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Metabolic stress-dependent regulation of the mitochondrial biogenic molecular response to high-intensity exercise in human skeletal muscle

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Key points

- Low-volume high-intensity exercise training promotes muscle mitochondrial adaptations that resemble those associated with high-volume moderate-intensity exercise training. These training-induced mitochondrial adaptations stem from the cumulative effects of transient transcriptional responses to each acute exercise bout.
- However, whether metabolic stress is a key mediator of the acute molecular responses to high-intensity exercise is still incompletely understood.
- Here we show that, by comparing different work-matched low-volume high-intensity exercise protocols, more marked metabolic perturbations were associated with enhanced mitochondrial biogenesis-related muscle mRNA responses.
- Furthermore, when compared with high-volume moderate-intensity exercise, only the low-volume high-intensity exercise eliciting severe metabolic stress compensated for reduced exercise volume in the induction of mitochondrial biogenic mRNA responses.
- The present results, besides improving our understanding of the mechanisms mediating exercise-induced mitochondrial biogenesis, may have implications for applied and clinical research that adopts exercise as a means to increase muscle mitochondrial content and function in healthy or diseased individuals.

Abstract The aim of the present study was to examine the impact of exercise-induced metabolic stress on regulation of the molecular responses promoting skeletal muscle mitochondrial biogenesis. Twelve endurance-trained men performed three cycling exercise protocols characterized by different metabolic profiles in a randomized, counter-balanced

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order. Specifically, two work-matched low-volume supramaximal-intensity intermittent regimes, consisting of repeated-sprint (RS) and speed endurance (SE) exercise, were employed and compared with a high-volume continuous moderate-intensity exercise (CM) protocol. Vastus lateralis muscle samples were obtained before, immediately after, and 3 h after exercise. SE produced the most marked metabolic perturbations as evidenced by the greatest changes in muscle lactate and pH, concomitantly with higher post-exercise plasma adrenaline levels in comparison with RS and CM. Exercise-induced phosphorylation of CaMKII and p38 MAPK was greater in SE than in RS and CM. The exercise-induced *PGC-1α* mRNA response was higher in SE and CM than in RS, with no difference between SE and CM. Muscle *NRF-2*, *TFAM*, *MFN2*, *DRP1* and *SOD2* mRNA content was elevated to the same extent by SE and CM, while RS had no effect on these mRNAs. The exercise-induced *HSP72* mRNA response was larger in SE than in RS and CM. Thus, the present results suggest that, for a given exercise volume, the initial events associated with mitochondrial biogenesis are modulated by metabolic stress. In addition, high-intensity exercise seems to compensate for reduced exercise volume in the induction of mitochondrial biogenic molecular responses only when the intense exercise elicits marked metabolic perturbations.

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Introduction

Endurance exercise training is a powerful stimulus for the expansion of the skeletal muscle mitochondrial network. This adaptive mechanism includes mitochondrial biogenesis, the cellular process promoting increases in mitochondrial volume and potential changes in mitochondrial composition. Consistent with the metabolic adaptations originating from the enlargement of the muscle mitochondrial pool (Holloszy & Coyle, 1984; Dudley *et al.* 1987), exercise training-induced mitochondrial biogenesis is thought to be a crucial adaptive event for the prevention or treatment of a range of metabolic disorders (Hood *et al.* 2011; Russell *et al.* 2014; Hesselink *et al.* 2016) and for improving exercise capacity (Yan *et al.* 2012; Lundby & Jacobs, 2016).

High-intensity interval training, defined as repeated intense work bouts separated by recovery periods, is broadly recognized as a time-efficient alternative to traditional endurance exercise training for promoting skeletal muscle remodelling and enhancing exercise performance (Laursen & Jenkins, 2002; MacInnis & Gibala, 2017). In recent years, a growing body of research has focused on specific forms of intense interval training characterized by short-duration maximal/supramaximal efforts (Bishop *et al.* 2011; Hostrup & Bangsbo, 2017). In this context, speed endurance exercise training, depicted as multiple prolonged "all-out" bouts (<40 s) separated by comparatively long resting periods (Iaia & Bangsbo, 2010), has been shown to promote increments in the activity and/or content of muscle mitochondrial enzymes together with improvements in endurance exercise performance (Burgomaster *et al.* 2005; Little *et al.* 2010; Hostrup *et al.* 2018). Likewise, repeated-sprint exercise training, characterized by shorter supramaximal efforts (<10 s) interspersed with relatively short (<60 s) recovery periods (Spencer *et al.* 2005), has recently been reported to upregulate the content of a series of mitochondrial proteins in human skeletal muscle (Serpiello *et al.* 2012). Therefore, it appears that both speed endurance and repeated-sprint exercise training promote muscle mitochondrial adaptations that resemble those induced by high-volume aerobic exercise training, implying that the high-metabolic demand elicited by intense intermittent exercise plays a putative role in the mitochondrial adaptive processes that occur within the exercising skeletal muscle.

The long-term mitochondrial adaptations to exercise training have been suggested to stem from the cumulative effects of the transient transcriptional responses to each acute exercise bout (Williams & Neufer, 1996). The peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α) is one factor proposed to contribute to initiating these early adaptive molecular responses by coactivating a broad range of transcription factors (Kelly & Scarpulla, 2004). Muscle-specific overexpression of PGC-1α in transgenic rodent models increased the mRNA and protein content of multiple mitochondrial proteins as well as elevated muscle mitochondrial enzyme activity and mitochondrial DNA (mtDNA) content (Lin *et al.* 2002; Calvo *et al.* 2008), supporting the purported role of *PGC-1α* as a key regulator of mitochondrial biogenesis. The observation that a single bout of exercise upregulates PGC-1α transcription and mRNA content in human skeletal muscle (Pilegaard *et al.* 2003), and that these

transcriptional responses precede increases in muscle mitochondrial proteins (Perry *et al.* 2010), substantiates the possibility that PGC-1α also mediates mitochondrial biogenesis in humans.

The molecular mechanisms through which *PGC-1α* is thought to coordinate exercise-induced mitochondrial biogenesis include regulation of mtDNA transcription and mitochondrial remodelling dynamics (Hood *et al.* 2016). Specifically, mtDNA transcription and replication are driven by the nuclear-encoded mitochondrial transcription factor A (TFAM), the expression of which is controlled by nuclear transcription factors, counting nuclear respiratory factor 1 and 2 (NRF-1 and -2) (Virbasius & Scarpulla, 1994). Consistent with a PGC-1α-mediated coactivation of these transcription factors, increased gene expression of muscle *NRF-1/2* and *TFAM* has been reported in concert with a higher *PGC-1α* mRNA content following acute exercise (Baar *et al.* 2002; Pilegaard *et al.* 2003; Perry *et al.* 2010; Saleem & Hood, 2013). However, other human studies failed to observe exercise-induced changes in the mRNA of these transcription factors despite an increase in *PGC-1α* mRNA (Stepto *et al.* 2012; Scribbans *et al.* 2017), suggesting that more research is needed to clarify the exercise-induced regulation of these mRNAs in human skeletal muscle. In addition, mtDNA integrity is preserved by the ability of mitochondria to constantly undergo structural changes via fusion and fission events, which alter mitochondrial morphology and contribute to mitochondrial quality control (Youle & van der Bliek, 2012; Hood *et al.* 2015). Despite the emerging importance of mitochondrial remodelling for the maintenance of a functional mitochondrial network, only a few human studies have investigated the impact of acute exercise on the mRNA response of proteins involved in mitochondrial fusion and fission dynamics (Cartoni *et al.* 2005; Ding *et al.* 2010; Granata *et al.* 2017).

PGC-1α gene expression has been reported to be regulated by multiple intracellular signalling kinases, including AMP-activated protein kinase (AMPK), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and p38 mitogen-activated protein kinase (p38 MAPK) (Puigserver *et al.* 2001; Jager *et al.* 2007; Zhang *et al.* 2014). Given that the contractile activity-induced activation of these signalling transduction pathways is governed by alterations in the AMP:ATP ratio, cytosolic Ca^{2+} and reactive oxygen species (ROS), metabolic stress is expected to be a key modulator of the signalling cascades converging on PGC-1α. In this direction, more marked induction of AMPK and CaMKII phosphorylation with corresponding greater *PGC-1α* mRNA abundance has been reported following high-intensity compared with isocaloric low-intensity continuous exercise (Egan *et al.* 2010). These differences were associated with greater plasma lactate accumulation and muscle glycogen depletion in the high-intensity compared with the low-intensity exercise protocol. In accordance, the exercise intensity-dependent increase in *PGC-1α* mRNA observed by Nordsborg *et al.* (2010) may be related to various degrees of metabolic challenges. Further evidence supporting the sensitivity of muscle PGC-1α transcription to the metabolic environment has been provided by studies using either blood flow-restricted exercise (Norrbom *et al.* 2004; Christiansen *et al.* 2018), nutritional means (Edge *et al.* 2015) or dietary interventions (Pilegaard *et al.* 2005) to manipulate the muscle metabolic state during or after exercise.

