PhD Thesis

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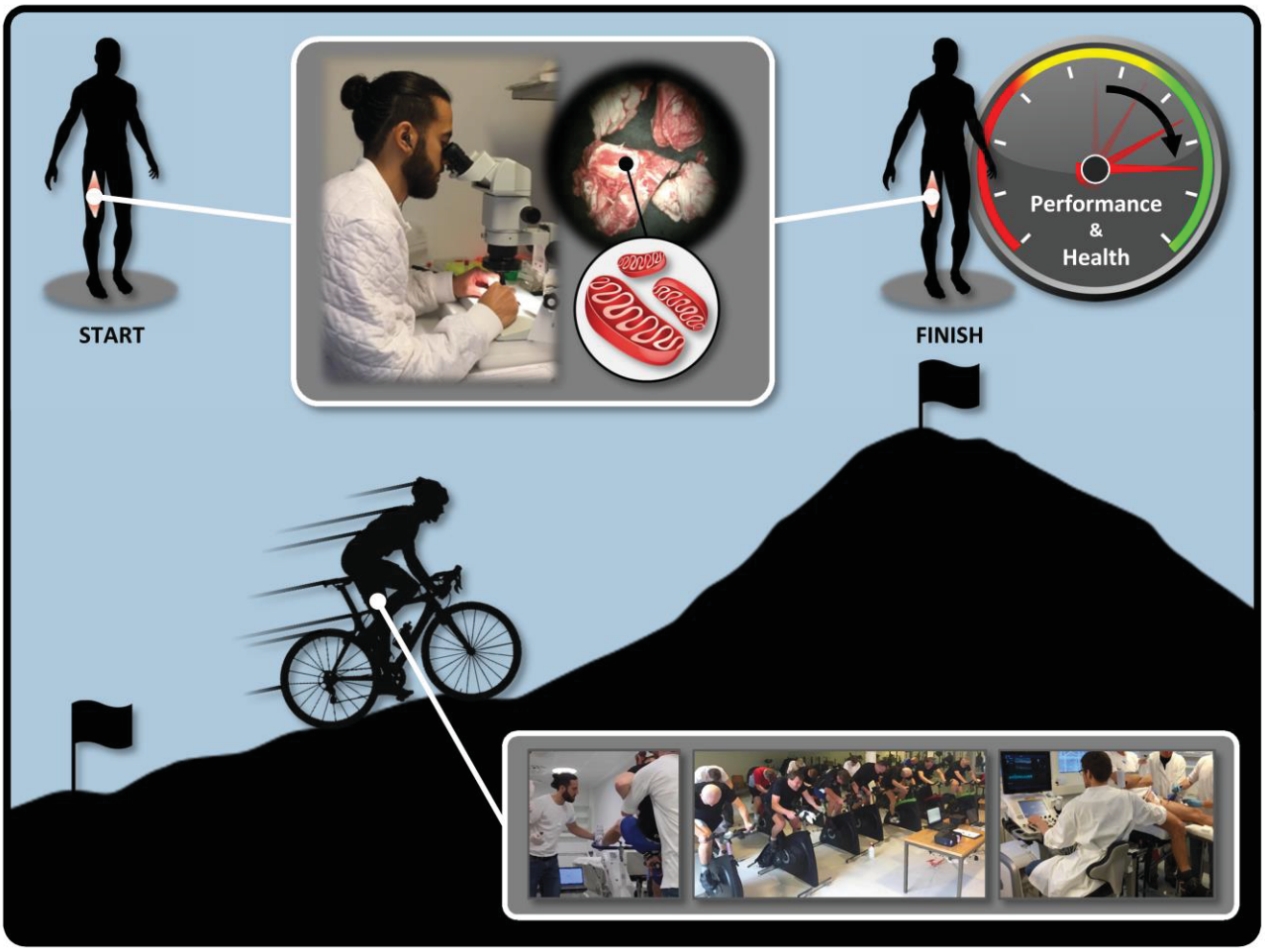

PHYSIOLOGICAL MECHANISMS UNDERLYING PERFORMANCE- AND HEALTH-ENHANCING EFFECTS OF HIGH-INTENSITY EXERCISE TRAINING IN HUMAN SKELETAL MUSCLE

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Insights into mitochondrial adaptive responses

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Title and subtitle:	Physiological mechanisms underlying performance- and health-enhancing effects of high-intensity exercise training in human skeletal muscle – Insights into mitochondrial adaptive responses
Topic description:	In recent years, high-intensity interval training (HIIT) has received considerable attention from the scientific community owing to its effectiveness in enhancing exercise performance and improving cardiovascular and metabolic health. HIIT promotes a number of adaptations resembling those associated with traditional endurance exercise, including quantitative and qualitative changes at the skeletal muscle mitochondrial level. However, the mechanisms underlying these similar adaptive responses in spite of divergent exercise stimuli are not completely clear. Hence, the examination of the skeletal muscle adaptive responses to HIIT with particular emphasis on the cellular pathways regulating mitochondrial quantity, quality and function would provide new insights into the mechanisms involved in the performance- and health-enhancing effects of HIIT. Thus, the overall purpose of this thesis was to investigate the acute and chronic effects of HIIT in humans with specific emphasis on the adaptations occurring in skeletal muscle mitochondria.
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LIST OF ARTICLES

The PhD thesis is based on the following articles and in the text they will be referred to by their roman numerals as “Study I-III”.

I. **Fiorenza M**, Gunnarsson TP, Hostrup M, Iaia FM, Schena F, Pilegaard H & Bangsbo J. Metabolic stress-dependent regulation of the mitochondrial biogenic molecular response to high-intensity exercise in human skeletal muscle. *J Physiol*. 2018;596(14):2823-2840.

II. **Fiorenza M**, Lemminger A, Marker M, Eibye K, Iaia FM, Bangsbo J & Hostrup M. High-intensity exercise training enhances mitochondrial oxidative phosphorylation efficiency in a temperature-dependent manner in human skeletal muscle: Implications for exercise performance. *FASEB J* (accepted for publication).

III. **Fiorenza M**, Gunnarsson TP, Ehlers TS & Bangsbo J. High-intensity exercise training ameliorates aberrant expression of markers of mitochondrial turnover but not oxidative damage in skeletal muscle of men with essential hypertension. *Acta Physiol (Oxf)*. 2019;225(3):e13208.

SUMMARY

In recent years, high-intensity interval training (HIIT) has received considerable attention from the scientific community owing to its effectiveness in enhancing exercise performance and improving cardiovascular and metabolic health. HIIT promotes a number of adaptations resembling those associated with traditional endurance exercise, including quantitative and qualitative changes at the skeletal muscle mitochondrial level. However, the mechanisms underlying these similar adaptive responses in spite of divergent exercise stimuli are not completely clear. Hence, the examination of the skeletal muscle adaptive responses to HIIT, with particular attention to the cellular pathways regulating mitochondrial quantity, quality and function, would provide new insights into the physiological mechanisms involved in the performance- and health-enhancing effects of HIIT.

Thus, the overall purpose of the present PhD thesis was to investigate the acute and chronic effects of HIIT in humans with specific emphasis on the adaptations occurring at the skeletal muscle mitochondrial level. The thesis is based on three human studies, including one acute exercise study (study I) and two training intervention studies (study II and III).

Study I explored the metabolic and molecular responses to an acute exercise bout in young healthy men. Specifically, the early adaptive molecular events evoked by two work-matched low-volume high-intensity interval exercise regimes and a high-volume moderate-intensity continuous exercise protocol were examined in concert with the exercise-induced metabolic perturbations. It was observed that for a given volume of high-intensity exercise, the initial events associated with mitochondrial biogenesis depended on the degree of metabolic stress and that high-intensity exercise could compensate for reduced exercise volume only when marked metabolic perturbations occurred.

Study II investigated the effects of a period of HIIT on exercise performance and skeletal muscle mitochondrial adaptations in young healthy men, with specific emphasis on mitochondrial respiratory function. Mitochondrial respiratory capacity and mitochondrial coupling efficiency were determined *ex vivo* under experimentally-induced normothermia and hyperthermia to mimic *in vivo* muscle temperature at rest and during intense exercise, respectively. While no substantial training-induced improvements in mitochondrial respiratory

function were detected at normothermia, both mitochondrial maximal respiratory capacity and coupling efficiency were enhanced under hyperthermia, indicating that HIIT improved mitochondrial bioenergetics in a temperature-specific manner. Furthermore, HIIT enhanced exercising skeletal muscle efficiency, which possibly contributed to improving exercise performance.

Study III investigated the health-enhancing effects along with the skeletal muscle adaptations elicited by a period of HIIT in sedentary middle aged/older men with essential hypertension. Given the complex interplay between dysregulated blood pressure, oxidative stress and mitochondrial dysfunction, it was questioned whether essential hypertension was associated with impaired muscle mitochondrial turnover and anti-oxidant defences, and whether HIIT was an effective strategy to ameliorate mitochondrial quality and anti-oxidant protection. It emerged that skeletal muscle from hypertensive individuals exhibited aberrant expression of markers of mitochondrial turnover and augmented oxidative damage, and that HIIT partly reversed hypertension-related impairments in muscle mitochondrial turnover but not oxidative damage. Most importantly, HIIT lowered blood pressure and improved cardiorespiratory fitness and body composition.

In conclusion, the present PhD thesis demonstrated that high-intensity training is a viable strategy to promote substantial improvements in performance- and health-related parameters in humans, with high-intensity exercise-induced metabolic stress likely playing a key role in the hormetic response culminating in multiple mitochondrial adaptations at the skeletal muscle level. Moreover, the thesis elaborated on specific cellular pathways contributing to enhancements in muscle mitochondrial quantity, quality and function, thus providing novel insights into the mechanisms underlying the beneficial effects of high-intensity exercise training. Taken together, the PhD thesis suggests a prominent role of skeletal muscle mitochondria in the performance- and health-enhancing effects of high-intensity exercise training in humans.

1 – INTRODUCTION

In recent years, much research has focused on the potential for high-intensity interval training (HIIT), defined as repeated intense work bouts separated by recovery periods (Laursen & Jenkins, 2002), to enhance exercise performance and improve cardiovascular and metabolic health. This scientific interest has been accompanied by an increased attention from the society, ranking HIIT among the top fitness trends worldwide.

HIIT promotes a number of physiological adaptations resembling those associated with traditional endurance exercise, including multiple changes within the skeletal muscle (MacInnis & Gibala, 2017). During the past 20 years, there has been a growing interest on specific forms of HIIT characterized by short-duration maximal/supramaximal efforts (Bishop *et al.*, 2011; Hostrup & Bangsbo, 2017). Supramaximal-intensity HIIT has been shown to promote substantial skeletal muscle remodelling, including marked adaptive responses at the mitochondrial level (Roberts *et al.*, 1982; Pilegaard *et al.*, 1999; Barnett *et al.*, 2004; Burgomaster *et al.*, 2005; Burgomaster *et al.*, 2006; Gibala *et al.*, 2006; Burgomaster *et al.*, 2007; Burgomaster *et al.*, 2008; Little *et al.*, 2010; Serpiello *et al.*, 2012; Jacobs *et al.*, 2013; Larsen *et al.*, 2015; Hostrup *et al.*, 2018). However, the mechanisms underlying such adaptive responses are still incompletely understood.

The term hormesis comes from the Greek hórmēsis, literally meaning “rapid motion”. In the fields of biology and medicine, the concept of hormesis, also known as “general adaptation syndrome”, refers to the process by which an organism adapts to a specific stress in an effort to return the body to normal homeostasis (Calabrese *et al.*, 2013). Exercise may elicit hormetic responses via the multitude of biochemical and physiological changes occurring at the cellular level, with skeletal muscle being among the tissues displaying the greatest remodelling capacity in response to various hormetic agents, including metabolic, oxidative, hypoxic, thermal and mechanical stress. Recently, the concept of hormesis has been applied specifically to the mitochondria (Yun & Finkel, 2014), the cellular organelles providing most of the energy necessary to sustain prolonged muscle contraction. Accordingly, mitochondrial hormesis (mitohormesis) has been proposed as a key process coordinating the adaptive responses to exercise, with exercise-induced perturbations in mitochondrial homeostasis purportedly

promoting nuclear and cytosolic adaptations to render the whole cell less susceptible to future challenges (Merry & Ristow, 2016).

Mitochondria are dynamic organelles undergoing a constant turnover coordinated by cellular pathways including biogenesis, remodelling dynamics and autophagy (Hood *et al.*, 2015). Mitochondrial biogenesis is the process promoting mitochondrial protein synthesis ultimately leading to an expansion in mitochondrial volume density. Mitochondrial remodelling dynamics by fusion and fission cycles is crucial for mitochondrial quality control, with fusion events restoring the functionality of defective organelles through the formation of a reticular network with neighbouring undamaged mitochondria, while fission events promote the segregation of severely damaged mitochondria from the mitochondrial network. To complete the mitochondrial quality control axis, mitochondria-specific autophagy (mitophagy) selectively degrades and recycles damaged mitochondria segregated by fission events. Taken together, effective mitochondrial turnover is essential for the maintenance of a high-quality and functional mitochondrial pool.

Early work from Holloszy demonstrated that rodents subjected to exercise training exhibited an increase in muscle mitochondrial enzyme activity along with a general increase in mitochondrial protein concentration following the training period (Holloszy, 1967). In addition, mitochondria from muscle of the trained rodents displayed higher capacity to oxidize pyruvate compared with muscle from untrained counterparts, indicating that exercise training promoted an increase in the capacity to produce ATP via oxidative phosphorylation. Notably, in view of earlier animal studies failing to detect substantial adaptive mitochondrial changes in response to moderate-intensity exercise training (Hearn & Wainio, 1956; Gould & Rawlinson, 1959), Holloszy adopted a strenuous exercise program including short vigorous efforts interspaced by longer periods at a relatively lower intensity with the purpose to induce a marked exercise stress (Holloszy, 1967). Overall, the findings from Holloszy highlighted the importance of utilizing exercise stimuli of an adequate intensity to elicit significant hormetic responses at the muscle mitochondrial level.

The remarkable hormetic response of mitochondria to exercise training was later confirmed in human skeletal muscle (Morgan *et al.*, 1971; Hoppeler *et al.*, 1973; Hoppeler *et al.*, 1985), paving the way for a multitude of human studies showing that the performance- and health-enhancing effects of physical exercise were accompanied by quantitative and qualitative changes in muscle mitochondria.

Thus, given that the nature of the mitochondrial hormetic responses to HIIT is still incompletely understood, the present PhD thesis elaborated on the acute and chronic effects of high-intensity exercise in human skeletal muscle and elucidated the potential role of mitochondria in the performance- and health-enhancing effects of HIIT.

The thesis includes the work conducted during a three-year PhD project, consisting of three research articles which aimed at answering the following questions:

1) Are the initial mitochondrial adaptive events elicited by high-intensity exercise mediated by the degree of exercise-induced metabolic stress? (Study I)
2) Are the performance-enhancing effects of high-intensity exercise training associated with improved muscle mitochondrial function in young healthy individuals? (Study II)
3) Are the health-enhancing effects of high-intensity exercise training associated with ameliorated muscle mitochondrial quality in middle aged/older individuals with essential hypertension? (Study III)

2 – RESULTS AND DISCUSSION

2.1 – Classification of high-intensity exercise/training

In the present thesis the term "exercise" will be used when referring to a single exercise session, whereas "training" will be used to describe a period comprising multiple exercise sessions.

High-intensity interval exercise is characterized by repeated bouts of relatively intense exercise interspersed by periods of low-intensity exercise or inactivity (Laursen & Jenkins, 2002). Specific forms of high-intensity interval exercise may involve very short-duration efforts performed at supramaximal intensity, i.e. greater than that eliciting the maximal oxygen consumption ($\dot{V}_{O_2max}$) (MacInnis & Gibala, 2017). These forms include speed endurance (or sprint interval) exercise, depicted as multiple prolonged "all-out" bouts (<40 s) separated by comparatively long resting periods (Iaia & Bangsbo, 2010), and repeated-sprint exercise, characterized by shorter "all-out" bouts (<10 s) interspersed with relatively short (<60 s) recovery periods (Spencer *et al.*, 2005; Bishop *et al.*, 2011).

Both speed endurance and repeated-sprint exercise were utilized acutely in **study I**. The speed endurance exercise protocol employed in **study I** was then adopted as a training intervention in **study II**. Finally, a speed endurance training period following the 10-20-30 training concept (Gunnarsson & Bangsbo, 2012) was implemented in **study III**.

2.2 – Acute physiological responses to high-intensity exercise

The analysis of the acute physiological responses to exercise is crucial for understanding the mechanisms involved in the long-term adaptive responses to exercise training (Coffey & Hawley, 2007). Consistent with the concept of hormesis, the acute perturbations in myocellular homeostasis elicited by a single exercise bout promote early adaptive molecular responses to render the cell less susceptible to future challenges. Mitochondria are key players in this process, but it is still incompletely understood whether the early adaptive mitochondrial responses evoked by high-intensity exercise depend on the degree of metabolic stress.

Thus, **study I** investigated whether different exercise-induced metabolic perturbations are associated with distinct early adaptive molecular responses at the skeletal muscle mitochondrial level.

2.2.1 – Metabolic responses

It is well-established that intense sustained muscle contraction elicits marked metabolic and ionic perturbations within the myocellular milieu, including depletion of energy substrates, accumulation of metabolic by-products as well as marked alterations in intracellular [P_i], interstitial [K^+] and cytosolic [Ca^{2+}]. In **study I**, repeated-sprint, speed endurance and moderate-intensity continuous exercise were employed with the purpose of inducing different metabolic stress levels. Specifically, moderate-intensity continuous exercise was adopted as traditional high-volume endurance exercise leading to mild, but prolonged metabolic disturbances, whereas repeated-sprint and speed endurance exercise were selected as low-volume supramaximal-intensity intermittent exercise regimes matched for work-volume and work-to-rest ratio (1:6), but provoking distinct degrees of metabolic stress. Indeed, muscle lactate and hydrogen ions (H+) accumulation were expected to occur with different patterns and magnitudes during repeated-sprint and speed endurance exercise. Based on prior evidence, repeated short-sprints (≤6 s) resulted in a gradual increase in muscle and blood lactate and a progressive drop in blood pH (Balsom et al., 1992; Gaitanos et al., 1993), whereas a single long-sprint (≥20 s) tended to markedly increase muscle lactate levels, which remained elevated after 2-4 min of recovery (Bogdanis et al., 1996; Bogdanis et al., 1998). Thus, for the same work volume, a higher degree of metabolic acidosis was likely induced by speed endurance than repeated-sprint exercise.

Consistently, the findings from **study I** indicated that speed endurance exercise 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 repeated-sprint and moderate-intensity continuous exercise. On the other hand, muscle glycogen was lowered to a similar extent in response to the three exercise protocols, but post-exercise glycogen levels were lower following moderate-intensity continuous exercise compared with the supramaximal-intensity intermittent protocols.

A comprehensive analysis of muscle and blood metabolic perturbations attained during repeated-sprint and speed endurance exercise has been presented in a separate study (Fiorenza *et al.*, 2019), whose results substantiate the metabolic stress-hypothesis of **study I**.

2.2.2 – Skeletal muscle molecular responses

The long-term mitochondrial adaptations elicited by 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 gamma coactivator 1-alpha (PGC-1α) is one factor proposed to contribute to initiating these early adaptive molecular responses by co-activating a broad range of transcription factors (Kelly & Scarpulla, 2004). Muscle-specific overexpression of PGC-1α in transgenic rodent models increased the messenger RNA (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 mediates mitochondrial biogenesis also in humans.

Exercise-induced PGC-1α mRNA responses

Training-induced mitochondrial biogenesis depends on both the duration and the intensity of the exercise stimulus (Hood, 2001). Thus, it is not surprising that *PGC-1α* mRNA is strongly up-regulated by both high-volume traditional endurance exercise (Pilegaard *et al.*, 2000; Pilegaard *et al.*, 2003; Leick *et al.*, 2010) and supramaximal-intensity intermittent exercise (Gibala *et al.*, 2009; Little *et al.*, 2011; Serpiello *et al.*, 2012) in human skeletal muscle. Moreover, existing evidence indicates the potency of low-volume supramaximal-intensity intermittent exercise to generate similar (Psilander *et al.*, 2010) or greater (Skovgaard *et al.*, 2016) muscle *PGC-1α* mRNA responses compared with high-volume submaximal exercise.

The findings from **study I** confirmed the high responsiveness of *PGC-1α* mRNA to low-volume supramaximal-intensity intermittent exercise. Moreover, the observation that *PGC-1α* mRNA was elevated by only 90-120 s of active exercise time, indicated that intense exercise of even shorter duration than the one employed by Psilander *et al.* (2010) and Skovgaard *et al.* (2016) (i.e. 180-240 s) was sufficient to increase *PGC-1α* mRNA, and therefore potentially induce transcriptional regulation of mitochondrial proteins, in endurance-trained skeletal muscle.

Metabolic perturbations underlying PGC-1α mRNA responses

Multiple 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 suggest the sensitivity of muscle PGC-1α transcription to the metabolic environment.

Study I supported the role of metabolic stress in mediating the magnitude of the exercise-induced *PGC-1α* mRNA response. Indeed, speed endurance exercise was associated with a more marked increase in *PGC-1α* mRNA than work-matched repeated-sprint exercise, suggesting that the greater metabolic perturbations with high muscle lactate and low muscle pH characterizing speed endurance exercise contributed to eliciting the enhanced *PGC-1α* mRNA response. In accordance, it has been shown that treatment with lactate elevates *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 blunts *PGC-1α* mRNA upregulation during early recovery from high-intensity exercise in humans (Edge *et al.*, 2015).

Another factor possibly contributing to exercise-induced increase in *PGC-1α* mRNA is the rise in plasma adrenaline levels, as injections of adrenaline (Chinsomboon *et al.*, 2009) or the beta-adrenergic agonist clenbuterol (Miura *et al.*, 2007) have been shown to induce an increase in *PGC-1α* mRNA content in mouse skeletal muscle. In this direction, the higher plasma adrenaline levels detected after speed endurance than repeated-sprint exercise in study I may also have caused the different *PGC-1α* mRNA responses. Conversely, the unaltered plasma adrenaline concentration found after moderate-intensity continuous exercise 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).

Muscle glycogen levels may affect the exercise–induced regulation of *PGC-1α* mRNA, with evidence indicating 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 **study I**, the observation that post-exercise muscle glycogen content was lower after high-volume moderate-intensity continuous exercise than after low-volume

supramaximal-intensity exercise may partly explain the higher *PGC-1α* mRNA response detected 3 h into recovery from moderate-intensity continuous exercise compared with repeated-sprint exercise.

Taken together, these findings support a metabolic stress-dependent regulation of *PGC-1α* mRNA and disagrees with the similar *PGC-1α* mRNA responses observed 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).

Exercise-induced activation of signalling kinases governing PGC-1α expression

PGC-1α gene expression is 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).

The cascade of events evoked by exercise-induced metabolic stress and culminating in *PGC-1α* mRNA up-regulation relies on the activation of these intracellular signalling pathways. In support, more marked induction of AMPK and CaMKII phosphorylation with corresponding greater *PGC-1α* mRNA abundance has been reported following high-intensity compared to 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 than the low-intensity exercise protocol.

In **study I**, phosphorylated AMPKα and phosphorylated ACC, a sensitive marker of AMPK activation (Chen *et al.*, 2003), were increased to the same extent by repeated-sprint, speed endurance and moderate-intensity continuous exercise, implying that the differences in the exercise-induced *PGC-1α* mRNA response were not explained by a differential activation of the AMPK signalling pathway. The exercise–induced regulation of the AMPK pathway observed in **study I** was consistent with the increased AMPKα phosphorylation documented after speed endurance (Gibala *et al.*, 2009) and traditional endurance exercise (Wojtaszewski *et al.*, 2000), and with the increased ACC phosphorylation reported after repeated-sprint exercise (Serpiello *et al.*, 2012).

In **study I**, the magnitude of CaMKII and p38 MAPK phosphorylation induced by speed endurance exercise was greater than that observed in response to repeated-sprint and

moderate-intensity continuous exercise, with no differences between these two protocols. This does not support that CaMKII and p38 MAPK determined the different elevation in *PGC-1α* mRNA. In addition, the greater CaMKII phosphorylation detected in response to speed endurance than moderate-intensity continuous exercise 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, no changes in phosphorylated CaMKII were observed immediately after the repeated-sprint trials in **study I,** which 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 finding that p38 MAPK phosphorylation was less robust after repeated-sprint and moderate-intensity continuous compared to speed endurance exercise was consistent with the lack of response of phosphorylated CaMKII to repeated-sprint and moderate-intensity continuous exercise. Furthermore, these observations were 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).

In light of evidence proposing class II histone deacetylases (HDACs) and activating transcription factor-2 (ATF-2) as intensity-dependent mediators of the exercise-induced PGC-1α mRNA response (Egan *et al.*, 2010), the phosphorylation of these proteins was examined in **study I**. The observation that phosphorylated HDAC4/5/7 was elevated by supramaximal-intensity, but not by submaximal-intensity exercise was in agreement with the exercise intensity–dependent inactivation of HDACs (Egan *et al.*, 2010). On the other hand, the lack of difference in HDACs phosphorylation between repeated-sprint and speed endurance exercise did 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 was 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). Thus, it is conceivable that other signalling events than those formerly proposed were responsible for the exercise–induced activation of HDACs and ATF-2 observed in **study I**.

