Title:
Repeated-ischaemic exercise enhances mitochondrial and ion transport gene adaptations in human skeletal muscle – Role of muscle redox state and AMPK

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Summary in key points (150 words):

- We investigated if ischaemia would augment the exercise-induced mRNA response of PGC-1α and Na⁺,K⁺-ATPase (NKA) isoforms (α₁-3, β₁-3, and FXYD1), and examined whether this effect could be related to oxidative stress and fibre type-dependent AMPK and CaMKII signalling in the skeletal muscle of trained men.

- Repeated-ischaemic exercise increased the mRNA content of PGC-1α total, -1α1, and -1α4, and of the NKA regulatory subunit FXYD1, whereas exercise in systemic hypoxia or alone was without effect on these genes.

- These responses to ischaemia were complemented by increased oxidative stress (as assessed by catalase and HSP70 mRNA) and ACC phosphorylation (an indicator of AMPK activation) in type I fibres. However, they were unrelated to CaMKII signalling, muscle hypoxia, and lactate accumulation.

- Thus, repeated ischaemic exercise augments the muscle gene response associated with mitochondrial biogenesis and ion homeostasis in trained men. This effect seems partly attributable to promoted oxidative stress and AMPK activation.
Abstract

This study assessed the effect of repeated-ischaemic exercise on the mRNA content of PGC-1α (total, 1α1, and 1α4) and Na⁺,K⁺-ATPase (NKA; α₁-3, β₁-3, and FXYD1) isoforms in human skeletal muscle, and studied some of the potential molecular mechanisms involved. Eight trained men (26 ± 5 y and 57.4 ± 6.3 mL·kg⁻¹·min⁻¹) completed three interval running sessions with (ISC) or without ischaemia (CON), or in hypoxia (HYP, ~3250 m), in a randomised, crossover fashion separated by 1 week. A muscle sample was collected from the dominant leg before (Pre) and after exercise (+0h, +3h) in all sessions to measure the mRNA content of PGC-1α and NKA isoforms, oxidative stress markers (i.e. catalase and HSP70 mRNA), muscle lactate, and phosphorylation of AMPK, ACC, CaMKII, and PLB protein in type I and II fibres. Muscle hypoxia (i.e. deoxygenated haemoglobin) was matched between ISC and HYP, which was higher than in CON (~90% vs. ~70%; p<0.05). The levels of PGC-1α total, -1α1, -1α4, and FXYD1 mRNA increased in ISC only (p<0.05). These changes were associated with increases in oxidative stress markers and higher p-ACCSer²²¹/ACC in type I fibres, but were unrelated to muscle hypoxia, lactate, and CaMKII and PLB phosphorylation. These findings highlight that repeated-ischaemic exercise augments the skeletal muscle gene response related to mitochondrial biogenesis and ion transport in trained men. This effect seems attributable, in part, to increased oxidative stress and AMPK activation, whereas it appears unrelated to altered CaMKII signalling, and the muscle hypoxia and lactate accumulation induced by ischaemia.

Abbreviations

ACC, Acetyl-CoA carboxylase; AMPK, 5' AMP-activated protein kinase subunit; β2M, β2 microglobulin; CaMKII, Ca2+-calmodulin-dependent protein kinase isoform II; CON, control session; C₇, cycle threshold; CV, coefficient of variation; FXYD1, phospholemman isoform 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GXT, graded exercise test; HHb, deoxygenated haemoglobin; HSP70, heat-shock protein 70; HYP, repeated-hypoxic exercise session; ISC, repeated-ischaemic exercise session; K⁺, potassium ion; LT, lactate threshold; MHC, myosin heavy chain; Na⁺, sodium ion; NIRS, near-infrared spectroscopy; NKA, Na⁺,K⁺-ATPase; OXPHOS, oxidative phosphorylation; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha; PLB, phospholamban; ROS, reactive oxygen species; SDS, sodium dodecyl sulphate; TBP, TATA-binding protein; VO₂max, maximum oxygen uptake.
Introduction

The movement pattern of many sports (e.g., football codes, handball, and tennis) requires athletes to possess a well-developed ability to repeatedly perform maximal or near-maximal intensity efforts (Povoas et al., 2012; Varley et al., 2013; Adriano Pereira et al., 2016). This ability appears limited by factors intrinsic to skeletal muscle (Bangsbo, 1994; Bishop, 2012; Bishop & Girard, 2013). For example, fatigue during this type of activity has been related to perturbations in muscle transmembrane sodium (Na\(^{+}\)) and potassium ion (K\(^{+}\)) gradients, which exceed the transport capacity of the Na\(^{+}\),K\(^{+}\)-ATPase (NKA) to sustain transmembrane ion equilibrium (McKenna, 1992; McKenna et al., 2008). As such, the NKA is important for performance during intense intermittent exercise. The NKA is composed of a catalytic α, a structural β, and a regulatory γ (phospholemman or FXYD) subunit (Clausen, 2013), all of which are expressed as different isoforms (α_{1-3}, β_{1-3} and FXYD1) in human skeletal muscle (Wyckelsma et al., 2015). The relative distribution of these isoforms determine, in part, the potential to generate active NKA complexes at the cell surface. Therefore, changes in the expression of different NKA isoforms could affect the muscle’s capacity for Na\(^{+}\)/K\(^{+}\) transport (Nielsen et al., 2004).

Another limiting factor for the ability to perform repeated, intense exercise is the muscle’s capacity to generate ATP via oxidative pathways (Thomas et al., 2004). Accordingly, increases with exercise training in both mitochondrial respiratory function and content (as assessed by citrate synthase activity) have been temporally associated with an enhanced exercise capacity (Bishop et al., 2014). A key determinant of these endurance-type adaptations is the transcriptional co-activator, the peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α). It has recently been reported in humans that one isoform of PGC-1α, namely the isoform 1 (PGC-1α1), orchestrates exercise-induced increases in the content of mitochondrial genes (e.g. OXPHOS), whereas another isoform, the isoform 4 (PGC-1α4), has been shown to regulate the expression of genes that influence muscle hypertrophy (Ruas et al., 2012). The expression of specific isoforms of PGC-1α could determine the nature of the responsiveness of skeletal muscle to various external stimuli, such as exercise.

Despite a scarcity of published research, it is believed that repeated, transient bursts in gene expression enhances the potential for increasing the protein abundance of NKA and PGC-1α isoforms in human skeletal muscle (Perry et al., 2010; Christiansen et al., 2017, unpublished). Identifying the cellular stressors involved in the transcription of these isoforms would therefore seem important. However, at present, the cellular stressors essential for the upregulation of NKA and PGC-1α isoforms in human muscle are poorly defined. What is known, primarily based on cell culture and animal models in vitro, is that the transcription of these isoforms...
might be initiated by ionic and metabolic perturbations, including shifts in K\(^+\) (Zhuang et al., 2000; Wang et al., 2007), lactate (Hashimoto et al., 2007), and Ca\(^{2+}\) concentrations (Ojuka et al., 2003; Norrbom et al., 2004), and by the formation of reactive oxygen species (ROS) (Wendt et al., 1998; Murphy et al., 2008; Irrcher et al., 2009). These cellular stressors have been shown to activate several signalling kinases, of which the AMP-stimulated protein kinase (AMPK), the Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaMK), or both, have been coupled to the transcriptional regulation of PGC-1\(\alpha\) (Wu et al., 2002; Irrcher et al., 2008) and NKA isoforms (Nordsborg et al., 2010a). Thus, strategies promoting perturbations in ionic gradients, metabolic and redox homeostasis, and ROS production, could prove useful for increasing the muscle’s expression of NKA- and PGC-1\(\alpha\)-isoform mRNA transcripts.

One potential strategy to augment these cellular stressors might be to exercise with reduced muscle blood flow (ischaemia), known as ischaemic exercise. Ischaemia has typically been invoked by inflation of an occlusion cuff fixed around the limb(s) proximal to locomotor muscles and has been applied during multiple exercise modes, including walking, cycling and resistance training (Abe et al., 2006; Abe et al., 2010; Cook et al., 2014). Inflation of the cuff has been reported to compromise both the arterial and venous flow (Sundberg & Kaijser, 1992; Horiuchi & Okita, 2012), resulting in an hypoxic and more acidic intramuscular environment (Sundberg & Kaijser, 1992). A successive deflation of the cuff can promote local reactive hyperaemia (Gundermann et al., 2012). In combination, these mechanisms seem a powerful stimulus for amplifying the transient bursts in skeletal muscle ROS production that accompany consecutive bouts of exercise (Slezak et al., 1995; Clanton, 2007). They could also affect K\(^+\) and calcium ion (Ca\(^{2+}\)) concentrations by modulating the function of ion channels and transport systems (Kourie, 1998; Juel et al., 2015). There is therefore evidence to suggest ischaemic exercise could be an effective strategy to promote the mRNA expression of NKA and PGC-1\(\alpha\) isoforms in human skeletal muscle. However, this hypothesis remain presently untested.

The primary aim of the present study was therefore to explore the effect of ischaemic exercise on the mRNA content of PGC-1\(\alpha\) (total, and isoforms 1\(\alpha\)1 and 1\(\alpha\)4) and NKA isoforms (\(\alpha\)1-3, \(\beta\)1-3, and FXYD1) in human skeletal muscle. Our second aim was to elucidate some of the potential molecular mechanisms involved. Our working hypotheses were: 1) Ischaemia would augment the effect of exercise on PGC-1\(\alpha\)- and NKA-isoform expression, and 2) increases in the expression of these isoforms would coincide with elevated levels of oxidative stress markers (i.e. catalase and HSP70 mRNA content) and Acetyl-CoA carboxylase (ACC; an indicator of AMPK activation) and CaMKII phosphorylation. Evidence from astrocytes in vitro suggests that the reperfusion phase, rather than hypoxia per se, may be the primary stimulus underlying increases in the mRNA content of NKA isoforms in response to hypoxia-reperfusion (Kasai et al., 2003). Thus, we also included a hypoxic condition (i.e. exercising in normobaric,
systemic hypoxia) to assess the hypothesis that 3) increases in isoform expression could not be attributed to the muscle hypoxia induced by occlusion.

