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THE EFFECT OF DIFFERENT EXERCISE REGIMENS ON MITOCHONDRIAL BIOGENESIS AND PERFORMANCE

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The effect of different exercise regimens on mitochondrial biogenesis and performance

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Endurance training is a powerful tool to improve both health and performance. Physical activity is now recognized as an effective treatment and prevention therapy for a wide range of diseases. One of the most profound adaptations to endurance training is increased mitochondrial function and content within the exercising muscles. Mitochondrial quality and quantity are closely related to several of the positive health effects reported after training. High mitochondrial content strongly correlates with muscle oxidative capacity and endurance performance. Even though it is well known that endurance training increases mitochondrial content, it is unclear which type of training is the most efficient to promote mitochondrial biogenesis. Therefore, the basis for current exercise recommendations relative to mitochondrial biogenesis is poor or absent. Thus, the main objective of this thesis was to evaluate the effect of different training strategies on mitochondrial biogenesis.

Recent developments in molecular methods have made it possible to study the initial adaptations to training through measurement of mRNA gene expression of exercise induced genes. One such gene is transcriptional coactivator peroxisome proliferator–activated receptor-γ coactivator-1α (PGC-1α). PGC-1α is a key regulator of mitochondrial biogenesis and the expression of PGC-1α can therefore be used as a marker of this process.

The first four studies presented in this thesis are acute exercise studies where two different exercise models were compared using a cross-over design. Muscle biopsies were obtained pre and post exercise and analysed for gene expression and glycogen, apart from study II. The final study was a long-term training study where muscle biopsies were obtained before and after the training period and analysed for mitochondrial enzyme activities and protein content.

Study I: The expression of PGC-1α and related genes were examined after 90 min of continuous and interval exercise in untrained subjects. The exercise protocols influenced the expression of genes involved in mitochondrial biogenesis and oxidative metabolism in a similar manner. Both interval and continuous exercise were potent training strategies for relatively sedentary individuals.

Study II: The expression of PGC-1α and related genes were examined after low-volume sprint interval (SIT) and high-volume interval (IE) exercise in highly trained cyclists. SIT induced a similar increase in PGC-1α expression as IE despite a much lower time commitment and work completed. Sprint interval exercise might, therefore, be a time efficient training strategy for highly trained individuals.

Study III: The expression of PGC-1α and related genes, as well as the activity of upstream proteins, were examined after concurrent (ER: cycling + leg press) and single-mode (E: cycling only) exercise in untrained subjects. PGC-1α expression doubled after ER compared

with E. It was concluded that concurrent training might be beneficial for mitochondrial biogenesis in untrained individuals.

Study IV: The expression of PGC-1α and related genes were examined after exercise performed with low (LG) and normal (NG) muscle glycogen in well-trained cyclists. PGC-1α expression increased approximately three times more after LG compared with NG. This finding suggested that low glycogen exercise is a potent inducer of mitochondrial biogenesis in well-trained individuals.

Study V: Mitochondrial enzyme activity, protein content and endurance performance were examined after eight weeks of concurrent (ES: cycling + leg press) or single-mode (E: cycling only) training in cyclists. ES did not affect enzyme activity, protein content or endurance performance differently than E. The beneficial effect previously observed in untrained subjects did not translate to higher numbers of mitochondria in trained individuals.

In three of the studies, I, III, and IV, both glycogen and PGC-1α expression were measured after exercise. These data were then pooled and examined. The highest PGC-1α mRNA expression levels were identified when glycogen levels were low, and vice versa. This suggests that low glycogen might play an important role in the regulation of mitochondrial biogenesis also during interval and concurrent strength and endurance exercise.

In conclusion, key markers of mitochondrial biogenesis can be effectively up-regulated by interval, concurrent and low glycogen exercise. A possible explanation for this might be that though the exercise protocols are quite divergent in nature, they all have a pronounced effect on muscle glycogen and/or perturbation in energetic stress.

LIST OF SCIENTIFIC PAPERS

I. Wang L, **PSILANDER N,** Tonkonogi M, Ding S, Sahlin K. Similar Expression of Oxidative Genes after Interval and Continuous Exercise. Med Sci Sports Exerc. 2009 Dec;41(12):2136-44.