Exercise-induced regulation of mtDNA transcription and mitochondrial remodelling dynamics

The molecular mechanisms whereby PGC-1α coordinates exercise-induced mitochondrial adaptations include regulation of mtDNA transcription and mitochondrial remodelling dynamics (Hood *et al.*, 2016). mtDNA transcription and replication are driven by the nuclear encoded mitochondrial transcription factor A (TFAM), whose expression is controlled by nuclear transcription factors, counting nuclear respiratory factor 1 and 2 (NRF-1 and -2) (Virbasius & Scarpulla, 1994). Consistent with the PGC-1α mediated co-activation 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 in spite of an increase in *PGC-1α* mRNA (Stepto *et al.*, 2012; Scribbans *et al.*, 2017). In view of these discrepancies, **study I** examined whether the exercise-induced mRNA responses of genes controlling mtDNA transcription corresponded with the proposed metabolic stress-mediated regulation of *PGC-1α* mRNA. The finding that *NRF-2* and *TFAM* mRNA content increased in response to moderate-intensity continuous exercise was in accordance with other studies (Pilegaard et al., 2003; Saleem & Hood, 2013), whereas the significant *NRF-2* and *TFAM* mRNA response observed with speed endurance but not with repeated-sprint exercise was novel. Taken together, this exercise protocol–specific regulation of *NRF-2* and *TFAM* mRNA corresponded with the *PGC-1α* responses, thus suggesting a common regulatory mechanism or a PGC-1α–mediated induction of these mRNAs, as previously proposed (Pilegaard et al., 2003).

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 dynamics for the maintenance of a functional mitochondrial network, only few human studies investigated the impact of exercise on the mRNA response of proteins involved in mitochondrial fusion and fission (Cartoni *et al.*, 2005; Ding *et al.*, 2010; Granata *et al.*, 2017). **Study I** indicated that mitofusin 2 (*MFN2*) and dynamin related protein 1 (*DRP1*) mRNA content was elevated after speed endurance and moderate-intensity continuous exercise, but not after repeated-sprint exercise, suggesting that regulation of mitochondrial dynamics depends on the nature of the exercise stimulus. The lack of response to repeated-sprint exercise may be related to the dampened *PGC-1α* mRNA response, as recent evidence demonstrated a PGC-1α–dependent regulation of both MFN2 (Zechner *et al.*,

2010) and DRP1 expression (Vainshtein *et al.*, 2015). Notably, 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 upregulation of *MFN2* mRNA may have occurred at a later stage into recovery from repeated-sprint exercise in **study I**. In accordance with the findings from **study I**, elevated *MFN2* and *DRP1* mRNA levels were observed immediately after speed endurance and continuous exercise (Granata *et al.*, 2017), supporting both the exercise intensity- and volume-dependent transcriptional regulation of mitochondrial fusion and fission proteins. This resembled the scenario depicted for PGC-1α, thus suggesting a close interplay between the complex mechanisms involved in the maintenance of a high-quality mitochondrial network.

2.2.3 – Summary

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

2.3 – Performance-enhancing effects of high-intensity training: What role for muscle mitochondria?

High-intensity interval training is an effective strategy to improve endurance exercise capacity. Endurance performance is determined by the product of $\dot{V}_{O_2max}$, lactate threshold and exercise efficiency (Bassett & Howley, 2000). While the beneficial impact of high-intensity training on both $\dot{V}_{O_2max}$ and lactate threshold is well documented, less is known about the high-intensity training-induced changes in exercise efficiency and the associated skeletal muscle adaptations.

Thus, **Study II** investigated the skeletal muscle mitochondrial adaptive mechanisms possibly underlying the performance-enhancing effects of high-intensity training in young healthy individuals, with particular emphasis on mitochondrial respiratory function and exercise efficiency.

2.3.1 – Performance-related adaptations

Low-volume high-intensity interval training has been shown to enhance anaerobic power (Bell & Wenger, 1988; Hellsten-Westing, 1993; Harmer et al., 2000; Barnett et al., 2004; Burgomaster et al., 2005; Burgomaster et al., 2006; Burgomaster et al., 2008; Hazell et al., 2010; Iaia et al., 2015) and intense intermittent exercise capacity (McKenna *et al.*, 1993; Ortenblad *et al.*, 2000; Mohr *et al.*, 2007; Ferrari Bravo *et al.*, 2008; Iaia *et al.*, 2008; Serpiello *et al.*, 2011; Gunnarsson *et al.*, 2012; Edge *et al.*, 2013; Faiss *et al.*, 2013; Ingebrigtsen *et al.*, 2013; Iaia *et al.*, 2015). The findings from **study II**, including a training-induced increase in peak power during a single sprint along with a lower magnitude of fatigue development during repeated sprints, confirmed the effectiveness of a period of high-intensity training for improving such peformance-related parameters.

In spite of a reduced training volume, high-intensity interval training may elicit substantial improvements in medium- to long-term endurance performance (Jensen et al., 2004; Nielsen et al., 2004; Burgomaster et al., 2005; Burgomaster et al., 2006; Gibala et al., 2006; Burgomaster et al., 2007; Bangsbo et al., 2009; Little et al., 2010). In addition, marked improvements in $\dot{V}_{O_2max}$, a major determinant of endurance performance, have been documented following low-volume high-intensity interval training (Weston *et al.*, 2014b). The low-volume high-intensity training intervention adopted in **study II** increased $\dot{V}_{O_2max}$ as well as peak power and time to exhaustion achieved during incremental exercise, thus pointing towards an improved exercise capacity.

Contrary to $\dot{V}_{O_2max}$, exercise efficiency appears less responsive to high-intensity interval training (Montero & Lundby, 2015). It is a common practice to estimate exercise efficiency by measurements of pulmonary gas exchanges during submaximal exercise. It is worth noting that, in light of evidence indicating that increments in pulmonary $\dot{V}_{O_2}$ closely reflect increments in the exercising muscle $\dot{V}_{O_2}$ (Poole *et al.*, 1992), training-induced changes in exercise efficiency are often inferred from measurements of pulmonary gas exchange during cycle ergometry. Notably, **study II** employed an experimental set-up including measurements of leg $\dot{V}_{O_2}$ during one-legged knee-extensor exercise, a methodological approach which allows for accurate assessments of efficiency of the contracting skeletal muscle by excluding possible increments in O_2 cost associated with processes occurring outside the exercising muscle (e.g. respiratory and cardiac muscle work). Consistent with the well-established importance of exercise efficiency for endurance performance (Bassett & Howley, 2000) and given the purported association between fatigue and muscle inefficiency during high-intensity exercise (Grassi et al., 2015), it is conceivable that the observed improvements in exercise efficiency along with the ~5% increase in $\dot{V}_{O_2max}$ partly explain the greater exercise capacity observed following the low-volume high-intensity training intervention in **study II**.

2.3.2 – Skeletal muscle adaptations

Mitochondria generate ATP via oxidation of substrates (oxidative phosphorylation), and skeletal muscle mitochondrial maximal oxidative phosphorylation (OXPHOS) capacity has been identified as the most significant determinant of endurance performance in humans (Jacobs *et al.*, 2011). Thus, maximization of skeletal muscle mitochondrial respiratory function by exercise training may result in robust improvements in exercise performance (Lundby & Jacobs, 2016). Also, given that overall exercise efficiency is determined by the efficiency of the processes providing energy (i.e. mitochondrial coupling) and converting energy to external work (i.e. contractile coupling) (Stainbsy *et al.*, 1980; Conley, 2016), it is conceivable that training-induced enhancements in mitochondrial coupling efficiency translate to a better efficiency of the contracting muscle during sustained exercise.

Mitochondrial respiratory function

Mitochondrial respiratory function can be assessed from rate of mitochondrial oxygen consumption. For such a purpose, *in vitro* polarographic measurements of oxygen consumption from isolated mitochondria have been utilized for over 60 years (Chance & Williams, 1955). However, recent advances in the field of high-resolution respirometry allows for in situ assessments of mitochondrial respiratory function from very small samples of permeabilized muscle fibres, being a more physiologically relevant measure of mitochondrial functional properties within an integrated cellular system (Kuznetsov et al., 2008; Pesta & Gnaiger, 2012). Existing evidence supports the beneficial impact of exercise training on mitochondrial respiratory function in humans (Granata et al., 2018), with training-induced enhancements in maximal OXPHOS capacity being associated with improvements in exercise performance (Walsh et al., 2001; Daussin et al., 2008; Pesta et al., 2011; Jacobs et al., 2013; Montero et al., 2015; Vincent et al., 2015; Granata et al., 2016a, b; MacInnis et al., 2017).

Mitochondrial oxidative phosphorylation system relies on the tight coupling between electron transport and ATP synthesis. However, a part of the energy generated by electron transport is uncoupled from ATP synthesis resulting in heat dissipation. Accordingly, mitochondrial OXPHOS coupling efficiency, defined as the molar amount of ATP produced per mole of atomic oxygen used (i.e. P/O ratio), or alternatively as the ratio of state 3 to state 4 respiration (i.e. respiratory control ratio (RCR)), is a critical feature of mitochondrial function. In contrast to maximal OXPHOS capacity, OXPHOS efficiency seems less responsive to exercise training, with most (Tonkonogi et al., 2000; Walsh et al., 2001; Bakkman et al., 2007; Porter et al., 2015; Vincent et al., 2015; Granata et al., 2016b, a; Robinson et al., 2017; Meinild Lundby et al., 2018), but not all (Daussin et al., 2008), human studies failing to report training-induced enhancements in indexes of mitochondrial efficiency.

A shortcoming of training studies conducted in humans is that muscle mitochondrial respiratory function was assessed at a standardized temperature of 37 °C. Indeed, while human muscle temperature at rest is within a range of 35-36 °C, it may rise up to 40 °C during prolonged intense exercise (Parkin et al., 1999; Morris et al., 2005). Notably, mitochondrial OXPHOS efficiency is highly affected by changes in muscle temperature (Willis & Jackman, 1994), with endurance training attenuating the decline in OXPHOS efficiency occurring at high assay temperature (42°C) in isolated rat skeletal muscle mitochondria (Zoladz et al., 2016). In view of these temperature-dependent alterations in mitochondrial respiratory function, **study II** examined the impact of high-intensity training on muscle mitochondrial

respiratory function assessed in permeabilized muscle fibres under experimentally-induced normothermia (35°C) and hyperthermia (40°C) to resemble muscle temperature at rest and during intense exercise, respectively. To date, **study II** is the first to show training-induced enhancements in OXPHOS efficiency in permeabilized fibres from human skeletal muscle. However, in view of the observed discrepancy between normothermic- and hyperthermic-related training adaptations, comparisons with other studies using different assay temperatures should be made carefully.

The training-induced increase in maximal OXPHOS capacity observed at 40°C in **study II** is in line with the increased $CI+CII_P$ (measured at 37°C) reported following a period of either high-intensity interval (Jacobs *et al.*, 2013; Vincent *et al.*, 2015; Granata *et al.*, 2016a; Robinson *et al.*, 2017; Dohlmann *et al.*, 2018), resistance training (Porter *et al.*, 2015) or a combination of different training modalities (Pesta *et al.*, 2011; Irving *et al.*, 2015; Robinson *et al.*, 2017). At the same time, the lack of training-induced changes in maximal OXPHOS capacity observed at 35°C is consistent with the absence of alterations in $CI+CII_P$ (measured at 37°C) documented following either moderate-intensity continuous (Irving *et al.*, 2015; Montero *et al.*, 2015; Granata *et al.*, 2016b; MacInnis *et al.*, 2017; Meinild Lundby *et al.*, 2018), high-intensity interval (Christensen *et al.*, 2016; Granata *et al.*, 2016b, a; MacInnis *et al.*, 2017) or resistance training (Pesta *et al.*, 2011; Irving *et al.*, 2015; Robinson *et al.*, 2017). Of note, the few studies utilizing a training regimen that included brief supramaximal-intensity efforts, as in the current study, reported either an increase in $CI+CII_P$ in permeabilized fibres (Granata *et al.*, 2016b) or a decrease in $CI+CII_P$ in isolated mitochondria (Larsen *et al.*, 2016), suggesting that between-study comparisons should account for the methodological approach employed for measuring mitochondrial OXPHOS capacity.

Overall, the enhancements in OXPHOS efficiency and maximal OXPHOS capacity were only apparent at 40°C, thus implying thermal-specificity of the training-induced adaptations in mitochondrial respiratory function. Taken together, the outcomes from **study II** not only support the effectiveness of high-intensity training in promoting mitochondrial qualitative changes, but also highlight that these adaptations may only occur or be detected within a thermal environment resembling that associated with intense muscle contraction. Thus, our results suggest that assay temperatures similar to those achieved by the exercising muscle should be utilized during *ex vivo* assessments of training-induced changes in mitochondrial respiratory function. From a physiological standpoint, our findings possibly indicate a

mitochondrial hormetic response to heat stress, whose magnitude is amplified under experimentally-induced hyperthermia.

Enzymes and proteins modulating mitochondrial function

Intrinsic mitochondrial respiratory capacity (mitochondrial respiratory capacity normalized to mitochondrial content) has been shown to be either unaltered or depressed in response to exercise training (Jacobs *et al.*, 2013; Granata *et al.*, 2016a, b; Dohlmann *et al.*, 2018; Meinild Lundby *et al.*, 2018), suggesting that training-induced improvements in mitochondrial respiratory function are related to an expansion of mitochondrial volume rather than improvements of mitochondrial function *per se*.

In **study II**, the high-intensity training period increased protein content of subunits from I, II, III and IV of the electron transport chain as well as citrate synthase (CS) activity, indicating that increments in indexes of mitochondrial content occurred in concert with the observed modifications in mitochondrial respiratory function. Such a finding, while being supported by reports showing concomitant increments in mitochondrial content and mitochondrial respiratory capacity (Jacobs et al., 2013; Granata et al., 2016a; MacInnis et al., 2017; Dohlmann et al., 2018; Meinild Lundby et al., 2018), points against the previously proposed dissociation between mitochondrial content and respiratory function (Rowe et al., 2013; Granata et al., 2016b).

Aside from adaptations in mitochondrial content, in **study II**, we observed that expression of MFN2 and DRP1 increased with the training intervention, possibly indicating an enhanced capacity for mitochondrial dynamic remodelling. These adaptations are in line with prior data indicating up-regulated *MFN2* and *DRP1* mRNA content in response to a single bout of high-intensity exercise in human skeletal muscle (Fiorenza *et al.*, 2018). In addition, while the observed training-induced increase in MFN2 is in agreement with other studies (Granata *et al.*, 2016b; MacInnis *et al.*, 2017; Meinild Lundby *et al.*, 2018), this is not the case for the training-induced increase in DRP1 (Granata *et al.*, 2016b), suggesting that the training intervention adopted in the current study enhanced the capacity of the mitochondria to undergo both fusion and fission events. On the other hand, we observed no apparent changes in BNIP3 with the training intervention, which may indicate that the capacity for mitophagy regulation was unaltered by the training undertaken. Other studies have observed increased BNIP3 levels following 8 weeks of exercise training in young healthy men (Brandt *et al.*, 2018), with the different response being possibly attributed to the shorter duration and the lower volume of the training intervention utilized in the present study.

Given that temperature-dependent impairments in mitochondrial coupling efficiency have been proposed to depend on hyperthermia-induced proton leak (Jarmuszkiewicz *et al.*, 2015), we explored whether the protein levels of ANT1 and UCP3, which are involved in mitochondrial proton leak (Divakaruni & Brand, 2011), were altered by the training intervention. We observed that the high-intensity training intervention increased content of ANT1, which is consistent with that reported in response to six weeks of endurance training (Fernstrom *et al.*, 2004). The training-induced concomitant increase in ANT1 and maximal OXPHOS capacity coincides with observations in other species showing a connection between rise in ANT1 and increase in OXPHOS capacity (Brand *et al.*, 2005). Indeed, upregulation of ANT1 abundance may serve as a protective mechanism to prevent excess ROS production via a mild uncoupling lowering the protonmotive force (Divakaruni & Brand, 2011). Moreover, it is relevant to point out that, besides being involved in proton leak, ANT1 affects mitochondrial ADP sensitivity by driving ADP inport across the inner mitochondrial membrane (Graham *et al.*, 1997). Thus, it may be that, in light of evidence indicating temperature-dependent changes in mitochondrial ADP sensitivity (Perry *et al.*, 2011), the observed hyperthermia-specific training-induced enhancement in OXPHOS coupling efficiency occurred via ANT1-mediated alterations in ADP sensitivity.

The observation from **study II** that UCP3 abundance was unaltered by the training intervention suggests that training-induced improvements in mitochondrial coupling efficiency were likely not related to a reduced UCP-mediated proton leak. Unaltered UCP3 content has been reported following a period of either endurance (Fernstrom *et al.*, 2004) or high-intensity intermittent exercise training (Iaia *et al.*, 2009). However, it cannot be excluded that the training intervention lowered UCP3 activity rather than abundance, as previously observed in rats (Zoladz *et al.*, 2016). Thus, future studies should investigate the importance of both content and activity of ANT1 and UCP3 for training-induced adaptations in mitochondrial respiratory function.

2.3.3 – Summary

Study II demonstrated that high-intensity training was an effective strategy to enhance both exercise efficiency and capacity in recreationally active healthy individuals, with such performance-related improvements being accompanied by mitochondrial qualitative and quantitative changes. Short-term high-intensity training attenuated the hyperthermia-induced decline in mitochondrial oxidative phosphorylation efficiency, suggesting that intense exercise

training may improve the bioenergetic efficiency of skeletal muscle during prolonged intense contractile activity. In addition, we showed training-induced quantitative changes in mitochondrial proteins, including an increased expression of markers of mitochondrial content as well as of proteins regulating mitochondrial dynamics and mitochondrial uncoupling.

2.4 – Health-enhancing effects of high-intensity training in patients: What role for muscle mitochondria?

Elevated blood pressure is among the leading risk factors contributing to the global burden of disease and to global mortality (Collaborators, 2017), with more than one fourth of the world's adult population predicted to be affected by hypertension in 2025 (Kearney *et al.*, 2005). Although hypertension is often viewed only as a cardiovascular disease, multiple lines of evidence indicate functional and morphological alterations in skeletal muscle from hypertensive human subjects and rodent models of essential hypertension (Gray *et al.*, 1994; Krotkiewski *et al.*, 1998; Bortolotto *et al.*, 1999; Quadrilatero & Rush, 2006, 2008; McMillan *et al.*, 2012; Bloemberg *et al.*, 2014; Bowen *et al.*, 2017).

Exercise training is a well-established antihypertensive therapeutic intervention (Cornelissen & Smart, 2013) and emerging evidence indicates that high-intensity interval training lowers blood pressure to a higher extent than traditional endurance training (Tjonna *et al.*, 2008; Molmen-Hansen *et al.*, 2012; Mitranun *et al.*, 2014). However, the physiological mechanisms underlying the antihypertensive effect of high-intensity interval training are still unclear.

Thus, **study III** investigated the skeletal muscle adaptive mechanisms whereby high-intensity training possibly ameliorates health-related outcomes in individuals with essential hypertension.

2.4.1 – Health-related adaptations

Blood pressure

The high-intensity training intervention adopted in **study III** lowered 24-h systolic and diastolic ambulatory blood pressure by 4.6±1.7 and 4.0±1.0 mm Hg, respectively, in hypertensive individuals, confirming the antihypertensive effect of intense exercise training (Cornelissen *et al.*, 2013) and underlining the potency of this training approach to improve blood pressure regulation regardless of the short-term nature of the intervention. Although the observed reductions in ambulatory blood pressure were modest compared to those reported in hypertensive individuals following 12 weeks of running-based high-intensity interval training (Molmen-Hansen *et al.*, 2012), they were in line with the mean training-induced reduction in ambulatory blood pressure computed by a recent meta-analysis (Sosner *et al.*, 2017). Most importantly, reductions in systolic blood pressure of 5 mmHg have been shown to decrease all-

cause mortality by 7% (Whelton *et al.*, 2002), highlighting the clinical relevance of the changes in blood pressure observed in **study III**.

Body composition and cardiorespiratory fitness

In **study III**, high-intensity training improved body composition and cardiorespiratory fitness in hypertensive individuals, thus supporting the mounting evidence advocating high-intensity interval training as a viable therapeutic strategy to reduce risk factors for cardiovascular disease (Kessler *et al.*, 2012; Weston *et al.*, 2014a). With specific regard to the training-induced changes in cardiorespiratory fitness, the hypertensive group increased $\dot{V}_{O_2max}$ to a lower extent than previously observed (Nyberg *et al.*, 2012), which may relate to the ~4-fold lower training volume as well as to the shorter duration of the training intervention used in the present study. Of note, the relatively small increase in cardiorespiratory fitness is still of high clinical relevance, as each 1 mL kg min^{-1} increase in $\dot{V}_{O_2max}$ has been shown to be associated with a 45-day increase in longevity (Clausen *et al.*, 2018).

2.4.2 – Skeletal muscle adaptations

In accordance with the proposed association between skeletal muscle abnormalities and vascular dysfunction (Gray *et al.*, 1994; Krotkiewski *et al.*, 1998; Bortolotto *et al.*, 1999; Quadrilatero & Rush, 2006, 2008; McMillan *et al.*, 2012; Bloemberg *et al.*, 2014; Bowen *et al.*, 2017), endothelium-derived nitric oxide (NO) is thought not only to modulate vascular tone but also to regulate skeletal muscle mitochondrial turnover (Valerio *et al.*, 2006; Lee-Young *et al.*, 2010), suggesting that impairments in NO bioavailability may negatively impact muscle mitochondrial quality and vice versa.

Dysfunctional mitochondrial generate excessive levels of reactive oxygen species (ROS) and mitochondria-derived ROS have been proposed to play a prominent role in the development of hypertension (Dikalov & Ungvari, 2013). In this direction, decrements in mitochondrial ROS production improved vascular relaxation and reduced blood pressure in experimental models of hypertension (Itani *et al.*, 2016), while increased mitochondrial ROS production and oxidative damage have been recently observed in skeletal muscle from patients with peripheral arterial disease (Hart *et al.*, 2018), a vascular disorder often associated with hypertension. In addition, oxidative stress, defined as a dysregulation between production and removal of ROS, while contributing to the development of multiple factors associated with essential hypertension

(Harrison *et al.*, 2007), is inextricably linked with mitochondrial dysfunction, as mitochondria are not only a source but also a target of ROS (Ballinger, 2005).

Muscle mitochondrial turnover

As discussed above, mitochondrial turnover via biogenic, fusion, fission and autophagic events is essential for the maintenance of a functional mitochondrial pool. Defective mitochondrial turnover leads to accumulation of dysfunctional organelles, which, via excess ROS generation, may contribute to hypertension-related endothelial dysfunction. Mitochondrial biogenesis may restrain endothelial dysfunction by the synthesis of new mitochondrial proteins which enhance organelle functional properties, thus limiting excess ROS formation. Accordingly, dysregulated mitochondrial biogenesis has been reported in concert with increased mitochondrial ROS generation in aged endothelial cells (Ungvari *et al.*, 2008). The observation from **study III** that muscle citrate synthase (CS) activity was lower in the hypertensive compared with the normotensive individuals is indicative of a reduced muscle mitochondrial volume in individuals with essential hypertension. It is noteworthy that, while CS activity has been shown to be associated with mitochondrial content in young healthy individuals (Larsen *et al.*, 2012), it needs to be determined whether this association occurs also in aged and diseased individuals. However, the finding that also protein levels of CS, the mitochondrial respiratory chain subunit COXIV, as well as the key regulator of mitochondrial biogenesis estrogen-related receptor alpha (ERRα) (Schreiber *et al.*, 2004), were lower in hypertensive compared with normotensive individuals supports the existence of hypertension-related alterations in proteins implicated in the mitochondrial biogenic pathway. High-intensity training partly reversed these alterations by normalizing CS activity/content to the levels of the normotensive individuals as well as by elevating COXIV content, implying a training-mediated induction of mitochondrial biogenesis.

A causative relationship has been proposed to exist between mitochondrial remodelling dynamics and ROS generation (Jheng *et al.*, 2012; Iqbal & Hood, 2014), with increased mitochondrial fission suggested to impair endothelial function via increased ROS formation (Shenouda *et al.*, 2011). The observation from **study III** that MFN2 levels were lower in hypertensive than healthy individuals with a concomitant lack of differences in mitofusin 1 (MFN1) and optic atrophy 1 (OPA1) implies protein-specific alterations in mitochondrial fusion with essential hypertension, thus highlighting the complexity of mitochondrial fusion machinery. In addition, the hypertensive patients exhibited lower levels of DRP1, suggesting an association between hypertension and a reduced capacity to segregate dysfunctional mitochondria. Overall,

while inconsistent results have been reported on the influence of ageing on mitochondrial fusion/fission (Bori *et al.*, 2012; Joseph *et al.*, 2012; Konopka *et al.*, 2014), our findings partly support hypertension-related alterations in muscle mitochondrial dynamics. In **study III**, high-intensity training increased MFN2, but not DRP1 expression in hypertensive individuals, possibly reflecting an increased fusion-to-fission ratio, and thus a potential decrease in mitochondrial fragmentation. Alternatively, the concomitant high-intensity training-induced decline in MFN1 and OPA1 expression points towards a differential training-induced regulation of specific fusion proteins. A possible explanation for the divergent response observed in MFN1 and MFN2 may be related to slightly different functions of mitofusins. Indeed, while both MFN1 and MFN2 play a role in mitochondrial fusion, MFN2 also regulate mitochondrial membrane potential, substrate oxidation, and oxidative phosphorylation, thereby affecting mitochondrial bioenergetics (Schrepfer & Scorrano, 2016). The decrease in OPA1 was in line with the training-induced down-regulation of MFN1, as OPA1-mediated mitochondrial fusion is functionally regulated by MFN1(Cipolat *et al.*, 2004). Thus, based on the divergent responses in fusion proteins, it was unclear whether the exercise training period stimulated mitochondrial fusion in the hypertensive individuals. Also, it can be argued that the observed increase in MFN2 occurred mainly as an adaptive mechanism to match the metabolic demands elicited by high-intensity training. However, electron microscopy imaging should be performed to confirm whether the observed hypertension- and training-related alterations in fusion/fission markers are associated with morphological changes of the mitochondrial network.