**Methods**

**Ethical Approval**

This study was approved by the Human Research Ethics Committee of Victoria University, Melbourne, Australia (HRE14-309), and was performed in accordance with the latest instructions in the *Declaration of Helsinki*. Participants provided oral and written informed consent before enrolment in the study.

**Participants**

Eight healthy men engaged in team sports (five in soccer, two in Australian-rules football, and one in basketball) participated in the study. Their physical characteristics are shown in Table 1. All participants were non-smokers and engaged in their sport 3-5 times per week.

**Randomisation and blinding**

The study was a randomised, counterbalanced, crossover experiment, and took place in the Exercise Physiology Laboratory at the Institute of Sport, Exercise and Active Living (ISEAL), Victoria University, Melbourne, Australia. All sessions were performed on a Katana Sport XL treadmill (Lode, Groningen, Netherlands) in 21.4 ± 1.1 °C and 40.8 ± 6.8 % humidity. Participants completed three main trials matched for total work, duration (34 min), and work:rest ratio. These trials were separated by one week and consisted of interval running with (ISC) or without ischaemia (CON), or in normobaric, systemic hypoxia (HYP). Each participant was allocated a trial order using a random-number generator (MS Excel 2013, Microsoft, USA). To minimise any perceived placebo effect (not to be confused with a true placebo effect) (Ernst & Resch, 1995), the participants were not informed about which trial was hypothesised to be of greatest value to the physiological response, and whether they were breathing hypoxic or normoxic air. A pneumatic tourniquet (Riester, Germany) was attached to the participants’ preferred kicking (dominant) leg by adhesive tape in all trials, but it was only inflated in ISC. In addition, the participants were informed that the study purpose was to evaluate the effect of different degrees of ischaemia. Information about what trial was to be performed on each occasion was given on the day of execution.
Pre-testing
Prior to the main trials, the participants visited the laboratory on four separate occasions interspersed by at least 48 h. On the first and fourth visit, participants performed a graded exercise test (GXT). This test was used to assess the participants' lactate threshold (LT) and maximum oxygen consumption ($\text{VO}_{2\text{max}}$). The LT from the fourth visit was used to determine individual running speed during the main trials (i.e. ISC, CON and HYP). On the second visit, participants performed the ISC trial with near-infrared spectroscopy (NIRS) probes placed over the vastus lateralis muscle belly of their dominant leg to assess muscle oxygen content (cf. section on Muscle deoxygenation), and to accustom the participants to ISC and the equipment. During the third visit, participants completed the same running protocol with NIRS probes attached. The first three exercise bouts during this visit were performed without ischaemia or hypoxia. The remaining six bouts were completed in normobaric, systemic hypoxia to accustom the participants to HYP and to allow estimation of individual inspired oxygen fraction ($F_i\text{O}_2$) to be used in HYP to match the level of muscle hypoxia during ISC (detailed in Ischaemia and hypoxia). The tourniquet was worn during both the second and third visit.

Main trials
On the days of the main trials, the participants reported to the laboratory between 8-9 am after 7.3 ± 1.1 h of sleep and after consuming a standardised dinner and breakfast (detailed in Diet and activity control) 15 h and 2.5 h, respectively, prior to arrival. After approximately 30 min of rest in the supine position, a catheter was inserted in an antecubital vein, allowing mixed-venous blood to be sampled. After an additional 15 min of rest, blood and muscle was sampled, also in the supine position. Next, the participants moved to the treadmill where they were instrumented with one pair of NIRS optodes on the belly of the vastus lateralis muscle of their preferred kicking (dominant) leg to reliably and non-invasively monitor muscle deoxygenation in vivo (Van Beekvelt et al., 2001). A belt was placed around their chest to measure heart rate. In a sitting position with the dominant leg unloaded, muscle deoxygenation was measured for at least 2 min until a plateau was reached and a stable baseline reading was recorded. Next, participants were fitted with a facemask covering the mouth and nose to enable them to breathe normoxic or hypoxic air. A pneumatic tourniquet was attached to the participants' dominant leg by adhesive tape. The tourniquet was inflated only in ISC before each bout of exercise and deflated upon termination of each bout. A time-aligned schematic representation of the experimental protocol is shown in Fig. 1. Every trial commenced with a 5-min warm-up (WU) at 75 % LT followed by 5 min of rest. At the third and fourth minute of the WU, a 5-s acceleration to ~110 % LT, followed by a 5-s deceleration to 75 % LT, was performed. Next, three series of three 2-min runs were executed at a fixed relative intensity...
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(105 % LT, 11.6 ± 1.7 km·h⁻¹; no incline). The runs were separated by 1 min, and the series by 5 min, of walking (~5 km·h⁻¹), respectively. This design was introduced to promote repeated, transient bursts in ROS formation (Slezak et al., 1995; Raedschelders et al., 2012), which is a key stimulus for increases in the mRNA content of NKA and PGC-1α isoforms in cell culture (Silva & Soares-da-Silva, 2007; Irrcher et al., 2009). The duration of runs and the work:rest ratio were based on pilot work and balanced to achieve the highest tolerable mean speed, by which the exercise regimen could be completed with the chosen magnitude of leg ischaemia (3.0 mmHg cm⁻¹). This training regimen (2:1 min) has been demonstrated to increase Na⁺-K⁺-ATPase content (as assessed by [³H]-ouabain binding) and enhance intense intermittent exercise performance (Edge et al., 2013). Antecubital venous blood was sampled at rest before exercise, prior to each series, immediately after each run, and at 3 and 6 min after termination of exercise. Muscle was sampled at rest in the supine position before (Pre), immediately after (64 ± 28 s; +0h), and 3 h post, exercise (+3h).

Ischaemia and hypoxia

In all trials, a pneumatic tourniquet made of nylon with a width of 13 cm (Riester, Germany) was externally applied to the most proximal part of the participants' preferred kicking leg. In ISC, 15 s prior to the onset of a run, the tourniquet was rapidly inflated over ~10 s to reach an end-pressure of 3.0 mmHg cm⁻¹ (i.e. relative to thigh circumference, TCF). The mean pressure was in the lower end of the range of pressures used in previous studies (~3-5 mmHg cm⁻¹) (Abe et al., 2005; Abe et al., 2006; Fujita et al., 2008; Abe et al., 2010; Kubota et al., 2011). The pressure during running ranged from (mean ± SD) 123 ± 12 (range: 109-139) mmHg in the float phase to 226 ± 24 (range: 200-260) mmHg in the landing phase. The difference between our predetermined (~175 mmHg) and actual (mean ± SD) pressure during the trials was -1 ± 8.5 mmHg. The tourniquet was deflated immediately after termination of exercise. After 15 min of recovery from exercise, the tourniquet was inflated to 320 mmHg until a maximum plateau in muscle deoxygenation. TCF was measured before exercise as one third of the distance midline from the inguinal crease to the proximal boarder of patella. This represented the site of tourniquet application. In HYP, the participants executed the exercise bouts in normobaric, systemic hypoxia with an F₂O₂ of 14.0 %, corresponding to an altitude of approximately 3250 m.

Muscle deoxygenation

Deoxygenation at the muscle level was measured by continuous-wave, near-infrared spectroscopy (NIRS), as described previously (Van Beekvelt et al., 2001). A pair of NIRS optodes was positioned over the distal part of the vastus lateralis muscle ~15 cm above the proximal boarder of patella. Optodes were fixed in a plastic spacer, which was attached to the skin by double-sided sticky disks to ensure direct contact between optodes and skin. A black
bandage was placed over the optodes and around the leg for further fixation and to shield against extraneous light, and to minimise loss of transmitted near-infrared light. The interoptode distance was 40 mm. Skinfold thickness was measured between the emitter and receiver optodes using a skinfold caliper (Harpenden Ltd.). Skinfold thickness ($8.2 \pm 2.7$ mm) was less than half the distance separating the optodes. Circumference of the plastic spacer was marked on the skin using an indelible pen and pictures were taken to ensure that optodes were placed at the same position in all trials. Light absorption signals were converted to HHb deoxygenation changes using a differential pathlength factor (DPF) calculated according to participants’ age. The DPF was the same across trials for each participant. Data were acquired at 10 Hz and subsequently filtered in R software (ver. x64 3.2.5, R Foundation for Statistical Computing) using a 10th order zero-lag, low-pass Butterworth filter with a cut-off frequency of 0.1. The optimal cut-off frequency (i.e. reducing over- and underestimation of local means) was predetermined by an iterative analysis of root-mean-squared residuals derived from the application of multiple filters by use of a range of cut-off frequencies (0.075-0.150). Filtered data was used for the final analysis. Time-alignment and normalisation to the signal range between baseline (resting) and maximum (full occlusion) readings were completed in Excel (Ver. 2013, MS Office, Microsoft, USA).