The intermittent pattern of interval exercise *per se* may also play a key role in evoking the signalling cascade towards mitochondrial biogenesis (Combes *et al.* 2015). On the other hand, the metabolic fluctuations characterizing interval exercise appeared not to have a putative role on the *PGC-1α* mRNA response following "all-out" exercise performed in either an intermittent or a continuous manner (Cochran *et al.* 2014; Taylor *et al.* 2016), implying that the pulsatile nature of exercise is not of importance when the exercise intensity is supramaximal. Consistent with these results, multiple studies highlighted the potency of supramaximal-intensity intermittent exercise to promote mitochondrial biogenesis (Gibala *et al.* 2009; Little *et al.* 2011; Serpiello *et al.* 2012) and generate similar (Psilander *et al.* 2010) or greater (Skovgaard *et al.* 2016) muscle *PGC-1α* mRNA responses compared with high-volume submaximal exercise, likely due to the effectiveness of intense exercise in exacerbating metabolic stress and thereby inducing enhanced molecular responses. Nevertheless, the exercise-induced elevation in *PGC-1α* mRNA has been observed to be similar after work-matched high-intensity interval and continuous exercise characterized by different post-exercise blood lactate levels (Bartlett *et al.* 2012), as well as following four diverse high-volume exercise protocols associated with distinct alterations in muscle metabolism and plasma adrenaline (Brandt *et al.* 2016). This suggests that the role of metabolic stress in the initial mitochondrial biogenic responses to acute exercise in human skeletal muscle needs to be further elucidated. Furthermore, there is limited information regarding the impact of intense exercise-associated metabolic stress on the regulation of genes modulating mtDNA transcription and mitochondrial remodelling dynamics.

Therefore, the main aim of the present study was to assess whether different exercise-induced metabolic perturbations were associated with distinct intracellular signalling and mRNA responses of genes implicated in mitochondrial biogenesis in human skeletal muscle. This was investigated by applying low-volume repeated-sprint and speed endurance exercise, as two work-matched supramaximal-intensity intermittent regimes inducing

pronounced but different metabolic stress levels, and high-volume submaximal-intensity continuous exercise generating a mild but constant bioenergetic stress. It was hypothesized that speed endurance exercise promotes more marked mRNA responses than repeated-sprint exercise due to more profound muscle metabolic perturbations, while continuous moderate-intensity exercise, by virtue of a prolonged metabolic flux, evokes acute molecular responses analogous to those elicited by low-volume intense exercise.

Methods

Human subjects and ethics

Thirteen healthy trained men were initially included of which 12 completed the study. Prior to inclusion, subjects were informed of risks and discomforts associated with the experimental procedures. Each subject gave his oral and written informed consent. Inclusion criteria were males, aged 18–40 years, a weekly training volume above 3 h, a maximal oxygen consumption ($\dot{V}_{O_2max}$) above 50 mL min^{-1} kg^{-1}, and a body mass index below 30 kg m^{-2}. In addition, only subjects who regularly performed cycling-based training for the past 6 years were included to limit the possibility that the cycling exercise-induced early adaptive responses occurred solely as a consequence of a new mechanical stimulus within the exercising muscle. Exclusion criteria were smoking and chronic disease. Subjects were amateur cyclists or triathletes, and their characteristics are presented in Table 1. The study was approved by the regional research ethics committee of Copenhagen, Denmark (H-16000378) and adheres to the principles of the *Declaration of Helsinki*, except for registration in a database.

Study design

A randomized, counter-balanced crossover design was used to compare the acute effects of three different exercise protocols conducted on experimental days separated by at least 1 week.

Prior to the commencement of the experimental period, an incremental test to exhaustion was performed on a mechanically braked cycle ergometer (LC6; Monark Exercise AB, Vansbro, Sweden) for determination of $\dot{V}_{O_2max}$. The test protocol consisted of two submaximal 4 min bouts at 150 and 225 W followed by an incremental ramp test with increments of 25 W min^{-1} until volitional exhaustion. Pulmonary oxygen consumption ($\dot{V}_{O_2}$) was measured breath-by-breath using an on-line gas analysis system (Oxycon Pro, Viasys Healthcare, Hoechberg, Germany). $\dot{V}_{O_2max}$ was determined as the highest value achieved during a 30 s period. Criteria used for achievement of $\dot{V}_{O_2max}$ were a plateau in $\dot{V}_{O_2}$ despite an increase in workload and a respiratory exchange ratio above 1.10. Heart rate was monitored throughout the test (Polar Team^2, Polar Electro Oy, Kempele, Finland) and maximal heart rate established as the highest value achieved during the test checked for spikes. A regression equation of $\dot{V}_{O_2}$ *vs.* power output was derived from the two submaximal workloads and was used to determine the individual workloads to be sustained during the exercise protocols.

Table 1. Subject characteristics

Age (years)	31.9 ± 2.5
Height (cm)	177 ± 2
Weight (kg)	74.8 ± 2.7
$\dot{V}_{O_2max}$ (mL min^{-1})	4541 ± 130
$\dot{V}_{O_2max}$ (mL kg^{-1} min^{-1})	61.1 ± 1.9

$\dot{V}_{O_2max}$, maximal oxygen consumption. Values are means ± SEM (n = 12).

Experiments

Exercise protocols. The exercise protocols were performed on a mechanically braked cycle ergometer (894E; Monark Exercise AB, Vansbro, Sweden). Saddle and handlebar height were recorded during the first trial and reused in subsequent tests. The exercise protocols consisted of either repeated-sprint (RS) or speed endurance (SE) exercise at supramaximal intensity or continuous exercise at moderate intensity (CM) (Fig. 1). In RS the subjects performed 18 × 5 s "all-out" efforts interspersed with 30 s of passive recovery, while SE comprised 6 × 20 s "all-out" efforts interspersed with 120 s of passive recovery, with an average mean power output of 902 ± 33 and 669 ± 26 W (mean ± SEM) during RS and SE, respectively. CM consisted of 50 min of continuous exercise at a relative intensity corresponding to 70% $\dot{V}_{O_2max}$ (218 ± 7 W).

The rationale behind the choice of these protocols was to induce different metabolic perturbations in the myocellular milieu by using three common training routines. CM was employed as traditional high-volume (640 ± 21 kJ) endurance exercise leading to mild, but prolonged metabolic disturbances, whereas RS and SE were selected as low-volume intense intermittent exercise regimes matched for work volume (RS: 79 ± 3 kJ; SE: 78 ± 3 kJ) and work-to-rest ratio (1:6), but provoking distinct degrees of metabolic stress. Specifically, muscle lactate and hydrogen ion (H^+) accumulation are expected to occur with different patterns and magnitudes during RS and SE. Indeed, while multiple short sprints (≤6 s) result in a gradual increase in muscle and blood lactate and a progressive drop in blood pH (Balsom *et al.* 1992; Gaitanos *et al.* 1993), a single long sprint (≥20 s) markedly

increases muscle lactate levels, which remain elevated after 2–4 min of recovery (Bogdanis *et al.* 1996, 1998). Thus, for the same work volume, a higher degree of metabolic acidosis is more likely to be induced by SE than by RS.

All protocols were preceded by a standardized warm-up consisting of 7 min of continuous cycling at a workload corresponding to 65% $\dot{V}_{O_2max}$ (199 ± 7 W) followed by 5 min at rest.

Prior to sprinting in RS and SE, subjects were instructed to begin pedalling as fast as possible and against no resistance for ∼2 s. The cycle ergometer was interfaced with a dedicated software (Monark Anaerobic Test Software 3.3, Monark Exercise AB, Vansbro, Sweden) set to apply the workload and start the timer once a cadence of 100 r.p.m. was reached. Braking forces corresponding to 0.90 and 0.75 N kg^{-1} body mass were used for RS and SE, respectively. Subjects were encouraged to maintain maximum pedalling speed throughout each sprint. During CM, the braking force was 0.33 ± 0.01 N kg^{-1} body mass and the subjects kept a cadence of 85 r.p.m. throughout the exercise.

Control procedures

To minimize potential variation in the exercise-induced response due to external factors, subjects were instructed to refrain from caffeine, alcohol and exercise for 24 h before each trial. In addition, before commencing the experimental period, subjects reported their food habits in a questionnaire. Then, an individual diet plan was developed to standardize food intake during the 48 h preceding each experimental trial. Daily carbohydrate, protein and fat intake in the 48 h prior to the experimental trials were 4.7 ± 0.2, 1.7 ± 0.1 and 0.8 ± 0.0 g kg^{-1} body mass, respectively.

During the experimental period, subjects continued their usual training routine, and their weekly training load was kept constant. Time commitment and distance covered during endurance-based training in the 6 days prior to each experimental trial were 455 ± 170 min and 222 ± 64 km (RS), 415 ± 173 min and 204 ± 76 km (SE), and 409 ± 182 min and 204 ± 79 km (CM), respectively.