II. **PSILANDER N**, Wang L, Westergren J, Tonkonogi M, Sahlin K. Mitochondrial gene expression in elite cyclists: effects of high intensity exercis. Eur J Appl Physiol. 2010 Oct;110(3):597-606.

III. Wang L, Mascher H, **PSILANDER N**, Blomstrand E, Sahlin K. Resistance exercise enhances the molecular signaling of mitochondrial biogenesis induced by endurance. J Appl Physiol. 2011 Nov;111(5):1335-44.

IV. **PSILANDER N**, Frank P, Flockhart M, Sahlin K. Exercise with low glycogen increases PGC-1a gene expression in human skeletal muscle. Eur J Appl Physiol. 2013 Apr;113(4):951-63.

V. **PSILANDER N**, Frank P, Flockhart M, Sahlin K. Adding strength to endurance training does not enhance aerobic capacity in cyclists. Accepted for publication in Scand J Med Sci Sports 2014.

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INTRODUCTION

It is well accepted that regular exercise has a positive effect on most organ systems and reduces the risk of several chronic diseases such as type 2 diabetes, cancer and cardiovascular disease. Exercise also increases endurance capacity. An untrained person who engages in regular endurance exercise, such as running, will after a few weeks be able to comfortably run a distance that before training was perceived as difficult. Increased endurance capacity is not only important for performance and general well being, but also strongly correlates with a reduced mortality risk (Blair et al., 1995; Kokkinos et al., 2008).

Skeletal muscles quickly adapt to exercise. Muscles from well-trained individuals have more capillaries, mitochondria and improved capacity to store carbohydrates and oxidize fat. One of the most profound effects of exercise is an increase in mitochondrial content (Holloszy, 1967). Increases up to 50% after only 6-8 weeks of training are not unusual in untrained subjects (Henriksson, 1977; Tarnopolsky et al., 2007; Tonkonogi et al., 2000). However, little is known about the optimal exercise prescription, and an ongoing debate of whether focus should be on low intensity/long duration or on high intensity/short duration training exists (Bishop et al., 2014).

BACKGROUND

Skeletal muscle cells (fibers)

Skeletal muscles are made up of long and thin muscle fibers cells. Depending on the muscle, individual fibers can have a length similar to the muscle in which they reside. The longest muscle fibers are found in the thigh (Sartorius muscle) and can be over 40 cm long (Ward et al., 2009). Several different types of skeletal muscle fibers exist. They are normally classified as type I, type IIa and type IIx. The type I fibers have the slowest contraction speed and lowest power output, but the highest endurance capacity. A major reason for this high endurance capacity is that type I fibers have a high mitochondrial content, and can therefore utilize oxygen to a greater extent during energy production (aerobic metabolism). Type IIx fibers are the most explosive fibers, with the fastest contraction speed, but their endurance capacity is low and they easily fatigue. Type IIx fibers have a relatively low mitochondrial content and get most of their energy from anaerobic metabolism. The characteristics of type IIa fibers are highly dependent on an individual's training status. In well trained subjects, type IIa fibers have similar metabolic properties as type I fibers, whereas in sedentary subjects, metabolically they fall between type I and IIx fibers (Saltin et al., 1977).

Muscle fiber type proportions differ both between individuals and muscle groups. Well-trained subjects generally have a different fiber type profile when compared with untrained subjects. Untrained individuals have approximately 50% type I, 35% type IIa and 15% type IIx fibers in the thigh muscles, whereas endurance trained individuals typically have > 60% type I, < 30% type IIa and < 5% type IIx, though there is individual variability (Saltin et al., 1977). The reason for muscle composition differences is both genetic and a consequence of training. It is well known that training can induce fiber type transformation, especially from type IIx to IIa (Andersen and Henriksson, 1977).