To complete the mitochondrial quality control axis, mitophagy (i.e. mitochondria-specific autophagy) is regulated by changes in ROS production in a feed-forward mechanism (Lee *et al.*, 2012) and may affect endothelial function (LaRocca *et al.*, 2012). The lower content of microtubule-associated protein 1 light chain 3 (LC3) observed in the hypertensive patients in **study III** may indicate an impaired capacity to recycle damaged organelles via the autophagic pathway in individuals with essential hypertension. In spite of the crucial role played by autophagy for cellular homeostasis, reports in human muscle are scarce and limited to evidence of an age-dependent decline in autophagy (Fry *et al.*, 2013; Gouspillou *et al.*, 2014; Distefano *et al.*, 2017). Notably, findings from **study III** were in line with both the lower LC3-II/LC3-I ratio and the unaltered protein levels of SQSTM1/p62 (p62) observed in hypertensive compared with normotensive rats (Bloemberg *et al.*, 2014). That the high-intensity training-induced increase in both LC3-II content and LC3-II/LC3-I ratio was greater in the normotensive than the hypertensive individuals could imply a blunted responsiveness of LC3 to exercise training with

essential hypertension. On the other hand, a training-induced increase in p62 was observed in both the hypertensive and the normotensive individuals, suggesting that the capacity for autophagy regulation increased with exercise training independent of hypertension. These findings are in contrast to the unaltered levels of LC3-II and p62 documented in either healthy young or elderly men following an exercise training period (Fritzen *et al.*, 2016; Arribat *et al.*, 2018; Brandt *et al.*, 2018), with such discrepancies possibly related to the different characteristics of the populations and the exercise training interventions employed. It should be noted that even though LC3 and p62 have been widely used as markers of autophagy, they are indicators of the rate of lysosome formation, but not degradation, which would provide a more specific measure of the autophagic flux (Rubinsztein *et al.*, 2009). Also, it may be that the observed increments in autophagy markers are due to training-induced shifts toward a more oxidative muscle phenotype, as higher autophagy flux and protein expression have been observed in oxidative compared with glycolytic muscles from rodents (Lira *et al.*, 2013).

In case of overwhelming oxidative damage, mitochondria are more prone to trigger apoptotic signalling pathways by releasing a variety of pro-apoptotic factors (Orrenius *et al.*, 2015), which could consequently target the endothelium inducing endothelial cell death (Matsunaga *et al.*, 2001). In **study III**, no differences were observed for markers of mitochondria-mediated apoptosis between the hypertensive and the normotensive individuals. While apoptosis-related data in human hypertension is absent, our findings were in contrast to the two-fold greater BAX/BCL2 ratio observed in hypertensive compared with normotensive rats (McMillan *et al.*, 2012), and do not support an association between essential hypertension and elevated apoptotic activity. In **study III**, high-intensity training increased BAX and BAX/BCL2 ratio in both hypertensive and normotensive individuals, a change in contrast to the reduced BAX/BCL2 ratio reported in hypertensive rats following 6 weeks of exercise training (McMillan *et al.*, 2012). On the other hand, our results were in line with evidence indicating up-regulated BAX content in both healthy individuals and mitochondrial myopathy patients in response to 12 weeks of endurance exercise training (Adhihetty *et al.*, 2007). Notably, although increased apoptotic signalling may lead to muscle wasting, thereby implying a maladaptive response to exercise training, data from a simple animal model indicates that activation of the intrinsic apoptotic pathway by excess mitochondrial ROS generation leads to an adaptive protective mechanism that keeps the organism alive under stressful conditions rather than solely promoting apoptotic cell death (Yee *et al.*, 2014). Nevertheless, further investigations are required to confirm whether this mechanism occurs in humans. Also, it should be noted that lean body mass

did not decrease with the training intervention in either the hypertensive or the normotensive group, suggesting that in spite of increased apoptotic susceptibility, muscle mass was not negatively affected by the training intervention.

Taken together, the findings from **study III** suggest impaired mitochondrial quality control, but not overregulated mitochondria-mediated apoptosis, in men with essential hypertension. On the basis of the training-induced changes in markers of mitochondrial turnover, high-intensity training seemed to promote mitochondrial biogenesis and to increase autophagy capacity while possibly improving mitochondrial bioenergetics via up-regulation of MFN2 in muscle of hypertensive individuals. However, additional research is needed to unravel whether the increase in apoptotic susceptibility elicited by high-intensity training represents a maladaptive response leading to apoptosis rather than degradation of damaged organelles (Mishra & Chan, 2014).

Muscle anti-oxidant protection and oxidative damage

In light of the purported role of redox imbalance in the pathophysiology of essential hypertension, it is conceivable that hypertensive individuals exhibit impaired anti-oxidant defences, including altered levels of anti- and pro-oxidant enzymes. The finding from **study III** that protein levels of anti- and pro-oxidant enzymes were similar in hypertensive and normotensive individuals was in line with the lack of a hypertension-related depression in superoxide dismutase 1 (SOD1) and 2 (SOD2) as well as catalase (CAT) content reported in rodents (Quadrilatero & Rush, 2008), but in contrast to the elevated NAD(P)H oxidase (NOX) levels detected in different animal models of hypertension (Paravicini & Touyz, 2008; Quadrilatero & Rush, 2008). It is worth mentioning that, in order to minimize the risks associated with interruption of antihypertensive drug therapy, only a short-term washout period was included prior to the experimental days in **study III**. Therefore, it is possible that the lack of between-group differences in proteins involved in redox state regulation was related to the effects of long-term treatment with antihypertensive drugs with anti-oxidant properties. In spite of normal content of proteins regulating redox homeostasis, **study III** showed that muscle protein carbonylation was greater in hypertensive than normotensive individuals along with a significant correlation between protein carbonyl levels and 24-h ambulatory blood pressure, thus supporting the purported link between oxidative stress and elevated blood pressure.

Besides promoting a healthier mitochondrial pool, exercise training may reverse hypertension-related endothelial dysfunction by increasing NO bioavailability. This may occur

via a decreased ROS scavenging of NO as a consequence of training-induced increments in activity and/or content of anti-oxidant enzymes (Gliemann *et al.*, 2013; Gliemann *et al.*, 2014). In **study III**, SOD2 content was up-regulated by high-intensity training regardless of blood pressure levels, confirming the responsiveness of the mitochondria-specific isoform of superoxide dismutase to high-intensity training, as observed in aged sedentary men (Gliemann *et al.*, 2013). On the other hand, protein levels of the cytosolic isoform of superoxide dismutase (SOD1) decreased with high-intensity training in hypertensive but not in normotensive individuals, implying a differential compartmentalization of the origin of muscle oxidative stress between healthy and hypertensive individuals, a phenomenon suggested to occur also with ageing (Gianni *et al.*, 2004). The same phenomenon may also underlie the observation that glutathione peroxidase 1 (GPX1) increased with the high-intensity training intervention in the normotensive, but not in the hypertensive individuals, while CAT increased in the hypertensive patients only. The observation from **study III** that high-intensity training promoted an increase in NOX content was consistent with a report in aged sedentary men (Gliemann *et al.*, 2013), and implies an enhanced capacity for ROS formation, which is essential for amplifying ROS-mediated adaptive responses to exercise training. The lack of training-induced changes in sirtuin 3 (SIRT3), which regulates mitochondrial redox homeostasis (Tang *et al.*, 2017), was in contrast to the elevated SIRT3 levels observed following eight weeks of endurance training in both young and old healthy individuals(Johnson *et al.*, 2015), with differences in the intensity and duration of the training intervention possibly explaining this discrepancy. The training-induced decrease in heat shock protein 70 (HSP70), which exerts a cyto-protective function against oxidative stress (Kregel, 2002), was consistent with the observed training-induced increase in apoptotic susceptibility, as HSP70 plays a role in preventing apoptosis by blocking BAX translocation (Beere, 2005). Interestingly, the elevated protein carbonyl levels observed in the hypertensive patients following the training intervention indicated increased levels of oxidative damage. This represented a novel finding in humans and was in accordance with the exacerbated protein carbonylation documented in senescent rats with exercise training (Thomas *et al.*, 2010). Notably, in view of a previous report showing a greater accumulation of carbonyls in oxidative compared with glycolytic muscle fibres (Thomas *et al.*, 2010), future studies including assessments of the muscle phenotype may clarify whether the increase in protein carbonyls observed in **study III** might be related to a training-induced increase in the proportion of type I muscle fibres.

2.4.3 – Summary

Study III provided the first evidence of reduced expression of markers of mitochondrial volume/biogenesis, mitochondrial fusion and fission, and autophagy in skeletal muscle of essential hypertensive humans, suggesting aberrant mitochondrial turnover and thus impaired mitochondrial quality with essential hypertension. On the other hand, the expression of muscle markers of anti-oxidant protection was not lower in hypertensive individuals in spite of increased levels of oxidative damage compared with their normotensive counterparts. Most importantly, **study III** demonstrated that short-term high-intensity training is a viable therapeutic intervention to lower ambulatory blood pressure and promote a healthier mitochondrial pool in hypertensive individuals.

3 – CONCLUSION AND PERSPECTIVES

The main aim of the PhD thesis was to explore the nature of the muscle mitochondrial adaptations possibly underlying the performance- and health-enhancing effects of high-intensity exercise training in humans. Overall, the thesis provides novel physiological insights into the role of muscle mitochondrial adaptations in the beneficial impact of high-intensity training on performance- and health-related parameters.

Study I demonstrated that, for a given low-volume of exercise, a high degree of exercise-induced metabolic stress was associated with enhanced mitochondrial biogenesis-related muscle mRNA responses. Furthermore, when compared with high-volume moderate-intensity exercise, only low-volume high-intensity exercise eliciting severe metabolic stress compensated for reduced exercise volume in the induction of mitochondrial biogenic mRNA responses. However, given that mRNA transcription represents only the first step towards protein synthesis, future studies assessing post-transcriptional regulating mechanisms may improve the comprehension of the molecular events underlying training-induced adaptations.

Study II demonstrated that high-intensity training was an effective strategy to promote enhancements in exercise efficiency in concert with mitochondrial qualitative changes in healthy individuals. Specifically, high-intensity training attenuated the hyperthermia-induced decline in mitochondrial oxidative phosphorylation efficiency, suggesting that high-intensity training improves the bioenergetic efficiency of skeletal muscle during sustained, intense contractile activity. These mitochondrial qualitative changes were associated with quantitative changes in enzymes and proteins important for mitochondrial function, including regulators of either mitochondrial dynamics or mitochondrial uncoupling. In perspective, future studies quantifying mitochondrial oxidative phosphorylation efficiency by direct measurements of the P/O ratio in permeabilized muscle fibres are warranted to confirm the observed training-induced adaptive responses in mitochondrial coupling efficiency and their temperature dependency. In addition, besides muscle temperature, other factors should be considered when modelling *in vivo* conditions during *ex vivo* respiratory assessments. Lastly, simultaneous assessments of changes in mitochondrial dynamics, ultrastructure (i.e. cristae density) and supercomplexes formation are warranted to unravel the mechanisms whereby exercise training enhances the efficiency of mitochondrial energy transduction.

Study III demonstrated that high-intensity training was a viable therapeutic intervention to lower blood pressure and to improve health-related parameters in individuals with essential hypertension. In addition, high-intensity training promoted a number of skeletal muscle mitochondrial adaptations possibly ameliorating mitochondrial turnover and quality in hypertensive individuals. Notably, the lack of training-induced substantial reductions in muscle oxidative damage in spite of enhanced mitochondrial quality highlights the need for supplementary studies providing new insights into the association between mitochondrial adaptations and alterations in redox homeostasis, with assessments of training-induced changes in mitochondrial respiratory function and mitochondrial ROS emission possibly shedding new light on these adaptive mechanisms. Lastly, given that measurements were made on whole muscle homogenates, of which endothelial cells make up only a small fraction, more evidence is required to confirm whether similar hypertension-related alterations and training-induced adaptations would be evident and would occur also within the endothelium.

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APPENDIX

Manuscripts included in the PhD thesis

I. **Fiorenza M**, Gunnarsson TP, Hostrup M, Iaia FM, Schena F, Pilegaard H & Bangsbo J. Metabolic stress-dependent regulation of the mitochondrial biogenic molecular response to high-intensity exercise in human skeletal muscle. *J Physiol*. 2018;596(14):2823-2840.

II. **Fiorenza M**, Lemminger A, Marker M, Eibye K, Iaia FM, Bangsbo J & Hostrup M. High-intensity exercise training enhances mitochondrial oxidative phosphorylation efficiency in a temperature-dependent manner in human skeletal muscle: Implications for exercise performance. *FASEB J* (accepted for publication).

III. **Fiorenza M**, Gunnarsson TP, Ehlers TS & Bangsbo J. High-intensity exercise training ameliorates aberrant expression of markers of mitochondrial turnover but not oxidative damage in skeletal muscle of men with essential hypertension. *Acta Physiol (Oxf)*. 2019;225(3):e13208.

Study I

Fiorenza M, Gunnarsson TP, Hostrup M, Iaia FM, Schena F, Pilegaard H & Bangsbo J. Metabolic stress-dependent regulation of the mitochondrial biogenic molecular response to high-intensity exercise in human skeletal muscle. *J Physiol*. 2018;596(14):2823-2840.

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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.

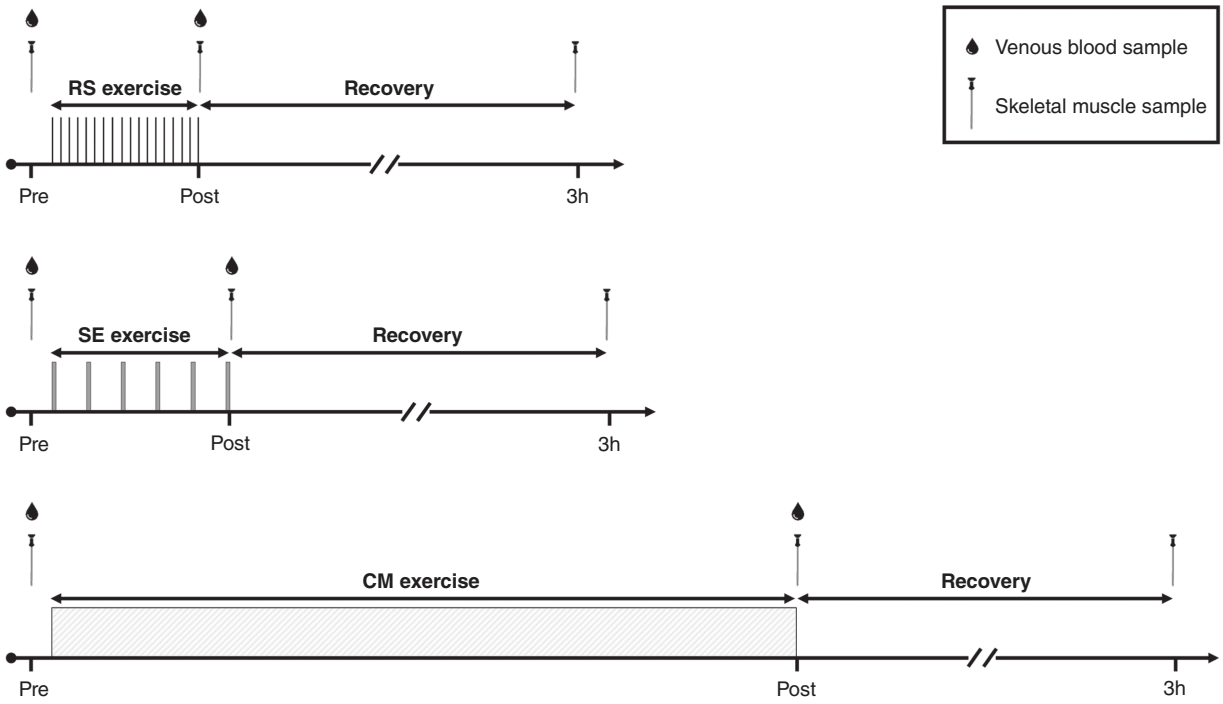

Figure 1. Schematic presentation of the experimental trials
Schematic presentation of the three experimental trials consisting of cycling-based repeated-sprint exercise (RS, 18 × 5 s "all-out" sprints against a breaking force of 0.90 N kg^{-1} interspersed with 30 s of recovery), speed endurance exercise (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), AMPKα^{Thr172} phosphorylation (no. 2531, Cell Signaling Technology), ATF-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.

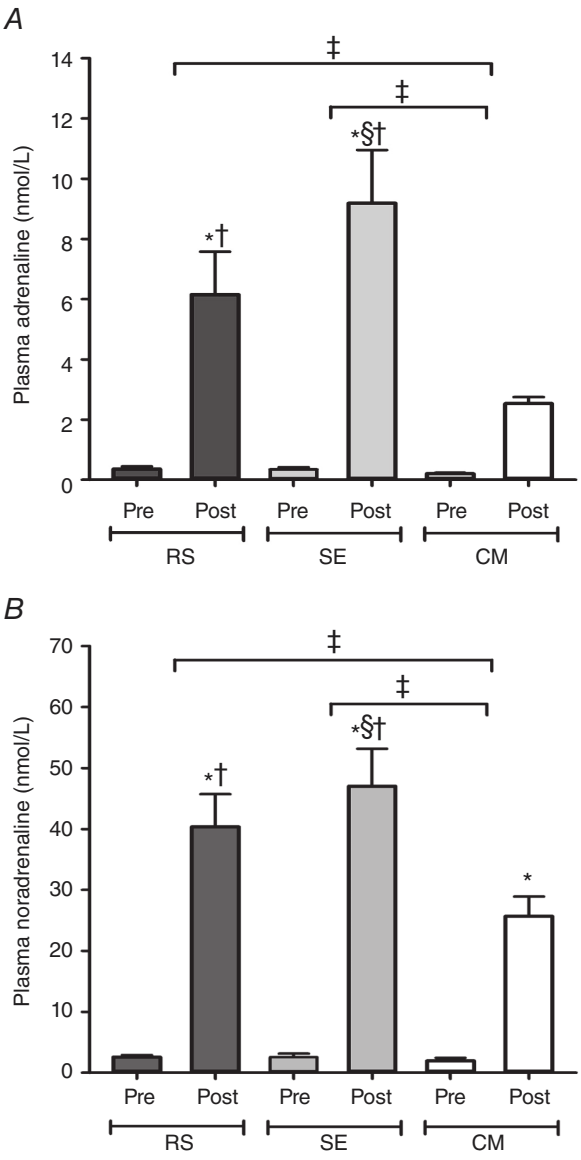

Figure 2. Plasma catecholamines
Venous plasma adrenaline (*A*) and noradrenaline (*B*) concentration before (Pre) and immediately after (Post) repeated-sprint (RS), speed endurance (SE) and continuous moderate-intensity (CM) exercise. Values are mean ± SEM ($n = 12$). *Significantly different from Pre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

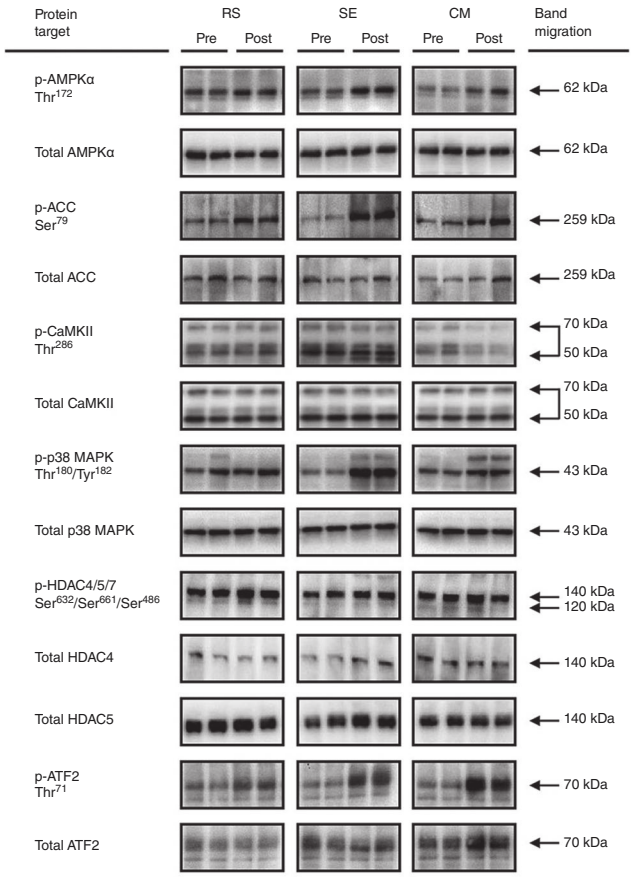

Figure 3. Representative western blots
Representative western blots corresponding to phosphorylated and total protein content measured before (Pre) and immediately after (Post) repeated-sprint (RS), speed endurance (SE) and continuous moderate-intensity (CM) exercise. The molecular mass of band migration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

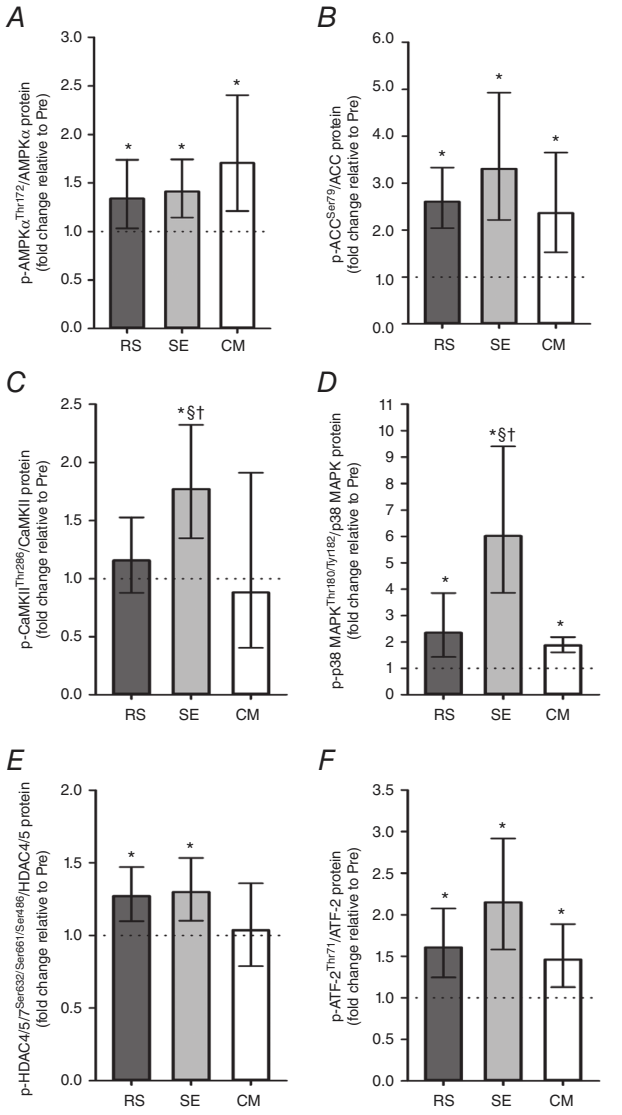

Figure 4. Exercise-induced skeletal muscle intracellular signalling
Repeated-sprint (RS), speed endurance (SE) and continuous moderate-intensity (CM) exercise-induced alterations in phosphorylated AMP-activated protein kinase alpha (AMPKα) protein, acetyl-CoA carboxylase (ACC) protein, Ca^{2+}/calmodulin-dependent 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.

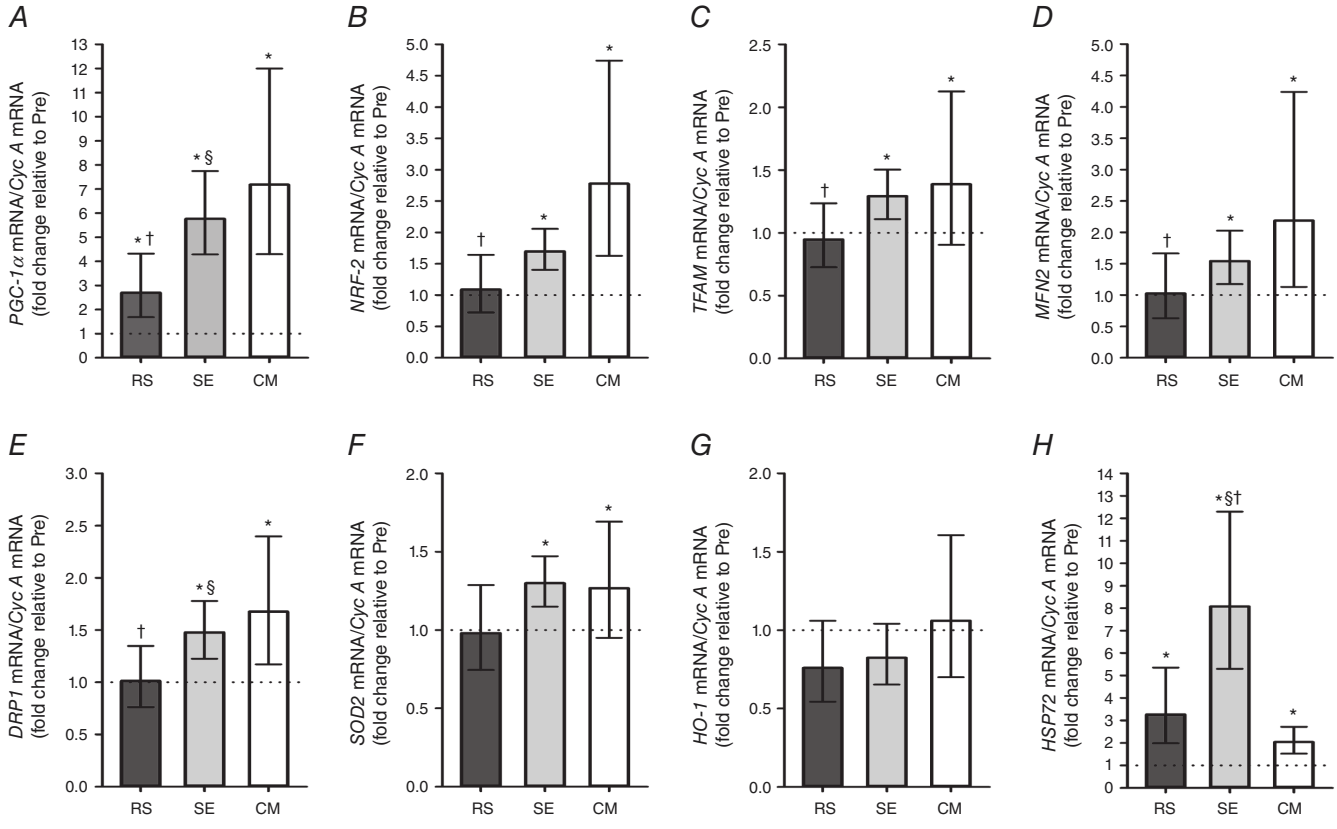

Figure 5. Exercise-induced skeletal muscle mRNA responses
Repeated-sprint (RS), speed endurance (SE) and continuous moderate-intensity (CM) exercise-induced mRNA responses of peroxisome proliferator-activated receptor-*γ* coactivator-1*α* (*PGC-1α*), nuclear respiratory factor 2 (*NRF-2*), mitochondrial 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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Study II

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High-intensity exercise training enhances mitochondrial oxidative phosphorylation efficiency in a temperature-dependent manner in human skeletal muscle: Implications for exercise performance

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Running title:

Training improves OXPHOS and exercise efficiency

Nonstandard abbreviations

OXPHOS, oxidative phosphorylation; CI, CII, CIII, CIV, CV, complexes I-V of the electron transport chain; L_N, normal leak respiration in absence of adenylates; FAO_P, fatty acid oxidative capacity; CI_P, maximal OXPHOS capacity through CI. $CI+CII_P$, maximal OXPHOS capacity through CI and CII combined; L_{Omy}, oligomycin-induced leak respiration; *ETS*, electron transport system respiratory capacity; CII_P, maximal OXPHOS capacity through CII; CS, citrate synthase; HAD, 3-hydroxyacyl-CoA dehydrogenase; MFN2, mitofusin 2; DRP1, dynamin related protein 1; BNIP3, Bcl-2/adenovirus E1B 19 kDa-interacting protein-3; ANT1, adenine nucleotide translocase 1; UCP3, uncoupling protein 3; HSP70, heat shock protein 70; SET, speed endurance training.