**Graded exercise test (GXT)**

Participants completed the GXT following a light standardised meal ~3 hours prior to arrival. The test consisted of 4-min runs punctuated by 1 min of rest. The first run commenced at 5.0 km·h⁻¹, and the second at 8 km·h⁻¹. The speed was then increased by 1 km·h⁻¹ at the onset of each subsequent run until volitional exhaustion, defined as an inability to maintain the required speed. This progression in speed allowed a minimum of seven running stages to be completed (range: 7-11). After 5 min of rest, participants commenced running at the speed of the last completed run, after which the speed was increased by 1 km·h⁻¹ per minute until volitional exhaustion. This incremental bout was performed to ascertain attainment of a maximum 30-s plateau in oxygen consumption. Before the test, a facemask was placed over the mouth and nose and connected to an online, gas-analysing system for measurement of inspired and expired gases. To determine LT, blood was sampled at rest and immediately after each running stage from a 20-gauge, antecubital venous catheter. The catheter was inserted at rest in a supine position on a laboratory bed at least 20 min prior to the test. The LT was calculated using the modified $D_{\text{max}}$ method as it has been shown to better discriminate between individuals in comparison with other methods (Bishop et al., 1998). $V_{\text{O}_2\text{max}}$ was determined as the mean of the two peak consecutive 15-s values recorded during the test.
Diet and activity control

Participants consumed a standardised dinner (55 kJ·kg\(^{-1}\) BM; 2.1 g carbohydrate·kg\(^{-1}\) BM, 0.3 g fat·kg\(^{-1}\) BM, and 0.6 g protein·kg\(^{-1}\) BM) and breakfast (41 kJ·kg\(^{-1}\) BM; 1.8 g carbohydrate·kg\(^{-1}\) BM, 0.2 g fat·kg\(^{-1}\) BM, and 0.3 g protein·kg\(^{-1}\) BM) 15 and 3 hours, respectively, before every main trial. They recorded their dietary pattern within 48 h prior to each laboratory visit and were asked to replicate the same nutritional intake as per before their first exercise trial. Participants were instructed to maintain their normal dietary pattern throughout the study and were free of anti-inflammatory drugs and supplements, as well as medicine. The participants were instructed to replicate their weekly routine physical activity throughout the study and to avoid activity beyond daily living in the 48 h prior to each visit. In the 3-h period from termination of exercise to the +3h biopsy, oral consumption was limited to *ad libitum* water.

Muscle sampling

Vastus lateralis muscle biopsies were collected from the dominant leg in all trials using the Bergström needle biopsy technique with suction, amounting to 9 biopsies per participant. To minimise bleeding, the biopsy in ISC was obtained immediately after deflation of the tourniquet. In preparation for a muscle sample, a small incision was made under local anaesthesia (5 ml, 1% Xylocaine) through the skin, subcutaneous tissue and fascia of the muscle. Incisions were separated by approximately 1-2 cm. Immediately after sampling, samples were rapidly blotted on filter paper to remove excessive blood, and instantly frozen in liquid nitrogen. The samples were stored at -80 °C until being analysed. The incisions were covered with sterile Band-Aid strips and a waterproof Tegaderm film dressing (3M, North Ryde, Australia).

Blood handling and analysis

To ensure blood samples accurately represented circulating blood, ~2 mL of blood was withdrawn and discarded before sampling of approximately 2 mL of blood per sample. After being drawn, samples were instantaneously placed on ice until being analysed for lactate, pH, and K\(^+\), concentrations after exercise on an ABL 800 Flex blood gas analyzer (Radiometer, Brønshøj, Denmark).

Arterial oxygen saturation (\(S_aO_2\))

For safety reasons, adhesive optodes were placed on the tip of the left index finger to monitor arterial oxygen saturation during the HYP trial by pulse oximetry (Nellcor N-600, Nellcor Inc., Hayward, CA). Data was recorded at rest in the standing position on the treadmill, and during the final minute of each bout of running.
RNA isolation and reverse transcription

Approximately 44 mg w.w. muscle per sample was homogenised (2 × 2 min at 30 Hz) in ~800 μL TRIzol reagent (Invitrogen, Carlsbad, CA) using an electronic homogeniser (FastPrep FP120 Homogenizer, Thermo Savant). After homogenisation, the supernatant was aspirated into a new, freshly autoclaved microfuge tube containing 250 μL chloroform (Sigma Aldrich, St. Louis, MO). After few manual inversions and 5 min on ice, the mixture was centrifuged (15 min at 13,000 rpm) at 4°C. After centrifugation, the superior phase was pipetted into a new, autoclaved microfuge tube, and 400 μL 2-isopropanol alcohol (Sigma-Aldrich, St Louis, MO) and 10 μL of 5 M NaCl were added. The samples were then stored at -20°C for 3 h to precipitate the amount of RNA. After cooling, the samples were centrifuged (20 min at 13,000 rpm) at 4°C, and the isopropanol aspirated. The RNA pellet was rinsed with 75 % ethanol made from DEPC-treated H₂O (Invitrogen Life Sciences), and centrifuged (8 min at 9000 rpm) at 4°C. After pipetting off the ethanol, the pellet was resuspended in 5 μL of heated (60 °C) DEPC-treated H₂O. The samples were stored at -80°C until reverse transcription. RNA purity (mean ± SD, 1.96 ± 0.24; 260nm/280nm) and concentration (mean ± SD, 1317.4 ± 1311.5 ng·µL⁻¹) were determined spectrophotometrically on a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE). In addition, RNA integrity was assessed in six randomly chosen samples on an electrophoresis station (Experion, BioRad) using the manufacturer’s RNA analysis kit (Experion RNA StdSens) and instructions. The RNA quality indicator (RQI) of the six samples was (mean ± SD) 8.1 ± 0.7. One microgram of RNA per sample was reverse-transcribed into cDNA on a thermal cycler (S1000™ Thermal Cycler, Bio-Rad, Hercules, CA) using a cDNA synthesis kit (iScript RT Supermix, #1708841; Bio-Rad, Hercules, CA). The following incubation profile was used with random hexamers and oligo dTs in accordance with the manufacturer’s instructions: 5 min at 25 °C, 20 min at 46 °C and 1 min at 95 °C. cDNA was stored at -20°C until real-time PCR.

Real-time RT-PCR

Real-time PCR was performed to determine the expression of target and reference genes. Reactions were prepared on a 384-well plate using an automated pipetting system (epMotion 5073l, Eppendorf, Hamburg, Germany). One reaction (5 μL) was composed of 2 μL diluted cDNA, 0.15 μL forward and reverse primer (100 μM concentration), 0.2 μL DEPC-treated H₂O, and 2.5 μL iTaq universal SYBR Green Supermix (#1725125; Bio-Rad, Hercules, CA). Real-time PCR was performed on a QuantStudio 7 Flex Real-Time PCR System (#4485701, Thermo Fisher Scientific, USA) using the following protocol: Denaturing at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 60 s. Reactions were run in duplicate on the same plate with four template-free and four RT-negative controls. The expression of target genes was normalised to that of three reference genes using the \(2^{-\Delta\Delta Ct}\) method (Livak &
Schmittgen, 2001). This correction has been shown to yield reliable and valid mRNA data (Vandesompele et al., 2002). Reference genes used were glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TATA-binding protein (TBP), and β2 microglobulin (β2M). The mean (± SD) coefficient of variation (CV) of duplicate reactions (C_{T}), along with the forward and reverse sequences for the primers are shown in Table 2. Criteria and procedure for the design of primers for NKA isoforms are presented elsewhere (Christiansen et al. 2017, in review, FASEB J). Primers for PGC-1α isoforms were identical to those previously used (Ruas et al., 2012). Primer specificity was confirmed by performing a melt curve analysis at the end of each PCR run.

Dissection and fibre typing of muscle fibres

All chemicals used for dot blotting and Western blotting were from Bio-Rad unless otherwise stated. Antibodies are detailed in Table 3.

One part of each muscle biopsy (49.6 ± 10.0 mg w.w.) was freeze dried for 40 h, yielding 11.6 ± 2.7 mg d.w. muscle tissue. From these freeze-dried portions, a minimum of 40 single-fibre segments per sample (range: 40-120; total n = 2750) were isolated under a dissecting microscope using fine jeweller’s forceps. The segments were placed in individual microfuge tubes and incubated instantly for 1 h at room temperature in 10-µL denaturing buffer (0.125 M Tris-HCl, 10% glycerol, 4% SDS, 4 M urea, 10% mercaptoethanol, and 0.001% bromophenol blue, pH 6.8), in accordance with previous procedure (Murphy, 2011). The denatured segments were stored at -80 °C until future use.

Fibre type of individual segments was determined using dot blotting, as recently described (Christiansen et al. 2017, in preparation). In brief, two 0.45-µm PVDF membranes were activated in 95 % ethanol and equilibrated for two minutes in cold transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, 20 % methanol), after which a 1.5 µL aliquot of denatured sample, corresponding to one seventh of a fibre segment, was spotted onto each membrane. The membranes were placed at room temperature on a dry piece of filter paper to dry completely (5-10 min), after which they were reactivated in the ethanol and re-equilibrated in transfer buffer. After a quick wash in Tris-buffered saline-Tween (TBST), membranes were blocked in 5 % non-fat milk in TBST (blocking buffer) for 5-15 min. One of the blocked membranes was incubated (1 in 200 in 1 % BSA with PBST) with MHCIIa antibody, and the other membrane with MHCIIa antibody for 2 h at room temperature with gentle rocking. After a quick wash in blocking buffer, membranes were incubated (concentration: 1:20.000) with goat anti-mouse IgG (MHCIIa, #PIE31430, ThermoFisher Scientific) or IgM (MHCII, #sc-2064, Santa Cruz Biotechnology, TX, USA) horse radish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature with rocking. Membranes were then quickly rinsed in TBST, exposed
to Clarity enhanced chemiluminescence reagent (Bio-Rad, Hercules, CA, USA), and imaged on a ChemiDoc MP (Bio-Rad). The membrane incubated with MHCIIa antibody was reprobed with MHCIIx antibody for 2 h with rocking at room temperature, after which it was exposed to the same secondary antibody as MHCI (#sc-2064, Santa Cruz Biotechnology, TX, USA) for 1 h at room temperature and imaged accordingly. The difference in the host immunoglobulin species of the MHCIIa (IgG) and MHCIIx (IgM) antibodies allowed both isoforms to be quantified on the same membrane.