Exercising muscles rarely use all of their muscle fibers. Type IIx fibers are only fully recruited during high intensity exercise, such as supramaximal sprints, or at the end of exhaustive training session when type I and IIa fibers run out of fuel (Gollnick et al., 1974; Vollestad et al., 1984). Type I fibers are primarily responsible for muscle contractions during intensities below ~60% of VO_2max, whereas both type I and IIa fibers are recruited to a similar extent during intensities above ~65%, in untrained and moderately trained subjects (Scribbans et al., 2014; Vollestad and Blom, 1985).

The mitochondria

Mitochondria are the power plants of the cell. They use carbohydrates, fat and proteins as fuel and produce energy in the form of adenosine triphosphate (ATP). Mitochondria produce ATP via oxidative phosphorylation, which requires oxygen. Energy is released when ATP is converted to adenosine diphosphate (ADP) and adenosine monophosphate (AMP), and this energy can be used for different cellular processes such as muscle contraction. The more dependent a cell is on a high, constant energy supply, the more mitochondria it contains. For example, cardiac muscle fibers can never rest and need a constant flow of ATP to keep the heart beating. These fibers have a mitochondrial fraction of more than 25% (Barth et al., 1992). Skeletal muscles of sedentary individuals have a mitochondrial content of 3-6%, whereas well trained individuals can have a content of up to 12% (Hoppeler, 1986). The mitochondrial content is normally ~20% lower in type IIa and ~50% lower in type IIx fibers, when compared with type I fibers in untrained subjects (Hoppeler, 1986). The more trained a person becomes, the less fiber types differ in mitochondrial content. Therefore, highly trained individuals often have similar contents in type I and type IIa muscle fibers (Saltin et al., 1977).

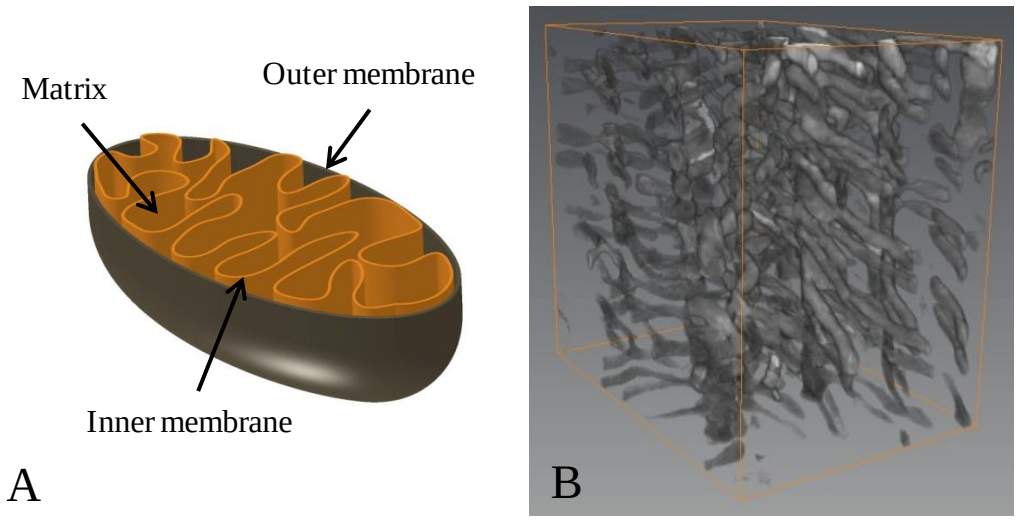

Figure 1. A. Mitochondria illustrated as an individual organelle. B. Three-dimensional reconstruction of mitochondria from a mouse soleus muscle displaying how the mitochondria form a reticular network throughout the muscle section. Michael Larsen is acknowledged for the 3D reconstruction and the picture was kindly provided by Prof. Robert Boushel.

Mitochondria should not be considered individual structures, as often illustrated in the literature (Fig. 1A), but rather as a reticular network within skeletal muscle cells (Fig. 1B) (Ogata and Yamasaki, 1997). This network is dynamic in nature, with mitochondria joining and separating from the network in processes termed fusion and fission (Bo et al., 2010). The mitochondrial content within the cell at any one time is a balance between mitochondrial biogenesis (growth) and mitophagy (degradation). As previously mentioned, mitochondrial content increases rapidly with training, up to 50% after 6-8 weeks, but also rapidly decreases when training stops. Only ~50% of the training induced increase remains two weeks after the

last exercise session. Further, six weeks after training ends, the mitochondrial content (SDH-activity) is back to pre-training level (Henriksson and Reitman, 1977).