Abstract

The purpose of the present study was to investigate whether exercise training-induced adaptations in human skeletal muscle mitochondrial bioenergetics are magnified under thermal conditions resembling sustained intense contractile activity, and whether training-induced changes in mitochondrial oxidative phosphorylation (OXPHOS) efficiency influence exercise efficiency. Twenty healthy men performed six weeks of high-intensity exercise training (SET; n=10) or maintained their usual lifestyle (CON; n=10). Before and after the intervention, mitochondrial respiratory function was determined *ex vivo* in permeabilized muscle fibres under experimentally-induced normothermia (35°C) and hyperthermia (40°C) mimicking *in vivo* muscle temperature at rest and during intense exercise, respectively. In addition, activity/content of muscle mitochondrial enzymes and proteins was quantified. Exercising muscle efficiency was determined *in vivo* by measurements of leg hemodynamics and blood parameters during one-legged knee-extensor exercise. SET enhanced maximal OXPHOS capacity and OXPHOS efficiency at 40°C, but not at 35°C, and attenuated hyperthermia-induced decline in OXPHOS efficiency. Furthermore, SET increased expression of markers of mitochondrial content and up-regulated content of MFN2, DRP1 and ANT1. Also, SET improved exercise efficiency and capacity. These findings indicate that muscle mitochondrial bioenergetics adapts to high-intensity exercise training in a temperature-dependent manner, and that enhancements in mitochondrial OXPHOS efficiency may contribute to improving exercise performance.

Keywords: mitochondrial respiratory function; mitochondrial efficiency; high-intensity interval training (HIIT); uncoupling proteins; exercise efficiency

Introduction

Exercise training promotes multiple changes in skeletal muscle cells (fibres), including substantial adaptations at the mitochondrial level which culminate in expanded mitochondrial volume and increased oxidative capacity (1). While the beneficial effects of exercise training on human skeletal muscle mitochondrial volume are well documented (2), less is known about training-induced adaptations in mitochondrial bioenergetics and their implications for exercise performance.

Mitochondria generate ATP via oxidation of substrates (oxidative phosphorylation). Thus, mitochondrial respiratory function can be assessed from rate of mitochondrial oxygen consumption. For such a purpose, *in vitro* polarographic measurements of oxygen consumption from isolated mitochondria have been utilized for over 60 years (3). However, recent advances in the field of high-resolution respirometry allows for *in situ* assessments of mitochondrial respiratory function from very small samples of permeabilized muscle fibres, being a more physiologically relevant measure of mitochondrial functional properties within an integrated cellular system (4, 5).

Skeletal muscle mitochondrial oxidative phosphorylation (OXPHOS) capacity has been identified as an important determinant of endurance performance in humans (6). Existing evidence supports the beneficial impact of exercise training on mitochondrial respiratory function (7), with training-induced enhancements in maximal OXPHOS capacity being associated with improvements in exercise performance (8-16). On the other hand, intrinsic mitochondrial maximal OXPHOS capacity (i.e. mitochondrial respiratory capacity normalized to mitochondrial content) has been shown to be either unaltered or depressed in response to exercise training (9-11, 17, 18), suggesting that training-induced enhancements in mitochondrial respiratory function are related to an expansion in mitochondrial volume rather than to improvements in mitochondrial respiration *per se*.

Mitochondrial oxidative phosphorylation system relies on the tight coupling between electron transport and ATP synthesis. However, a part of the energy generated by electron transport is uncoupled from ATP synthesis resulting in heat dissipation. Accordingly, mitochondrial OXPHOS coupling efficiency, defined as the molar amount of ATP produced per mole of atomic oxygen consumed (i.e. P/O ratio), or alternatively as the ratio of state 3 to state 4 respiration (i.e. respiratory control ratio (RCR)), is a critical feature of mitochondrial function. In contrast to maximal OXPHOS capacity, OXPHOS efficiency seems less responsive to exercise training, with most (9, 10, 15-17, 19-22), but not all (8), human studies failing to report training-induced enhancements in indeces of mitochondrial coupling efficiency.

Notably, OXPHOS efficiency is highly affected by changes in muscle temperature (23). In mitochondria isolated from rat skeletal muscle, both P/O ratio and RCR decrease with increasing temperature (24). In view of these temperature-dependent alterations, a recent study from Zoladz, et al. (25) demonstrated that endurance training attenuated the decline in OXPHOS efficiency occurring at high assay temperature (42°C) in isolated rat skeletal muscle mitochondria. A shortcoming of studies conducted in humans is that muscle mitochondrial respiratory function is commonly assessed at a standardized temperature of 37°C. While human muscle temperature at rest is within a range of 35-36°C, it may rise up to

40°C during intense exercise (26-28). Accordingly, it should be investigated whether changes in mitochondrial respiratory function attained by a period of exercise training are temperature-dependent in human skeletal muscle. In addition, in light of the exercise intensity-dependency of the training-induced mitochondrial qualitative changes (8, 10, 12), and in view of the transient heat stress incurred during high-intensity exercise (29), it is conceivable that high-intensity exercise training promotes adaptations attenuating the hyperthermia-induced decline in OXPHOS efficiency.

Lastly, given that the overall efficiency of the exercising muscle is determined by the efficiency of the processes providing energy (i.e. mitochondrial coupling) and converting energy to external work (i.e. contractile coupling) (30, 31), it is relevant to examine whether training-induced enhancements in OXPHOS efficiency translate to a better efficiency of the contracting muscle during sustained exercise.

Thus, in the present study, we investigated the effect of high-intensity exercise training on skeletal muscle mitochondrial respiratory function under experimentally-induced hyperthermia resembling muscle temperature during intense exercise. In addition, to examine whether muscle mitochondrial adaptations attained by high-intensity exercise training are associated with changes in exercise efficiency, we integrated *ex vivo* measurements of mitochondrial respiratory function with *in vivo* assessments of exercising muscle efficiency. Our hypotheses were that high-intensity exercise training would improve muscle mitochondrial respiratory capacity and coupling efficiency in a temperature-specific manner, and that these adaptations would be accompanied by improvements in both exercise efficiency and capacity.

Material and Methods

Human subjects and ethics

Twenty-one healthy men were initially included of which 20 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 maximal oxygen consumption ($\dot{V}_{O_2max}$) comprised between 45 and 55 $mL \cdot min^{-1} \cdot kg^{-1}$ and a body mass index of 19-26 $kg \cdot m^{-2}$. Exclusion criteria were abnormal ECG, chronic disease, ongoing pharmacological treatment and smoking. Included subjects were allocated in either a control (CON; n=10) or training (SET; n=10) group. All subjects were recreationally active and their characteristics are presented in Table 1. The study was approved by the regional research ethics committee of Copenhagen, Denmark (H-17004045) and adheres to the principles of the Declaration of Helsinki. The study was registered in clinicaltrials.gov (NCT03317704).

Study design and preliminary tests

This study was designed as a longitudinal randomized controlled trial (Figure 1). Prior to enrolment in the study, each subject underwent an examination for assessment of eligibility criteria, including an incremental test to exhaustion performed on a mechanically braked cycle-ergometer (LC4; Monark Exercise AB, Vansbro, Sweden) for determination of $\dot{V}_{O_2max}$. The test protocol consisted of a 4-min bout at 100 W followed by an incremental graded test with increments of 25 $W \cdot min^{-1}$ until volitional exhaustion. Pulmonary gas exchanges were 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 pulmonary oxygen consumption ($\dot{V}_{O_2}$) despite an increase in workload, and a respiratory exchange ratio above 1.10. Then, subjects returned to the laboratory on a different day to perform an incremental graded test with one leg in a one-legged knee-extensor ergometer for determination of the time to exhaustion and incremental peak power output (iPPO). The test started at 12 W and progressed with increments of 6 $W \cdot min^{-1}$. Subjects were instructed to keep a constant cadence of 60 rpm and the test was terminated when cadence dropped below 55 rpm.

Experimental setup

Two experimental days, separated by 2-4 days, were conducted before (Pre) and after (Post) a 6-week intervention period, with the experimental days at Post being carried out within 5 days after the last training session in SET (Figure 1). Subjects refrained from caffeine, alcohol and exercise for 24 h prior to the experimental days. In addition, subjects were asked to record their food intake for the 48 h before experimental day 1 so that they could replicate the same food intake during the 48 h preceding experimental day 2 as well as during the 48 h preceding experimental day 1 and 2 at Post.

Experimental day 1

Subjects reported to the laboratory in the morning after an overnight fast. After 10 min of supine rest, subjects were whole-body dual-energy X-ray absorptiometry (DXA) scanned. After the scan, subjects were provided with a standardized breakfast consisting of 18.7 g of carbohydrates, 6.8 g of fats, 17.0 g of proteins, and water *ad libitum*. After 15 min of rest in the supine position, a 3-mm incision was made over the lateral portion of the experimental thigh under local anesthesia (2 mL lidocaine without epinephrine, 20 $mg \cdot mL^{-1}$ Xylocain, AstraZeneca, Copenhagen, Denmark) and a biopsy was obtained from the *vastus lateralis* muscle by using a percutaneous Bergstrom needle with suction. At this point, subjects included in SET went through an experimental procedure aimed at determining exercising muscle efficiency.

After local anesthesia (2 mL lidocaine without epinephrine, 20 $mg \cdot mL^{-1}$ Xylocain), catheters (20 gauge; Arrow, Reading, PA) were placed in the femoral artery and vein of the experimental leg. The catheters were placed 2-3 cm below the inguinal ligament and advanced 10 cm in the proximal direction. The correct placement of the catheters was verified by ultrasound Doppler (Vivid E9; GE Healthcare). After ~60 min of rest, subjects were seated in a semi-recumbent position with a hip-angle fixed at ~110° and completed a one-legged knee-extensor exercise test. The test protocol included three exercise bouts at low- (LI_{ex}; 5 min at 30% iPPO), moderate- (MI_{ex}; 5 min at 50% iPPO) and high-intensity (HI_{ex}; 4 min at 80% iPPO). Upon completion of the high-intensity bout, an incremental test was carried out with increments of 6 $W \cdot min^{-1}$. Subjects were instructed to keep a constant cadence of 60 rpm throughout the test, and the incremental test terminated when subjects were not anymore able to maintain a cadence above 55 rpm. Blood was drawn from the femoral artery and vein during the last minute of the three submaximal exercise bouts. In order to account for the transit time of blood from the artery through the muscle capillary bed and to the collection point at the vein (32), the arterial samples were taken ~5 s before the venous samples. In addition, femoral artery blood flow was measured at rest and during the last minute of each submaximal exercise bout.

Subjects included in CON completed the one-legged knee-extensor exercise test as described for SET, but without assessments of exercising muscle efficiency.

Experimental day 2

Subjects reported to the laboratory on a second occasion. Upon arrival in the morning, after an overnight fast, subjects were provided with the same standardized breakfast consumed on experimental day 1. 30 min after the ingestion of the meal, subjects rested in the supine position for 10 min to measure pulmonary gas exchanges at rest. After that, subjects performed a two-legged cycling exercise test on a mechanically braked cycle-ergometer (LC4; Monark Exercise AB). The test protocol included 12 min of warm-up followed by an incremental graded test with increments of 20 $W \cdot min^{-1}$ until volitional exhaustion. During the incremental test, pulmonary gas exchanges were measured breath-by-breath using an on-line gas analysis system (Oxycon Pro, Viasys Healthcare). $\dot{V}_{O_2max}$ was determined as described for the preliminary visit. In SET, time to exhaustion and incremental peak power output (iPPO) were also recorded. Heart rate was

monitored throughout the test and maximal heart rate (HR_{max}) was established as the highest value achieved during the test.

Exercise training intervention

Subjects included in CON were instructed to maintain their usual lifestyle and not to change their physical activity level.

Subjects included in SET performed 6 weeks of cycling-based supramaximal-intensity interval training (i.e. speed endurance training (SET)) consisting of repeated 20-s "all-out" efforts interspersed with 2 min of recovery. Subjects were instructed to pedal as fast as possible during the 20-s sprints and to cycle at low-intensity (50-100 W) during the recovery periods. The number of sprints performed throughout a training session was progressively increased from week 1 to week 5, while it was reduced during week 6 to reduce accumulated fatigue induced by the intense training period while maximizing physiological and performance adaptations (Figure 2). SET was chosen in view of evidence indicating its effectiveness to increase mitochondrial respiratory capacity, up-regulate mitochondrial proteins and enhance performance (7, 33, 34). All training sessions were preceded by a 7-min warm-up at a relative intensity corresponding to ~70% HR_{max}. During the first and the last training session the power outputs during each sprint were recorded, as previously described (35). All training sessions were supervised and subjects wore heart rate monitors (TEAM2 Wearlink+, Polar, Kempele, Finland) throughout the whole training session. Overall, subjects completed 17 training sessions, with training compliance being 100% aside from one subject who missed one training session. On average, the mean heart rate during a training session (i.e. exercise and recovery intervals included) was 81±1% of HR_{max} and peaked at 91±1% HR_{max} (Figure 2).

Measurements and data analysis

Body composition

Whole body muscle mass, fat mass and fat percentage were measured by dual-energy x-ray absorptiometry (DXA) (Lunar iDXA, GE Healthcare Lunar, Belgium). The scanner was calibrated prior to measurements in accordance with the manufacturer's guidelines. Before each scan, subjects rested for 10 min in the supine position to accommodate fluid distribution thereby minimizing estimation error of muscle mass (36, 37). In addition, the composition of the experimental thigh, defined as the area from ischial tuberosity to the patellar groove, was determined using the DXA software (GE Healthcare, encore Forma, V. 15). All scans and analyses were conducted by the same operator.

Muscle biopsies

Sampled biopsies were divided in two portions, the first portion was immediately placed in ice-cold biopsy preservation solution and prepared for assessment of mitochondrial respiratory function, as previously described (5). The second biopsy portion was snap-frozen in liquid N_2 and stored at -80°C ready to be freeze-dried. Muscle specimens were weighed before and after freeze-drying to determine the water content. After freeze-drying the samples, connective tissue, visible fat, and blood were carefully dissected away.

Then, the dissected muscle samples were stored at -80°C until analysis for enzymatic activity and protein content. Both enzymatic activity and protein content were determined in duplicates, i.e. two different samples were obtained from the same muscle specimen after dissection and the mean value of the two samples was used as result. One subject from the control group did not undertake the muscle biopsy sampling at Post due to discomfort associated with the procedure.

Mitochondrial respiratory function

Mitochondrial respiratory function was measured in permeabilized muscle fibres by high-resolution respirometry (Oxygraph-2k, Oroboros, Austria). A substrate-uncoupler-inhibitor titration (SUIT) protocol was applied to measure specific features of mitochondrial respiration. Respirometric analyses were carried out in duplicate at a chamber temperature of either 35°C or 40°C (i.e. 4 respiration chambers were used, two chambers at 35°C and two chambers at 40°C). Instrumental and chemical oxygen background fluxes were calibrated as a function of oxygen concentration and subtracted from the total volume-specific oxygen flux (Datlab v.6.1 software, Oroboros Instruments). Oxygen levels were maintained between 200 and 500 μM to prevent potential oxygen diffusion limitation.

Mitochondrial respiratory capacity. The SUIT protocol was specific for the analysis of multiple mitochondrial respiratory states induced via separate titrations, as previously described (38). The SUIT protocol started with addition of malate (2 mM) and octanoyl carnitine (0.2 mM) to induce normal leak respiration (L_N) in absence of adenylates. Then, ADP (5 mM) without the addition of Mg^{2+} was titrated to determine fatty acid oxidative capacity (FAO_P). Pyruvate (5 mM) and glutamate (10 mM) were added for measurement of maximal OXPHOS capacity through complex I (CI_P). Succinate (10 mM) was then added to induce maximal OXPHOS capacity through complex I and II combined ($CI+CII_P$), corresponding to maximal state 3 respiration. Addition of cytochrome *c* (10 μM) allowed for assessment of mitochondrial outer membrane intactness and was used as an internal control for compromised integrity of the mitochondrial preparation. If mitochondrial respiratory capacity increased more than 10% after addition of cytochrome *c* the measurements were not used in the analyses. Oligomycin (1 μM) was then added to inhibit ATP synthase and to measure oligomycin-induced leak respiration (L_{Omy}), which is the leak state corresponding to $CI+CII_P$ and is comparable with state 4 respiration (39). After that, a series of stepwise carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) titrations (1.5-3.0 μM) were carried out to reach the electron transport system respiratory capacity (*ETS*) state. Rotenone (0.5 μM) was then added to inhibit complex I, so that maximal OXPHOS capacity through CII (CII_P) could be measured. Lastly, antimycin A (2.5 μM) was added to terminate respiration and allow for the determination and correction of residual oxygen consumption (ROX), indicative of non-mitochondrial oxygen consumption in the chamber. One experiment from the control group was not used in the analyses due to high cytochrome *c* response.

Mitochondrial OXPHOS efficiency. The respiratory control ratio (RCR), as determined by the ratio of state 3 (i.e. $CI+CII_P$) to state 4 (i.e. L_{Omy}) respiration, was used as an index of OXPHOS coupling efficiency. For statistical and conceptual reasons, the RCR was replaced by $1\text{-}RCR^{-1} = 1\text{-}L_{Omy}/CI+CII_P$ (40).

Enzymatic activity

Maximal enzyme activity of citrate synthase (CS) and 3-hydroxyacyl CoA dehydrogenase (HAD) were quantified in muscle homogenates using fluorometric method (Fluoroscan Ascent; Thermo Scientific, Waltham, MA, USA) at 25°C, as previously described (41). Enzymatic activity was determined in duplicates from the same muscle specimen. Enzymatic activity was normalized to mg of total protein.

Protein content in homogenate lysate

Protein content in muscle homogenate lysates was determined by SDS-PAGE and Western blot analyses. In short, freeze-dried muscle samples (~2 mg dw) were homogenized for 1 min at 29 Hz (Qiagen Tissuelyser II, Retsch GmbH, Haan, Germany) in a fresh-batch of ice-cold buffer (10% glycerol, 20 mM Na-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 PMSF, 1 mM EDTA (pH 8), 1 mM EGTA (pH 8), 10 $\mu g \cdot mL^{-1}$ aprotinin, 10 $\mu g \cdot mL^{-1}$ 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 double distilled H_2O to reach equal protein concentration. Equal amounts of protein (range: 6-12 µg) were loaded in each well of either 4-15% or 16.5% pre-cast gels (Bio-Rad Laboratories, Hercules, CA, USA). Samples from each subject were loaded on the same gel, with the samples from before training (Pre) being placed adjacent to the samples after training (Post). The same pool of a mixed human muscle standard lysate was loaded in three different wells per gel and the average intensity of these samples was used for normalization to allow gel-to-gel comparison, as previously described (42). Proteins were separated according to their molecular weight by SDS page gel electrophoresis and semi-dry transferred to a PVDF membrane (Millipore, Denmark). The membranes were blocked in either 2-5% skim milk or 3% BSA in TBST before being incubated overnight at 4°C in primary antibody diluted in either 2-5% skim milk or 3% BSA (see Table S1). After washing in a Tris-buffered saline, membranes were incubated with a secondary antibody for ~1 h at room temperature. The secondary horseradish peroxidase-conjugated antibodies used were diluted 1:5000 in 2-5% skim milk or 3% BSA depending on the primary antibody (P-0447, P-0448, and P-0449; DakoCytomation). 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).

Blood variables, leg $\dot{V}_{O_2}$ and leg $\dot{V}_{CO_2}$

<u>*Blood sample analyses*</u>**.** Femoral arterial and venous blood samples were drawn in heparinized tubes for immediate analyses of P_{O_2}, P_{CO_2}, O_2 saturation (S_{O_2}), haemoglobin concentration ([Hb]), bicarbonate concentration ($[HCO_3^-]$) and pH using an ABL800 Flex (Radiometer, Copenhagen, Denmark).

Femoral artery blood flow. Femoral artery blood flow was measured with ultrasound Doppler (Vivid E9; GE Healthcare) equipped with a linear probe operating at an imaging frequency of 8.0 MHz and Doppler frequency of 3.1 MHz as previously described (43).

Calculations. Leg O_2 consumption ($\dot{V}_{O_2}$) and CO_2 release ($\dot{V}_{CO_2}$) were calculated using Fick's principle:

$$\dot{V}_{O_2} = F \times (Ca_{O_2} - Cv_{O_2})$$

$$\dot{V}_{CO_2} = F \times (Ca_{CO_2} - Cv_{CO_2})$$

Where F is the femoral artery blood flow times the arteriovenous difference in content of the given gas.

Content of O_2 and CO_2 in arterial and venous blood were computed using the equations from Siggaard-Andersen, et al. (44).

Exercise efficiency

Gross and net efficiency during one-legged knee-extensor exercise were determined as follows:

$$Gross\ efficiency\ (\%) = [Work_{(kcal/min)} / EEE_{(kcal/min)}] \times 100$$

$$Net\ efficiency\ (\%) = [Work_{(kcal/min)} / (EEE_{(kcal/min)} - REE_{(kcal/min)})] \times 100$$

Where EEE and REE represent the energy expenditure during exercise and at rest, respectively.

REE was calculated from the resting values of leg $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ using the table of nonprotein respiratory quotient proposed by Peronnet and Massicotte (45). To account for the glycogen oxidation occurring during exercise, EEE was calculated from the leg $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ measured during the final minute of LI_{ex}, MI_{ex} and HI_{ex} using the equations proposed by Jeukendrup and Wallis (46), as follows:

$$EEE_{LI_{ex}} = 0.575 \times \dot{V}_{CO_2} - 4.435 \times \dot{V}_{O_2}$$

$$EEE_{MI_{ex},\, HI_{ex}} = 0.550 \times \dot{V}_{CO_2} - 4.471 \times \dot{V}_{O_2}$$

Statistics

Between-group differences in subject characteristics at baseline and after the intervention period were determined using a linear mixed model with group (CON, SET) and time (Pre, Post) as fixed factors and subjects as random factor. To estimate between-group differences in the training-induced changes, as well as within-time (Pre vs. Post) and between-group (CON vs. SET) differences, a linear mixed model was used with group-time interaction as a fixed factor, subjects as random factor, and with baseline $\dot{V}_{O_2max}$ and baseline value of the outcome variable included as covariates. For mitochondrial respiratory function measurements, a linear mixed model was used with group-time-temperature interaction as a fixed factor, subjects as random factor, and baseline $\dot{V}_{O_2max}$ and baseline value of the outcome variable included as covariates. To estimate differences in the hyperthermia-induced changes in mitochondrial OXPHOS capacity and efficiency, a linear mixed model was used with group-time-temperature interaction as a fixed factor, subjects as random factor, and baseline $\dot{V}_{O_2max}$ and 35°C value of the outcome variable included as covariates. For exercise efficiency measurements, a linear mixed model was used with time as a fixed factor, subjects as random factor, and baseline $\dot{V}_{O_2max}$ and baseline value of the outcome variable included as

covariates. Model checking was based on Shapiro Wilk's test and Q-Q plots. In 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- and within-group differences. The level of significance for all analyses was defined as $P<0.05$. Statistical analyses were carried out with R ver. 3.4.1 and the extension packages *lme4* and *multcomp*. Absolute values are presented as means ± SEM. Fold-changes are presented as means ± 95% confidence intervals.

Results

Anthropometrics and cardiorespiratory fitness

Age, weight, height, body mass index and $\dot{V}_{O_2max}$ were not different between the high-intensity exercise training (SET) and the control (CON) group at baseline (Pre) (Table 1). Muscle mass and fat percentage of the experimental thigh increased by 0.2±0.0 kg (P=0.001) and decreased by 0.8±0.3 %-points (P=0.015), respectively, with training in SET, whereas no changes were observed in CON (Table 1). Pulmonary $\dot{V}_{O_2max}$ ($mL \cdot min^{-1} \cdot kg^{-1}$) increased by 10±1 % with training in SET (P<0.001) and remained unchanged in CON, with the change being greater in SET than in CON (P<0.001) (Table 1).

Mitochondrial respiratory capacity

Mitochondrial respiration values are reported in Table 2, while training- and hyperthermia-induced changes in mitochondrial respiratory function are presented in Figure 3.

In SET, L_N decreased with training when analyzed at 40°C (P<0.001), but not at 35°C, with the change at 40°C being greater than that observed at 35°C (temperature×time interaction; P<0.001) as well as than that observed in CON (group×time interaction; P<0.001) (Figure 3B). In addition, temperature-dependent training-induced change in L_N was different between SET and CON (group×time×temperature interaction; P<0.001). In SET, $CI+CII_P$ increased with training at 40°C only (P<0.001), with the change being greater than that observed in CON (group×time interaction; P=0.004) and with temperature-dependent training-induced change being different between SET and CON (group×time×temperature interaction; P=0.005). In SET, L_{Omy} increased with training at both 35°C (P=0.002) and 40°C (P=0.008).