The remnant of each denatured fibre segment (7 µL) was grouped according to MHC expression to form samples of type I (MHCI) and type II (MHCIIa) fibres for each biopsy, in line with previous procedure (Kristensen et al., 2015). The number of fibre segments included in each group of muscle fibres per biopsy was (mean ± SD) n = 12 ± 6 (range: 5-27) for type I, and n = 16 ± 5 (range 7-33) for type IIa, fibres. Hybrid fibres (expressing multiple MHC isoforms) and type IIx fibres (classified by absence of MHCI and MHCIIa, but presence of MHCIIx protein), both constituting 3.1 % of the total pool of fibres, were excluded from analysis.

**Immunoblotting**

Fibre-type specific protein abundance and phosphorylation status of the signalling proteins AMPK and CaMKII, and their downstream targets ACC and PLB, respectively, were determined by Western blotting. Fifteen micrograms of protein per sample (~5 µL) were separated (45 min at 200 V) on 26-wells, 4-15 % Criterion TGX stain-free gels (Bio-Rad, Hercules, CA). Each gel was loaded with all samples from one participant, two calibration curves (a four- and a three-point to improve the reliability) and two protein ladders (PageRuler, Thermo Fischer Scientific). Calibration curves were of human whole-muscle crude homogenate with a known protein concentration, which was predetermined as described previously (Christiansen et al. 2017, in review, The Journal of Physiology). After electrophoresis, gels were UV activated (5 min) on a Criterion stain-free imager (Bio-Rad). Proteins were wet-transferred to 0.45 µm nitrocellulose membrane (30 min at 100 V) in a circulating bath at 4 °C in transfer buffer (25 mM Tris, 190 mM glycine and 20 % methanol). Membranes were then incubated (10 min) in antibody extender solution (Pierce Miser, Pierce, Rockford, IL, USA), washed in double-distilled H2O, and blocked for 2 h in blocking buffer (5 % non-fat milk in Tris-buffered saline Tween, TBST) at room temperature with rocking. To allow multiple proteins to be quantified on the same membrane, the membranes were cut horizontally at appropriate molecular masses using the two protein ladders as markers prior to probing with the primary antibodies overnight at 4 °C, and for 2 h at room temperature with constant, gentle rocking. Antibody details are presented in Table 3. Primary antibodies were diluted in 1 % bovine serum albumin (BSA) in phosphate-buffered saline with 0.025% Tween
(PBST) and 0.02% NaN₃. After washing in TBST and probing with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-mouse immunoglobulins or goat anti-rabbit immunoglobulins; Pierce, Rockford, IL, USA) for 1 h with rocking at room temperature, chemiluminescent images of membranes were captured on a ChemiDoc Touch (Bio-Rad), followed by densitometry using Image Lab (Ver. 5.2.1, Bio-Rad). Protein ladders were captured under white light prior to chemiluminescent imaging without moving the membranes. Band densities for proteins were quantified by reference to the linear calibration curves on the same gel and normalised to the total amount of protein in each lane on the stain-free gel image. The same researcher with substantial experience with the techniques was responsible for performing all muscle analyses.

**Muscle ATP and lactate**

A portion of each freeze-dried muscle sample (2 mg d.w.) was dissected free of connective tissue, blood and fat before being powdered using a Teflon pestle. The content of ATP and lactate in each sample was extracted using precooled perchloric acid/EDTA and KHCO₃, and analysed fluorometrically using a modification of a method previously described (Harris et al., 1974), where samples are analysed in a 96-well plate format. All samples from each participant, along with two standards of either ATP or lactate, a four-point NADH standard curve, and blanks (i.e. double distilled H₂O) were analysed in triplicate on the same plate. Absorbance readings of samples were normalised to the standards and subtracted blanks.

**Statistics**

Data was firstly assessed for normality using the Shapiro-Wilk test. An appropriate transformation of data was applied, if necessary, to obtain a normal distribution prior to subsequent statistical analyses. A two-way repeated-measures analysis of variance (RM ANOVA) was used to test the null-hypothesis of no effect of time (Pre, +3h) and condition (CON, ISC, HYP) for mRNA data (using the 2^ΔΔCt expression), and for blood and metabolite data. The sample size used for gene analyses was n = 8 for the CON. Due to contamination (and a Cₒ >35) of some samples, the sample size was reduced to n = 6 and n = 5 for the ISC and HYP, respectively. A one-way RM ANOVA was used to test the null-hypothesis of no effect of condition for mRNA changes with time using ΔmRNA values (i.e. difference between Pre and +3h). A two-way RM ANOVA was used to test the null-hypothesis of no effect of time (Pre, +0h) and fibre type (type I and type II) within condition for the content and phosphorylation of proteins and to evaluate conditional interactions with time (Pre, +0h) within fibre type. Data normalised to total protein, and not relative changes, was used for protein analyses. For NIRS data, a two-way RM ANOVA was used to test the null-hypothesis of no time and condition effects using the Butterworth-filtered data. Multiple pairwise, post hoc
analyses used the Tukey test. Interpretation of effect size ($d$) was based on Cohen’s conventions, where <0.2, 0.2-0.5, >0.5-0.8 and >0.8 were considered as trivial, small, moderate and large effect, respectively (Cohen, 1988). Data are reported as means ± SEM unless otherwise stated. The α-level was set at $p \leq 0.05$. Statistical analyses were performed in Sigma Plot (Ver. 11.0, Systat Software, CA).

Results

PGC-1α gene transcripts

PGC-1α total mRNA increased from Pre to +3h in ISC ($p = 0.007$, $d = 1.32$), but was not significantly altered in CON ($p = 0.447$, $d = 1.06$) or in HYP ($p = 0.166$, $d = 0.97$) (Fig. 2A).

PGC-1α1 mRNA increased from Pre to +3h in ISC ($p = 0.003$, $d = 1.36$), but was not significantly altered in CON ($p = 0.649$, $d = 0.29$) or in HYP ($p = 0.702$, $d = 0.32$). The increase in ISC was greater compared to that in CON ($p = 0.017$, $d = 1.45$) and in HYP ($p = 0.040$, $d = 1.21$) (Fig. 2B). PGC-1α4 mRNA increased from Pre to +3h in ISC ($p = 0.002$, $d = 1.25$), but was not significantly increased in CON ($p = 0.333$, $d = 1.12$) or in HYP ($p = 0.272$, $d = 0.86$). The increase in ISC was greater than in CON ($p = 0.037$, $d = 1.15$) and in HYP ($p = 0.057$, $d = 0.77$) (Fig. 2C).

Na$^+$/K$^+$-ATPase (NKA) and FXYD1 gene transcripts

NKAα1 mRNA remained unchanged in ISC ($p = 0.799$, $d = 0.44$), CON ($p = 0.648$, $d = 0.54$), and in HYP ($p = 0.557$, $d = 0.32$) (Fig. 3A). NKAα2 mRNA increased from Pre to +3h in ISC ($p = 0.050$, $d = 0.90$) and in HYP ($p = 0.004$, $d = 0.61$), but there was no significant change in CON ($p = 0.089$, $d = 1.07$) (Fig. 3B). NKAα3 mRNA was not significantly altered in all conditions ($p \geq 0.169$, $d = 0.57$, 0.63 and 0.58 in ISC, CON and HYP, respectively) (Fig. 3C). NKAβ1 mRNA increased in HYP ($p = 0.027$, $d = 0.68$), but there was no significant change in ISC ($p = 0.268$, $d = 0.79$) or in CON ($p = 0.539$, $d = 1.19$) (Fig. 4A). NKAβ2 mRNA was not significantly altered in all conditions ($p \geq 0.276$, $d = 0.24$, 0.40 and 0.24 in ISC, CON and HYP, respectively) (Fig. 4B). NKAβ3 mRNA was also not significantly changed all conditions (ISC: $p = 0.909$, $d = 0.34$; CON: $p = 0.859$, $d = 0.55$; HYP: $p = 0.328$, $d = 0.52$) (Fig. 4C). FXYD1 mRNA increased in ISC ($p = 0.016$, $d = 1.10$). This increase was greater than in CON ($p = 0.039$, $d = 1.05$). There were no changes in the remaining conditions for this gene (CON: $p = 0.915$, $d = 0.20$; HYP: $p = 0.305$, $d = 0.32$) (Fig. 4D).

Muscle deoxygenation and oxidative stress markers

Muscle deoxygenation, as assessed by deoxygenated haemoglobin concentration (muscle HHb), was higher ($p < 0.05$) during exercise in ISC and HYP, relative to CON, except for the seventh bout of exercise ($p > 0.05$). During the recovery from the first, fourth and fifth bout,
Running head: Ischaemic exercise augments gene adaptations and redox signalling

Muscle HHb was higher in ISC, relative to CON (p < 0.05). During the recovery from the second bout, muscle HHb was higher in ISC and HYP, compared to CON (p > 0.05; Fig. 5A). No significant differences were detected between ISC and HYP at any time point (p > 0.05). Catalase mRNA content increased significantly in ISC (p = 0.024, d = 0.70), but there was no significant changes in CON (p = 0.881, d = 0.34) or in HYP (p = 0.505, d = 0.15). HSP70 mRNA increased in ISC (p = 0.057, d = 0.86), but there was no significant changes in CON (p = 0.669, d = 0.18) or in HYP (p = 0.176, d = 0.95).

Muscle metabolites
Muscle ATP remained unchanged in all conditions (p > 0.05; Fig. 6A). Muscle lactate increased in ISC and in HYP, but not in CON (p > 0.05). The increase in ISC and HYP was greater than CON (p < 0.05; Fig. 6B).

Venous blood lactate, pH and potassium ion concentration
In ISC, blood lactate concentration ([lac-]) increased (p < 0.05) after the third bout of exercise and remained elevated throughout the trial compared to rest. Blood [lac-] was higher (p < 0.05) in ISC than in CON after the third bout, the fifth to ninth bout, and after 3 min of recovery. In HYP, blood [lac-] increased (p < 0.05) after the third, fifth, sixth, eighth and ninth bout and in recovery, compared to rest. Blood [lac-] was higher (p < 0.05) in HYP than in CON after the third, fifth and ninth bout of exercise. In CON, blood [lac-] remained unchanged throughout the trial, compared to rest (p > 0.05).