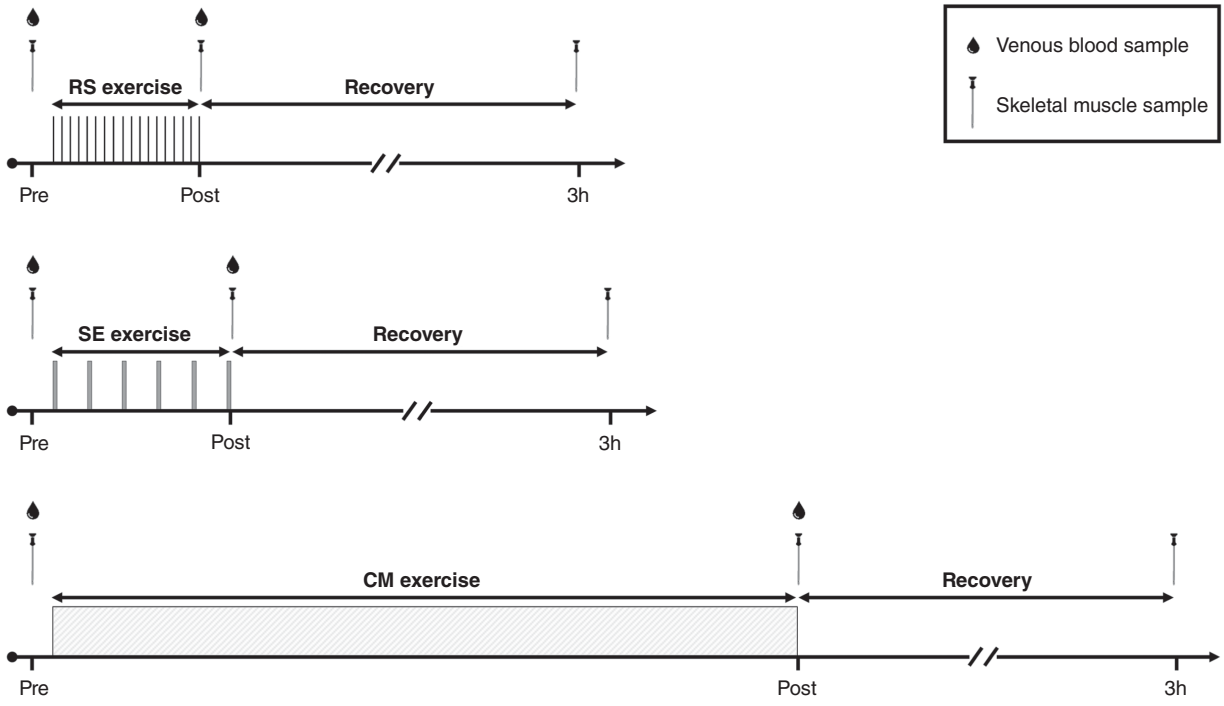

Figure 1. Schematic presentation of the experimental trials
Schematic presentation of the three experimental trials consisting of cycling-based repeated-sprint exercise (RS, 18 × 5 s "all-out" sprints against a breaking force of 0.90 N kg^{-1} interspersed with 30 s of recovery), speed endurance exercise (SE, 6 × 20 s "all-out" sprints against a breaking force of 0.75 N kg^{-1} interspersed with 120 s of recovery) and continuous moderate-intensity exercise (CM, 50 min at 70% $\dot{V}_{O_2max}$). Muscle metabolites, pH and protein content were determined in the biopsies obtained at rest (Pre) and immediately after exercise (Post). Muscle mRNA content was determined in the biopsies obtained at Pre and 3 h after exercise (3h). Plasma catecholamines were determined in blood samples taken at Pre and Post.

Experimental procedures

On the experimental days, subjects reported to the laboratory 120 min after ingesting their last meal. After 15 min of rest in the supine position, two 3 mm incisions were made over the lateral portion of the left thigh under local anaesthesia (1 mL lidocaine without epinephrine, 20 mg mL^{-1} Xylocain, AstraZeneca). A biopsy was obtained from the vastus lateralis muscle at rest before warm-up (Pre) and immediately after exercise (Post) using a percutaneous Bergstrom needle with suction through separate incisions. An additional muscle biopsy was obtained from the right thigh 3 h into recovery (3h), during which subjects remained in the laboratory and were allowed to consume only water *ad libitum*. The muscle biopsy samples were immediately frozen in liquid nitrogen and stored at −80°C until further analyses. A blood sample was taken from a catheter inserted in the right antecubital vein prior to and immediately after exercise.

Blood analyses. Blood samples were collected in 2 mL syringes and transferred to an Eppendorf tube containing 30 μL EDTA (0.2 M), after which they were spun at 20,000 *g* for 3 min to collect plasma, which was stored at −20°C until analysis. Plasma catecholamines were determined by using an enzyme immunoassay kit (2-CAT Plasma ELISA High Sensitive BA E-4500; LDN, Nordhorn, Germany).

Muscle analyses. Approximately 20 mg of the muscle samples were frozen separately for mRNA analyses. The remaining part of the muscle sample was freeze-dried for 48 h and dissected free of blood, fat and connective tissue. Dissection was performed under a stereo microscope with an ambient temperature of ∼18°C and a relative humidity <30%. After dissection, muscle tissue was weighed and separated into different tubes for analysis. Muscle metabolites, pH, protein content and phosphorylation were determined in the biopsies sampled before (Pre) and immediately after (Post) exercise, while mRNA content was determined in the muscle samples obtained before (Pre) and 3 h after (3h) exercise.

Muscle metabolites and pH. Muscle ATP, phosphocreatine (PCr) and lactate were determined on freeze-dried muscle tissue (∼2.0 mg dry weight (DW)). Determination was made by extraction in 3 M perchloric acid, neutralization to pH 7.0 with 2.2 M $KHCO_3$, followed by fluorometric analyses as previously described (Lowry & Passonneau, 1972). Muscle glycogen was measured in DW muscle tissue (∼1.5 mg) by acid hydrolysis at 100°C for 3 h followed by determination of glycosyl units as previously described (Lowry & Passonneau, 1972). Muscle pH was measured by a small glass electrode (Radiometer GK2801, Copenhagen, Denmark) after homogenizing ∼2 mg DW in a non-buffering solution containing 145 mM KCl, 10 mM NaCl and 5 mM iodoacetic acid.

Protein content in muscle lysate. Protein content was determined by SDS-PAGE and western blotting analyses. Briefly, freeze-dried muscle samples (∼3 mg DW) were homogenized for 1 min at 29 Hz (TissueLyser II, Qiagen, Valencia, CA, USA) in a fresh batch of ice-cold buffer (10% glycerol, 20 mM sodium pyrophosphate, 150 mM NaCl, 50 mM HEPES (pH 7.5), 1% NP-40, 20 mM β-glycerophosphate, 2 mM Na_3VO_4, 10 mM NaF, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA (pH 8), 1 mM EGTA (pH 8), 10 μg mL^{-1} aprotinin, 10 μg mL^{-1} leupeptin and 3 mM benzamidine). Afterwards, samples were rotated end over end for 1 h at 4°C followed by centrifugation at 17,500 *g* for 20 min at 4°C. The supernatant (lysate) was collected and total protein concentration in each sample was determined by a bovine serum albumin (BSA) standard kit (Thermo Scientific, Waltham, MA, USA), assayed in triplicate. Then, each lysate sample was mixed with 6 × Laemmli buffer (7 mL 0.5 M Tris-base, 3 mL glycerol, 0.93 g DTT, 1 g SDS and 1.2 mg bromophenol blue) and ddH_2O to reach equal protein concentration. Equal amounts of total protein (range: 12–24 μg) were loaded for each sample in precast gels (Bio-Rad Laboratories, Hercules, CA, USA). All samples from each subject were loaded on the same gel, with the sample from before exercise (Pre) being placed adjacent to the sample after exercise (Post) for each exercise protocol. Proteins were separated by SDS-PAGE and semi-dry transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories). The membranes were blocked in either 2% skimmed milk or 3% BSA in Tris-buffered saline with Tween 20 buffer (TBST) before being incubated overnight at 4°C in primary antibody diluted either in 2% skimmed milk: AMP-activated protein kinase α (AMPKα) (no. 2532, Cell Signaling Technology, Leiden, The Netherlands), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (611293, BD Transduction, San Jose, CA, USA), p38 mitogen-activated protein kinase (p38 MAPK) (no. 9212, Cell Signaling Technology), acetyl-CoA carboxylase $(ACC)^{Ser79}$ phosphorylation (07-303, Merck Millipore, Darmstadt, Germany), $CaMKII^{Thr286}$ phosphorylation (no. 3361, Cell Signaling Technology); or in 3% BSA: ACC (p0397, Agilent Dako, Santa Clara, CA, USA), activating transcription factor-2 (ATF-2) (no. 9226, Cell Signaling Technology), class II histone deacetylase (HDAC) 4 (no. 2072, Cell Signaling Technology), HDAC5 (no. 20458, Cell Signaling Technology), $AMPK\alpha^{Thr172}$ phosphorylation (no. 2531, Cell Signaling Technology), $ATF\text{-}2^{Thr71}$ phosphorylation (no. 9221, Cell Signaling Technology), $HDAC4/5/7^{Ser632/Ser661/Ser486}$ phosphorylation (no. 3424,

Cell Signaling Technology), p38 MAPK$^{Thr180/Tyr182}$ phosphorylation (no. 9211, Cell Signaling Technology).