Mitochondrial respiratory capacity did not change in CON at either 35°C or 40°C during the 6 weeks.

Mitochondrial OXPHOS efficiency

OXPHOS efficiency (i.e. $1-L_{Omy}/CI+CII_P$) increased with training in SET when analyzed at 40°C, but not at 35°C, and was unchanged in CON, with the training-induced change in OXPHOS efficiency at 40°C being greater than that observed at 35°C (temperature×time interaction; P=0.001) as well as than that observed in CON (group×time interaction; P<0.001) (Figure 3C). In addition, temperature-dependent training-induced change in OXPHOS efficiency was greater in SET than CON (group×time×temperature interaction; P=0.039) (Figure 3C).

OXPHOS efficiency was lower at 40°C than at 35°C in SET and CON at both Pre and Post (P<0.01) (Figure 3D). In SET, hyperthermia-induced decrease in OXPHOS efficiency was smaller at Post than Pre (temperature×time interaction; P=0.038), and temperature-dependent training-induced change was greater in SET than CON (group×time×temperature interaction; P=0.001) (Figure 3D).

Enzymes and proteins modulating mitochondrial function

Maximal activity of CS and HAD increased with training in SET (P<0.001 and P=0.017) and did not change in CON, and the changes in SET were larger than in CON (P=0.001 and P=0.003) (Table 3).

Protein content of subunits from the electron transport chain complexes I, II, III and IV increased with training in SET (P<0.001, P=0.003, P=0.001, P=0.007) and were unchanged in CON, with changes in subunits from CI, CII and CIII being greater in SET than in CON (P<0.001, P=0.005 and P=0.030) (Figure 4). IDH did not change with the intervention in either SET or CON. MFN2 and DRP1 increased with training in SET (P=0.001 and P=0.004), but not in CON, with the changes being greater in SET than in CON (P=0.041 and P=0.017). BNIP3, UCP3 and HSP70 were unaffected by the intervention in either SET or CON, while ANT1 was up-regulated in SET only (P=0.029).

Exercise efficiency

Femoral artery blood flow during one-legged knee-extensor exercise at low-, moderate- and high-intensity was lower after than before the training intervention in SET (all P<0.05) (Table 4). Leg $\dot{V}_{O_2}$ (mL·min^{-1}) and leg $\dot{V}_{O_2}$ relative to thigh muscle mass (mL·min^{-1}·kg muscle^{-1}) during one-legged knee-extensor exercise at low-, moderate- and high-intensity decreased with training in SET (all P<0.001). Gross (GE) and net efficiency (NE) during one-legged knee-extensor exercise at low-, moderate-, and high-intensity improved with training in SET (GE, P=0.001, P<0.001, P<0.001; NE, P=0.003, P=0.001, P<0.001).

Exercise performance

Time to exhaustion and iPPO achieved during incremental one-legged knee-extensor exercise increased with training in SET (all P<0.001) and were unchanged in CON, with the change being greater in SET than CON (all P<0.001) (Table 5). In SET, the training intervention increased time to exhaustion and iPPO achieved during incremental two-legged cycling exercise from 526±26 to 612±18 s (P<0.001) and from 355±14 to 384±12 W (P<0.001), respectively.

In SET, the peak and the mean power output achieved during the first versus the last training session increased from 1025±55 to 1094±56 W (P=0.021) and from 796±44 to 827±39 W (P=0.030), respectively. Likewise, the mechanical work completed during the first versus the last training session increased from 48.6±3.4 to 53.3±3.1 kJ (P<0.001).

Discussion

Herein we demonstrate that six weeks of high-intensity exercise training promote an increase in mitochondrial maximal OXPHOS capacity and OXPHOS efficiency under experimentally-induced hyperthermia (40°C), but not in normothermia (35°C). Furthermore, the high-intensity exercise training attenuated hyperthermia-induced decline in OXPHOS efficiency. The training period increased expression of muscle markers of mitochondrial content and up-regulated abundance of MFN2, DRP1 and ANT1. Hyperthermia-specific training-induced enhancements in mitochondrial maximal OXPHOS capacity and OXPHOS efficiency were accompanied by improvements in gross and net efficiency during low-, moderate- and high-intensity one-legged knee-extensor exercise, as well as by improvements in performance during both incremental one-legged knee-extensor and two-legged cycling exercise.

Temperature-specific training-induced adaptations in muscle mitochondrial respiratory function

To the best of our knowledge, the present study is the first to show training-induced enhancements in OXPHOS efficiency in permeabilized fibres from human skeletal muscle, with prior evidence failing to detect substantial changes in indeces of mitochondrial coupling efficiency following a period of exercise training (9, 10, 15, 17, 19, 20). However, in view of the observed discrepancy between normothermia- and hyperthermia-related training adaptations, comparisons with other studies using different assay temperatures should be made carefully.

The training-induced increase in maximal OXPHOS capacity observed at 40°C is in line with the increased $CI+CII_P$ (measured at 37°C) reported following a period of either high-intensity interval (9, 11, 15, 18, 20), resistance training (19) or a combination of different training modalities (14, 20, 47). At the same time, the lack of training-induced changes in maximal OXPHOS capacity observed at 35°C is consistent with the absence of alterations in $CI+CII_P$ (measured at 37°C) documented following either moderate-intensity continuous (10, 12, 13, 17, 47), high-intensity interval (9, 10, 12, 48) or resistance training (14, 20, 47). Of note, the few studies utilizing a training regimen that included brief supramaximal-intensity efforts, as in the current study, reported either an increase in $CI+CII_P$ in permeabilized fibres (10) or a decrease in $CI+CII_P$ in isolated mitochondria (49), suggesting that between-study comparisons should account for the methodological approach employed for measuring mitochondrial OXPHOS capacity.

Overall, the enhancements in OXPHOS efficiency and maximal OXPHOS capacity were only apparent at 40°C, thus implying thermal-specificity of the training-induced adaptations in mitochondrial respiratory function.

Besides training-induced alterations in OXPHOS capacity and efficiency, our results show that normal leak respiration (L_N) decreased with training under hyperthermia only, whereas oligomycin-induced leak respiration (L_{Omy}) increased with training irrespective of assay temperature. As L_N represents mitochondrial O_2 consumption in absence of adenylates, thereby resembling mitochondrial respiration rate in a resting state, it can be argued that the training intervention promoted an adaptive response possibly lowering energy dissipation when resting muscle is exposed to heat stress. Conversely, given that L_{Omy}

represents leak respiration associated with maximal OXPHOS capacity, it appears that the training intervention was ineffective in reducing energy dispertion of the contracting muscle at either normothermic or hyperthermic conditions. However, it should be noted that, under hyperthermia, the training-induced increase in L_{Omy} was paralleled by an increase in maximal OXPHOS capacity ($CI+CII_P$), resulting in higher OXPHOS efficiency, as previously discussed. In spite of training-induced enhancements in $CI+CII_P$, the high-intensity exercise training did not affect maximal OXPHOS capacity through either complex I (CI_P) or complex II (CII_P) as well as maximal fat oxidation capacity (FAO_P) and electron transport system capacity (*ETS*), a finding partly consistent with studies reporting a mismatch between changes in these respiratory states and increments in $CI+CII_P$ (10, 11, 15, 18). From a methodological point of view, it is worth noting that *ETS* was lower than $CI+CII_P$ (see Table 2); an observation that may be related to the inhibitory effect of oligomycin on maximal uncoupled respiration (50, 51).

A new aspect of the present study was to investigate the effects of experimentally-induced hyperthermia *per se* on mitochondrial respiratory function in permeabilized fibres from human skeletal muscle. The observation that maximal OXPHOS capacity ($CI+CII_P$) did not increase with increasing assay temperature (see Figure 3D) contrasts with the hyperthermia-induced increments in state 3 respiration documented in rodents (24, 25, 52). This dissimilarity, besides possibly depending on inter-species differences, may be attributed to the different methodological approaches employed to measure mitochondrial respiration (i.e. high-resolution respirometry in permeabilized fibres vs. Clark-type electrodes in isolated mitochondria). It is worth mentioning that while maximal OXPHOS capacity decreased with hyperthermia in SET at baseline, this was not the case for CON. Such a finding, considering that SET exhibited a trend for a lower maximal OXPHOS capacity than CON in normothermia at baseline (see Table 2), suggests that skeletal muscle characterized by lower maximal OXPHOS capacity are more prone to be affected by heat stress. This assumption is further supported by the observation that hyperthermia-induced impariments in maximal OXPHOS capacity disappeared concomitantly with an increase in maximal OXPHOS capacity following the intervention in SET.

The observed hyperthermia-induced decrease in OXPHOS efficiency is in line with the decline in P/O ratio and RCR occurring at high temperature in isolated rat skeletal muscle mitochondria (24, 25, 52). Overall, the present temperature-dependent alterations in mitochondrial respiratory function are in accordance with studies in isolated mitochondria from rat cardiac and hepatic tissue reporting no hyperthermia-induced increments in maximal OXPHOS capacity in the heart (53) and hyperthermia-induced decrements in mitochondrial coupling in both the heart and the liver (53, 54).

Notably, we observed that the high-intensity exercise training attenuated the hyperthermia-induced decline in OXPHOS efficiency, an adaptation in accordance with the dampened hyperthermia-induced impairments in OXPHOS efficiency documented following a period of endurance exercise training in rats (25).

Taken together, the present outcomes not only support the effectiveness of high-intensity exercise training in promoting mitochondrial qualitative changes, but also highlight that these adaptations may only occur or be detected within a thermal environment resembling that associated with sustained

intense muscle contraction. Thus, our results suggest that assay temperatures similar to those achieved by the exercising muscle should be utilized during *ex vivo* assessments of training-induced changes in mitochondrial respiratory function. From a physiological standpoint, our findings possibly indicate a mitochondrial hormetic response to heat stress, whose magnitude is amplified under experimentally-induced hyperthermia.

High-intensity exercise training up-regulates muscle proteins modulating mitochondrial function

The high-intensity exercise training period increased protein content of subunits from complexes I, II, III and IV of the electron transport chain as well as CS activity, indicating that increments in mitochondrial content occurred in concert with the observed modifications in mitochondrial respiratory function. Such a finding, while being supported by reports showing concomitant increments in mitochondrial content and mitochondrial respiratory capacity (9, 11, 12, 17, 18), points against the previously proposed dissociation between mitochondrial content and respiratory function (10, 55). On the other hand, we observed no training-induced changes in content of IDH; an observation in contrast to other research showing an increase in IDH following a period of intense interval training (34).

Aside from adaptations in mitochondrial content, we observed that expression of MFN2 and DRP1 increased with the training intervention, possibly indicating an enhanced capacity for mitochondrial dynamic remodelling. These adaptations are in line with prior data indicating up-regulated MFN2 and DRP1 mRNA content in response to a single bout of high-intensity exercise in human skeletal muscle (56). In addition, while the observed training-induced increase in MFN2 is in agreement with other studies (10, 12, 17), this is not the case for the training-induced increase in DRP1 (10), suggesting that the training intervention adopted in the current study enhanced the capacity of the mitochondria to undergo both fusion and fission events. On the other hand, we observed no apparent changes in BNIP3 with the training intervention, which may indicate that the capacity for mitophagy regulation was unaltered by the training undertaken. Other studies have observed increased BNIP3 levels following 8 weeks of exercise training in young healthy men (57), with the different response being possibly attributed to the shorter duration and the lower volume of the training intervention utilized in the present study.

Given that temperature-dependent impairments in mitochondrial coupling efficiency have been proposed to depend on hyperthermia-induced proton leak (52), we explored whether the protein levels of ANT1 and UCP3, which are involved in mitochondrial proton leak (58), were altered by the training intervention. We observed that the high-intensity exercise training intervention increased content of ANT1, which is consistent with that reported in response to six weeks of endurance training (59). The training-induced concomitant increase in ANT1 and maximal OXPHOS capacity coincides with observations in other species showing a connection between rise in ANT1 and increase in OXPHOS capacity (60). Indeed, upregulation of ANT1 abundance may serve as a protective mechanism to prevent excess ROS production via a mild uncoupling lowering the protonmotive force (58). Moreover, it is relevant to point out that, besides being involved in proton leak, ANT1 affects mitochondrial ADP sensitivity by driving ADP inport

across the inner mitochondrial membrane (61). Thus, it may be that, in light of evidence indicating temperature-dependent changes in mitochondrial ADP sensitivity (62), the observed hyperthermia-specific training-induced enhancement in OXPHOS efficiency occurred via ANT1-mediated alterations in ADP sensitivity.

The observation that UCP3 abundance was unaltered by the training intervention suggests that training-induced improvements in mitochondrial coupling efficiency were likely not related to a reduced UCP-mediated proton leak. Unaltered UCP3 content has been reported following a period of either endurance (59) or high-intensity intermittent exercise training (63). However, it cannot be excluded that the training intervention lowered UCP3 activity rather than abundance, as previously observed in rats (25). Thus, future studies should investigate the importance of both content and activity of ANT1 and UCP3 for training-induced adaptations in mitochondrial respiratory function.

To further explore the potential mechanisms underlying the temperature-specificity of the observed training-induced mitochondrial adaptations, we quantified changes in HSP70, a molecular chaperone and cytoprotective protein mediating enhancement of mitochondrial respiratory capacity in diseased animal models (64, 65). Interestingly, protein levels of HSP70 were not altered by exercise training in the present study, suggesting a minor role for HSP70 in modulating changes in mitochondrial bioenergetics in healthy skeletal muscle.

High-intensity exercise training enhances exercise efficiency and performance

The present study shows that enhancements in mitochondrial OXPHOS efficiency elicited by high-intensity exercise training are accompanied by improvements in exercise efficiency, and that this occurs independently of reductions in abundance of uncoupling proteins. This is in contrast to studies indicating that improvements in mitochondrial coupling and exercise efficiency are associated with reductions in protein content of ANT1 (66) and that higher exercise efficiency positively correlates with lower mRNA and protein content of UCP3 (67-71).

Although a lack of association between mitochondrial coupling efficiency and cycling efficiency has been reported in a cross-sectional study comparing untrained and trained individuals (67), the concomitant enhancements in mitochondrial and exercise efficiency observed in the present study suggest that training-induced changes in mitochondrial respiratory function may have contributed to augment exercise efficiency. Given that increments in pulmonary $\dot{V}_{O_2}$ closely reflect increments in the exercising muscle $\dot{V}_{O_2}$ (72), training-induced changes in exercise efficiency are commonly inferred from measurements of pulmonary gas exchange during cycle ergometry (73). However, in the present study, we measured leg $\dot{V}_{O_2}$ during one-legged knee-extensor exercise; a methodological approach that allows for accurate assessments of efficiency of the contracting skeletal muscle by excluding possible increments in O_2 cost associated with processes occurring outside the exercising muscle (e.g. respiratory and cardiac muscle work).

Consistent with the well-established importance of exercise efficiency for endurance performance (74) and given the purported association between fatigue and muscle inefficiency during high-intensity exercise (75),

it is conceivable that the observed improvements in exercise efficiency along with the ~5% increase in $\dot{V}_{O_2max}$ partly explain the greater peak power and time to exhaustion achieved during the incremental exercise. Taken together, the mitochondrial qualitative changes elicited by the high-intensity exercise training period may have played a prominent role in enhancing exercise performance.

Summary and perspectives

The present study demostrates that high-intensity exercise training promotes beneficial adaptations in human muscle mitochondrial oxidative phosporylation capacity and efficiency, but only when *ex vivo* mitochondrial respiratory function is assessed under experimentally-induced hyperthermia mimicking *in vivo* muscle temperature during intense exercise. These findings, not only indicate temperature-dependency of training-induced mitochondrial qualitative changes, but also provide novel methodological insights into assessments of mitochondrial respiratory function in human permeabilized muscle fibres. Furthermore, the exercise training period attenuated the hyperthermia-induced decline in mitochondrial coupling efficiency, suggesting that high-intensity exercise training improves the bioenergetic efficiency of skeletal muscle during sustained intense contractile activity. These mitochondrial qualitative changes were associated with quantitative changes in enzymes and proteins important for mitochondrial function, including regulators of either mitochondrial dynamics or mitochondrial uncoupling. Importantly, we report the novel observation that the mitochondrial qualitative and quantitative changes elicited by the high-intensity exercise training were accompanied by enhancements in both exercise efficiency and capacity, thus highlighting the physiological relevance of the observed mitochondrial adaptations (Figure 5).

Given that the P/O ratio can only be directly measured in isolated mitochondria, and in view of the intended integrative approach of the present study, mitochondrial oxidative phosphorylation efficiency was assessed in a preserved myocellular system (i.e. permebabilized fibre bundles) via a methodological approach indirectly determining P/O ratio by assuming absolute ATP production from a known amount of ADP. In view of this limitation, alternative methods to quantify P/O ratio in permeabilized muscle fibres have been recently proposed (76), however further methodological development is necessary to account for other factors possibly affecting P/O ratio, including ATP/ADP recycling, proton leak and slip, cation cycling and ROS production.

In addition, it should be noted that muscle temperature represents only one of the physiological factors to be considered when modelling *in vivo* conditions during *ex vivo* respiratory assessments (77). Thus, future studies should evaluate the impact of further parameters (i.e. O_2 availability, metabolite and ion concentrations) in mimicking *in vivo* skeletal muscle conditions. Lastly, simultaneous assessments of changes in mitochondrial dynamics, ultrastructure (i.e. cristae density) and supercomplexes formation are warranted to unravel the specific mechanisms whereby exercise training enhances the efficiency of mitochondrial energy transduction.

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Author contributions

M. Fiorenza, A. K. Lemminger, J. Bangsbo and M. Hostrup designed the study. M. Fiorenza, A. K. Lemminger and K. Eibye conducted the experiments. M. Marker conducted the training and collected data. M. Fiorenza and A. K. Lemminger analyzed data. M. Fiorenza, A. K. Lemminger, J. Bangsbo and M. Hostrup drafted the manuscript. All authors contributed to data interpretation, critically revised the manuscript for important intellectual content, and approved the final version of the manuscript. All authors agree to be accountable for all aspects of the manuscript in ensuring that questions related to the accuracy or integrity of any part of the manuscript are appropriately investigated and resolved.

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Figures

Figure 1. Schematic presentation of the study design

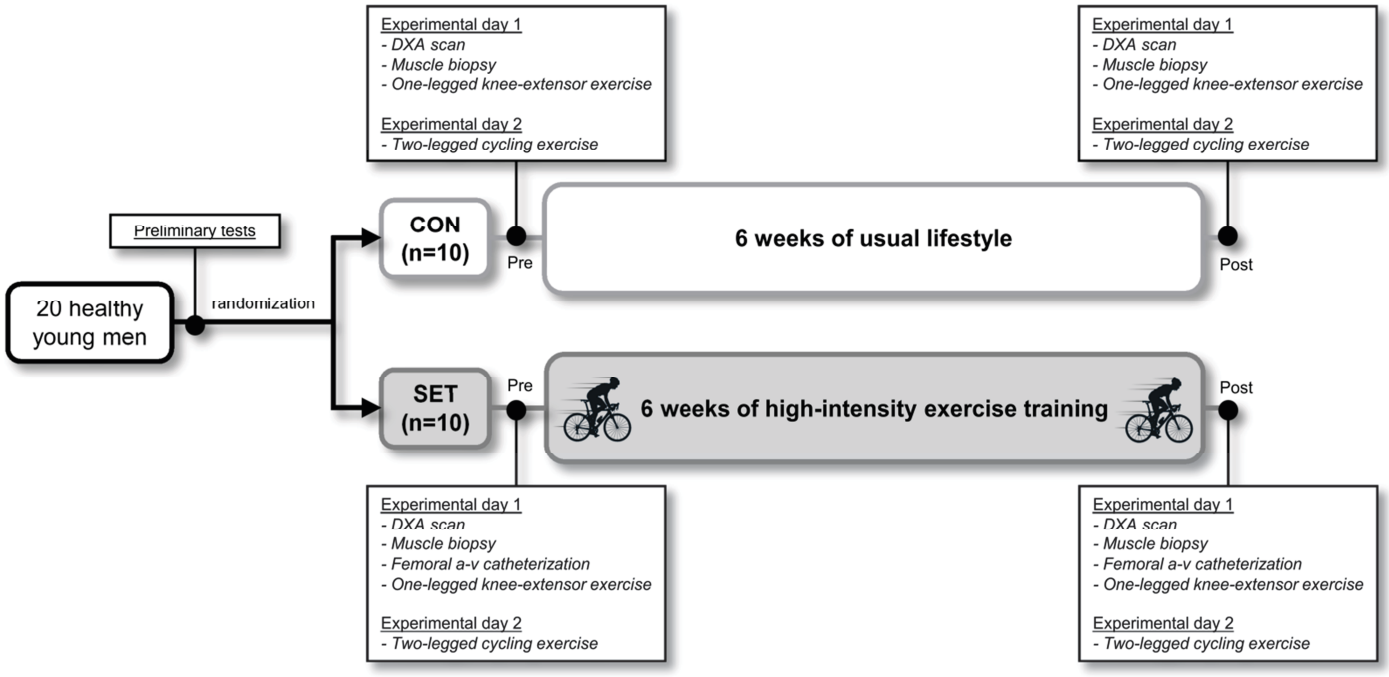
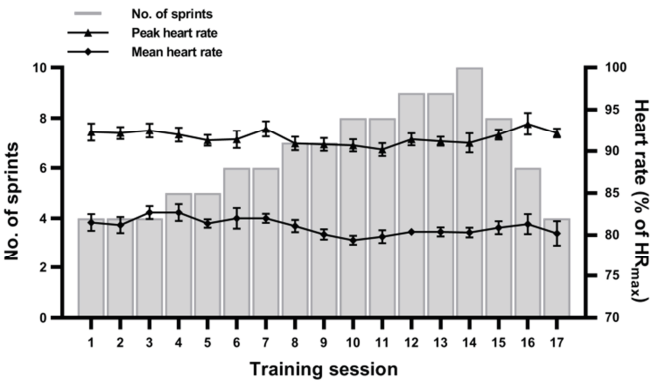
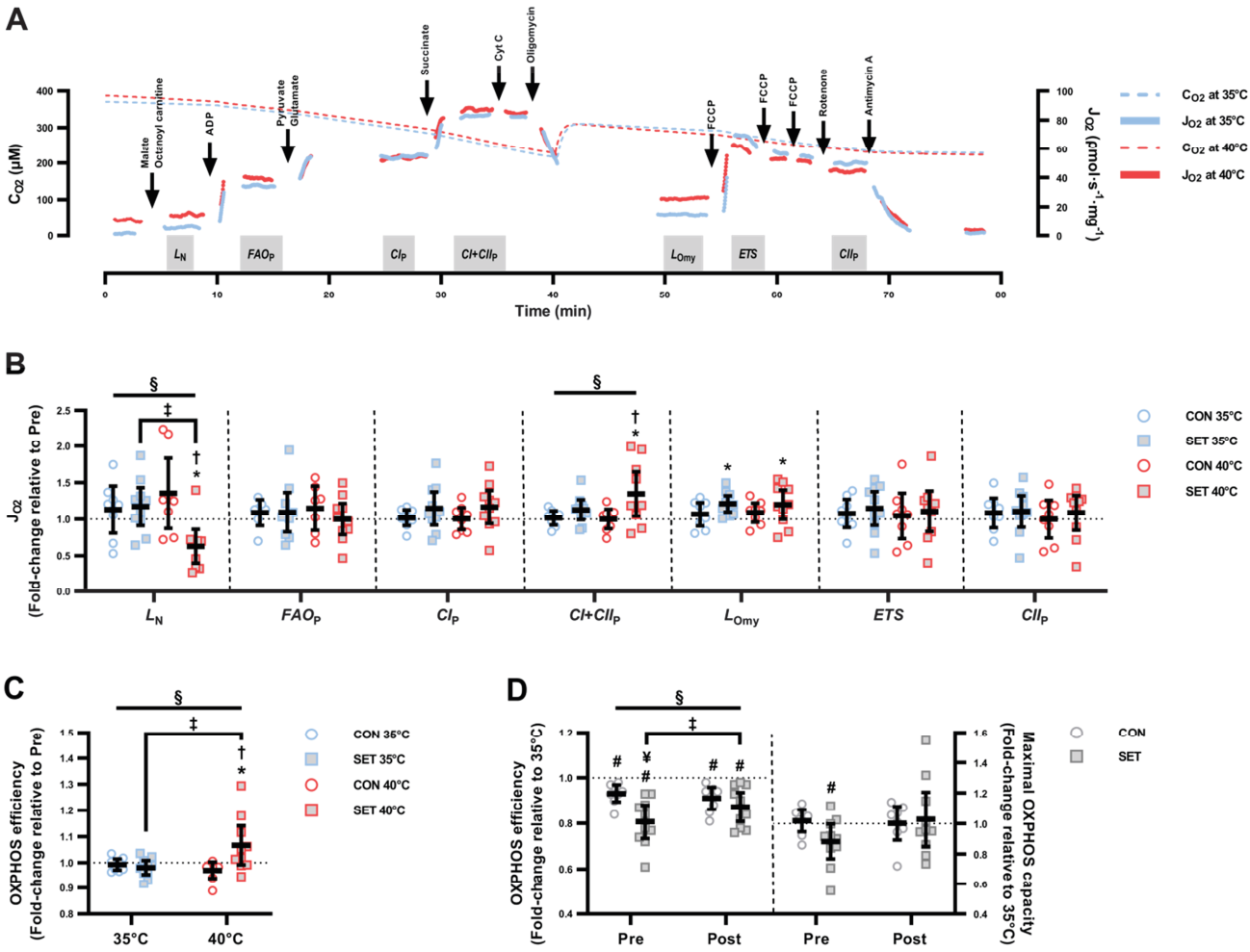


Figure 2. Number of sprints and heart rate during the high-intensity exercise training period



HR_{max}, maximal heart rate. Heart rate values are means ± SEM.