In ISC, blood pH dropped (p < 0.05) following the first bout of exercise and remained lower (p < 0.05) compared to rest throughout the trial, and 3 min into recovery, but returned to resting level after 6 min of recovery (p > 0.05). The drop in pH in ISC was lower, relative to CON, after the sixth, before the seventh, and after the eighth and ninth, bout relative to CON (p < 0.05). In HYP, blood pH was lower, compared to rest, following the third, fifth, and sixth bout, and before the seventh bout, but returned to resting level after the seventh bout, from where it remained unchanged. The drop in pH in HYP was lower following the sixth and before the seventh bout, relative to CON (p < 0.05). In CON, blood pH remained unchanged throughout the trial (p > 0.05).

In all trials, blood potassium ion concentration ([K+]') increased after warm-up, and after the first to eighth bout of exercise, compared to rest. In CON, blood [K+]’ was also elevated (p < 0.05) after the ninth bout of exercise, relative to rest. Compared to CON, blood [K+]’ was lower (p < 0.05) in ISC 4 min into recovery from the third bout, and 6 min into recovery from the ninth bout of exercise, with no differences at other time points (p > 0.05), nor between HYP and CON at all time points (p > 0.05).
AMPK and ACC protein abundance and phosphorylation

Representative blots for AMPK and ACC are shown in Fig. 8A.

In HYP, α-AMPK protein abundance decreased (p = 0.023) from Pre to +0h in type I, but did not change significantly in type II (p = 0.112) fibres. In ISC and CON, α-AMPK protein abundance was not significantly altered in both fibre types (p ≥ 0.423). The abundance was significantly higher in both fibre types in ISC relative to HYP at +0h (p ≤ 0.002) (Fig. 9A). The α-AMPK protein abundance was significantly higher in type II vs. type I fibres in all conditions (main effect of fibre type, p ≤ 0.027). In HYP, the phosphorylation of α-AMPK at Thr\(^{172}\) relative to total α-AMPK protein abundance (α-AMPK Thr\(^{172}\)/α-AMPK) increased significantly in type I (p = 0.003), but not in type II (p = 0.558) fibres. In ISC and CON, there was no significant change in α-AMPK Thr\(^{172}\)/α-AMPK in both fibre types (p ≥ 0.112). The α-AMPK Thr\(^{172}\)/α-AMPK was significantly higher in type II vs. type I fibres in all conditions (main effect of fibre type, p ≤ 0.017) (Fig. 9B).

ACC protein abundance was not significantly altered in both fibre types in all conditions (p ≥ 0.168), and overall it was higher in type II vs. type I fibres (main effect of fibre type, p ≤ 0.015; Fig. 9C). The phosphorylation of ACC at Ser\(^{79}\) to total ACC protein (ACC Ser\(^{79}\)/ACC) increased significantly from Pre to +0h in type I fibres in ISC (p ≤ 0.020), with the increase being significantly higher relative to CON (p = 0.052). In the same condition, there was no significant change in ACC Ser\(^{79}\)/ACC in type II fibres (p = 0.260). No changes in ACC Ser\(^{79}\)/ACC occurred in CON and HYP (p ≥ 0.213) (Fig. 9D).

CaMKII and phospholamban (PLB) protein abundance and phosphorylation

Representative blots for CaMKII and PLB are shown in Fig. 8B.

CaMKII protein abundance did not change significantly in both fibre types in all conditions (p ≥ 0.108). In ISC and CON, it was significantly higher in type I vs. type II fibres (main effect of fibre type, p ≤ 0.038), but not significantly different between fibre types in HYP (p = 0.200) (Fig. 10A). Phosphorylation of CaMKII at Thr\(^{287}\) to total CaMKII protein (CaMKII Thr\(^{287}\)/CaMKII) decreased significantly in type II fibres in CON (p = 0.023), and tended to decrease in ISC (p = 0.056), but did not change significantly in HYP (p = 0.746). No significant changes in CaMKII Thr\(^{287}\)/CaMKII occurred in type I fibres in all conditions (p ≥ 0.213), CaMKII Thr\(^{287}\)/CaMKII was significantly higher in type II vs. type I fibres in all conditions (main effect of fibre type, p ≤ 0.023) (Fig. 10B).

In type I fibres, PLB protein abundance decreased significantly in CON (p = 0.037), whereas it did not change significantly in ISC (p = 0.786) or in HYP (p = 0.428) in the same fibre type. The PLB abundance did not change significantly in type II fibres in all conditions (p ≥ 0.288). The PLB abundance was lower in type II vs. type I fibres (main effect of fibre type, p ≤ 0.050)
(Fig. 8C). The phosphorylation of PLB at Ser\textsuperscript{16} to total PLB protein (PLB Ser\textsuperscript{16}/PLB) increased significantly in type I fibres in CON (p = 0.023) and in ISC (p = 0.010), but it remained unchanged in HYP in the same fibre type (p = 0.412). In type II fibres, PLB Ser\textsuperscript{16}/PLB increased in ISC (p ≤ 0.026) and in HYP (p = 0.025), but did not change significantly in CON (p = 0.350) (Fig. 8D).
Discussion

The main novel findings, which are summarised in Fig. 11, were that repeated bouts of ischaemic exercise substantially elevated the mRNA content of PGC-1α (~4.3 fold), PGC-1α1 (~2.3 fold) and PGC-1α4 (~6 fold), and of the NKA regulatory subunit gene, FXYD1 (~2.7 fold), in trained human skeletal muscle. These changes coincided with increased levels of oxidative stress markers (catalase and HSP70 mRNA, 1.5-1.9 fold), and were temporally preceded by elevated (~1-2 fold) ACC phosphorylation (Ser\textsuperscript{79}) in both muscle fibre types. These results suggest the effect of ischaemia on exercise-induced changes in PGC-1α and FXYD1 mRNA levels was mediated, at least in part, through potentiation of ROS formation and activation of associated signalling networks, of which AMPK could be one (contributing) pathway (Irrcher \textit{et al.}, 2009). Despite a similar exercise-induced reduction in muscle oxygen content in the ischaemic and hypoxic trials (ISC vs. HYP, Fig. 5), we found no impact of exercising in systemic hypoxia on either the mRNA content of PGC-1α isoforms and FXYD1, or on markers of oxidative stress and ACC phosphorylation. These findings support that perturbations in redox homeostasis, rather than a critical threshold of muscle hypoxia \textit{per se}, were key to the transcriptional induction of PGC-1α isoforms and FXYD1 in response to the repeated-ischaemic exercise (Stoner \textit{et al.}, 2007).

Repeated-ischaemic exercise increases PGC-1α total and -isoform mRNA levels in the skeletal muscle of trained men

In a previous human study, a decrease of ~15-20 % in muscle blood perfusion (Eiken & Bjurstedt, 1987) augmented the rise (~5.5 fold) in muscle \textit{PGC-1α} (total) mRNA content in response to knee-extensor exercise (45 min at 26 % of one-leg peak load) (Norr bom \textit{et al.}, 2004). In accordance, in the present study, ischaemic exercise induced a 4.3-fold rise (i.e. 2.5-fold greater increase vs. CON) in total \textit{PGC-1α} expression (Fig. 2). Furthermore, the mRNA levels of both the \textit{PGC-1α1} (2.5-fold) and \textit{PGC-1α4} (6.0 fold) were elevated only after the ischaemic exercise. Our results highlight for the first time the effectiveness of this training modality to enhance the exercise-induced response of PGC-1α-isoform transcripts in humans.

Oral consumption of antioxidants (i.e. vitamin C and E) have been shown to blunt the exercise-induced increase in \textit{PGC-1α} mRNA content in the skeletal muscle of young men (Ristow \textit{et al.}, 2009), suggesting ROS could be important for the upregulation of \textit{PGC-1α} mRNA content in human muscle. Accordingly, the increases in PGC-1α-isoform mRNA levels in ISC coincided with elevated \textit{catalase} and \textit{HSP70} mRNA levels, both shown to be specifically induced by hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) in myocytes (Kemp \textit{et al.}, 2003; Zhong \textit{et al.}, 2011). These data provide the first evidence in humans that facilitated ROS production may be one mechanism underlying the increases in \textit{PGC-1α1} and -1α4 mRNA transcript levels. In support,
several in vitro studies have suggested ROS may be implicated in PGC-1α transcription. For example, in C2C12 cells, incubation with H₂O₂ elevated both PGC-1α mRNA content (1.4 fold) and promoter activity (Ircher et al., 2009), whereas pre-incubation with the ROS scavenger, N-acetylcysteine (NAC) abolished the H₂O₂-evoked rise in promoter activity (Zhang et al., 2014) and mRNA content (Ircher et al., 2009). Together, our results suggest that the effect of ischaemia was mediated, in part, by promotion of ROS generation in skeletal muscle.

Association between increases in PGC-1α mRNA transcripts and ACC phosphorylation

In line with our second working hypothesis, the ischaemia-evoked increases in PGC-1α mRNA transcripts were preceded by elevated ACC phosphorylation, indicative of higher AMPK activity (Chen et al., 2000). This suggests AMPK could be one signalling kinase through which the initial cellular signals are transduced to regulate the content of multiple PGC-1α mRNA transcripts in human muscle. This hypothesis is consistent with data from cell culture studies. In one of these studies, treatment of C2C12 cells with the AMPK activator, 5-aminimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR), raised PGC-1α mRNA content (2.2 fold) coincidently with augmented PGC-1α promoter activity (3.5 fold). In that study, several AICAR-sensitive PGC-1α promoter sites were identified (Ircher et al., 2008), suggesting AMPK may directly facilitate PGC-1α transcription. In a more recent experiment, transcriptional activation of PGC-1α by ROS coincided with promoted AMPK activation (Ircher et al., 2009). Thus, in vitro, ROS-induced activation of PGC-1α transcription could be mediated in part via AMPK.