After washing in TBST, membranes were incubated with a secondary horseradish peroxidase-conjugated antibody for ∼1 h at room temperature. The membrane staining was visualized by incubation with a chemiluminescent horseradish peroxidase substrate (Millipore, Denmark) before image digitalization on a Chemi Doc MP (Bio-Rad Laboratories). Western blot band intensity was determined by densitometry quantification (total band intensity adjusted for background intensity) using Image Lab v.4.0 (Bio-Rad Laboratories). Phosphorylation levels and protein content were determined on separate membranes in separate analyses and none of the membrane was stripped before protein quantification.

Phosphorylated and total protein content were determined from duplicates, i.e. the biopsies were divided and kept in two parts after dissection, giving two results for the same muscle biopsy sample. The two samples were run on the same gel, and the mean signal intensity of the two samples was used as the result. Exercise-induced changes in phosphorylated protein content are presented in graphical form as the geometric mean of the fold changes at Post normalized to their respective Pre values.

RNA isolation, reverse transcription and real-time PCR. Total RNA was isolated from 15–20 mg of muscle tissue (wet weight) by a modified guanidinium thiocyanate–phenol–chloroform extraction method as described previously (Pilegaard *et al.* 2000) except for the use of a TissueLyser (TissueLyser II, Qiagen) for homogenization.

Superscript II RNase H$^-$system and Oligo dT (Invitrogen, Carlsbad, CA, USA) were used to reverse transcribe the mRNA to cDNA. Quantification of cDNA as a measure of mRNA content of a given gene was performed by real-time PCR using an ABI 7900 sequence-detection system (Applied Biosystems, Foster City, CA, USA). Primers and TaqMan probes were designed from human specific databases from ensemble (www.ensembl.org/Homo_sapiens/Info/Index) and Primer Express (Applied Biosystems) and are presented in Table 2. Self-designed TaqMan probes were labelled with 5′-6-carboxyfluorescein (FAM) and 3′-6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine (TAMRA) and obtained from TAG Copenhagen (Copenhagen, Denmark). *Cyclophilin A*, *β-actin* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA were amplified using a 5′-VIC- and 3′-TAMRA-labelled predeveloped assay reagent (Applied Biosystems). Real-time PCR was performed in triplicates in a total reaction volume of 10 μL using Universal Mastermix with uracil-*N* glycosylase (UNG;

Table 2. Primers and TaqMan probe sequences used for real-time PCR

Gene	Forward primer	Reverse primer	TaqMan probe
PGC-1α	5′ CAAGCCAAACCAACAACTTTATCTCT 3′	5′ CACACTTAAGGTGCGTTCAATAGTC 3′	5′ AGTCACCAAATGACCCCAAGGGTTCC 3′
NRF-2	5′ GAAATTGAGATTGATGGAACAGAGAA 3′	5′ TATGGCCTGGCTTACACATTCA 3′	5′ AAGCATTGTAGAACAAACCTACGCGCCAG 3′
TFAM	5′ AGATTCCAAGAAGCTAAGGGTGATT 3′	5′ TTTCAGAGTCAGACAGATTTTTCCA 3′	5′ CCGCAGGAAAAGCTGAAGACTGTAAAGGA 3′
MFN2	5′ CCTCTCTGTACTGGTGGACGATT 3′	5′ TGGCGGTGCAGCTCATT 3′	5′ CCAGATGGACTTCCACCCTTCTCCAGTAG 3′
DRP1	5′ CGGCAAATCAAACGTCTAGAAGA 3′	5′ AAGTTTAGGAAATCGTAACAATTCCT 3′	5′ CCCAGCCTCCGCTGTGTGGAAC 3′
SOD2	5′ CGCGGCCTACGTGAACAA 3′	5′ CTATCTGGGCTGTAACATCTCCCTT 3′	5′ CACCGAGGAGAAGTACCAGGAGGCGTT 3′
HO-1	5′ GCCAGCAACAAAGTGCAAGAT 3′	5′ AGTGTAAGGACCCATCGGAGAA 3′	5′ AGAGGGAAGCCCCCACTCAACACC 3′
HSP72	5′ GCGTGATGACTGCCCTGAT 3′	5′ CGCCCTCGTACACCTGGAT 3′	5′ TCCCCACCAAGCAGACGCAGATCT 3′
Cyc A	5′ GGTCTCTTTGGGCGGAAGAC 3′	5′ CTCTCCCCAGATGATGCCTTT 3′	5′ CCCTGGATACTCTTACACAGCCGCCAA 3′

PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; *NRF-2*, nuclear respiratory factor 2; *TFAM*, mitochondrial transcription factor A; *MFN2*, mitofusin-2; *DRP1*, dynamin-related protein 1; *SOD2*, superoxide dismutase 2; *HO-1*, heme oxygenase-1; *HSP72*, heat shock protein 72; *Cyc A*, cyclophilin A.

Table 3. mRNA content of reference genes before (Pre) and 3 h after (3h) repeated-sprint (RS), speed endurance (SE) and continuous moderate-intensity (CM) exercise

	Pre			3h		
	RS	SE	CM	RS	SE	CM
Cyc A	0.47 ± 0.03	0.49 ± 0.04	0.44 ± 0.04	0.54 ± 0.06	0.53 ± 0.03	0.51 ± 0.05
β-Actin	0.34 ± 0.05	0.31 ± 0.04	0.23 ± 0.04	0.48 ± 0.05†	0.62 ± 0.07*	0.65 ± 0.12*
GAPDH	0.53 ± 0.08†	0.41 ± 0.07	0.29 ± 0.07	0.53 ± 0.09	0.60 ± 0.09*	0.56 ± 0.11*

Values are means ± SEM ($n = 12$), *Significantly different from Pre ($P < 0.05$), †Significantly different from CM ($P < 0.05$).

Applied Biosystems). The obtained cycle threshold values reflecting the initial content of the specific transcript in the samples were converted to a relative amount by using standard curves constructed from serial dilution of a pooled sample made from all samples. *Cyclophilin A*, *β-actin* and *GAPDH* were analysed as reference genes. *β-Actin* and *GAPDH* changed with exercise, whereas *cyclophilin A* was unaffected by exercise and did not change between trials (Table 3). Thus, *cyclophilin A* was used as an endogenous control, i.e. for each cDNA sample, the mRNA content of the given target mRNA was normalized to *cyclophilin A* mRNA. Exercise-induced changes in mRNA content are presented in graphical form as the geometric mean of the fold changes at 3 h of recovery normalized to their respective Pre values.

Statistics

A priori sample size determination was performed for the primary outcome measure "*PGC-1α* mRNA" based on data from a previous study with a similar experimental design (Brandt *et al.* 2016). Power was set to 0.8 and significance level was set to 0.05.

To estimate between-trial differences in the exercise-induced changes across sampling time, as well as within- and between-trial differences, a linear mixed model was used with trial and sampling time included as fixed factors and subjects as random factor for a full factorial design. Baseline (i.e. Pre) values were included as covariates in the mixed model. Model checking was based on residual and Q–Q plots. In the case of heteroscedasticity (i.e. unequal variance), log-transformation was applied prior to analysis. Model-based *t* tests were used in pairwise comparisons to identify between-trial differences in the exercise-induced changes across sampling time. In addition, within- and between-trial differences were compared pairwise using model-based *t* tests. Multiple linear regression analysis was used to determine the individual relationships between change of metabolic and molecular variables with low-volume supramaximal-intensity intermittent exercise. Fold changes in muscle ATP, PCr, lactate, $[H^+]$ and glycogen, and plasma adrenaline, were initially included in the model as predictors of the fold change in mRNA and phosphorylated protein content. Then, for each mRNA and protein, the stepwise backward method was used to determine the metabolic variables that best predicted the exercise-induced response (i.e. best-fitting model). Multicollinearity was assessed using the variance inflation factor (VIF), which indicates whether a predictor has a strong linear relationship with another predictor. The coefficient of determination of the multiple linear regression is presented as the R^2 and adjusted R^2. The level of significance for all analyses was defined as $P < 0.05$. The statistical analysis was carried out with R version 3.4.1 and the extension packages *lme4* and *multcomp*. Exercise-induced changes in protein and mRNA content are presented as geometric means ± 95% confidence interval (CI) of the geometric mean, while all other results are reported as means ± SEM.

Results

Muscle metabolites and pH

Muscle metabolite concentrations and pH levels before and immediately after exercise are reported in Table 4. Exercise reduced muscle ATP levels in RS and SE ($P < 0.001$). Muscle PCr decreased with exercise in all trials ($P < 0.001$), with greater PCr depletion in RS and SE than in CM ($P = 0.003$ and $P = 0.004$).

Muscle lactate increased with exercise in all trials ($P < 0.001$), with lactate accumulation being larger in SE than in RS and CM ($P < 0.001$), and larger in RS than in CM ($P < 0.001$). Muscle pH decreased in RS and SE ($P < 0.001$), and was unchanged in CM ($P = 0.478$). Moreover, muscle pH decreased more in SE than in RS and CM ($P < 0.001$), and more in RS than in CM ($P < 0.001$).

Muscle glycogen was lowered in all trials ($P < 0.001$), with post-exercise glycogen levels being lower in CM than in RS ($P = 0.003$) and SE ($P = 0.040$).

