keap1 are also compatible with increased RONS-mediated signalling.

5.2. Skeletal muscle total Nrf2 and its Ser⁴⁰ phosphorylation are increased by exhaustive exercise in humans with similar responses in normoxia and severe acute hypoxia

Nrf2 abundance was elevated at exhaustion suggesting increased de novo synthesis or reduced proteasomal degradation during exercise. The observed activation of AMPKα may have prevented the degradation of Nrf2 by stimulating the p62-mediated autophagy of Keap1 [54–56] or by phosphorylating and inhibiting glycogen synthase kinase-3-β (GSK3-β), which phosphorylates and activates β-transducin repeat-containing E3 ubiquitin-protein ligase (β-TrCP) resulting in Nrf2 ubiquitination and proteasomal degradation [57, 58]. In agreement with p62-mediated autophagy of Keap1 elicited by AMPK phosphorylation of its serine 349 [56], the fractional phosphorylation of p62 was associated with that of Thr¹⁷² AMPKα in the present investigation. Besides, p62 protein was significantly reduced after exercise, suggesting co-degradation with Keap1 [59]. The fact that Keap1 protein content was lowered supports a reduction of Nrf2 proteasomal degradation during intense exercise, as indicated by the inverse association between the two observed here. Nevertheless, the marked increase of the Nrf2-to-Keap1 ratio also indicates stimulation of Nrf2 de novo synthesis.

It must be emphasized that the level of hypoxia utilized here is close to the limit that humans can tolerate without altitude acclimatization [60]. RONS production in skeletal muscle is exacerbated by exercise in
hypoxia, likely due to higher activation of the anaerobic metabolism 
[19]. In fact, we have previously shown increased protein carbonylation 
in human skeletal muscle during prolonged sprint exercise performed at 
this level of hypoxia [19]. Despite the latter, and in contrast with our 
hypothesis, Nrf2 accumulation and Keap1 reduction were similar at 
exhaustion in normoxia and hypoxia, indicating that the stimulation of 
Nrf2 signalling was already maximal in normoxia or that the additional 
reduction in cellular PO$_2$ during exercise in hypoxia was not sufficient as 
to stimulate Nrf2 accumulation or Keap1 reduction further, perhaps 
counteracted by unknown mechanisms.

Serine$^{40}$ phosphorylation of Nrf2 by the ROS-sensitive kinase PKC$\delta$ 
[27] is thought to facilitate its nuclear translocation [61] and gene
The present investigation shows that pSer transactivation [62], although experimental evidence is not conclusive.

Fig. 8. Schematic model of the regulation of Nrf2 and Keap1 signalling in human skeletal muscle immediately after incremental exercise to exhaustion in normoxia and severe hypoxia. Under basal conditions, Keap1 continuously targets Nrf2 for ubiquitination and degradation, allowing for minimal levels of Nrf2. The production of RONS during exhaustive exercise stimulates the activation of AMPK and CaMKII. Concomitantly, CaMKII acts indirectly as an upstream AMPK activator (by an unknown mechanism). AMPK promotes the increase of Nrf2 levels by two main mechanisms. Firstly, by phosphorylating p62 at Ser349, which stimulates the p62-mediated degradation of Keap1 via autophagy; secondly, by phosphorylating and blocking GSK3-β, which activates β-TrCP (an E3 ubiquitin-protein ligase) which tags Nrf2 for proteasomal degradation (not measured here). RONS may also activate PKCδ which phosphorylates Nrf2 at its Serine 40 promoting its nuclear translocation and gene transactivation. The lowered levels of Keap1 and the reduced amount of p62 observed here are suggestive of co-degradation following exercise. Overall, the augmented Nrf2 total and phosphorylated protein expression together with the rise in the Nrf2-to-Keap1 ratio elicited by exhausting exercise should be sufficient to enhance the Nrf2-mediated antioxidant response. A central role of Catalase is manifested by a remarkable increase in its protein content following exercise, which was exacerbated during exercise in severe acute hypoxia, likely as a response to increased H2O2 production, by superoxide dismutases. This process is facilitated in hypoxia due to the upregulation of SOD1. No acute changes in SOD2 protein expression were observed. Most changes evoked by the exhaustive exercise bout were almost entirely reverted to baseline in less than 60 s by an O2-dependent mechanism. Activating/inhibiting actions are represented by blue/red connecting lines (dashed if the effect is indirect). Changes on cellular locations are presented with black dashed lines. The arrows and symbols depicted inside dashed grey boxes and located beside the specific markers illustrate the overall protein expression changes in this investigation, as follows: Thin arrows in green (phosphorylated form) and black (total form) depict the overall direction of the outcomes (increase/decrease) for the particular muscle protein; thick arrows in darker green represent the overall effect on stimulation/inhibition of Nrf2 signalling; the symbol $\downarrow$ indicates a significant difference between the biopsies taken 60 s after the end of the exercise, i.e., between the legs recovering with and without ischaemia. A differential modulation due to F2O2 is illustrated by the presence of arrows in red (normoxia) and blue (severe hypoxia). The size of each arrow is commensurate with the magnitude of the change. Abbreviations not defined in the text: ARE, antioxidant response element; OS: oxidative stress. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