Figure 3. Mitochondrial respiratory function



(A) Representative track of oxygen concentration (C_{O_2}) and mass-specific mitochondrial respiration (O_2 flux, J_{O_2}) measured in permeabilized fibres from human *vastus lateralis* at 35°C and 40°C.

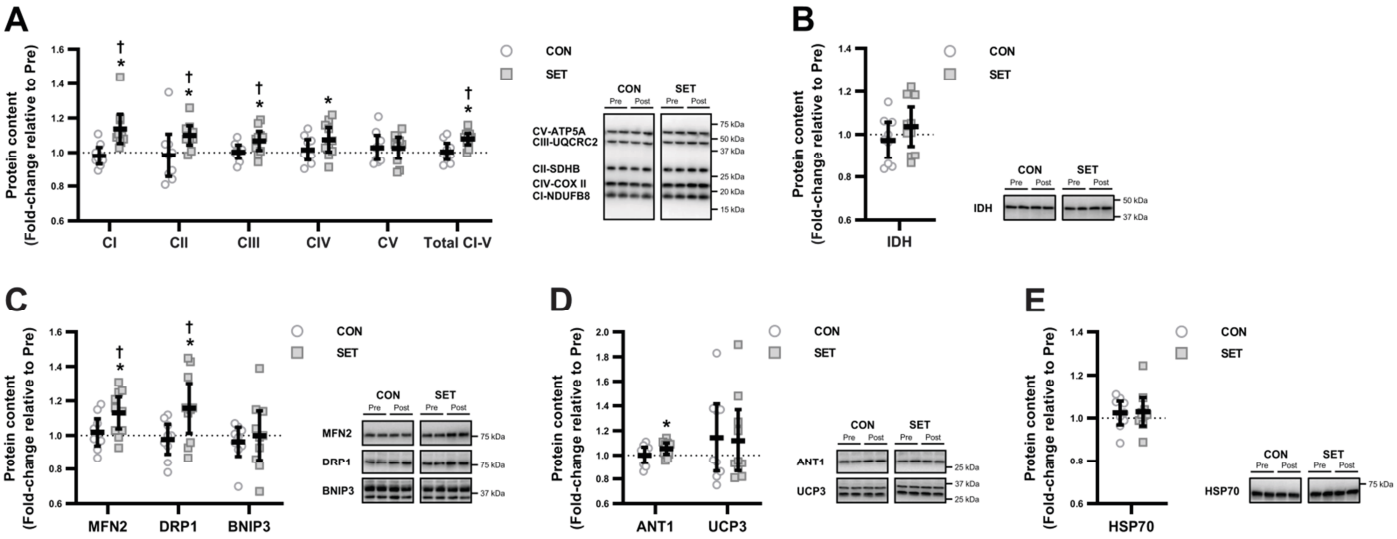
(B) Training-induced changes in mass-specific mitochondrial respiratory capacity measured at 35°C and 40°C.

(C) Training-induced changes in OXPHOS efficiency (1-L_{Omy}/CI+CII_P) measured at 35°C and 40°C.

(D) Hyperthermia-induced changes in OXPHOS efficiency (1-L_{Omy}/CI+CII_P) and mass-specific maximal OXPHOS capacity (CI+CII_P) before (Pre) and after (Post) the control (CON) and the high-intensity exercise training (SET) period.

n=8 (CON) and n=10 (SET); Data presented as individual values with means ± 95% confidence intervals. *Significant difference between Pre and Post (P < 0.05). †Significant difference between CON and SET in the training-induced change (group×time interaction) (P < 0.05). ‡Significant difference between 35°C and 40°C in the training-induced change (temperature×time interaction) (P < 0.05). §Significant difference between CON and SET in the temperature-dependent training-induced change (group×time×temperature interaction) (P < 0.05). #Significant difference between 35°C and 40°C (P < 0.05). ¥Significant difference between CON and SET in the hyperthermia-induced change (group×temperature interaction) (P < 0.05).

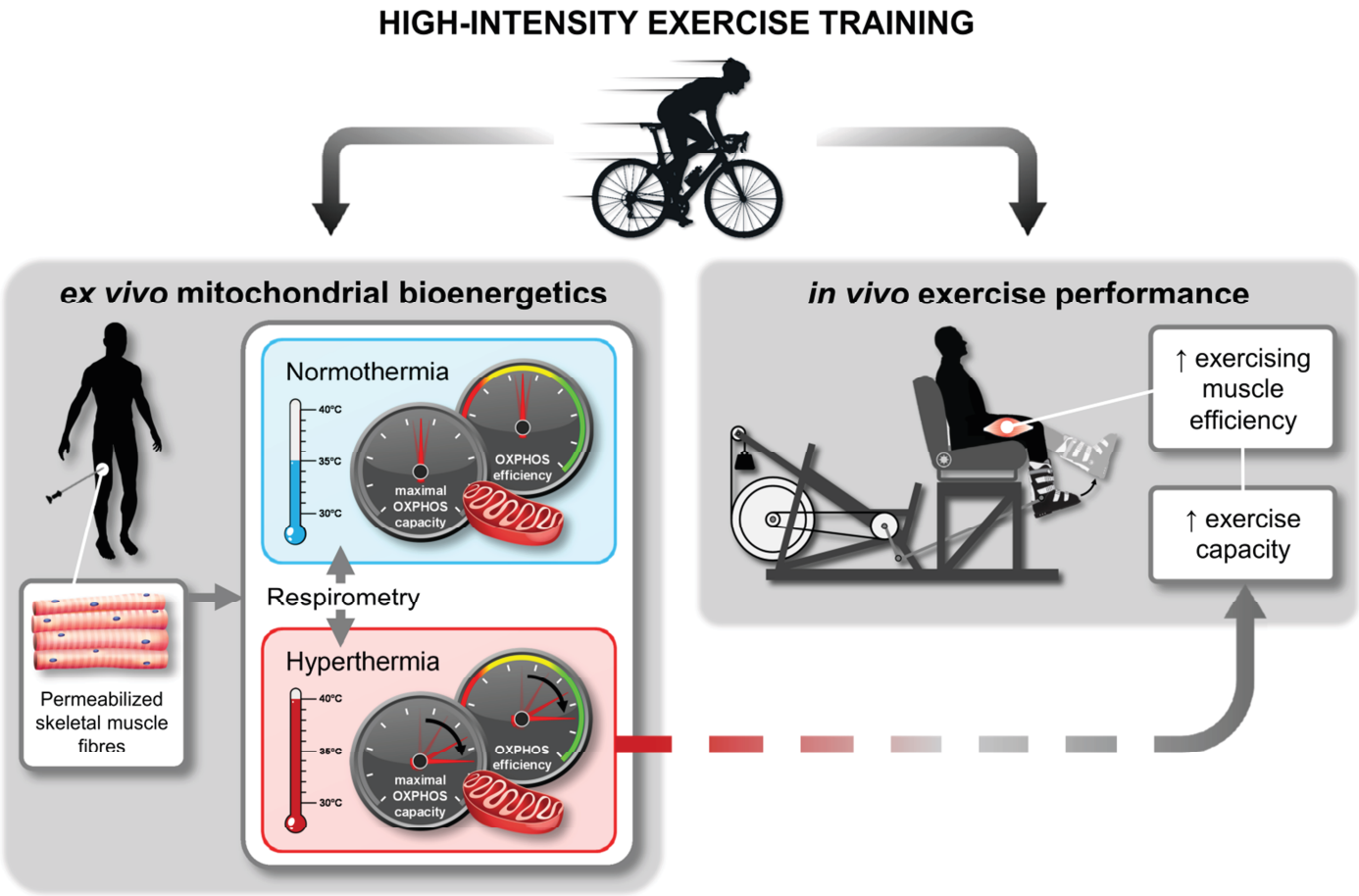
Figure 4. Proteins modulating mitochondrial function



(A-D) Training-induced changes in protein content of subunits of the electron transport chain complexes I-V (A), isocitrate dehydrogenase (B), markers of mitochondrial turnover (C), uncoupling proteins (D), and heat shock protein 70 (E).

n=9 (CON) and n=10 (SET); Data presented as individual values with means ± 95% confidence intervals. *Significant difference between Pre and Post ($P < 0.05$). †Significant difference between CON and SET in training-induced change (group×time interaction) ($P < 0.05$).

Figure 5. Proposed association between high-intensity exercise training-induced adaptations in muscle mitochondrial bioenergetics and exercise performance.



The effects of high-intensity exercise training on *ex vivo* mitochondrial respiratory function are magnified under experimentally-induced hyperthermia mimicking *in vivo* muscle temperature during intense exercise. The hyperthermia-specific training-induced changes in mitochondrial OXPHOS efficiency are accompanied by enhancements in overall efficiency of the exercising muscle, which possibly contribute to improving the capacity for sustained exercise.

Tables

Table 1. Subject characteristics before (Pre) and after (Post) the control (CON) and the high-intensity exercise training (SET) period.

	CON (n=10)		SET (n=10)	
	Pre	Post	Pre	Post
Age (years)	25.7 ± 1.2		23.0 ± 1.1	
Height (m)	1.84 ± 0.02		1.86 ± 0.02	
Weight (kg)	76.0 ± 2.5	75.9 ± 2.6	79.6 ± 3.8	79.5 ± 3.7
Body mass index ($kg \cdot m^{-2}$)	22.4 ± 0.5	22.4 ± 0.5	23.0 ± 0.8	23.0 ± 0.8
Whole body muscle mass (kg)	61.5 ± 2.7	61.5 ± 2.7	60.4 ± 2.8	60.8 ± 2.8
Whole body fat mass (kg)	11.7 ± 1.1	11.5 ± 1.1	16.2 ± 1.7 †	15.7 ± 1.5 †
Whole body fat (%)	15.5 ± 1.6	15.3 ± 1.6	20.0 ± 1.5 †	19.5 ± 1.5
Experimental thigh muscle mass (kg) §	7.05 ± 0.37	7.03 ± 0.36	6.94 ± 0.40	7.13 ± 0.39 *
Experimental thigh fat mass (kg)	1.21 ± 0.12	1.20 ± 0.13	1.61 ± 0.17	1.57 ± 0.17
Experimental thigh fat (%)	14.8 ± 1.6	14.7 ± 1.6	18.6 ± 1.4	17.9 ± 1.4 *
Energy expenditure at rest ($kcal \cdot min^{-1}$)	1.50 ± 0.10	1.53 ± 0.06	1.65 ± 0.11	1.80 ± 0.14
$\dot{V}_{O_2max}$ ($mL \cdot min^{-1}$) §	4053 ± 262	3932 ± 206	3976 ± 192	4377 ± 184 *
$\dot{V}_{O_2max}$ ($mL \cdot min^{-1} \cdot kg^{-1}$) §	52.8 ± 2.0	51.5 ± 1.5	50.2 ± 1.4	55.3 ± 1.4 *

$\dot{V}_{O_2max}$, maximal pulmonary oxygen consumption. *Significantly different from Pre ($P < 0.05$). †Significantly different from CON ($P < 0.05$). §Significant group×time interaction ($P < 0.05$). Values are means ± SEM.

Table 2. Mitochondrial respiration values

		Normothermia (35°C)		Hyperthermia (40°C)	
		CON (n=8)	SET (n=10)	CON (n=8)	SET (n=10)
J_{O_2} (pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$)					
L_N	Pre	6.7 ± 0.7	4.4 ± 0.5 †	9.0 ± 1.1	9.2 ± 0.7 #
	Post	7.0 ± 0.5	4.9 ± 0.5	10.9 ± 1.0 #	5.6 ± 0.9 *†
FAO_P	Pre	31.8 ± 3.3	31.3 ± 4.1	35.4 ± 3.1 #	30.6 ± 3.9
	Post	34.0 ± 3.4	31.4 ± 2.6	39.3 ± 3.6 #	28.5 ± 2.8 †#
CI_P	Pre	64.8 ± 6.3	47.5 ± 4.6 †	61.7 ± 5.4	41.7 ± 4.4 †#
	Post	65.3 ± 5.9	52.4 ± 4.3	61.1 ± 4.9	45.8 ± 3.6 #
$CI+CII_P$	Pre	97.7 ± 8.7	79.3 ± 7.0	98.4 ± 7.9	70.0 ± 7.5 †#
	Post	98.4 ± 8.1	88.0 ± 6.8	97.3 ± 7.5	87.0 ± 5.4 *
L_{Omy}	Pre	17.4 ± 1.6	15.7 ± 1.0	23.0 ± 1.9 #	22.9 ± 1.0 #
	Post	18.5 ± 2.0	19.0 ± 1.4 *	24.5 ± 1.5 #	27.2 ± 1.9 *#
ETS	Pre	63.6 ± 5.5	71.1 ± 7.5	50.4 ± 3.4 #	56.1 ± 4.5 #
	Post	68.7 ± 8.1	76.7 ± 5.4	52.0 ± 6.4 #	59.3 ± 4.7 #
CII_P	Pre	38.1 ± 3.9	48.1 ± 3.9 †	32.8 ± 2.9	39.7 ± 2.7 #
	Post	40.4 ± 4.1	50.8 ± 3.5 †	32.1 ± 3.6 #	41.8 ± 3.3 †#
OXPHOS efficiency					
$1-L_{Omy}/CI+CII_P$	Pre	0.82 ± 0.01	0.80 ± 0.01	0.76 ± 0.02 #	0.64 ± 0.03 †#
	Post	0.81 ± 0.01	0.78 ± 0.01	0.74 ± 0.02 #	0.68 ± 0.02 *†#

*Significantly different from Pre (P < 0.05). †Significantly different from CON (P < 0.05). #Significantly different from 35°C (P < 0.05). Values are means ± SEM

Table 3. Maximal activity of mitochondrial enzymes before (Pre) and after (Post) the control (CON) and the high-intensity exercise training (SET) period.

	CON (n=9)		SET (n=10)	
	Pre	Post	Pre	Post
CS (µmol·min^{-1}·g $protein^{-1}$) §	77.8 ± 6.1	74.1 ± 6.5	76.2 ± 6.1	90.1 ± 4.7 *†
HAD (µmol·min^{-1}·g $protein^{-1}$) §	27.3 ± 2.1	24.5 ± 1.9	27.5 ± 2.0	31.0 ± 1.6 *†

CS, citrate synthase; HAD, 3-hydroxyacyl-CoA dehydrogenase. *Significantly different from Pre (P < 0.05). †Significantly different from CON (P < 0.05). §Significant group×time interaction (P < 0.05). Values are means ± SEM.

Table 4. Leg hemodynamics, $\dot{V}_{O_2}$ and efficiency during one-legged knee-extensor exercise before (Pre) and after (Post) the high-intensity exercise training (SET) period.

	SET (n=10)	
	Pre	Post
Low-intensity exercise (30% iPPO)		
Workload (W)	22 ± 1	
Femoral artery blood flow (L·min^{-1})	3.04 ± 0.21	2.65 ± 0.12 *
a-v $_{O_2}$ difference (mL·L^{-1})	151 ± 6	147 ± 3
Leg $\dot{V}_{O_2}$ (mL·min^{-1})	456 ± 30	391 ± 23 *
Leg $\dot{V}_{O_2}$ (mL·min^{-1}·kg muscle^{-1})	65.6 ± 1.9	55.0 ± 1.5 *
Leg RQ	0.71 ± 0.06	0.72 ± 0.06
GE (%)	17.8 ± 1.3	20.5 ± 1.1 *
NE (%)	19.4 ± 1.4	22.5 ± 1.3 *
Moderate-intensity exercise (50% iPPO)		
Workload (W)	37 ± 2	
Femoral artery blood flow (L·min^{-1})	3.80 ± 0.21	3.59 ± 0.19 *
a-v $_{O_2}$ difference (mL·L^{-1})	167 ± 6	158 ± 4 *
Leg $\dot{V}_{O_2}$ (mL·min^{-1})	635 ± 44	570 ± 37 *
Leg $\dot{V}_{O_2}$ (mL·min^{-1}·kg muscle^{-1})	91.7 ± 4.6	79.8 ± 2.8 *
Leg RQ	0.82 ± 0.03	0.83 ± 0.05
GE (%)	21.1 ± 0.9	23.5 ± 0.8 *
NE (%)	22.4 ± 1.0	25.1 ± 1.0 *
High-intensity exercise (80% iPPO)		
Workload (W)	59 ± 3	
Femoral artery blood flow (L·min^{-1})	4.56 ± 0.23	4.08 ± 0.21 *
a-v $_{O_2}$ difference (mL·L^{-1})	178 ± 6	174 ± 4
Leg $\dot{V}_{O_2}$ (mL·min^{-1})	808 ± 45	714 ± 48 *
Leg $\dot{V}_{O_2}$ (mL·min^{-1}·kg muscle^{-1})	115.1 ± 5.6	100.5 ± 5.3 *
Leg RQ	0.97 ± 0.03	0.93 ± 0.04
GE (%)	27.8 ± 0.8	30.3 ± 0.9 *
NE (%)	29.2 ± 0.8	31.9 ± 1.0 *

a-v $_{O_2}$ difference, arteriovenous difference of O_2; RQ, respiratory quotient; GE, gross efficiency; NE, net efficiency. *Significantly different from Pre ($P < 0.05$). Values are means ± SEM.

Table 5. One-legged knee-extensor exercise performance before (Pre) and after (Post) the control (CON) and the high-intensity exercise training (SET) period.

	CON (n=10)		SET (n=10)	
	Pre	Post	Pre	Post
Time to exhaustion (s) [§]	315 ± 15	306 ± 14	315 ± 34	451 ± 37 *[†]
iPPO (W·kg muscle^{-1}) [§]	10.1 ± 0.9	10.0 ± 0.8	11.4 ± 0.9	14.6 ± 1.1 *[†]

iPPO, peak power output during the incremental test. *Significantly different from Pre ($P < 0.05$). [†]Significantly different from CON ($P < 0.05$). [§]Significant group×time interaction ($P < 0.05$). Values are means ± SEM.

Study III

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ORIGINAL ARTICLE

ACTA PHYSIOLOGICA

High-intensity exercise training ameliorates aberrant expression of markers of mitochondrial turnover but not oxidative damage in skeletal muscle of men with essential hypertension

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Abstract

Aim: To examine whether hypertensive individuals exhibit altered muscle mitochondrial turnover and redox homeostasis compared with healthy normotensive counterparts, and whether the antihypertensive effect of high-intensity exercise training is associated with improved mitochondrial quality and enhanced anti-oxidant defence.

Methods: In a cross-sectional and longitudinal parallel design, 24 essential hypertensive (HYP) and 13 healthy normotensive (NORM) men completed 6 weeks of high-intensity interval training (HIIT). Twenty four-hour ambulatory blood pressure, body composition, cardiorespiratory fitness, exercise capacity and skeletal muscle characteristics were examined before and after HIIT. Expression of markers of mitochondrial turnover, anti-oxidant protection and oxidative damage was determined in *vastus lateralis* muscle biopsies. Muscle protein levels of eNOS and VEGF, and muscle capillarity were also evaluated.

Results: At baseline, HYP exhibited lower expression of markers of mitochondrial volume/biogenesis, mitochondrial fusion/fission and autophagy along with depressed eNOS expression compared with NORM. Expression of markers of anti-oxidant protection was similar in HYP and NORM, whereas oxidative damage was higher in HYP than in NORM. In HYP, HIIT lowered blood pressure, improved body composition, cardiorespiratory fitness and exercise capacity, up-regulated markers of mitochondrial volume/biogenesis and autophagy and increased eNOS and VEGF protein content. Furthermore, in HYP, HIIT induced divergent responses in markers of mitochondrial fusion and anti-oxidant protection, did not affect markers of mitochondrial fission, and increased apoptotic susceptibility and oxidative damage.

Conclusion: The present results indicate aberrant muscle mitochondrial turnover and augmented oxidative damage in hypertensive individuals. High-intensity exercise training can partly reverse hypertension-related impairments in muscle mitochondrial turnover, but not redox imbalance.

KEYWORDS

apoptosis, autophagy, endothelium nitric oxide synthase (eNOS), mitochondrial biogenesis, mitochondrial dynamics, oxidative stress

1 | INTRODUCTION

Elevated blood pressure is among the leading risk factors contributing to the global burden of disease and to global mortality,[1] with more than one fourth of the world's adult population predicted to be affected by hypertension in 2025.[2] Oxidative stress, defined as a dysregulation between production and removal of reactive oxygen species (ROS), is thought to contribute to the development of multiple factors associated with essential hypertension, including vascular resistance, increased sympathetic outflow, altered baroreflex sensitivity and nephron damage.[3] A primary mechanism responsible for oxidative stress-induced vascular resistance is the decrease of nitric oxide (NO) bioavailability due to scavenging by excessive levels of ROS,[4] ultimately leading to endothelial dysfunction. ROS originate from sources located in endothelial, smooth muscle and skeletal muscle cells, counting xanthine oxidase, NAD(P)H oxidase, and mitochondria, which interact with each other in a crosstalk fashion.[5–7] Mounting evidence supports a prominent role of mitochondria-derived ROS in the development of hypertension,[8] with decreases in mitochondrial ROS production recently shown to improve vascular relaxation and reduce blood pressure in experimental models of hypertension.[9] Oxidative stress is inextricably linked with mitochondrial dysfunction, as mitochondria are not only a source but also a target of ROS.[10] Specifically, mitochondrial DNA, lipids and proteins are particularly vulnerable to oxidative damage, resulting in dysfunctional organelles, which further contribute to an elevation in ROS production by a ROS-induced ROS release mechanism.[11] This scenario generates a vicious cycle that, in aging or pathological circumstances, may exacerbate a pre-existing state of oxidative stress and mitochondrial dysfunction.

Mitochondria are dynamic organelles undergoing a constant turnover regulated by cellular pathways including biogenesis, remodelling dynamics and autophagy, which ensure the maintenance of a functional mitochondrial pool.[12] Defective mitochondrial turnover leads to accumulation of dysfunctional organelles, which, by excess ROS generation, may contribute to hypertension-related endothelial dysfunction. Mitochondrial biogenesis may restrain endothelial dysfunction by the synthesis of new mitochondrial proteins which enhance organelle functional properties, thus limiting excess ROS formation. Accordingly, dysregulated mitochondrial biogenesis has been reported in concert with increased mitochondrial ROS generation in aged endothelial cells.[13] Mitochondrial remodelling by fusion/fission cycles is crucial for mitochondrial quality control, with fusion events restoring the functionality of defective organelles through the formation of a reticular network with neighbouring undamaged mitochondria, while fission events promote the segregation of severely damaged mitochondria from the mitochondrial network. A causative relationship has been proposed to exist between mitochondrial remodelling dynamics and ROS generation,[14,15] with increased mitochondrial fission suggested to impair endothelial function via increased ROS formation.[16] To complete the mitochondrial quality control axis, mitophagy (i.e. mitochondria-specific autophagy) selectively degrades organelles segregated by fission events, a process regulated by changes in ROS production in a feed-forward mechanism[17] and reported to affect endothelial function.[18] Lastly, in case of overwhelming oxidative damage, mitochondria are more prone to trigger apoptotic signalling pathways by releasing a variety of pro-apoptotic factors,[19] which could consequently target the endothelium and induce endothelial cell death.[20] Collectively, the complex interplay between mitochondrial turnover, redox state and endothelial function suggests a critical role for mitochondria in the development and progression of essential hypertension.

Interestingly, although hypertension is often viewed only as a cardiovascular disease, multiple lines of evidence indicate functional and morphological alterations in skeletal muscle from hypertensive human subjects and rodent models of essential hypertension,[21–28] implying a potential crosstalk between organelles in vascular and skeletal muscle cells. In support of this, endothelium-derived NO is thought not only to modulate vascular tone but also to regulate skeletal muscle mitochondrial turnover,[29,30] suggesting that impairments in NO bioavailability may negatively impact muscle mitochondrial quality and vice versa. Furthermore, increased mitochondrial ROS production and oxidative damage have been recently observed in skeletal muscle from patients with peripheral arterial disease,[31] a vascular disorder often associated with hypertension.

Exercise training is a well-established antihypertensive therapeutic intervention[32] and has been documented to reduce oxidative stress and increase NO bioavailability in animal models of essential hypertension.[33–35] In addition, exercise training promotes a number of beneficial muscle mitochondrial adaptations, including increased mitochondrial volume and enhanced expression of proteins regulating mitochondrial fusion (i.e. MFN1/2, OPA1), fission (i.e. DRP1, FIS1) and autophagy (i.e. LC3, p62).[36] Emerging evidence indicates that high-intensity interval training (HIIT) lowers blood pressure to a higher extent than traditional endurance exercise training.[37–39] Also, HIIT prevents oxidant-mediated muscle dysfunction and normalizes endothelial function while protecting against elevated oxidative stress in hypertensive mice.[28] However, the physiological mechanisms underlying the greater antihypertensive effect of HIIT are still unclear. Consistent with the concept of mitohormesis,[40] one explanation could be that the transient marked ROS bursts generated during HIIT activate signalling pathways promoting mitochondrial

adaptive responses to render the whole cell less susceptible to future perturbations in redox homeostasis. Thus, HIIT may amplify the endurance exercise training-induced reduction in oxidative stress via more marked improvements in mitochondrial quality and anti-oxidant capacity. Taken together, in spite of the putative involvement of mitochondria-derived oxidative stress in the pathophysiology of essential hypertension and the potential crosstalk between vascular and skeletal muscle mitochondria, it is still unknown whether human hypertension is associated with defective muscle mitochondrial turnover and/or impaired muscle redox balance, and whether the blood pressure-lowering effect of HIIT is associated with changes in the capacity to regulate these processes.

Thus, the aim of the present study was to test the hypothesis that skeletal muscle of hypertensive individuals is characterized by altered expression of markers of mitochondrial volume/biogenesis, mitochondrial dynamics, autophagy, mitochondria-mediated apoptosis, anti-oxidant protection and oxidative damage compared with healthy normotensive controls. Moreover, it was hypothesized that HIIT would improve muscle mitochondrial turnover and anti-oxidant defences while reducing oxidative damage in skeletal muscle of sedentary men with essential hypertension.

2 | RESULTS

2.1 | Compliance and physiological response to training

Compliance to training was ~98% for both groups. On average, NORM and HYP completed 15.8 and 15.7 out of 16 planned training sessions, respectively.