The coincident increases in oxidative stress markers, ACC phosphorylation, and PGC-1α-isoform mRNA content with ischaemia in the present study underline that a similar signal transduction axis involving ROS and AMPK may exist in humans. However, factors other than ROS may, in part, account for the higher ACC phosphorylation with ischaemia. For example, circulating norepinephrine can stimulate AMPK activity in skeletal muscle cells via α₁-adrenoceptors (Hutchinson & Bengtsson, 2006), and ischaemia has been shown to exacerbate exercise-induced increases in circulating norepinephrine concentration (Sundberg, 1994). Moreover, the increase in ACC phosphorylation was most prominent in type I fibres (Fig. 9D), consistent with an accelerated glycogenolysis in this fibre type during repeated contractions with circulatory occlusion (250 mmHg) (Greenhaff et al., 1993). These findings support that the mRNA responses to ischaemic exercise could be fibre type-dependent. Future work is required to examine this further by measuring PGC-1α-isoform mRNA and AMPK activity in different muscle fibre types. Inconsistent with the changes in ACC phosphorylation, phosphorylation of AMPKα remained unaltered in ISC and CON, and as such, was dissociated from the changes in PGC-1α transcript levels. The latter finding is in line with previous observations in humans (Brandt et al., 2016; Taylor et al., 2016).
dissociation of AMPK and ACC phosphorylation is likely explained by the extent to which phosphorylation of different AMPK heterotrimer complexes affects muscle AMPK activity (Birk & Wojtaszewski, 2006).

**Increases in PGC-1α-isof orm mRNA content are unrelated to modulation of CaMKII phosphorylation in human muscle fibre types**

In myocytes in vitro, inhibition of CaMK has been reported to abolish Ca^{2+}-induced increases in PGC-1α expression (Ojuka et al., 2003). Thus, we explored the association between changes in PGC-1α mRNA levels and those of CaMKII phosphorylation at Thr^{287}, which in human muscle has been correlated with CaMKII autonomous activity ($r^2 = 0.884$) (Rose et al., 2006). A novel observation was that CaMKII phosphorylation decreased in type II fibres only in ISC ($p = 0.056$) and CON. This fibre type-specific modulation of CaMKII phosphorylation was abolished by systemic hypoxia, indicating that decreased arterial oxygen saturation may affect contraction-stimulated Ca^{2+} signalling in type II human muscle fibres. This effect of hypoxia, however, was dissociated from alterations in PGC-1α levels. Changes in CaMKII phosphorylation, regardless of fibre type, were unrelated to those of PGC-1α in the ischaemic-exercised leg. Together, these data imply that promotion of human muscle PGC-1α-isof orm mRNA levels does not require changes in CaMKII autonomous activity per se, although our observations are limited to immediately post exercise. In mouse fast-twitch skeletal muscle fibres, CaMKII partly controls SR Ca^{2+} release, but not SR Ca^{2+} uptake (Tavi et al., 2003). It may therefore be speculated whether the decrease in CaMKII phosphorylation in type II fibres in ISC and CON (Fig. 10) may have reflected a reduced SR Ca^{2+} leak, although this cannot be resolved from the present data alone. Nevertheless, ischaemia may have affected type-II fibre SR Ca^{2+} uptake, as ischaemia invoked an increase in PLB phosphorylation in this fibre type (Fig. 10D). In agreement, relieving the inhibition of PLB by phosphorylation at Ser^{16} promotes SR Ca^{2+} uptake in both fibre types, with the effect being most pronounced in predominantly glycolytic fibres (Briggs et al., 1992). A similar fibre type-dependent effect on PLB phosphorylation was evident with systemic hypoxia. Based on our observations, modulation of SR Ca^{2+} kinetics may not have been a primary mechanism underlying the effect of ischaemia on PGC-1α mRNA levels in the present study.

**Increases in human muscle PGC-1α-isof orm mRNA content are unrelated to muscle hypoxia**

Unlike ischaemia, exercise alone was without impact on the levels of the investigated PGC-1α transcripts. This may be explained by a low relative exercise intensity (105 % LT) given the high training status of our participants, the moderate running speed (~12 km·h⁻¹), and the positive relation between exercise intensity and exercise-induced increases in muscle PGC-1α mRNA content previously reported (Egan et al., 2010; Nordsborg et al., 2010b). However,
absence of an effect of exercise alone was unlikely related to muscle metabolic stress per se. Accordingly, we found no effect of systemic hypoxia on PGC-1α levels, despite exacerbated metabolic by-product accumulation comparable to that induced by ischaemic exercise (Fig. 6 and 7). This is consistent with a previous human study, in which contraction-stimulated increases in leg muscle PGC-1α mRNA content was unaffected by promoted systemic levels of epinephrine and lactate invoked by simultaneous arm exercise (Brandt et al., 2016). The present observation of unaltered PGC-1α levels after exercise in systemic hypoxia agrees with a previous human study that reported unchanged PGC-1α mRNA content after a single exercise session at simulated altitude (3000 m) in recreationally active men (Slivka et al., 2014). Since deoxygenated HHb was similar during exercise with ischaemia and in systemic hypoxia (Fig. 5A), this lack of a hypoxic effect may not be ascribed to cellular hypoxia per se. As such, the severity of exercise-induced muscle hypoxia was not decisive for the ischaemia-mediated increases in PGC-1α transcript levels in the present study. Given the comparable fluctuations in muscle lactate and blood metabolites in HYP and ISC (Fig. 6 and 7), inadequate metabolic by-product accumulation may also not account for the lack of a hypoxic effect. As we detected no change in oxidative stress markers with hypoxia, it may rather be explained by insufficient ROS accumulation, although a single factor may not fully account for this absence of effect given the cross-talk and redundancy of ionic and metabolic perturbations, and ROS formation (Kourie, 1998).

Possible involvement of ROS in the regulation of FXYD1 mRNA content in human muscle

Another novel result was that FXYD1 mRNA content increased (2.7 fold) due to ischaemia (Fig. 4D). Despite similar increases in deoxygenated HHb between HYP and ISC (Fig. 5A), systemic hypoxia was without effect on FXYD1 expression. Thus, cellular hypoxia was also unlikely a primary stimulus for the ischaemia-induced increase in FXYD1. Nor may the increase be related to the severity of metabolic stress, as changes in muscle lactate and phosphocreatine were similar in ISC and HYP (Fig. 6). The rise in FXYD1 mRNA expression, however, was accompanied by increases in markers of oxidative stress (Fig. 5). Therefore, accumulation of ROS may be an important mechanism underlying the effect of ischaemia on FXYD1 transcription. Accordingly, FXYD1 overexpression protected myocytes against ROS-induced NKA dysfunction (Liu et al., 2013), suggesting a ROS-protective effect of elevated FXYD1 content. It has also been established in cell culture that AMPK can be activated by ROS (Ircher et al., 2009), and this regulates FXYD transcription in mouse glycolytic skeletal muscles (Nilsson et al., 2006). In accordance, the increases in FXYD1 and oxidative stress markers were paralleled by elevated ACC phosphorylation (Fig. 9). Therefore, regulation of human muscle FXYD1 expression could involve both ROS production and AMPK activation. Measurement of AMPK activity, along with FXYD1 mRNA content, should be performed in...
future studies to verify these initial results. In addition, alterations in FXYD1 expression were dissociated from those of CaMKII and PLB phosphorylation (Fig. 10), suggesting transcriptional induction of FXYD1 mRNA content in human muscle does not require alterations in CaMKII autonomous activity.

**Increases in human muscle NKA α₁ and β₃ mRNA content are unrelated to oxidative stress and metabolic perturbations**

In the present study, NKAα₁ and -β₃ mRNA content was unaffected by exercise with ischaemia, in systemic hypoxia, or by exercise alone (Fig. 3A and 4C), despite substantial differences amongst conditions in changes in markers of oxidative stress, muscle lactate, and blood metabolites. As such, the level of these mRNA transcripts are neither influenced by the nature of metabolic and ionic fluctuations, nor by the degree of oxidative stress, in human skeletal muscle. In support, raising the metabolic stress by performing simultaneous arm exercise was without effect on the increases in α₁ and β₃ mRNA content after isolated knee-extensions (Nordsborg et al., 2005). Based on the individual changes for these isoforms in the present study (Fig. 3A and 4C), they seem to be regulated similarly independent of condition. For example, the same two individuals that decreased their α₁ mRNA content in ISC also reduced their β₃ expression in the same condition. Accordingly, we have recently shown parallel increases in only α₁ and β₃ mRNA levels following sprint interval exercise (Christiansen et al. 2017, unpublished). In another human study, NKAα₁ and -β₃ were the only transcripts of those investigated (α₁-3 and β₁-3) that remained unaltered in response to 45 min cycling at 71 % VO₂max (Murphy et al., 2008). Together, these results highlight that α₁ and β₃ are likely regulated at the mRNA level by the same cellular signals, different from those important to changes in the mRNA expression of other NKA isoforms (e.g. compare the individual changes for α₁ and α₂; Fig. 3A and 3B).