transactivation [62], although experimental evidence is not conclusive [35]. The present investigation shows that pSer40 Nrf2 expression is increased during high-intensity exercise to a similar extent when the exercise is performed in normoxia and severe acute hypoxia. This may have facilitated nuclear translocation and gene transactivation, as supported by the increased protein expression of Catalase.

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

No previous study has determined acute changes in Catalase protein expression with acute exercise in humans. The few studies measuring this protein in humans have focussed on basal levels, reporting either an increased expression of the protein [63] or no change [36]. Three days after a 20 min high-intensity intermittent exercise session, basal levels of Catalase and SOD2 protein expression were increased in the human vastus lateralis muscle [64]. Likewise, increased SOD2 mRNA expression has been reported in human skeletal muscle 3 h after high-intensity and prolonged continuous exercise for 50 min [17]. Catalase is localized principally in peroxisomes but is also present in mitochondria [65]. The fast increase in Catalase protein expression (within minutes of exercise and within seconds during ischaemia after the incremental exercise in hypoxia) is likely necessary to counteract an increased H2O2 produced during exercise and ischaemia. This experimental observation concurs with the proposed role of H2O2 as a crucial signal driving the skeletal muscle adaptations to exercise [66]. Interestingly, cardiomyocyte overexpression of either Catalase or SOD2 results in increased lethality when transgenic mice are submitted to a forced-swimming program [67]. In these mice, the overexpression of SOD2 increases H2O2 production exceeding the detoxifying capacity of Catalase and peroxidases during exercise, leading to pathological levels of oxidative stress [67]. Overexpression of Catalase may result in hampering of signalling events necessary for the normal adaptation to exercise [66], causing maladaptation and increased death in transgenic
mice submitted to repeated forced swimming [67]. Here we have observed a transient increase of Catalase expression partly reverted within seconds after exercise and no significant changes of SOD2. This contrasts with the increased SOD2 protein content observed after endurance training in human skeletal muscle [36,68]. However, we cannot rule out a delayed SOD2 increase in our study since no additional muscle biopsies were performed to check for changes in the following hours after the exercise.

Thus, we have demonstrated that skeletal muscle can increase the amount of critical antioxidant enzymes acutely, likely via RONS-stimulated Nrf2 activation. When the exercise is stopped the excess antioxidant capacity build-up during the exercise is to a large extent, if not wholly, reversed to restore the redox balance to pre-exercise levels avoiding the risks of excessive reductive capacity [69].