The mitochondrial reticulum is a very unique intracellular compartment because it contains DNA (mtDNA). All other DNA is located in the nucleus and most mitochondrial genes are actually found there. A possible explanation for this is that during evolution, approximately $1.5x10^9$ years ago, anaerobic eukaryotic cells absorbed aerobic bacteria and established an endosymbiotic relationship (Cavalier-Smith, 1987; Gray et al., 1999). Thereafter, an extensive transfer of genes to the nuclear genome took place and few genes remained (Andersson et al., 2003). Despite low numbers, these genes are essential for proper function of the oxidative phosphorylation process. To expand the mitochondrial reticulum, a coordinated activation of both the mitochondrial and nuclear genome must take place (Taanman, 1999). A key regulator of this process is the transcriptional coactivator PGC-1α (peroxisome proliferative–activated receptor-γ coactivator 1α) that will be discussed in detail below.

Mitochondrial content/function and health

Disorders in muscle mitochondrial content or function are closely related to various chronic diseases, and to numerous health issues that face our ageing population (Russell et al., 2014). For example, reduced mitochondrial content and functionality might contribute to the onset and/or severity of type 2 diabetes, sarcopenia, Alzheimer's and amyotrophic lateral sclerosis (ALS) (Calvani et al., 2013; Russell et al., 2014). Also, mitochondrial dysfunction seem to be heavily implicated in the ageing process (Trifunovic and Larsson, 2008). Exercise is a promising treatment for patients with mitochondrial dysfunction. Studies show that prescribing regular exercise is an efficient way to improve cellular and whole body health (van Tienen et al., 2012).

Mitochondrial content and endurance capacity

There is a close relationship between mitochondrial content and endurance capacity. Highly trained individuals have a mitochondrial content that is 3-4 times higher than that of untrained (Costill et al., 1976; Hoppeler, 1990). But why is high mitochondrial content important for endurance capacity?

Maximal oxygen consumption (VO_2max) and the % of VO_2max that can be sustained during prolonged exercise (%VO_2max) are two crucial factors for endurance performance. The VO_2max is the highest amount of oxygen that the body can utilize during exhaustive exercise. Elite runners and cross-country skiers generally have a VO_2max of 70-80 ml/kg/min (Losnegard and Hallen, 2014). This can be compared with sedentary individuals that normally range between 30-40 ml/kg/min (Ekblom-Bak et al., 2009). VO_2max is mainly determined by the capacity of the heart and blood to transport oxygen to the working muscles. Mitochondrial content has minor effects on VO_2max under normal conditions

(Bassett and Howley, 2000). Well-trained individuals not only have a high VO_2max, but also a high fractional utilization of VO_2max (%VO_2max). For example, elite marathon runners can run a marathon at an average intensity corresponding to ~85% of their VO_2max (Sjodin and Svedenhag, 1985). On the other hand, untrained individuals can only exercise for a similar period of time (~2 h) at 35-40% of their VO_2max (Bassett and Howley, 2000). The %VO_2max is closely related to the muscles aerobic capacity and mitochondrial content. This is because high mitochondrial content will increase fat utilization and reduce the formation of lactic acid and other fatigue related substances at a given submaximal workload (Bassett and Howley, 2000; Stisen et al., 2006). Coaches and athletes usually refer to this phenomenon as improved lactate threshold because there is a strong relationship between a high %VO_2max and the exercise intensity at which lactate accumulation in the blood accelerates, as a result of increasing lactate production and decreasing lactate removal (Miller et al., 2002).

Mitochondrial biogenesis

The regulation of mitochondrial biogenesis is a complex process that not only requires the synthesis of mitochondrial building blocks, but also the import and incorporation of these proteins into the existing mitochondrial reticulum (Hood, 2009; Scarpulla, 2011). The growing reticulum also requires regulation of factors controlling the fusion and fission processes (Bo et al., 2010). How are all of these processes regulated and coordinated within the skeletal muscle cell?