**Regulation of other NKA-isoform mRNA transcripts by repeated-ischaemic exercise**

The NKA α₂ isoform is limiting for a muscle’s contractile performance (Radzyukevich et al., 2013) and forms up to 90 % of NKA complexes in adult rat skeletal muscles (Orlowski & Lingrel, 1988). Together with the β₁ isoform, it forms the largest NKA pool in this tissue. Understanding the cellular signals that regulate α₂ and β₁ expression is therefore fundamental. In the present study, significant increases in the mRNA content of NKAα₂ and -β₁ were evident in HYP and ISC, and in HYP alone, respectively, whereas CON was without effect on these genes. This suggests a hypoxic and/or a metabolically perturbed cellular milieu could be beneficial for increasing the mRNA content of these isoforms in human muscle. However, taking into account the individual values for α₂ and β₁ (Fig. 3B), there appears no obvious difference in the changes in both α₂ and β₁ levels between CON, ISC, and HYP. This raises
the possibility of a statistical type II error (in CON for $\alpha_2$ and in CON and ISC for $\beta_1$). This precludes us from unequivocally interpreting the present data related to these isoforms. More research appears required to elaborate on the idea that cellular hypoxia and/or metabolic stress per se are important stimuli for increases in $\alpha_2$- and $\beta_1$-isoform expression with contractile activity. In addition, no alterations in the levels of $NKA_2$ and -$\beta_2$ transcripts were found for any condition (Fig. 3 and 4). We have previously observed no change in $\alpha_3$ mRNA content after repeated-intense exercise of short duration (Christiansen et al. 2017, in review, FASEB). Conversely, exercise-induced increases in $\alpha_3$ expression have been reported in other human studies. In these studies, the changes in $\alpha_3$ mRNA occurred at exercise termination, with the level returning to basal state after 3 h of recovery (Murphy et al., 2004; Aughey et al., 2007; Murphy et al., 2008). Thus, our time point of mRNA measurement may have been decisive for the present outcome, and as such is a limitation of the present study. The effect of exercise on $\beta_2$ mRNA expression is controversial with human studies reporting both increased, decreased, or unchanged, expression 3 h into recovery. In the present study, the level of $\beta_2$ mRNA remained unchanged. The reason for these conflicting findings is not clear (Christiansen et al. 2017, unpublished), and further mechanistic studies are needed to understand how $\beta_2$ expression is regulated in human muscle.

**Conclusion and perspectives**

In summary, repeated bouts of ischaemic exercise potently raised the mRNA levels of PGC-1$\alpha$, -1$\alpha_1$ and -1$\alpha_4$, and of the NKA regulatory subunit, FXYD1, in skeletal muscle of trained men. The molecular mechanisms likely involved were promoted ROS generation and AMPK activation. The effect of ischaemia was, however, unrelated to the severity of muscle hypoxia, lactate accumulation, and fibre type-specific modulation of CaMKII signalling. Thus, repeated-ischaemic exercise is a potent strategy to enhance the muscle’s gene response associated with mitochondrial biogenesis and K$^+$ handling in trained individuals. Based on this work, future studies should evaluate whether the muscle’s capacity for oxidative ATP generation and potassium ion regulation would be improved by a period of repeated-ischaemic training.
Legends

Figure 1. Time-aligned, schematic representation of the experimental protocol. The participants performed three exercise trials separated by one week consisting of running without (control) or with the muscle blood flow partially occluded (ischaemia), or in normobaric, systemic hypoxia (hypoxia). The intensity of exercise was set according to the participants’ individual lactate threshold (LT). Muscle was sampled at rest before, immediately post, and after 3 h of recovery from, each trial. Blood was sampled from an antecubital vein at the time points indicated.

Figure 2. PGC-1α total and -isoform mRNA responses to repeated exercise with ischaemia or in systemic hypoxia. A) PGC-1α total, B) PGC-1α1, and C) PGC-1α4, mRNA content. Individual changes from before (Pre) to 3 h after exercise (+3h) are displayed on the left with each symbol representing one participant across trials and figures. On the right are bars representing mean (± SEM) changes relative to Pre in the dominant leg for exercise alone (CON, white), with ischaemia (ISC occluded, black), or in systemic hypoxia (HYP, meshed). * p ≤ 0.05, different from Pre; † p ≤ 0.05, different from CON and HYP.

Figure 3. NKA-α-isoform mRNA responses to repeated exercise with ischaemia or in systemic hypoxia. A) α1, B) α2, and C) α3, mRNA content. Individual changes from before (Pre) to 3 h after exercise (+3h) are displayed on the left with each symbol representing one participant across trials and figures. On the right are bars representing mean (± SEM) changes relative to Pre in the dominant leg for exercise alone (CON, white), with ischaemia (ISC occluded, black), or in systemic hypoxia (HYP, meshed). * p ≤ 0.05, different from Pre.

Figure 4. NKA-β-isoform and FXYD1 mRNA responses to repeated exercise with ischaemia or in systemic hypoxia. A) β1, B) β2, C) β3, and D) FXYD1, mRNA content. Individual changes from before (Pre) to 3 h after exercise (+3h) are displayed on the left with each symbol representing one participant across trials and figures. On the right are bars representing mean (± SEM) changes relative to Pre in the dominant leg for exercise alone (CON, white), with ischaemia (ISC occluded, black), or in systemic hypoxia (HYP, meshed). * p < 0.05, different from Pre; † p < 0.05, different from CON.

Figure 5. Changes in muscle deoxygenation and oxidative stress markers in response to repeated exercise with ischaemia or in systemic hypoxia. A) Muscle deoxygenation (i.e. deoxygenated haemoglobin, Muscle HHb) as assessed by near-infrared spectroscopy during exercise alone (CON, ○), with ischaemia (ISC, ■), or in systemic hypoxia (HYP, Δ). Hashed bars represent exercise bouts. # p ≤ 0.05, ISC and HYP different from CON. * p ≤ 0.05, ISC different from CON. B) Catalase, and C) heat-shock protein 70 (HSP70), mRNA expression. Individual changes from before (Pre) to 3 h after exercise (+3h) are displayed on the left with each symbol representing one participant across trials and figures. On the right are bars representing mean (± SEM) changes relative to Pre in the dominant leg for exercise alone (CON, white), with ischaemia (ISC occluded, black), or in systemic hypoxia (HYP, meshed). Data are means ± SEM. * p ≤ 0.05, different from Pre.

Figure 6. Changes in muscle ATP and lactate concentration in response to repeated exercise with ischaemia (ISC) or in systemic hypoxia (HYP). A) ATP, and B) lactate concentration before (Pre, white) and immediately after exercise (+0h, black). Data are means ± SEM. * p < 0.05, different from Pre. † p < 0.05, different from CON.

Figure 7. Changes in venous blood lactate, pH, and potassium ion (K+) concentration in response to exercise with ischaemia or in systemic hypoxia. A) Lactate, B) pH, and C) K+ concentration during exercise alone (CON, ○), with ischaemia (ISC, ■), or in systemic hypoxia
(HYP, Δ). Hashed bars represent exercise bouts. Data are means ± SEM. * p < 0.05, different from rest; † p < 0.05, ISC different from CON; # p < 0.05, HYP different from CON.

**Figure 8.** Representative blots for AMPKα, ACC, CaMKII, and phospholamban (PLB) protein abundance and phosphorylation in type I and II human skeletal muscle fibres. A) AMPK and ACC, and B) CaMKII and PLB, protein abundance and phosphorylation in the dominant leg in response to exercise alone (CON), with ischaemia (ISC occluded), or in systemic hypoxia (HYP) before (Pre) and immediately after (+0h) exercise. Total protein was determined in each lane from the stain-free gel images obtained after electrophoresis. CaMKII isoforms (βM and σ/γ) are indicated in B.

**Figure 9.** Changes in AMPKα and ACC protein abundance and phosphorylation in type I and II human skeletal muscle fibres in response to repeated exercise with ischaemia or in systemic hypoxia. A) AMPKα protein, B) AMPKα phosphorylation at Thr172 normalised to AMPKα protein, C) ACC protein, and D) ACC phosphorylation at Ser79 normalised to ACC protein in type I (white bars) and type II (black bars) fibres before (Pre) and immediately after (+0h) exercise. Data are means ± SEM. * p < 0.05, different from rest within condition and fibre type; † p ≤ 0.05, ISC different from HYP in A, and from CON in D.

**Figure 10.** Changes in CaMKII, and phospholamban (PLB) protein abundance and phosphorylation in type I and II human skeletal muscle fibres in response to repeated exercise with ischaemia or in systemic hypoxia. A) CaMKII protein, B) CaMKII phosphorylation at Thr287 normalised to CaMKII protein, C) PLB protein, and D) PLB phosphorylation at Ser16 normalised to PLB protein in type I (white bars) and type II (black bars) fibres before (Pre) and immediately after (+0h) exercise. Data are means ± SEM. * p < 0.05, different from rest within condition and fibre type.

**Figure 11.** Summary of key findings. The effect of aerobic interval (CON), repeated-ischaemic (ISC) and –hypoxic exercise (ISC) on the mRNA content of PGC-1α (total, 1α1, 1α4) and Na⁺,K⁺-ATPase (NKAα1, NKAβ1-3, FXYD1) isoforms, oxidative stress markers (catalase and heat-shock protein 70, HSP70, mRNA content), muscle hypoxia (i.e. deoxygenated haemoglobin as measured by near-infrared spectroscopy), lactate concentration, and AMPK and CaMKII signalling in the skeletal muscle of trained men. *p-“ denotes phosphorylation; ACC, Acetyl-CoA carboxylase; AMPK, 5' AMP-activated protein kinase; CaMKII, Ca2+/calmodulin-dependent protein kinase II; PLB, phospholamban; LT, lactate threshold.
Figures:

Figure 1

Muscle: Rest
Blood: Rest
NIRS: Ischaemia/hypoxia:

105 % LT
105 % LT
105 % LT

75 % LT
Walk
Walk

Time (min): -10 -5 0 3 6 8 13 16 19 21 26 29 32 34 37 40 214

320 mmHg
Figure 4

A. 

B. 

C. 

D. 