Table 4. Muscle metabolites (mmol kg^{-1} DW) and pH before (Pre) and immediately after (Post) repeated-sprint (RS), speed endurance (SE) and continuous moderate-intensity (CM) exercise

	Pre			Post			Δ		
	RS	SE	CM	RS	SE	CM	RS	SE	CM
ATP	25.8 ± 1.8	25.1 ± 1.5	24.4 ± 2.3	19.2 ± 1.8*†	16.7 ± 1.4*†	21.9 ± 2.0	−6.6 ± 1.9†	−8.4 ± 1.5†	−2.6 ± 0.5
PCr	79.8 ± 5.8	79.8 ± 3.0	79.0 ± 4.7	38.4 ± 5.3*†	39.3 ± 6.9*†	62.4 ± 4.5*	−41.4 ± 7.7†	−40.5 ± 6.9†	−16.7 ± 3.5
Lactate	5.8 ± 1.1	5.5 ± 0.7	5.0 ± 0.5	41.4 ± 7.2*†	79.9 ± 8.4*§†	11.4 ± 1.9*	35.6 ± 6.4†	74.4 ± 8.5§†	6.4 ± 1.7
Glycogen	783 ± 39	722 ± 45	708 ± 48	652 ± 35*†	576 ± 38*†	513 ± 49*	−130 ± 29	−146 ± 19	−194 ± 26
pH	7.26 ± 0.02	7.25 ± 0.02	7.27 ± 0.02	7.02 ± 0.05*†	6.74 ± 0.03*§†	7.25 ± 0.04	(47 ± 10) × 10^{-7}†	(132 ± 16) × 10^{-7}§†	(5 ± 5) × 10^{-7}

Values are means ± SEM (n = 12). Δ, difference between Post and Pre. Differences in pH are presented as changes in [H$^+$], *Significantly different from Pre ($P < 0.05$, †Significantly different from CM ($P < 0.05$), §Significantly different from RS ($P < 0.05$).

Plasma catecholamines

Plasma adrenaline was increased by exercise in RS and SE ($P < 0.001$) (Fig. 2*A*). Post-exercise adrenaline was ∼2.5- and 3.5-fold higher in RS and SE, respectively, than in CM ($P = 0.005$ and $P < 0.001$), and was higher in SE than in RS ($P = 0.011$). Furthermore, the exercise-induced change in plasma adrenaline was greater in RS and SE than in CM ($P = 0.042$ and $P = 0.001$).

Plasma noradrenaline increased with exercise in all trials ($P < 0.001$) (Fig. 2*B*). In RS and SE, post-exercise noradrenaline was higher than in CM ($P = 0.001$ and $P < 0.001$), with concomitant differences in the magnitude of change between RS and CM ($P = 0.027$), and between SE and CM ($P = 0.001$).

Muscle protein phosphorylation

Representative blots for each protein analysed are presented in Fig. 3.

Phosphorylated AMPKα increased immediately after exercise in all trials (RS, $P = 0.006$; SE, $P = 0.001$; CM, $P < 0.001$), with no difference between trials (Fig. 4*A*). Similarly, phosphorylated ACC increased to a similar extent in all trials ($P < 0.001$) (Fig. 4*B*).

Phosphorylated CaMKII content was elevated by SE ($P < 0.001$), but remained unchanged in RS ($P = 0.310$) and CM ($P = 0.373$), resulting in a higher level of phosphorylation in SE than in RS ($P < 0.036$) and CM ($P = 0.006$) (Fig. 4*C*).

Phosphorylated p38 MAPK increased in all trials (RS, $P < 0.001$; SE, $P < 0.001$; CM, $P = 0.004$), but the change within SE was larger than observed within RS ($P = 0.002$) and CM ($P < 0.001$) (Fig. 4*D*).

Phosphorylated HDAC4/5/7 increased in RS ($P = 0.005$) and SE ($P = 0.002$), but was unaltered in CM ($P = 0.676$), with no difference in the change between SE and CM ($P = 0.059$) (Fig. 4*E*).

Phosphorylated ATF-2 increased with exercise in all trials (RS, $P = 0.001$; SE, $P < 0.001$; CM, $P = 0.018$), with no difference in the change between SE and CM ($P = 0.066$) (Fig. 4*F*).

Muscle mRNA

Muscle *PGC-1α* mRNA content was elevated 3 h after exercise relative to Pre in all trials ($P < 0.001$), with SE and CM inducing a greater response than RS ($P = 0.006$ and $P < 0.001$) (Fig. 5*A*). Muscle *NRF-2* mRNA levels were elevated in SE ($P = 0.004$) and CM ($P < 0.001$), but were unchanged in RS ($P = 0.640$), with the response being larger in CM than in RS ($P < 0.001$) (Fig. 5*B*). *TFAM* mRNA content was upregulated by SE ($P = 0.043$) and CM ($P = 0.010$), while it did not change in RS ($P = 0.667$), and

CM induced a more marked response than RS ($P = 0.033$) (Fig. 5*C*).

Mitofusin-2 (*MFN2*) mRNA abundance increased in SE ($P = 0.041$) and CM ($P < 0.001$), with no changes observed in RS ($P = 0.905$), resulting in a greater mRNA response in CM than in RS ($P = 0.012$) (Fig. 5*D*). Dynamin related protein 1 (*DRP1*) mRNA content increased with exercise in SE ($P = 0.002$) and CM ($P < 0.001$), while it was unaltered in RS ($P = 0.935$). Moreover, the *DRP1* mRNA response was more marked in SE and CM than in RS ($P = 0.037$ and $P = 0.005$, respectively) (Fig. 5*E*).

Muscle mRNA content of superoxide dismutase 2 (*SOD2*), the gene encoding the mitochondrial anti-oxidant enzyme MnSOD, increased 3 h into recovery in SE ($P = 0.017$) and CM ($P = 0.031$), with no change observed in RS ($P = 0.856$) (Fig. 5*F*). Muscle mRNA abundance of the ROS-sensitive gene heme oxygenase-1 (*HO-1*) was not altered by exercise in either of the trials (Fig. 5*G*). Muscle mRNA content of heat shock protein 72 (*HSP72*), a protein exerting a cyto-protective function against oxidative stress, was upregulated by exercise in all trials ($P < 0.001$), and the change within SE was larger than the change within RS ($P = 0.001$) and CM ($P < 0.001$) (Fig. 5*H*).

Relationships between change in metabolic and molecular variables

Multiple linear regression analysis showed that change in muscle PCr, $[H^+]$ and glycogen, and plasma adrenaline, predicted the *PGC-1α* mRNA response to RS and SE (Table 5), with change in muscle glycogen ($P = 0.032$) and plasma adrenaline ($P = 0.014$) adding significantly to the model, whereas change in muscle PCr ($P = 0.103$) and $[H^+]$ ($P = 0.055$) did not contribute significantly to the model.

Moreover, change in metabolic variables predicted change in *NRF-2*, *TFAM*, *MFN2* and *DRP1* mRNA, as well as in phosphorylated CaMKII and phosphorylated p38 MAPK in response to RS and SE (Table 5).

Discussion

The major finding in the present study was that the *PGC-1α* mRNA response to low-volume intense intermittent exercise was greater when exercise induced the highest muscle lactate accumulation, the greatest drop in muscle pH, and the highest plasma adrenaline levels. On the other hand, although supramaximal-intensity intermittent exercise resulted in more marked metabolic perturbations than continuous moderate-intensity exercise (CM), *PGC-1α* mRNA was elevated to a lower or similar extent in response to repeated-sprint (RS) and speed endurance (SE) exercise, respectively, compared with CM. Moreover, the severe metabolic stress elicited by SE was associated with higher exercise-induced phosphorylation of CaMKII and p38 MAPK, while no differences in the exercise-induced signalling were detected between RS and CM. Only SE and CM upregulated the mRNA abundance of *NRF-2*, *TFAM*, *MFN2*, *DRP1* and *SOD2*. In accordance with the different exercise-induced metabolic challenges, SE elevated the mRNA content of *HSP72* more than RS and CM.