During HIIT (warm-up not included), average heart rate was 80 ± 1% of HR_{max} in both groups (Figure 1). Relative training time spent above 80% and 90% of HR_{max} was 62 ± 3% and 16 ± 4% respectively, in NORM, and 56 ± 3% and 11 ± 2% respectively, in HYP.

2.2 | Blood pressure, body composition, cardiorespiratory fitness and exercise capacity

Twenty four-hour systolic, diastolic and mean ambulatory blood pressure were higher in HYP than in NORM both before (Pre) and after (Post) HIIT ($P < 0.001$) (Table 1). Twenty four-hour ambulatory blood pressure decreased with HIIT in HYP only ($P < 0.01$).

Body weight, fat free mass, fat mass, fat percentage and visceral fat mass were not different between HYP and NORM at either Pre or Post. Body weight, fat mass, fat percentage and visceral fat mass decreased with HIIT in both HYP and NORM ($P < 0.05$), whereas fat free mass increased with HIIT in HYP only ($P < 0.01$).

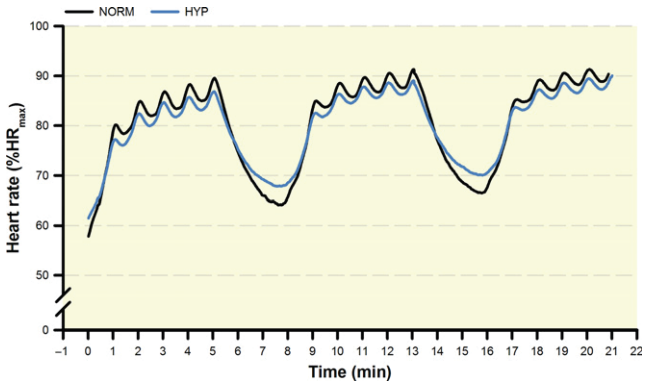

FIGURE 1 Heart rate response to training. Heart rate during the high-intensity exercise training (i.e. 3 × 5 min bouts based on the 10-20-30 training concept) in healthy normotensive (NORM, n = 13) and hypertensive (HYP, n = 24) men

Cardiorespiratory fitness and exercise capacity were not different between groups at Pre. $\dot{V}_{O2max}$ (mL min^{-1}) and $\dot{V}_{O2max}$ relative to body weight (mL kg^{-1} min^{-1}) increased with HIIT in both HYP and NORM ($P < 0.05$). $\dot{V}_{O2}$ during low- and moderate-intensity exercise decreased with HIIT in both HYP and NORM ($P < 0.05$). Time to exhaustion and peak power output during the incremental test (iPPO) increased with HIIT in both HYP and NORM ($P < 0.001$).

2.3 | Muscle markers of mitochondrial turnover

Maximal citrate synthase (CS) activity was lower in HYP than in NORM at Pre ($P < 0.01$), and increased with HIIT in HYP only ($P < 0.001$), with a greater HIIT-induced change compared with NORM ($P < 0.05$) (Figure 2A). Maximal 3-hydroxyacyl-CoA dehydrogenase (HAD) activity was not different between groups at either Pre or Post, and increased with HIIT in HYP only ($P < 0.01$).

Protein content of CS, cytochrome C oxidase subunit IV (COXIV) and estrogen-related receptor alpha (ERRα) was lower in HYP than in NORM at Pre ($P < 0.05$) (Figure 2B). CS and COXIV increased with HIIT in HYP only ($P < 0.01$).

Protein content of mitofusin 1 (MFN1) and optic atrophy 1 (OPA1) was not different between groups at either Pre or Post and was down-regulated by HIIT in HYP ($P < 0.01$). Protein content of mitofusin 2 (MFN2) and dynamin related protein 1 (DRP1) was lower in HYP than in NORM at both Pre and Post ($P < 0.05$), and MFN2 was up-regulated by HIIT in HYP ($P < 0.01$) (Figure 2C).

Protein content of LC3-II (i.e. lipidated active form of microtubule-associated protein 1 light chain 3 (LC3)) was lower in HYP than in NORM at both Pre and Post ($P < 0.01$), and increased with HIIT in NORM only ($P < 0.05$), resulting in a greater HIIT-induced change compared with HYP ($P < 0.05$) (Figure 2D). Similarly, LC3-II/LC3-I ratio increased with HIIT in NORM only ($P < 0.01$),

TABLE 1 Subject characteristics

	NORM (n = 13)		HYP (n = 24)	
Variable	**Pre**	**Post**	**Pre**	**Post**
Age (y)	58.4 ± 2.5		60.8 ± 1.5	
Height (m)	1.81 ± 0.02		1.77 ± 0.01	
Weight (kg)	84.8 ± 3.4	83.5 ± 3.3*	88.0 ± 2.0	87.3 ± 2.1*
24-h ambulatory blood pressure[a]				
Systolic (mm Hg)	137.2 ± 2.6	136.1 ± 3.0	153.9 ± 1.7†	148.9 ± 2.2*†
Diastolic (mm Hg)§	80.5 ± 1.0	81.0 ± 1.6	92.9 ± 1.1†	89.2 ± 1.3*†
Mean (mm Hg)§	99.4 ± 1.4	99.8 ± 6.0	113.2 ± 1.1†	109.1 ± 1.5*†
Body composition[b]				
Fat free mass (kg)	56.9 ± 2.3	57.2 ± 2.2	56.5 ± 1.1	57.2 ± 1.2*
Fat mass (kg)	24.9 ± 1.7	23.1 ± 1.8*	27.9 ± 1.1	26.3 ± 1.1*
Fat percentage (%)	30.2 ± 1.5	28.5 ± 1.6*	32.9 ± 0.8	31.3 ± 0.8*
Visceral fat mass (g)	1373 ± 173	1208 ± 174*	2010 ± 134	1841 ± 151*
Cardiorespiratory fitness and exercise capacity[c]				
$\dot{V}_{O2max}$ (mL min^{-1})	3085 ± 149	3266 ± 168*	2930 ± 103	3018 ± 114*
$\dot{V}_{O2max}$ (mL kg^{-1} min^{-1})	36.3 ± 1.1	39.0 ± 1.3*	32.7 ± 1.0	34.0 ± 1.1*†
$\dot{V}_{O2}$ at 50% $\dot{V}_{O2max}$ (mL min^{-1})	1545 ± 105	1443 ± 113*	1378 ± 56	1262 ± 57*
$\dot{V}_{O2}$ at 65% $\dot{V}_{O2max}$ (mL min^{-1})	2098 ± 129	1994 ± 146*	1842 ± 75	1685 ± 84*
Time to exhaustion (min)	9.7 ± 0.8	11.1 ± 0.8*	8.0 ± 0.4	9.1 ± 0.4*
iPPO (W)	277 ± 16	306 ± 17*	231 ± 9	260 ± 10*

Characteristics of healthy normotensive (NORM) and hypertensive (HYP) subjects before (Pre) and after (Post) 6 weeks of high-intensity exercise training. $\dot{V}_{O2}$, pulmonary oxygen consumption; $\dot{V}_{O2max}$, maximal oxygen consumption; iPPO, peak power output during the incremental test. Values are mean ± SEM.

*Significantly different from Pre ($P < 0.05$).

†Significantly different from NORM ($P < 0.05$).

§Significant difference in the change ($P < 0.05$).

[a]HYP, n = 23.

[b]HYP, n = 21.

[c]HYP, n = 22.

resulting in a greater HIIT-induced change compared with HYP ($P < 0.01$). SQSTM1/p62 (p62) was not different between groups at either Pre and Post and increased with HIIT in both HYP ($P < 0.001$) and NORM ($P < 0.05$).

Protein content of the pro-apoptotic factor bcl-2-like protein 4 (BAX) and the anti-apoptotic factor B-cell lymphoma 2 (BCL2) was not different between groups at Pre, but BAX was lower in HYP than in NORM at Post ($P < 0.01$) (Figure 2E). BAX increased with HIIT in both HYP ($P < 0.001$) and NORM ($P < 0.05$), while BCL2 was down-regulated by HIIT in HYP only ($P < 0.01$). BAX/BCL2 ratio was similar in HYP and NORM at Pre, but not at Post ($P < 0.05$), and increased with HIIT in both groups ($P < 0.001$).

2.4 | Muscle markers of anti-oxidant protection

Protein content of superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), glutathione peroxidase 1 (GPX1), catalase (CAT) and NAD(P)H oxidase (NOX) was similar in HYP and NORM at Pre (Figure 3A). SOD1 was lowered by HIIT in HYP ($P < 0.01$), with a different HIIT-induced change compared with NORM ($P < 0.05$). SOD2 increased with HIIT in both HYP and NORM ($P < 0.05$). GPX1 increased with HIIT in NORM only ($P < 0.05$), while CAT increased with HIIT in HYP only ($P < 0.05$). NOX increased with HIIT in both HYP and NORM ($P < 0.05$).

Protein content of sirtuin 3 (SIRT3), uncoupling protein 3 (UCP3) and heat shock protein 70 (HSP70) was not different between groups at either Pre or Post (Figure 3B). SIRT3 and UCP3 were not affected by HIIT in either group, whereas HSP70 decreased with HIIT in HYP ($P < 0.01$), with a different HIIT-induced change compared with NORM ($P < 0.01$).

2.5 | Muscle markers of oxidative damage

Protein carbonylation in skeletal muscle was evaluated as a biomarker of ROS-induced oxidative damage.[41] Protein

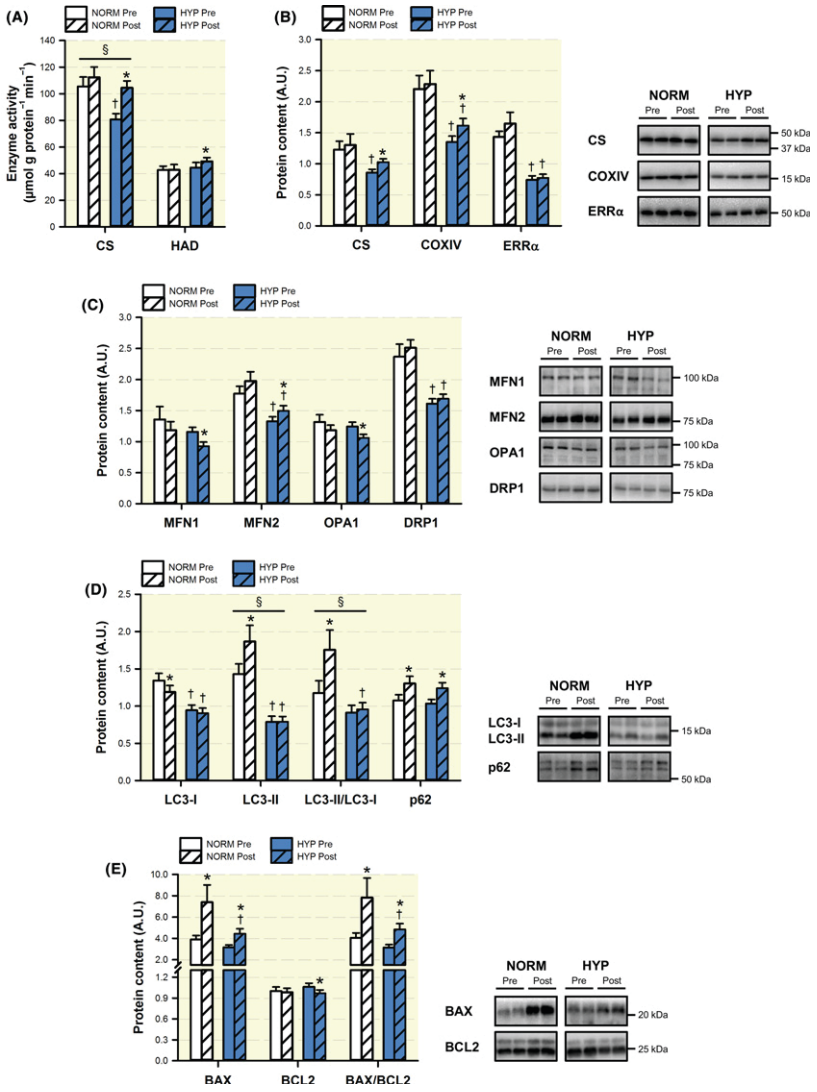

FIGURE 2 Muscle markers of mitochondrial turnover and mitochondria-mediated apoptosis. Mitochondrial enzyme activity (A), protein content of markers of mitochondrial volume/biogenesis (B), mitochondrial dynamics (C), autophagy (D), and mitochondria-mediated apoptosis (E) in skeletal muscle of healthy normotensive (NORM, n = 13) and hypertensive (HYP, n = 24) men before (Pre) and after (Post) 6 weeks of high-intensity exercise training. Values are mean ± SEM. †Significantly different from NORM ($P < 0.05$). *Significantly different from Pre ($P < 0.05$). §Significant difference in the change ($P < 0.05$)

carbonyl content was higher in HYP than in NORM at both Pre and Post ($P < 0.001$) (Figure 4A). Protein carbonyls increased with HIIT in HYP ($P < 0.01$), whereas were not affected by HIIT in NORM. At baseline, protein carbonyl content was related to 24-hour ambulatory blood pressure (systolic: $r = 0.45$, $P < 0.001$; diastolic: $r = 0.57$ $P < 0.001$; mean arterial pressure: $r = 0.56$, $P < 0.001$) (Figure 4B).

2.6 | Muscle protein levels of eNOS and VEGF

Protein content of endothelial nitric oxide synthase (eNOS) was lower in HYP than in NORM at both Pre and Post ($P < 0.001$), and increased with HIIT in both HYP and NORM ($P < 0.01$) (Figure 5). Protein content of vascular endothelial grow factor (VEGF) was not different between groups at either Pre or Post, and was up-regulated by HIIT in both HYP and NORM ($P < 0.01$).

2.7 | Muscle capillarity

Capillary-to-fibre ratio and capillary density were not different between groups at either Pre or Post, but increased with HIIT in HYP only ($P < 0.01$), with a greater HIIT-induced change in capillary-to-fibre ratio in HYP than in NORM ($P < 0.01$) (Table 2). Fibre cross sectional area was not different between groups at either Pre or Post, and was not affected by HIIT.

3 | DISCUSSION

The major findings of the present study were that the individuals with essential hypertension had lower expression of markers of mitochondrial volume/biogenesis, mitochondrial fusion/fission, and autophagy along with depressed eNOS content in skeletal muscle compared with healthy normotensive controls. Instead, no differences were observed for markers of mitochondria-mediated apoptosis and anti-

oxidant protection, albeit the hypertensive individuals had higher protein carbonyl levels than their normotensive counterparts. Six weeks of high-intensity exercise training lowered ambulatory blood pressure, and increased the expression of markers of mitochondrial volume/biogenesis and autophagy while up-regulating eNOS and VEGF content and enhancing muscle capillarity in the hypertensive individuals. At the same time, the high-intensity exercise training intervention induced divergent responses in different proteins regulating either mitochondrial fusion or the anti-oxidant defence system, did not affect markers of mitochondrial fission and increased markers of mitochondria-mediated apoptosis and oxidative damage in skeletal muscle of the individuals with essential hypertension.

3.1 | Muscle markers of mitochondrial turnover

The observation that muscle CS activity was lower in the hypertensive compared with the normotensive group at baseline is indicative of a reduced muscle mitochondrial volume in individuals with essential hypertension. It is noteworthy that, while CS activity has been shown to be associated with mitochondrial content in young healthy individuals,[42] it needs to be determined whether this association is evident also in aged and diseased individuals. However, the finding that also protein levels of CS, the mitochondrial respiratory chain subunit COXIV, as well as the key regulator of mitochondrial biogenesis ERRα,[43] were lower in the hypertensive group at baseline supports the existence of hypertension-related alterations in proteins implicated in the mitochondrial biogenic pathway. The high-intensity exercise training partly reversed these alterations by normalizing CS activity and content to the levels of the normotensive group and by elevating COXIV content, reflecting a training-mediated induction of mitochondrial biogenesis.

The lack of between-group differences in MFN1 and OPA1 along with the lower MFN2 levels detected in the hypertensive group implies protein-specific alterations in mitochondrial fusion with essential hypertension, thus highlighting the complexity of the mitochondrial fusion machinery. The lower DRP1 levels observed in the hypertensive group suggest that hypertension might be associated with a reduced capacity to segregate dysfunctional mitochondria. Overall, while inconsistent results have been reported on the influence of ageing on mitochondrial fusion/fission,[44–46] our findings partly support hypertension-related alterations in muscle mitochondrial dynamics. The observation that MFN2 increased, while DRP1 did not change, in response to the high-intensity exercise training in the hypertensive group, may indicate an increased fusion-to-fission ratio, and thus a potential decrease in mitochondrial fragmentation. Alternatively, the decline in MFN1 and OPA1 content observed in the hypertensive group following the training period points to a differential training-induced regulation of specific fusion proteins. A possible explanation for the divergent response observed in MFN1 and MFN2 might be related to slightly different functions of the mitofusins. Indeed, while both MFN1 and MFN2 play a role in mitochondrial fusion, MFN2 also regulate mitochondrial membrane potential, substrate oxidation, and oxidative phosphorylation, thereby affecting mitochondrial bioenergetics.[47] The decrease in OPA1 is in line with the training-induced down-regulation of MFN1, as OPA1-mediated mitochondrial fusion is functionally regulated by MFN1.[48]

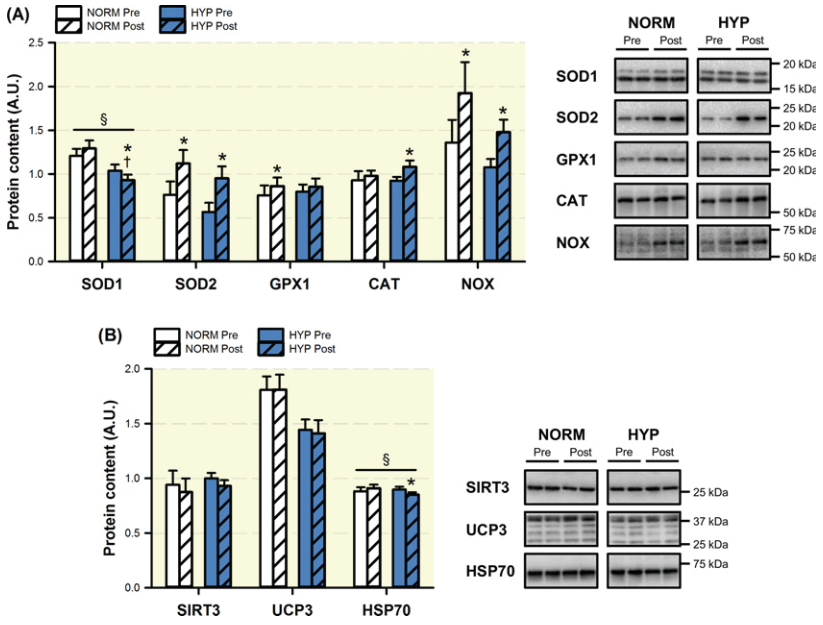

FIGURE 3 Muscle markers of anti-oxidant protection. Protein content of anti- and pro-oxidant enzymes (A), and content of proteins conferring protection against oxidative stress (B) in skeletal muscle of healthy normotensive (NORM, n = 13) and hypertensive (HYP, n = 24) men before (Pre) and after (Post) 6 weeks of high-intensity exercise training. Values are mean ± SEM. †Significantly different from NORM ($P < 0.05$). *Significantly different from Pre ($P < 0.05$). §Significant difference in the change ($P < 0.05$)

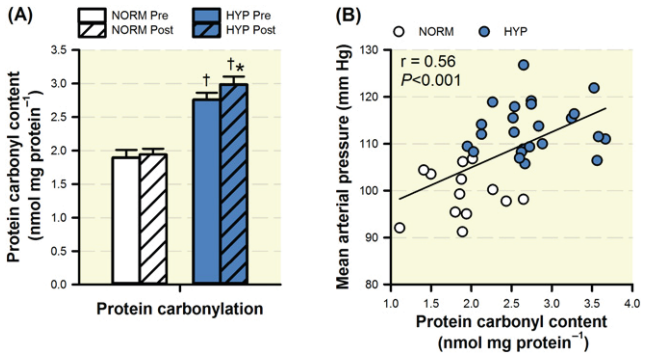

FIGURE 4 Muscle markers of oxidative damage. Protein carbonyl content in skeletal muscle of healthy normotensive (NORM, n = 13) and hypertensive (HYP, n = 24) men before (Pre) and after (Post) 6 weeks of high-intensity exercise training (A). Values are mean ± SEM. †Significantly different from NORM ($P < 0.05$). *Significantly different from Pre ($P < 0.05$). Correlation between protein carbonyl content in skeletal muscle and mean arterial pressure at baseline (B)

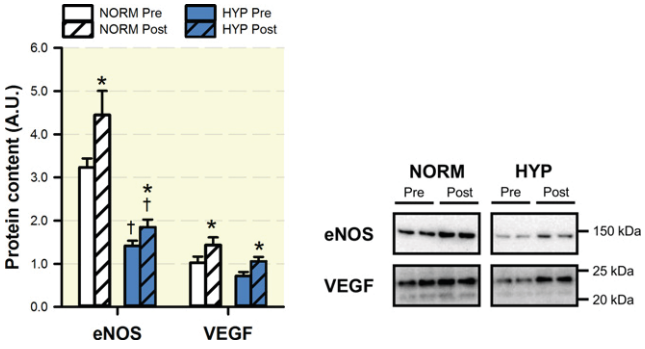

FIGURE 5 Protein content of eNOS and VEGF in skeletal muscle of healthy normotensive (NORM, n = 13) and hypertensive (HYP, n = 24) men before (Pre) and after (Post) 6 weeks of high-intensity exercise training. Values are mean ± SEM. †Significantly different from NORM ($P < 0.05$). *Significantly different from Pre ($P < 0.05$)

Thus, based on the divergent responses in fusion proteins, it is unclear whether the exercise training period stimulated mitochondrial fusion in the hypertensive individuals. Also, it can be argued that the increase in MFN2 occurred mainly as an adaptive mechanism to match the metabolic demands elicited by the high-intensity exercise training. However, electron microscopy imaging should be performed to confirm whether the observed hypertension- and training-related alterations in fusion/fission markers are associated with morphological changes of the mitochondrial network.

The lower LC3 levels observed in the hypertensive group may indicate an impaired capacity to recycle damaged organelles via the autophagic pathway in individuals with essential hypertension. In spite of the crucial role played by autophagy for cellular homeostasis, reports in human muscle are scarce and limited to evidence of an age-dependent decline in autophagy.[49–51] Nevertheless, the present novel findings in humans are in line with both the lower LC3-II/LC3-I ratio and the unaltered p62 protein levels observed in hypertensive compared with normotensive rats.[24] That the high-intensity exercise training-induced increase in both LC3-II content and the LC3-II/LC3-I ratio was greater in the normotensive than in the hypertensive group could imply a blunted responsiveness of LC3 to exercise training with essential hypertension. On the other hand, the increase in p62 observed following the training period in both the hypertensive and the normotensive group suggests that the capacity for autophagy regulation increased with exercise training independent of hypertension. Overall, the present findings are in contrast to the unaltered levels of LC3-II and p62 documented in either healthy young or elderly men following an exercise training period,[52–54] with such discrepancies possibly related to the different characteristics of the populations and the exercise training interventions employed. It should be noted that even though LC3 and p62 have been widely used as markers of autophagy, they are indicators of the rate of lysosome formation, but not degradation, which would provide a more specific measure of the autophagic flux.[55] Also, it may be that the observed increments in autophagy markers are due to training-induced shifts toward a more oxidative muscle phenotype, as higher autophagy flux and protein expression have been observed in oxidative compared with glycolytic muscles from rodents.[56]

TABLE 2 Muscle capillarity

	NORM		HYP	
	Pre	Post	Pre	Post
Capillary-to-fibre ratio	2.05 ± 0.15	2.19 ± 0.23	1.79 ± 0.07	1.97 ± 0.11*
Capillary density (cap mm^{-2})§	322 ± 15	302 ± 18	293 ± 12	326 ± 14*
Fibre cross sectional area (μm^2)	6608 ± 656	7285 ± 569	6315 ± 371	6147 ± 360

Capillarity in skeletal muscle of healthy normotensive (NORM, n = 13) and hypertensive (HYP, n = 24) men before (Pre) and after (Post) 6 weeks of high-intensity exercise training. Values are mean ± SEM.

*Significantly different from Pre ($P < 0.05$).

§Significant difference in the change ($P < 0.05$).

No differences were observed for markers of mitochondria-mediated apoptosis between the hypertensive and the normotensive group at baseline. While apoptosis-related data in human hypertension is absent, the present finding differs from the two-fold greater BAX/BCL2 ratio observed in hypertensive compared with normotensive rats,[25] and do not support an association between essential hypertension and elevated apoptotic activity. Furthermore, the current finding that the high-intensity exercise training increased BAX and BAX/BCL2 ratio in both the hypertensive and normotensive group suggests a training-induced increase in apoptotic susceptibility regardless of hypertension, and is in contrast to the reduced BAX/BCL2 ratio reported in hypertensive rats following 6 weeks of exercise training.[25] On the other hand, our results are in line with evidence of up-regulated BAX content in both healthy individuals and mitochondrial myopathy patients in response to 12 weeks of endurance exercise training.[57] Notably, although increased apoptotic signalling may lead to muscle wasting, thereby implying a maladaptive response to exercise training, evidence from a simple animal model indicates that activation of the intrinsic apoptotic pathway by excess mitochondrial ROS generation leads to an adaptive protective mechanism that keeps the organism alive under stressful conditions rather than solely promoting apoptotic cell death.[58] Nevertheless, further investigations are required to confirm whether this mechanism occurs in humans. Also, it should be noted that fat free mass did not decrease with the training intervention in either the hypertensive or the normotensive group, suggesting that in spite of the increased apoptotic susceptibility, muscle mass was not negatively affected by the training intervention.