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

Previous studies indicate that SOD1 is constitutively expressed with limited regulation by external stimuli [70]. SOD1 is located in the cytoplasm, nucleus and outer mitochondrial membrane, while Catalase is a predominantly extramitochondrial protein, but also found in the mitochondrial matrix [67,70]. SOD1 was slightly increased during exercise in hypoxia, facilitating the dissmutation of superoxide generated by extramitochondrial oxidases [71]. This response concurs with the observed increased RONS production during high-intensity exercise in hypoxia [3], which has a significant cytoplasmatic component [66,71]. In the presence of higher levels of SOD1, the production of H2O2 is likely increased during exercise in severe hypoxia, requiring a higher amount of Catalase to avoid unchecked oxidative damage. In agreement with this explanation, it has been reported that H2O2 may induce SOD1 gene transcription by an Nrf2-independent mechanism [72]. Besides, it has been shown that H2O2 promotes SOD1 nuclear localization, where it acts as a transcription factor promoting the expression of antioxidant enzymes [73]. The remarkable acute increase of Catalase expression after exercise in hypoxia and during ischaemia is likely necessary to counteract an excessive H2O2 production during exercise and after the application of ischaemia [74,75]. The latter was accompanied by the expected, although non-statistically significant, changes in the protein expression of Ser40 Nrf2, Nrf2 total, Nrf2 total/Keap1, and Keap1. The fact that the antioxidant enzymes increased in response to exercise and ischaemia are located mostly outside the mitochondria is compatible with a predominant extramitochondrial production of RONS during exercise [71], which may be even more marked during exercise in hypoxia and ischaemia.

5.5. Keap1 levels recover rapidly after the cessation of contractile activity in an O2-dependent mechanism

In the present study, subjects performed exercise until their limit, and upon exhaustion, a pneumatic cuff was instantaneously inflated to 300 mmHg to fully occlude the circulation in less than 2 s in one leg, while the other leg recovered without circulatory restraint. During the first 3–5 s of the occlusion, the O2 stores (O2 trapped in capillary blood and bound to myoglobin) are depleted by oxidative phosphorylation, which is strongly stimulated [2,42]. This was evidenced by the fast reduction and plateauing of muscle oxygenation measured by near-infrared spectroscopy (NIRS), as previously reported [42]. The first post-exercise muscle biopsy was obtained 10 s after the end of the exercise, preventing potential effects of early oxygenation at exhaustion on muscle signalling. Then, the leg biopsied first remained occluded and, after 60 s another two muscle biopsies were obtained from the occluded and non-occluded leg simultaneously. This allowed a direct comparison of the occluded and non-occluded leg. During the 60 s of occlusion, the energy metabolism remained active in the occluded leg, utilizing the energy supplied by the glycolysis, leading to a higher accumulation of lactate, H+, Pi and Cr. In contrast, the concentration of ATP remained at the same level reached at exhaustion, i.e. ~80% of the concentration observed before exercise [42]. Despite the increased build-up of glycolytic metabolites during the 60 s occlusion, no further increase of Nrf2 or reduction of Keap1 was detected in the occluded leg. Although we cannot rule out some RONS production from 10 to the 60 s of ischaemia [74,75], its magnitude should have been small as indirectly indicated by stability during this period of both pThr287 CaMKII, pSer40 Nrf2 and Keap1, which are sensitive to RONS.

In the leg recovering with free circulation, Nrf2, Keap1 and the Nrf2-to-Keap1 ratio returned to pre-exercise levels within 60 s after the end of exercise, even though muscle lactate and H+ remained at the same level reached at exhaustion [42]. In contrast, Pi and Cr were reduced, and PCr increased during the 60 s of recovery with free circulation, without reaching pre-exercise values. This also indicates that the glycolytic metabolites accumulated during exercise do not play an essential role in either eliciting or maintaining Nrf2 activation. Nevertheless, the massive increase of Pi, which led to almost depletion of PCr during ischaemia may have inhibited the phosphatases [29], keeping the phosphorylation levels during ischaemia.