Legend:
- CON
- ISC
- HYP

Notes:
- Pre: Pre-exercise
- +3h: 3 hours post-exercise

Analysis:
- Fold change in Na^+ - K^+ ATPase β1 mRNA
- Fold change in Na^+ - K^+ ATPase β2 mRNA
- Fold change in Na^+ - K^+ ATPase β3 mRNA
- Fold change in FXYD1 mRNA

Significance:
- *: p < 0.05
- †: p < 0.01
Figure 6

A

![Bar chart showing muscle ATP levels.](chart_A)

B

![Bar chart showing muscle lactate levels.](chart_B)
Figure 8

A

<table>
<thead>
<tr>
<th>Fibre type:</th>
<th>Pre</th>
<th>+0h</th>
<th>Pre</th>
<th>+0h</th>
<th>Pre</th>
<th>+0h</th>
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<td><img src="image2.png" alt="Image" /></td>
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<td><img src="image4.png" alt="Image" /></td>
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<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
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<tr>
<td>Total protein</td>
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<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
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<tr>
<td>p-ACC Ser\textsuperscript{79} (257 kDa)</td>
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<tr>
<td>AMPK (63 kDa)</td>
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<td><img src="image23.png" alt="Image" /></td>
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<td>Total protein</td>
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<td><img src="image28.png" alt="Image" /></td>
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</tr>
<tr>
<td>p-AMPK Thr\textsuperscript{172} (63 kDa)</td>
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<td><img src="image30.png" alt="Image" /></td>
<td><img src="image31.png" alt="Image" /></td>
<td><img src="image32.png" alt="Image" /></td>
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<td></td>
</tr>
</tbody>
</table>

B

| Total protein | ![Image](image33.png) | ![Image](image34.png) | ![Image](image35.png) |
| 70 kDa | ![Image](image36.png) | ![Image](image37.png) | ![Image](image38.png) |
| 55 kDa | ![Image](image39.png) | ![Image](image40.png) | ![Image](image41.png) |
| δ/γ | ![Image](image42.png) | ![Image](image43.png) | ![Image](image44.png) |
| Total protein | ![Image](image45.png) | ![Image](image46.png) | ![Image](image47.png) |
| 70 kDa | ![Image](image48.png) | ![Image](image49.png) | ![Image](image50.png) |
| p-βM | ![Image](image51.png) | ![Image](image52.png) | ![Image](image53.png) |
| 55 kDa | ![Image](image54.png) | ![Image](image55.png) | ![Image](image56.png) |
| Total protein | ![Image](image57.png) | ![Image](image58.png) | ![Image](image59.png) |
| PLB (25 kDa) | ![Image](image60.png) | ![Image](image61.png) | ![Image](image62.png) |
| Total protein | ![Image](image63.png) | ![Image](image64.png) | ![Image](image65.png) |
| p-PLB Ser\textsuperscript{16} (25 kDa) | ![Image](image66.png) | ![Image](image67.png) | ![Image](image68.png) |
Figure 9

A. Main effect of fibre type ($p \leq 0.027$)
† Different from HYP within fibre type ($p \leq 0.002$)

B. Main effect of fibre type ($p \leq 0.017$)
* Effect of time within fibre type ($p = 0.003$)

C. Main effect of fibre type ($p \leq 0.015$)

D. * Effect of time within fibre type ($p \leq 0.020$)
† Different from CON within fibre type ($p = 0.052$)
### Figure 11

<table>
<thead>
<tr>
<th></th>
<th>Repeated, aerobic exercise (CON)</th>
<th>Repeated-ischaemic exercise (ISC)</th>
<th>Repeated-hypoxic exercise (HYP)</th>
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<tr>
<td><strong>Muscle hypoxia</strong></td>
<td>~70 %</td>
<td>~90 %</td>
<td>~90 %</td>
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<tr>
<td><strong>Cuff pressure</strong></td>
<td>0 mmHg</td>
<td>~175 mmHg</td>
<td>0 mmHg</td>
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<tr>
<td><strong>Blinding</strong></td>
<td>Face mask/cuff</td>
<td>Face mask/cuff</td>
<td>Face mask/cuff</td>
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<tr>
<td><strong>Workload</strong></td>
<td>105 % LT</td>
<td>Matched</td>
<td>Matched</td>
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<tr>
<td><strong>Oxidative stress markers</strong></td>
<td>-</td>
<td>Catalase mRNA, HSP70 mRNA</td>
<td>-</td>
</tr>
<tr>
<td><strong>Metabolic by-products</strong></td>
<td>-</td>
<td>Muscle lactate</td>
<td>Muscle lactate</td>
</tr>
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<td><strong>AMPK signalling</strong></td>
<td>-</td>
<td>p-ACC/ACC, type I fibres</td>
<td>p-AMPK/AMPK, type I fibres</td>
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<tr>
<td><strong>Ca²⁺ signalling pathways</strong></td>
<td>p-CaMKII/CaMKII, type II fibres</td>
<td>p-CaMKII/CaMKII, type II fibres</td>
<td>-</td>
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<tr>
<td></td>
<td>p-PLB/PLB, type I fibres</td>
<td>p-PLB/PLB, type I + II fibres</td>
<td>p-PLB/PLB, type II fibres</td>
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<tr>
<td><strong>Mitochondrial genes</strong></td>
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<td>PGC-1α total, PGC-1α1, PGC-1α4</td>
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<tr>
<td><strong>Ion transport genes</strong></td>
<td>-</td>
<td>NKAα₂, FXYD1</td>
<td>NKAα₂, NKAβ₁</td>
</tr>
</tbody>
</table>
Running head: Ischaemic exercise augments gene adaptations and redox signalling

Tables

**Table 1.** Participant characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>177.3 ± 7.6</td>
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<tr>
<td>Body mass (kg)</td>
<td>74.3 ± 7.2</td>
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<tr>
<td>Body mass index (kg·m⁻²)</td>
<td>23.6 ± 1.3</td>
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<tr>
<td>Upper thigh circumference</td>
<td>57.5 ± 3.0 / 57.9 ± 2.8</td>
</tr>
<tr>
<td>(relaxed/contracted; cm)</td>
<td></td>
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<tr>
<td>Upper thigh skinfold (mm)</td>
<td>8.2 ± 2.7</td>
</tr>
<tr>
<td>VO₂max (mL O₂·min⁻¹)</td>
<td>4243 ± 408</td>
</tr>
<tr>
<td>VO₂max (mL O₂·kg⁻¹·min⁻¹)</td>
<td>57.4 ± 6.2</td>
</tr>
<tr>
<td>Peak treadmill speed during the GXT (km·h⁻¹)</td>
<td>14.9 ± 1.8</td>
</tr>
<tr>
<td>Lactate threshold (running speed in km·h⁻¹)</td>
<td>11.1 ± 1.6</td>
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</tbody>
</table>

Data are presented as mean ± SD. Lactate threshold was determined using the modified Dₘₐₓ method. Skinfold was measured over the vastus lateralis muscle belly and is the mean of three consecutive measurements. Peak treadmill speed was calculated as the sum of the last completed stage and the product of the fractional time at the last stage and the increment (1 km·h⁻¹). GXT, graded exercise test (2nd).
Table 2. Forward and reverse primer sequences used in real-time PCR, their amplification efficiency, and the coefficient of variation (CV) of duplicates.

<table>
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<tr>
<th>Gene</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
<th>Efficiency</th>
<th>CV (%)</th>
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<td>Na⁺,K⁺-ATPase</td>
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<tr>
<td>α₁</td>
<td>CGACAGAGAATCAGAGTGTTGT</td>
<td>GCCCTGTTACAAAGACCTGC</td>
<td>1.79</td>
<td>0.7 ± 0.6</td>
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<tr>
<td>α₂</td>
<td>ACATCTCCGTGTCTAAGCGG</td>
<td>AGCCACAGGAGGCTCAATG</td>
<td>2.25</td>
<td>0.7 ± 0.5</td>
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<td>α₃</td>
<td>ACTGAGGACCAGTCAGGGAC</td>
<td>CTTGAAGAGACGCGGATTG</td>
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<td>3.4 ± 2.4</td>
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<td>β₁</td>
<td>CTGACCCGGCCATCGCC</td>
<td>TAGAAGGATCTTTAAACCACTG</td>
<td>1.76</td>
<td>0.5 ± 0.4</td>
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<td>β₂</td>
<td>TTCGCCCAAGACTGAGAAC</td>
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<td>β₃</td>
<td>TCATCTACAACCCGACCACC</td>
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<td>0.8 ± 0.6</td>
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<td>FXYD1</td>
<td>AGCGAGCAGAATTTCCTCCAG</td>
<td>GCAGGGACTGGTAGTCTGTAAG</td>
<td>1.97</td>
<td>1.4 ± 1.6</td>
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<td>Total</td>
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<td>1α₁</td>
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<td>1α₄</td>
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<td>Catalase</td>
<td>CTCAGGTGCGGGGATTCTAT</td>
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<td>1.2 ± 1.0</td>
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<td>HSP70</td>
<td>GGGCTTTCCCAAGATTGCTG</td>
<td>TGCAACACAGGAATTTGAGAAT</td>
<td>1.92</td>
<td>0.9 ± 0.5</td>
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<td>GAPDH</td>
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<td>β2M</td>
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The efficiency of the primer set for the Na⁺,K⁺-ATPase α₅ could not be determined due to the low expression of this transcript in human skeletal muscle (Cₚ values ~34, i.e. upper end of detection range).
Table 3. Primary antibodies used for dot blotting and western blotting

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<thead>
<tr>
<th>Protein</th>
<th>Primary antibody and supplier</th>
<th>Host species and isotype (antibody type)</th>
<th>Concentration</th>
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<td>ACC</td>
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<td>p-AMPK-α Thr^{172}</td>
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<td>PLB</td>
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<td>MHC I</td>
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<td>1:200</td>
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<td>Mouse, IgM (monoclonal)</td>
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<td>~200</td>
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</tbody>
</table>

Antibodies were diluted in 1% bovine serum albumin in 1 x phosphate-buffered saline with 0.02% sodium azide and 0.025% Tween.
Running head: Ischaemic exercise augments gene adaptations and redox signalling

References


Running head: Ischaemic exercise augments gene adaptations and redox signalling


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