Taken together, our findings suggest of an impaired mitochondrial quality control, but not overregulated mitochondria-mediated apoptosis, in men with essential hypertension. On the basis of the training-induced changes in markers of mitochondrial turnover, the high-intensity exercise training seemed to promote mitochondrial biogenesis and to increase autophagy capacity while possibly improving mitochondrial bioenergetics via up-regulation of MFN2 in muscle of hypertensive individuals. However, additional research is needed to unravel whether the increase in apoptotic susceptibility elicited by the high-intensity exercise training represents a maladaptive response leading to apoptosis rather than degradation of damaged organelles.[59]

3.2 | Muscle markers of anti-oxidant protection and oxidative damage

The finding that baseline protein content of anti- and pro-oxidant enzymes was similar in the hypertensive and the normotensive group is in line with the lack of a hypertension-related depression in SOD1, SOD2 and catalase levels reported in rats,[27] but in contrast to the elevated NOX content detected in different animal models of hypertension.[27,60] Multiple proteins other than anti-oxidant enzymes contribute to the anti-oxidant defence system, and may thereby be altered with essential hypertension. Nevertheless, protein levels of SIRT3, which regulates mitochondrial redox homeostasis,[61] UCP3, which may limit excess mitochondrial ROS production,[62] and HSP70, which exerts a cyto-protective function against oxidative stress,[63] were similar in the hypertensive and the normotensive group. While providing novel data about unaltered UCP3 and HSP70 levels in human hypertension, the observation that muscle SIRT3 content was not different between the hypertensive and the normotensive group is in contrast to the reduced SIRT3 expression observed in peripheral blood mononuclear cell fractions from hypertensive patients.[64] It is worth mentioning that, in order to minimize the risks associated with interruption of antihypertensive drug therapy, only a short-term washout period was included prior to the experimental days in the current study. Therefore, it is possible that the lack of between-group differences in proteins involved in redox state regulation is related to the effects of long-term treatment with antihypertensive drugs having anti-oxidant properties.

In spite of normal content of proteins regulating redox homeostasis, the findings that muscle protein carbonylation was greater in the hypertensive group and that baseline protein carbonyl levels and 24-hour ambulatory blood pressure were correlated support the purported link between oxidative stress and elevated blood pressure.

Besides promoting a healthier mitochondrial pool, exercise training may reverse hypertension-related endothelial dysfunction by increasing NO bioavailability. This may occur via a decreased ROS scavenging of NO as a consequence of training-induced increments in activity and/or content of anti-oxidant enzymes.[65,66] In the present study, SOD2 content was up-regulated by the exercise training intervention regardless of blood pressure levels, confirming the responsiveness of the mitochondria-specific isoform of superoxide dismutase to high-intensity exercise training, as observed in aged sedentary men.[66] On the other hand, the finding that content of SOD1, the cytosolic isoform of superoxide dismutase, decreased with the high-intensity exercise training period in the hypertensive but not in the normotensive group may be related to a differential compartmentalization of the origin of muscle oxidative stress between healthy and hypertensive individuals, a phenomenon suggested to occur also with ageing.[67] The same phenomenon may also underlie the observation that GPX1 increased with the training

intervention in the normotensive, but not in the hypertensive group, while CAT increased in the hypertensive group only. The observed training-induced increase in NOX content is consistent with a report in aged sedentary men,[66] and implies an enhanced capacity for ROS formation, which is essential for amplifying ROS-mediated adaptive responses to exercise training. The lack of change in SIRT3 content with the exercise training intervention is in contrast to the elevated SIRT3 levels observed following 8 weeks of endurance training in both young and old healthy individuals,[68] with differences in the intensity and duration of the training intervention possibly explaining this discrepancy. The decrease in HSP70 with the training intervention in the hypertensive group is consistent with the observed training-induced increase in apoptotic susceptibility, as HSP70 has a role in preventing apoptosis by blocking BAX translocation.[69] Interestingly, the elevated protein carbonyl levels observed in the hypertensive group following the training intervention indicates increased levels of oxidative damage. This represents a novel finding in humans and is in accordance with the exacerbated protein carbonylation documented in senescent rats with exercise training.[70] Notably, in light of evidence showing a greater accumulation of carbonyls in oxidative compared with glycolytic muscle fibres,[70] future studies including assessments of the muscle phenotype may clarify whether the increase in protein carbonyls observed in the hypertensive group might be related to a training-induced increase in the proportion of type I muscle fibres.

3.3 | Muscle eNOS expression

The lower muscle eNOS protein content detected in the hypertensive compared with the normotensive group is in line with one study in mild hypertensive subjects,[71] and supports the critical role of defective endothelium-derived NO production in the pathophysiology of essential hypertension. Also, the association between depressed eNOS expression and aberrant mitochondrial turnover observed in the hypertensive group is consistent with a study reporting impaired vascular mitochondrial turnover in eNOS null mice.[72] A novel finding was that the high-intensity exercise training up-regulated eNOS expression regardless of hypertension, which differs from the unaltered eNOS levels reported in both hypertensive and normotensive individuals following 16 weeks of moderate-intensity exercise training.[71] This, not only indicates that high-intensity exercise might be needed to up-regulate eNOS expression in human skeletal muscle but also suggests up-regulation of eNOS expression as a putative mechanism underlying the antihypertensive effect of the high-intensity exercise training.

3.4 | Muscle VEGF expression and capillarity

The observation that muscle VEGF protein content was similar in the hypertensive and the normotensive group is in contrast with observations in untreated hypertensive individuals,[73] but in agreement with observations in medicated hypertensive patients.[74] The high-intensity exercise training up-regulated VEGF levels to the same extent in the two groups, as previously observed in response to 8 weeks of moderate-intensity exercise training.[74] Furthermore, the lack of alterations in muscle capillarity with hypertension is in agreement with prior observations,[74] and does not support the documented association between elevated blood pressure and capillary rarefaction.[73] However, it is possible that differences in severity and duration of hypertension account for these contrasting observations. Here, the exercise training intervention promoted an increase in capillary-to-fibre ratio in the hypertensive, but not in the normotensive group, as previously reported.[73,74] This, along with the training-induced increase in capillary density observed in the hypertensive group only, suggests that the high-intensity exercise training had a greater angiogenic potential in the hypertensive than in the normotensive individuals. However, in view of evidence indicating alterations in capillary structure with hypertension,[74] it is conceivable that the training-induced increase in capillarity observed in the hypertensive group occurred as an adaptive response compensating for hypertension-related structural and/or functional abnormalities in the capillary bed.

3.5 | Blood pressure and health-related outcomes

The training intervention adopted in the present study lowered 24-hour systolic and diastolic ambulatory blood pressure by 4.6 ± 1.7 and 4.0 ± 1.0 mm Hg, respectively, in the hypertensive group, confirming the antihypertensive effect of intense exercise training[75] and underlining the potency of this training approach to improve blood pressure regulation regardless of the short-term nature of the intervention. Moreover, although the observed reductions in ambulatory blood pressure are modest compared with those elicited by 12 weeks of running-based high-intensity interval training,[38] they are in line with the mean training-induced reduction in ambulatory blood pressure computed by a recent meta-analysis.[76] Most importantly, reductions in systolic blood pressure of 5 mm Hg have been shown to decrease all-cause mortality by 7%,[77] highlighting the clinical relevance of the present finding. Furthermore, the observation that the high-intensity exercise training improved body composition, cardiorespiratory fitness and exercise

capacity in both the hypertensive and normotensive group supports the mounting evidence advocating high-intensity interval training as a viable therapeutic strategy to reduce risk factors for cardiovascular disease.[78,79] With specific regard to the exercise training-induced changes in cardiorespiratory fitness, the hypertensive group increased $\dot{V}_{O2max}$ to a lower extent than previously observed,[80] which may relate to the ~4-fold lower training volume as well as to the shorter duration of the training intervention used in the present study. Of note, the relatively small increase in cardiorespiratory fitness is still of high clinical relevance, as each 1 mL kg min^{-1} increase in $\dot{V}_{O2max}$ has been recently shown to be associated with a 45-day increase in longevity.[81]

3.6 | Summary and perspectives

This study provides the first evidence of reduced expression of markers of mitochondrial volume/biogenesis, mitochondrial fusion/fission, and autophagy in skeletal muscle of essential hypertensive humans, suggesting aberrant mitochondrial turnover and impaired mitochondrial quality with essential hypertension. On the other hand, the expression of muscle markers of anti-oxidant protection was not lower in hypertensive individuals in spite of increased levels of oxidative damage compared with their normotensive counterparts. On the basis of these findings, future studies addressing mitochondrial function, including ex vivo measurements of mitochondrial respiration and mitochondrial ROS emission, will be important to provide further information regarding the specific role of mitochondria in the pathophysiology of essential hypertension. Also, in order to clarify the role of muscle mitochondrial dysfunction in the development of endothelial dysfunction, the potential cross-talk between mitochondria in the vascular endothelial and skeletal muscle cells should be further investigated.

The present study shows that short-term high-intensity exercise training is a viable therapeutic intervention to lower ambulatory blood pressure and promote a healthier mitochondrial pool in hypertensive individuals. On the other hand, the lack of training-induced substantial reductions in muscle oxidative damage in spite of enhanced mitochondrial quality highlights the need for supplementary studies providing new insights into the association between mitochondrial adaptations and alterations in redox homeostasis. Lastly, given that current data is based on measurements made on whole muscle homogenates, of which endothelial cells make up only a small fraction, more evidence is required to confirm whether similar hypertension-related alterations and training-induced adaptations would be evident and would occur also in endothelial cells.

4 | MATERIAL AND METHODS

4.1 | Subjects

Twenty four men with essential hypertension and thirteen age-matched healthy men were involved in the study. Prior to inclusion, subjects were informed of risks and discomforts associated with the experimental procedures. After giving oral and written informed consent, all potential subjects underwent a medical screening examination with a physician. All subjects were males, aged above 40 years and habitually inactive (<1.5 hours of physical activity per week). Exclusion criteria were other chronic diseases, smoking, excessive alcohol consumption, body mass index above 30 kg m^{-2}, and treatment with blood thinners. Females were excluded to avoid any confounding effect of different menopausal phases on blood pressure and muscle mitochondrial adaptations to exercise training.[82] Included subjects were allocated in either a normotensive (NORM) or hypertensive (HYP) group. Subject characteristics are presented in Table 1. Thirteen of the hypertensive subjects were under treatment with ACE-inhibitors (n = 5), angiotensin II-receptor blockers (n = 7), Ca^{2+} antagonist (n = 2) and/or diuretics (n = 8). The study was approved by the Ethics Committee of Copenhagen and Frederiksberg communities (H-4-2014-100) and conducted in accordance with the guidelines of the Declaration of Helsinki, except for registration in a database.

4.2 | Study design

This study was designed as a cross-sectional and longitudinal parallel study, and was a part of a larger study on the effects of exercise training on the vascular, nervous and muscular mechanisms underlying elevated blood pressure (Figure 6). For the purpose of the present study, only the muscular-related measurements were considered.

4.3 | Screening examination

Prior to enrolment in the study, each subject underwent a screening examination consisting of a medical history review, medication use, and a physical exam including lung and heart auscultation, assessments of resting electrocardiography and resting blood pressure, and fasting blood samples. Blood pressure was measured six consecutive times by an automatic upper-arm blood pressure monitor (M7; OMRON, Vernon Hills, IL, USA) after at least 15 minutes of rest in the supine position (NORM, 127 ± 3/76 ± 2 mm Hg; HYP, 141 ± 2/87 ± 1 mm Hg (mean ± SEM)). It should be noted that thirteen hypertensive subjects were under treatment with antihypertensive

medication during the screening examination. After the physical exam, an incremental test to exhaustion was carried on a mechanically braked cycle-ergometer (LC6; Monark Exercise AB, Vansbro, Sweden) for determination pulmonary maximal oxygen consumption ($\dot{V}_{O2max}$). The test protocol consisted of two submaximal 4-minute bouts at 40 and 80 W followed by an incremental ramp test with increments of 20 W min^{-1} until volitional exhaustion. Pulmonary oxygen consumption ($\dot{V}_{O2}$) was measured breath-by-breath using an on-line gas analysis system (Oxycon Pro, Viasys Healthcare, Hoechberg, Germany). $\dot{V}_{O2max}$ was determined as the highest value achieved during a 30-s period. Criteria used for achievement of $\dot{V}_{O2max}$ were a plateau in $\dot{V}_{O2}$ despite an increase in workload and a respiratory exchange ratio above 1.10. A regression equation of $\dot{V}_{O2}$ vs power output was derived from the two submaximal workloads and was used to determine the individual workloads to be sustained during the cycling test performed on experimental day 2.

4.4 | Experimental setup

Two experimental days separated by 2-4 days were conducted before (Pre) and after (Post) a 6-week exercise training intervention period, with the post-experimental days being carried out within 5 days after the last training session. The hypertensive subjects were instructed to stop their medication 4 days prior to the experimental days at both Pre and Post the training intervention period. During the training intervention, the hypertensive subjects were treated with their regular antihypertensive medication. Subjects refrained from caffeine, alcohol and exercise for 24 hours prior to both experimental day 1 and 2.

4.4.1 | Experimental day 1

Subjects reported to the laboratory between 8 and 9 AM, 2 hours after eating breakfast, which was recorded and replicated after the training intervention. At this point, a part of the subjects went through a procedure aimed at determine vascular function with intravenous infusion of vasoactive compounds, whereas other subjects were tested for muscle sympathetic nervous activity (MSNA) by the microneurography technique. After the vascular- or the MSNA-based measurements, a 3-mm incision was made over the lateral portion of the thigh under local anesthesia (2 mL lidocaine without epinephrine, 20 mg mL^{-1} Xylocain, AstraZeneca, Denmark) and a biopsy was obtained from the *vastus lateralis* muscle by using a percutaneous Bergstrom needle with suction.

4.4.2 | Experimental day 2

Subjects reported to the laboratory in the morning after an overnight fast, and were whole-body dual-energy X-ray absorptiometry (DXA) scanned. After the scan subjects were provided with a standardized breakfast consisting of ~75 g of carbohydrates, ~25 g of fats, ~15 g of proteins, and water ad libitum. Ninty minutes after ingestion of the meal, subjects performed a cycling incremental test to exhaustion for determination of $\dot{V}_{O2max}$ and exercise capacity. The test protocol included two submaximal 4-minute bouts at a relative workload corresponding to 50% and 65% $\dot{V}_{O2max}$. After 3 minutes of passive recovery, an incremental ramp test with increments of 20 W min^{-1} until volitional exhaustion was performed. Pulmonary $\dot{V}_{O2}$ and $\dot{V}_{O2max}$ were determined as described for the screening

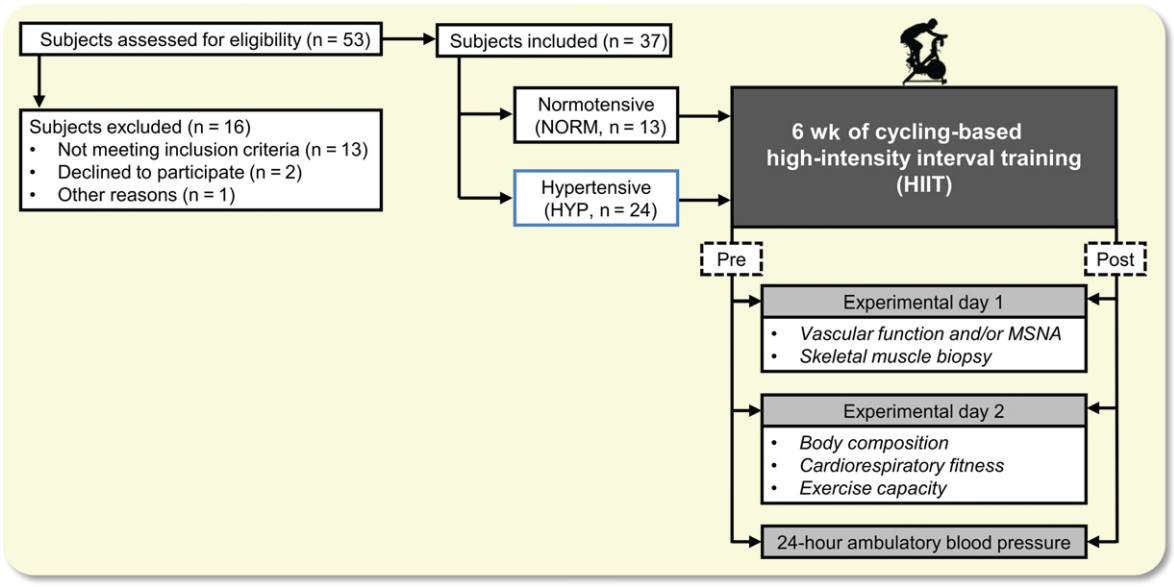

FIGURE 6 Schematic presentation of the study design

visit. The time to exhaustion and the peak power output during the incremental test (iPPO) were recorded. Heart rate was monitored throughout the test and maximal heart rate (HR_{max}) established as the highest value achieved during the test checked for spikes.

4.5 | Exercise training intervention

The training intervention consisted of 6 weeks of cycling-based high-intensity interval training (HIIT) following the 10-20-30 training concept.[83] 10-20-30 training was characterized by five consecutive 1-minute intervals divided into 30, 20, and 10 seconds at an intensity corresponding to ~30%, ~50% and ~100% of maximal intensity, respectively. Each training session lasted for 20-28 minutes and comprised 7 minutes of warm-up at moderate intensity, 10-15 minutes of 10-20-30 training with 3 minutes of recovery between each 5-minute bout. During the first two weeks, subjects completed 2 × 5-minute bouts, whereas, from week 3, training volume was increased to 3 × 5-minute bouts per training session. Likewise, weekly training sessions were increased from two (week 1 and 2) to three (week 3-6). Subjects were instructed to pedal as hard and fast as possible during the 10-second sprints. All training sessions were supervised and subjects wore heart rate monitors (TEAM2 Wearlink$^+$, Polar, Kempele, Finland) throughout the whole training session.

4.6 | Measurements and analyses

4.6.1 | Blood pressure

Ambulatory blood pressure was measured over 24 hours using a Boso TM-2430 PC2 monitor (BOSCH + SOHN, Germany). All subjects were instructed to begin the blood pressure measurements 24 hours after the end of experimental day 2 and to do their habitual daily activities, which were noted on a diary. Monitors were programmed to measure blood pressure at 15-minute intervals during day-time (awake) and at 30-minute intervals during night-time (asleep) periods. Day-time and night-time periods were based on the subjects' time from getting into and out of bed. Ambulatory blood pressure data were analyzed as average 24-hour systolic, diastolic and mean blood pressure.

4.6.2 | Body composition

Whole body fat free mass, fat mass, fat percentage and visceral fat mass were measured by dual-energy X-ray absorptiometry (DXA) (Lunar iDXA, GE Healthcare Lunar, Belgium), calibrated in accordance with the manufacturer's guidelines.

4.6.3 | Muscle biopsies

Sampled biopsies were divided in two portions, the first portion was mounted on an embedded medium (OCT Compound Tissue-Tek; Sakura Finetek, Zoeterwoude, The Netherlands), frozen in isopentane cooled to the freezing point in liquid N_2 and stored at −80°C until immunohistochemical analysis for capillaries and fibre cross-sectional area. The second biopsy portion was snap-frozen in liquid N_2 and stored at −80°C ready to be freeze-dried. Muscle specimens were weighed before and after freeze-drying to determine the water content. After freeze-drying the samples, connective tissue, visible fat, and blood were carefully dissected away. Then, the dissected muscle samples were stored at −80°C until analysis for enzymatic activity and protein content. Both enzymatic activity and protein content were determined in duplicates, i.e. two different samples were obtained from the same muscle specimen after dissection and the mean value of the two samples was used as result.

Enzymatic activity

Maximal enzyme activity of citrate synthase (CS) and 3-hydroxyacyl CoA dehydrogenase (HAD) was quantified in muscle homogenates using fluorometric method (Fluoroscan Ascent; Thermo Scientific, Waltham, MA, USA), as previously described.[84] Enzymatic activity was determined in duplicates from the same muscle specimen.

Protein content in homogenate lysate

Protein content in muscle homogenate lysates was determined by SDS-PAGE and Western blotting analyses. In short, freeze-dried muscle samples (~2 mg dry weight) were homogenized for 1 minute at 29 Hz (Qiagen Tissuelyser II, Retsch GmbH, Haan, Germany) in a fresh-batch of ice-cold buffer (10% glycerol, 20 mmol L^{-1} Na-pyrophosphate, 150 mmol L^{-1} NaCl, 50 mmol L^{-1} HEPES (pH 7.5), 1% NP-40, 20 mmol L^{-1} β-glycerophosphate, 2 mmol L^{-1} Na_3VO_4, 10 mmol L^{-1} NaF, 2 mmol L^{-1} PMSF, 1 mmol L^{-1} EDTA (pH 8), 1 mmol L^{-1} EGTA (pH 8), 10 μg mL^{-1} aprotinin, 10 μg mL^{-1} leupeptin, and 3 mmol L^{-1} benzamidine). Afterwards, samples were rotated end over end for 1 hour at 4°C, followed by centrifugation at 17 500 *g* for 20 minutes 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 mol L^{-1} Tris-base, 3 mL glycerol, 0.93 g DTT, 1 g SDS, and 1.2 mg bromophenol blue) and double distilled H_2O to reach equal protein concentration.

Equal amounts of protein (range: 6-15 μg) were loaded in each well of either 4-15%, 7.5%, 12% or 18% pre-cast gels (Bio-Rad Laboratories, Hercules, CA, USA). Samples from each subject were loaded on the same gel, with the samples from before training (Pre) being placed adjacent to the samples after training (Post). The same pool of a mixed human muscle standard lysate was loaded in three different wells per gel and the average intensity of these samples was used for normalization to allow gel-to-gel comparison, as previously described.[85] Proteins were separated according to their molecular weight by SDS page gel electrophoresis and semi-dry transferred to a PVDF membrane (Merck Millipore, Darmstadt, Germany). The membranes were blocked in either 2% skim milk, 3% BSA or 5% BSA in TBST before being incubated overnight at 4°C in primary antibody diluted in either 2% skim milk, 3% BSA, or 5% BSA (see Table S1). After washing in a Tris-buffered saline, membranes were incubated with a secondary antibody for ~1 hour at room temperature. The secondary horseradish peroxidase-conjugated antibodies used were diluted 1:5000 in 2% skim milk, 3% BSA or 5% BSA depending on the primary antibody (P-0447, P-0448, and P-0449; DakoCytomation). The membrane staining was visualized by incubation with a chemiluminescent horseradish peroxidase substrate (Millipore) 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).

Protein carbonylation

Protein carbonylation was determined in homogenates made in a 0.3 mol L^{-1} phosphate buffer (pH 7.7) containing 0.05% BSA using an OxiSelectTM ELISA-kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's protocol, with absorbance measured at 450 nm (Multiscan; Thermo Scientific). Based on a serially diluted oxidized/reduced BSA standard, the absorbance was converted to protein carbonyl concentration and normalized to protein content in the samples determined by a BSA standard kit (Thermo Scientific). Protein carbonyl content was determined in duplicates from the same muscle specimen and the mean signal intensity of the two samples was used as result.

Immunohistochemistry and imaging

The embedded muscle samples were cut using a cryostat, and transverse sections 8 μm in thickness were mounted on glass slides. To verify the cross-sectional orientation of the individual muscle fibre, multiple samples were cut and examined under light microscopy until a cross section of desirable size, orientation, and uniform polygonal appearance was visible. For immunohistochemical staining, the cross sections were fixed for 2 minutes in phosphate-buffered saline (pH 7.2, Gibco 70013-016; Life Technologies Denmark, Nærum, Denmark) containing 2% formaldehyde followed by a washing sequence in a 1:10 wash buffer (Dako S3006, Glostrup, Denmark). Staining targets were visualized pairwise, and each step was followed by a washing sequence. All antibodies were diluted in antibody diluent (Dako S0809). First, capillaries were visualized using biotinylated Ulex europaeus agglutinin I lectin (1:100; VECTB-1065, VWR, Bie and Berntsen, Herlev, Denmark). Second, myofibre borders were visualized using an antibody against laminin (1:500; Dako Z0097). Specific secondary antibodies were applied to each primary antibody. The specificity of the staining was assessed by single staining, and by staining without the primary antibody. Visualization was performed on a computer screen using a light microscope (Carl Zeiss, Germany), and all morphometric analyses were performed using a digital analysis program (ImageJ, National Institutes of Health). Two or more separate sections of a cross section were used for analysis, and only sections without artifacts or tendency to longitudinal cuts were analysed. The mean fibre cross-sectional area was determined by manually drawing the perimeter around each selected section which was subsequently divided by the number of muscle fibres within the selected section. The number of capillaries within each section was counted, and capillary supply was subsequently expressed as capillary-to-fibre ratio and capillary density (cap mm^{-2}). All analyses were conducted by the same blinded experienced investigator.

4.7 | Statistics

Between-group differences at baseline and after the training intervention were determined using a linear mixed model with group (NORM, HYP) and time (Pre, Post) as fixed factors and subjects as random factor. In addition, given that age, fitness status and adiposity have been shown to affect myocellular function by altering the content of muscle proteins regulating mitochondrial turnover and redox homeostasis,[49,86] age, baseline relative $\dot{V}_{O2max}$ and baseline body fat percentage were included as covariates to limit the confounding influence of other factors than essential hypertension. To estimate the effect of the training intervention within each group, a linear mixed model was used with time as fixed factor, subjects as random factor, and with age, baseline relative $\dot{V}_{O2max}$ and baseline value of the outcome variable included as covariates. In addition, between-group differences in the training-induced changes across time were estimated by using a linear mixed model with group-time interaction as fixed factor, subjects as random factor, and with age, baseline relative $\dot{V}_{O2max}$, and baseline

value of the outcome variable included as covariates. Model checking was based on Shapiro Wilk's test and Q-Q plots. In 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- and within-group differences. Linear regression analysis was used to determine the Pearson's correlation coefficient (r) between baseline oxidative damage and blood pressure. The level of significance for all analyses was defined as $P < 0.05$. Statistical analyses were carried out with R ver. 3.4.1 and the extension packages *lme4* and *multcomp*. Data are presented as mean ± SEM.

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CONFLICT OF INTEREST

The authors have no conflict of interest that relates to the content of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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