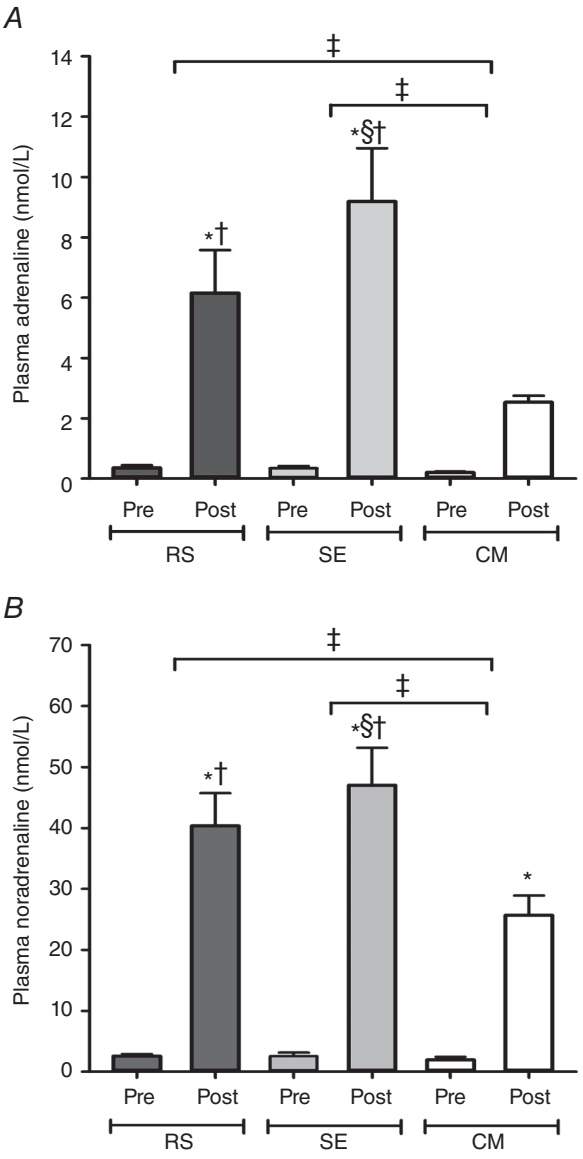

Figure 2. Plasma catecholamines
Venous plasma adrenaline (*A*) and noradrenaline (*B*) concentration before (Pre) and immediately after (Post) repeated-sprint (RS), speed endurance (SE) and continuous moderate-intensity (CM) exercise. Values are mean ± SEM ($n = 12$). *Significantly different from Pre ($P < 0.05$). †Significantly different from CM ($P < 0.05$). §Significantly different from RS ($P < 0.05$). ‡Significant difference in the exercise-induced change ($P < 0.05$).

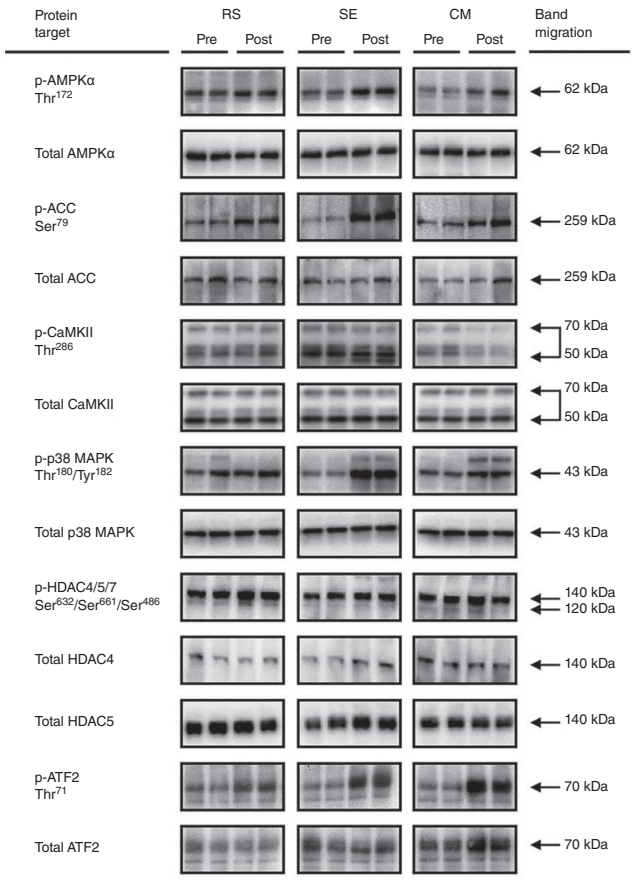

Figure 3. Representative western blots
Representative western blots corresponding to phosphorylated and total protein content measured before (Pre) and immediately after (Post) repeated-sprint (RS), speed endurance (SE) and continuous moderate-intensity (CM) exercise. The molecular mass of band migration of the given protein has been included.

The present finding that both RS and SE upregulated *PGC-1α* mRNA content highlights the potential impact of low-volume intense intermittent exercise on mitochondrial biogenesis in well-trained skeletal muscle. While the high responsiveness of *PGC-1α* mRNA to SE has been demonstrated in trained individuals (Psilander *et al.* 2010; Skovgaard *et al.* 2016), the present study provides novel evidence that RS also increases muscle *PGC-1α* mRNA abundance in well-trained subjects. Moreover, the observation that *PGC-1α* mRNA was elevated by only 90–120 s of active exercise time indicates that intense exercise of even shorter duration than that employed by Psilander *et al.* (2010) and Skovgaard *et al.* (2016) (i.e. 180–240 s) is sufficient to increase *PGC-1α* mRNA, and therefore potentially induce transcriptional regulation of mitochondrial proteins, in endurance-trained skeletal muscle.

The observation that SE was associated with a more marked increase in *PGC-1α* mRNA than work-matched RS, suggests that the greater metabolic perturbations with high muscle lactate and low muscle pH characterizing SE contributed to eliciting the enhanced *PGC-1α* mRNA response. Accordingly, treatment with lactate elevated *PGC-1α* mRNA in myotubes (Hashimoto *et al.* 2007) and mouse skeletal muscle (Kitaoka *et al.* 2016), and a restricted exercise-induced decline in muscle pH blunted

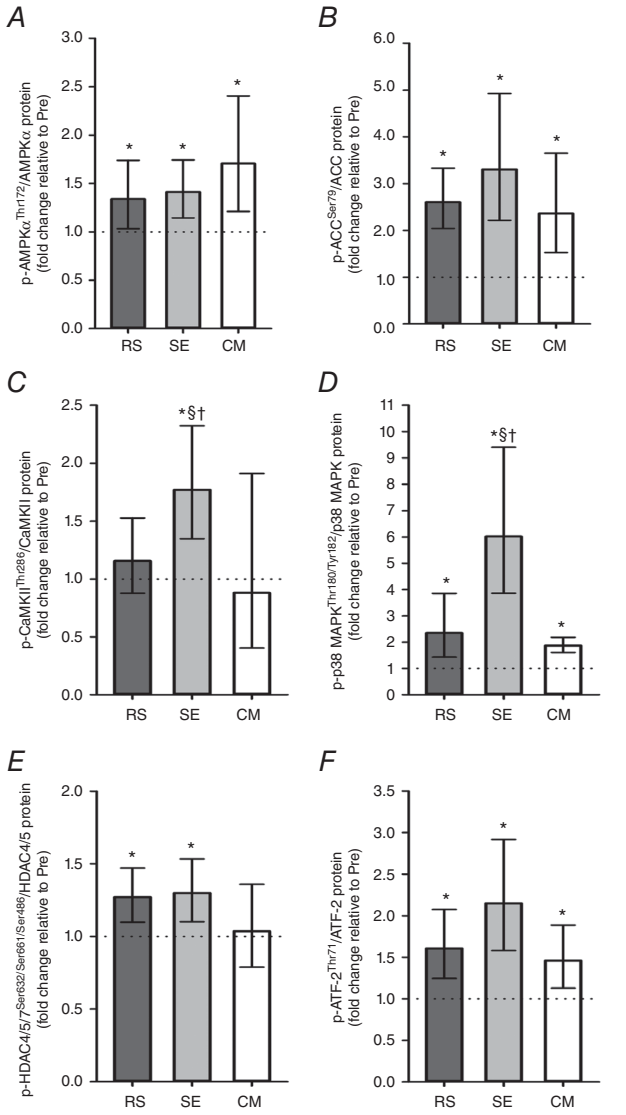

Figure 4. Exercise-induced skeletal muscle intracellular signalling
Repeated-sprint (RS), speed endurance (SE) and continuous moderate-intensity (CM) exercise-induced alterations in phosphorylated AMP-activated protein kinase alpha (AMPKα) protein, acetyl-CoA carboxylase (ACC) protein, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) protein, p38 mitogen-activated protein kinase (p38 MAPK) protein, class II histone deacetylase 4/5/7 (HDAC4/5/7) protein and activating transcription factor-2 (ATF-2) protein normalized to protein content of the given protein. Values are geometric means ± 95% CI ($n = 12$). *Significantly different from Pre ($P < 0.05$). †Significantly different from CM ($P < 0.05$). §Significantly different from RS ($P < 0.05$).

the *PGC-1α* mRNA upregulation during the early recovery from high-intensity exercise in humans (Edge *et al.* 2015).

The higher plasma adrenaline levels detected after SE compared with RS may also have caused the different *PGC-1α* mRNA responses, as injections of adrenaline (Chinsomboon *et al.* 2009) or the β-adrenergic agonist clenbuterol (Miura *et al.* 2007) have been shown to induce an increase in *PGC-1α* mRNA content in mouse skeletal muscle. Conversely, the unaltered plasma adrenaline concentration found after CM was not associated with a less robust *PGC-1α* mRNA response compared with intense intermittent exercise. This suggests that the time frame with elevated adrenaline during intense intermittent exercise may not have been sufficiently long to influence the exercise-induced regulation of *PGC-1α* mRNA, as also proposed by a recent human study (Brandt *et al.* 2016). In addition, muscle glycogen levels have been reported to affect the exercise-induced regulation of *PGC-1α* mRNA, with studies showing that exercise with low glycogen promoted a greater increase in *PGC-1α* mRNA than exercise with normal or high muscle glycogen content (Bartlett *et al.* 2013; Psilander *et al.* 2013). Likewise, low muscle glycogen concentration during recovery from exercise has been reported to prolong the exercise-induced elevation in *PGC-1α* mRNA (Pilegaard *et al.* 2005). Thus, although pre-exercise muscle glycogen content did not differ between the trials in the current study, the observation that post-exercise muscle glycogen content was lower after high-volume submaximal exercise than after low-volume supramaximal exercise may partly explain the higher *PGC-1α* mRNA response detected 3 h into recovery from CM compared with RS.