The principal difference between ischaemic and free circulation recoveries is the presence of O2. Femoral vein PO2, a surrogate of mean capillary PO2, is rapidly increased after the cessation of contractile activity as it was reflected by the NIRS signal captured at the end of exercise in the perfused leg [42]. The production of ATP by oxidative phosphorylation is likely mandatory to reactivate the de novo synthesis of Keap1 in skeletal muscle. Despite the shortage of energy during ischaemia, mainly when applied at exhaustion following exercise in severe acute hypoxia, Catalase protein content was almost doubled after the IE in Hyp, indicating that even in ischaemia the synthesis of some proteins is still active [76]. The reason why Nrf2 levels were not reduced to pre-exercise values during ischaemia may be, in part, explained by the attenuation of global protein degradation in anoxia [77]. In the leg recovering with free circulation, Nrf2 was reduced to the pre-exercise level already after only 1 min, likely through proteasomal and autophagy degradation facilitated by the fast increase of Keap1 during recovery.

It has been suggested that once modified by oxidants or electrophiles, Keap1 is committed to p62-mediated autophagy [59]. Keap1 gene harbours an ARE promoter which may be stimulated by Nrf2 to promote SOD1 nuclear localization, where it acts as a transcription factor promoting the expression of antioxidant enzymes [73]. The remarkable acute increase of Catalase expression after exercise in hypoxia and during ischaemia is likely necessary to counteract an excessive H2O2 production during exercise and after the application of ischaemia [74,75]. The latter was accompanied by the expected, although non-statistically significant, changes in the protein expression of Ser40 Nrf2, Nrf2 total, Nrf2 total/Keap1, and Keap1. The fact that the antioxidant enzymes increased in response to exercise and ischaemia are located mostly outside the mitochondria is compatible with a predominant extramitochondrial production of RONS during exercise [71], which may be even more marked during exercise in hypoxia and ischaemia.

5.6. Practical implications

The current experiments have two practical implications. First, we have demonstrated that some signalling events triggered by exercise recovered with rather fast kinetics. The importance of obtaining fast muscle biopsies during exercise was already pointed out by exercise physiologists in the 70–80s to capture metabolic changes with rapid recovery kinetics [80,81]. Secondly, we have shown, as a novelty, that post-exercise ischaemia allows maintaining the end exercise metabolic conditions during the first seconds of recovery, facilitating the capture of signals that otherwise will escape detection.

5.7. Limitations

In the present research, subjects were free to ingest their usual diet. Some components of the food may influence Nrf2 basal levels [82] and perhaps Nrf2 signalling responses to exercise. Although we asked our subjects to maintain their habitual diet the week before an experiment...
and reproduce the same dinner before the two experimental conditions, we cannot rule out a potential confounding effect of small differences in the composition of the diet. Nevertheless, the fact that the basal expression of Nrf2 and antioxidant enzymes were similar between basal conditions.

In the present investigation rather than measuring oxidative stress markers, we focused on assessing signalling events that are predominantly elicited by ROS. Although this approach is more sensitive to changes in RONS, it is also less specific, because both CaMKII and AMPK may be activated by mechanisms independent from RONS [37,68]. However, the reduction of Keap1 seems more specific as a marker of changes in RONS, it is also less specific, because both CaMKII and AMPK increases in elderly individuals [52,84]. Another limitation of the present experiments is that our findings are limited to the antioxidant enzymes assessed, i.e., catalase, SOD1 and SOD2 and to the time frame of our experiments.

In summary, this study shows that, during incremental exercise to exhaustion, Nrf2 signalling is upregulated to a similar extent in normoxia and severe acute hypoxia, by a mechanism connected to the decrease in Keap1 protein that promotes a remarkable elevation of nuclear factor kappaB activation, J. Physiol. 586 (16) (2008) 3979–3990.

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