In summary, while the similar *PGC-1α* mRNA responses to CM and SE are in line with previous findings (Psilander *et al.* 2010) and underline the ability of high-intensity exercise-induced metabolic stress to counteract a reduced volume of exercise, the lower elevation in *PGC-1α* mRNA detected in response to RS than both SE and CM is novel and indicates that the metabolic perturbations induced by RS did not provide sufficient stimuli to compensate for the low work volume.

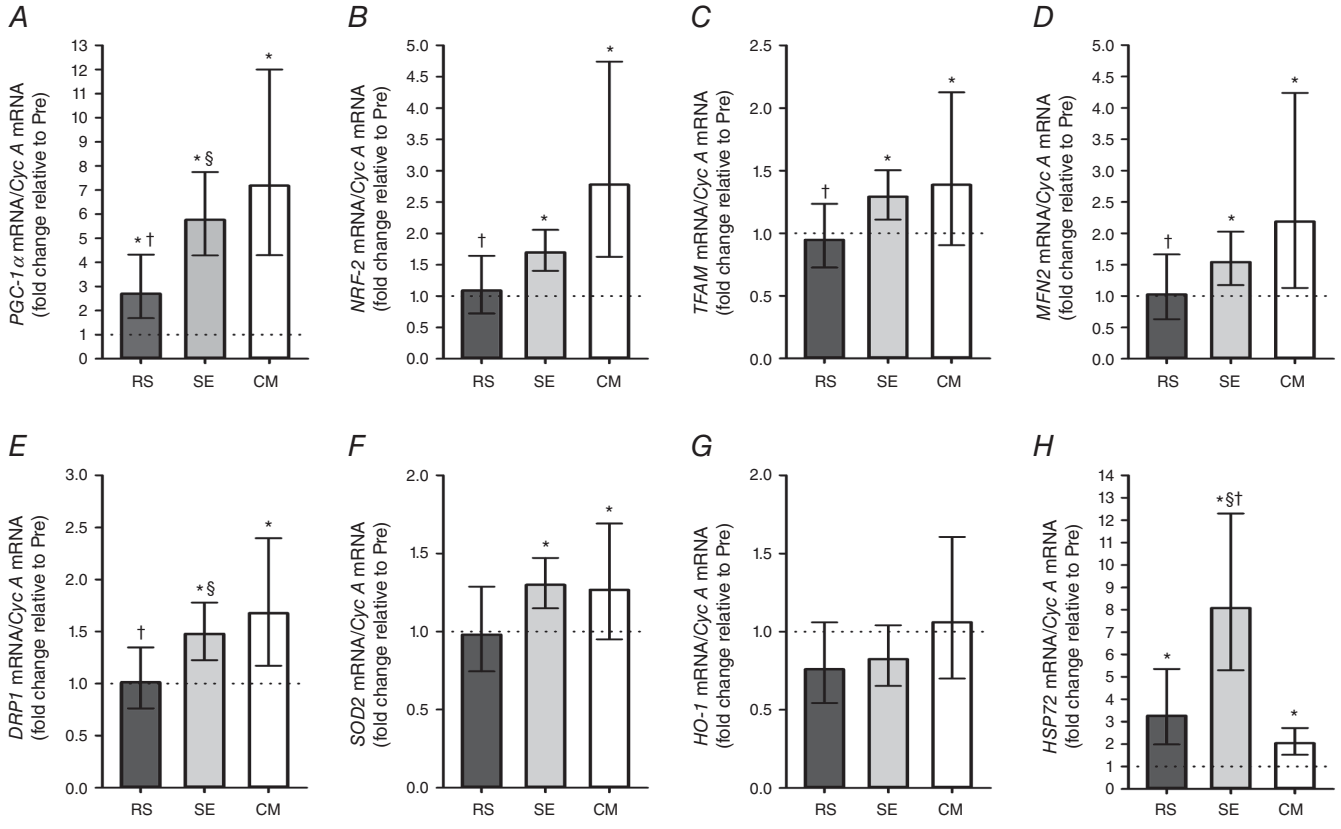

Figure 5. Exercise-induced skeletal muscle mRNA responses
Repeated-sprint (RS), speed endurance (SE) and continuous moderate-intensity (CM) exercise-induced mRNA responses of peroxisome proliferator-activated receptor-γ coactivator-1α (*PGC-1α*), nuclear respiratory factor 2 (*NRF-2*), mitochondrial transcription factor A (*TFAM*), mitofusin-2 (*MFN2*), dynamin-related protein 1 (*DRP1*), superoxide dismutase 2 (*SOD2*), heme oxygenase-1 (*HO-1*) and heat shock protein 72 (*HSP72*) mRNA content normalized to cyclophilin A (*Cyc A*) mRNA content. Values are geometric means ± 95% CI ($n = 12$). *Significantly different from Pre ($P < 0.05$). †Significantly different from CM ($P < 0.05$). §Significantly different from RS ($P < 0.05$).

Table 5. Individual relationships between change of metabolic and molecular variables with repeated-sprint (RS) and speed endurance (SE) exercise

	Metabolic variables included in the model	Multiple R^2	Adjusted R^2	*P* value
mRNA				
PGC-1α	PCr, [H^+], glycogen, adrenaline	0.41	0.28	0.034
NRF-2	PCr, adrenaline	0.32	0.25	0.018
TFAM	PCr, [H^+], glycogen, adrenaline	0.54	0.45	0.004
MFN2	PCr, adrenaline	0.45	0.39	0.002
DRP1	PCr, [H^+], adrenaline	0.46	0.38	0.005
SOD2	PCr, [H^+], adrenaline	0.27	0.15	0.071
HO-1	ATP, PCr, adrenaline	0.22	0.10	0.168
HSP72	Lactate	0.34	0.31	0.003
Phosphorylated protein				
AMPKα	PCr, [H^+], glycogen	0.12	−0.02	0.474
ACC	Adrenaline, glycogen	0.19	0.11	0.114
CaMKII	Lactate, [H^+], glycogen	0.39	0.30	0.017
p38 MAPK	ATP, lactate	0.58	0.54	<0.001
HDAC	[H^+]	0.01	−0.03	0.639
ATF-2	Lactate	0.16	0.12	0.054

mRNA abbreviations are defined in Table 2. Phosphorylated protein: AMPKα, AMP-activated protein kinase α; ACC, acetyl-CoA carboxylase; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; p38 MAPK, p38 mitogen-activated protein kinase; HDAC, histone deacetylase; ATF-2, activating transcription factor-2.

The finding that the mRNA content of genes controlling mtDNA transcription increased in response to CM is in accordance with other studies (Pilegaard *et al.* 2003; Saleem & Hood, 2013), whereas the significant *NRF-2* and *TFAM* mRNA response observed with SE but not with RS has not been shown before. Moreover, this exercise protocol-specific regulation of *NRF-2* and *TFAM* mRNA corresponds with the *PGC-1α* responses, which may suggest a common regulatory mechanism or a *PGC-1α*-mediated induction of these mRNAs, as previously proposed (Pilegaard *et al.* 2003). Likewise, the observation that *MFN2* and *DRP1* mRNA content was elevated after SE and CM, but not after RS, indicates that regulation of mitochondrial morphology depends on the nature of the exercise stimulus. The lack of response to RS may be related to the dampened *PGC-1α* mRNA response, as recent evidence indicates a *PGC-1α*-dependent regulation of both *MFN2* (Zechner *et al.* 2010) and *DRP1* expression (Vainshtein *et al.* 2015). It should be noted that a study involving trained cyclists reported elevated *MFN1* and *MFN2* mRNA levels 24 h, but not 2 h after exercise (Cartoni *et al.* 2005), suggesting that an upregulation of *MFN2* mRNA may have occurred at a later stage into recovery from RS in the current study. In accordance with the present observation, elevated *MFN2* and *DRP1* mRNA levels have been observed immediately after both speed endurance and continuous exercise (Granata *et al.* 2017), supporting both an exercise intensity- and volume-dependent transcriptional regulation of mitochondrial fusion and fission proteins. This resembles the scenario depicted for *PGC-1α*, indicating a close interplay between the complex mechanisms involved in the maintenance of a high-quality mitochondrial network.

Interestingly, the results of multiple linear regression analysis suggest that change in muscle metabolic variables, along with change in plasma adrenaline, significantly contributed to the change in *PGC-1α*, *NRF-2*, *TFAM*, *MFN2* and *DRP1* mRNA induced by RS and SE. Thus, the relationship between metabolic stress and the mitochondrial biogenic molecular response to intense intermittent exercise was significant not only at the group but also at the individual level.

The finding that both phosphorylated AMPKα and phosphorylated ACC, a sensitive marker of AMPK activation (Chen *et al.* 2003), were increased to the same extent by RS, SE and CM, implies that the current differences in the exercise-induced *PGC-1α* mRNA response may not be explained by a differential activation of the AMPK signalling pathway. The present exercise-induced regulation of the AMPK pathway is consistent with the observed increase in AMPKα phosphorylation immediately after both speed endurance (Gibala *et al.* 2009) and endurance exercise (Wojtaszewski *et al.* 2000), and with the increased ACC phosphorylation reported immediately after repeated-sprint exercise (Serpiello *et al.* 2012).

The observation that SE increased CaMKII and p38 MAPK phosphorylation more than both RS and CM, with no differences between these two protocols, does

not support that CaMKII and p38 MAPK determined the different elevation in *PGC-1α* mRNA. The greater CaMKII phosphorylation detected in response to SE compared with CM is in line with studies reporting an exercise intensity-dependent phosphorylation of CaMKII (Rose *et al.* 2006; Egan *et al.* 2010), which in turn is consistent with the more marked alterations in sarcoplasmic Ca^{2+} concentration likely to occur during exercise characterized by a heavy recruitment of type II muscle fibres (Baylor & Hollingworth, 2003). In spite of the high-intensity nature of repeated-sprint exercise, the lack of change in phosphorylated CaMKII immediately after the RS trial may be explained by the very short-lasting exercise stimulus. Similarly, Serpiello *et al.* (2012) observed no change in CaMKII phosphorylation immediately after repeated short sprints, while an increase was detected 1 h into recovery. The current finding that p38 MAPK phosphorylation was less robust after RS and CM than after SE is in line with the lack of response of phosphorylated CaMKII to RS and CM. Furthermore, these observations are in agreement with a study indicating that increments in Ca^{2+} flux are involved in p38 MAPK activation, and that p38 MAPK may be a downstream target of CaMKII (Wright *et al.* 2007). Notably, multiple linear regression analysis showed that intense intermittent exercise-induced phosphorylation of CaMKII and p38 MAPK could be partly explained by change in muscle metabolic variables, suggesting a metabolic stress-dependent activation of these kinases. As class II histone deacetylases (HDACs) and activating transcription factor-2 (ATF-2) have been proposed as intensity-dependent mediators of the exercise-induced *PGC-1α* mRNA response (Egan *et al.* 2010), the phosphorylation of these proteins was examined. The present observation that phosphorylated HDAC4/5/7 was elevated by supramaximal-intensity, but not by submaximal-intensity exercise is in agreement with the previously reported exercise intensity-dependent inactivation of HDACs (Egan *et al.* 2010). On the other hand, the lack of difference in HDAC phosphorylation between RS and SE does not seem to depend on CaMKII as shown in cell cultures (Backs *et al.* 2008). Phosphorylated ATF-2 was upregulated to the same extent by all trials, which is in contrast to the observed differential phosphorylation of CaMKII and p38 MAPK, as these signalling kinases have been suggested to regulate ATF-2 phosphorylation (Wright *et al.* 2007). Hence, the current data indicate that signalling events other than the ones formerly proposed may be responsible for the exercise-induced phosphorylation of HDACs and ATF-2 in human skeletal muscle.

Given the proposed importance of ROS in mediating the signalling cascades towards mitochondrial biogenesis (Gomez-Cabrera *et al.* 2005; Irrcher *et al.* 2009; Kang *et al.* 2009; Morales-Alamo *et al.* 2013; Place *et al.* 2015), the exercise-induced mRNA responses of proteins implicated in muscle anti-oxidant capacity were assessed. The finding that *SOD2* mRNA abundance increased in response to SE and CM, but not following RS, may indicate different adaptive responses in mitochondrial ROS-scavenging capacity. Furthermore, the lack of change in *SOD2* mRNA content after RS is in accordance with the attenuated *PGC-1α* mRNA response, as previous evidence has suggested a *PGC-1α*-mediated regulation of *SOD2* (Leick *et al.* 2008; Geng *et al.* 2010). The observation that *HO-1* mRNA was unaffected by exercise is in contrast with the elevated *HO-1* mRNA levels reported after endurance exercise (Pilegaard *et al.* 2000). However, the well-trained status of the subjects, the sample time point and the exercise protocols employed may explain the unresponsiveness of *HO-1* mRNA to acute exercise observed in the current study. Moreover, in agreement with the present findings, no alterations in *HO-1* mRNA content were observed in a moderately trained population after 90 min of interval exercise (Ballmann *et al.* 2014), suggesting that further studies are needed to clarify the exercise-induced regulation of *HO-1* mRNA in human skeletal muscle. While increased levels of *HSP72* mRNA have been demonstrated following traditional endurance exercise (Morton *et al.* 2009), the present study shows for the first time that low-volume intense intermittent exercise upregulated *HSP72* mRNA to a similar or even higher extent than high-volume submaximal exercise. This finding may be explained not only by the greater muscle metabolic stress, but perhaps also by a higher degree of oxidative stress elicited by SE than RS and CM. On the other hand, similar *HSP72* mRNA responses were reported following work-matched high-intensity interval and submaximal continuous exercise in spite of different metabolic demands (Bartlett *et al.* 2012), implying that the exercise-induced *HSP72* mRNA response may be further enhanced only by very high levels of metabolic stress, as occurred during SE in the current study.

A limitation of the present study is the different durations of the exercise protocols employed. Indeed, the long-lasting continuous submaximal exercise may have induced a prolonged activation of the signalling pathways mediating *PGC-1α* transcription. In fact, AMPK, ACC and CaMKII phosphorylation have been shown to increase shortly after the onset of submaximal exercise (Rose & Hargreaves, 2003; Rose *et al.* 2005), implying that the activation of these signalling pathways lasted longer with the long-term continuous exercise than with the short-term intermittent exercise. This might explain not only the smaller *PGC-1α* mRNA response observed following RS than CM despite similar degrees of intracellular signalling, but also the lack of differences in the *PGC-1α* mRNA response between SE and CM, although CaMKII and p38 MAPK phosphorylation levels

were different. Moreover, post-exercise mRNA levels were determined in the muscle biopsy sampled 3 h into recovery, which corresponded to a sampling time of 3 h 10 min, 3 h 12 min and 3 h 50 min after the effective onset of RS, SE and CM, respectively. The exercise-induced mRNA response of regulators of genes encoding mitochondrial proteins has been shown to peak within 2–8 h into recovery from exercise (Pilegaard *et al.* 2003; Booth & Neufer, 2005), which supports our choice of measuring mRNA content 3 h after the cessation of exercise. However, further research is warranted to elucidate whether a time frame of ∼40 min may underlie significantly different degrees of elevation in mRNA abundance.

Although the current results have important practical implications for effective exercise training prescription, we recognize that mRNA transcription represents only the first step towards protein synthesis, and thus the mRNA response to acute exercise may not accurately predict the skeletal muscle adaptations to chronic exercise training (Cochran *et al.* 2014). In this context, the assessment of post-transcriptional regulating mechanisms (e.g. microRNA) may improve the comprehension of the molecular events underlying the training-induced adaptations (Miller *et al.* 2016). On the other hand, our findings are supported by long-term studies showing that repeated-sprint, speed endurance and continuous submaximal exercise training promote mitochondrial biogenesis in skeletal muscle from moderately trained individuals (MacDougall *et al.* 1998; Gibala *et al.* 2006; Serpiello *et al.* 2012). Nevertheless, more evidence is needed to confirm whether such long-term mitochondrial adaptations are also evident in a well-trained population.

In conclusion, the present study demonstrates that, for a given low-volume of exercise, a high degree of exercise-induced metabolic stress is associated with a greater *PGC-1α* mRNA response. The exercise-induced mRNA responses of proteins implicated in mtDNA transcription and mitochondrial remodelling dynamics seem to follow the same pattern delineated for *PGC-1α* mRNA. In addition, mRNA responses related to mitochondrial turnover are induced by both low-volume supramaximal-intensity intermittent and high-volume continuous moderate-intensity exercise in skeletal muscle from well-trained individuals, but increased intensity appears to compensate for reduced volume only when the intense exercise promotes remarkably pronounced alterations in the intracellular metabolic milieu, with a substantial activation of the CaMKII and p38 MAPK signalling pathway. Together these findings suggest a metabolic stress-mediated regulation of the initial events that promote the development and maintenance of a high-quality mitochondrial pool in human skeletal muscle.

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Additional information

Competing interests

None declared.

Author contributions

M.F. designed the study, conducted the human experiments, participated in data collection, analysis and interpretation, and drafted the manuscript. T.P.G. participated in designing the study, as well as in data collection and interpretation, and in revising the manuscript. M.H. participated in data collection, analysis and interpretation, as well as in revising the manuscript. F.M.I. and F.S. participated in designing the study, as well as in revising the manuscript. H.P. participated in data analysis and interpretation, as well as in revising the manuscript. J.B. participated in designing the study, and drafted the manuscript. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. All experiments were carried out at the Department of Nutrition, Exercise and Sports at the University of Copenhagen, Denmark.

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