As previously mentioned, the genes that are needed to construct new mitochondria are located both in the nuclear and mitochondrial genome. The genes located in the mitochondrial genome are controlled by three transcription factors, mitochondrial transcription factor A (TFAM), B1 (TFBM1) and B2 (TFBM2) (Kelly and Scarpulla, 2004). The genes located on the nuclear genome are regulated by several different transcription factors, of which the nuclear respiratory factors 1 and 2 (NRF-1 and 2) are central. These two transcription factors regulate a large number of key mitochondrial proteins such as cytochrome C, succinate dehydrogenase and cytochrome oxidase. In addition to these proteins, the NRFs also regulates mitochondrial transcription factors and are thereby indirectly involved in the regulation of the mitochondrial genome (Scarpulla, 2008).

PGC-1α has emerged as a key regulator of mitochondrial biogenesis since its discovery in 1998. PGC-1α belongs to a family of proteins that also includes PGC-1β and PGC-1-related coactivator (PRC). These two family members have similarities and differences in function when compared with PGC-1α, but their precise roles are still under investigation (Scarpulla, 2011). PGC-1α interacts with the majority of the transcription factors known to regulate mitochondrial biogenesis (Kang and Li Ji, 2012). Included among these are the NRFs. PGC-1α can thereby control the expression of several important genes located on both the nuclear and mitochondrial genome. Recent studies also show that PGC-1α can bind to Tfam and directly influence the transcription of genes located on the mtDNA (Aquilano et al., 2010).

PGC-1α not only regulates the synthesis of mitochondrial proteins, but is also involved in the regulation of fusion and fission processes (Soriano et al., 2006).

Further evidence for the important role of PGC-1α in the regulation of mitochondrial biogenesis comes from animal studies where the PGC-1α gene has been manipulated. If the gene is "knocked out," mice will have reduced muscle mitochondrial content and endurance performance. If the gene is instead overexpressed, the mice will have increased mitochondrial content and enhanced endurance performance (Calvo et al., 2008; Leone et al., 2005).

Even though PGC-1α is mostly associated with mitochondrial biogenesis, it has other regulatory roles. For example, PGC-1α is known to bind to members of the peroxisomal proliferator activator receptor family (PPARs) and can thereby regulate the expression of genes involved in fat metabolism, such as cluster of differentiation 36 (CD36) and carnitine palmitoyl transferase (CPT1) (Vega et al., 2000). PGC-1α also regulates carbohydrate metabolism by binding to estrogen-related receptor-α (ERRα), a transcription factor that controls the expression of the pyruvate dehydrogenase kinase 4 (PDK4) gene (Olesen et al., 2010).

Exercise is a very potent stimulator of mitochondrial biogenesis and it does so mainly through PGC-1α (Holloszy, 2008). Exercise both upregulates PGC-1α gene expression and protein activity, as well as the migration of PGC-1α to the nucleus and mitochondria (Baar, 2014; Little et al., 2011). But how does exercise regulate PGC-1α?

Molecules and ions markedly affected by exercise are potent signaling substances that can mediate disturbances in homeostasis to adaptive processes such as mitochondrial biogenesis. During exercise there are increased intracellular levels of calcium ions (Ca^{2+}), energy regulating molecules, such as ADP or AMP, free phosphate groups (Pi) and reactive oxygen species (ROS) (Baar, 2014). These substances are potent signaling transducers and have been shown to activate protein kinases such as calcium/calmodulin-dependent protein kinases (CaMK), AMP-activated protein kinase (AMPK) and p38 mitogen-activated kinase (p38-MAPK). These in turn are known to regulate both PGC-1α transcription and activity (Kang and Li Ji, 2012). In addition, it has been suggested that extensive cross-talk exists between the different protein kinases. Studies show that CaMK can alter PGC-1α expression indirectly through regulation of p38-MAPK and AMPK (Fig. 2) (Zhang et al., 2014).

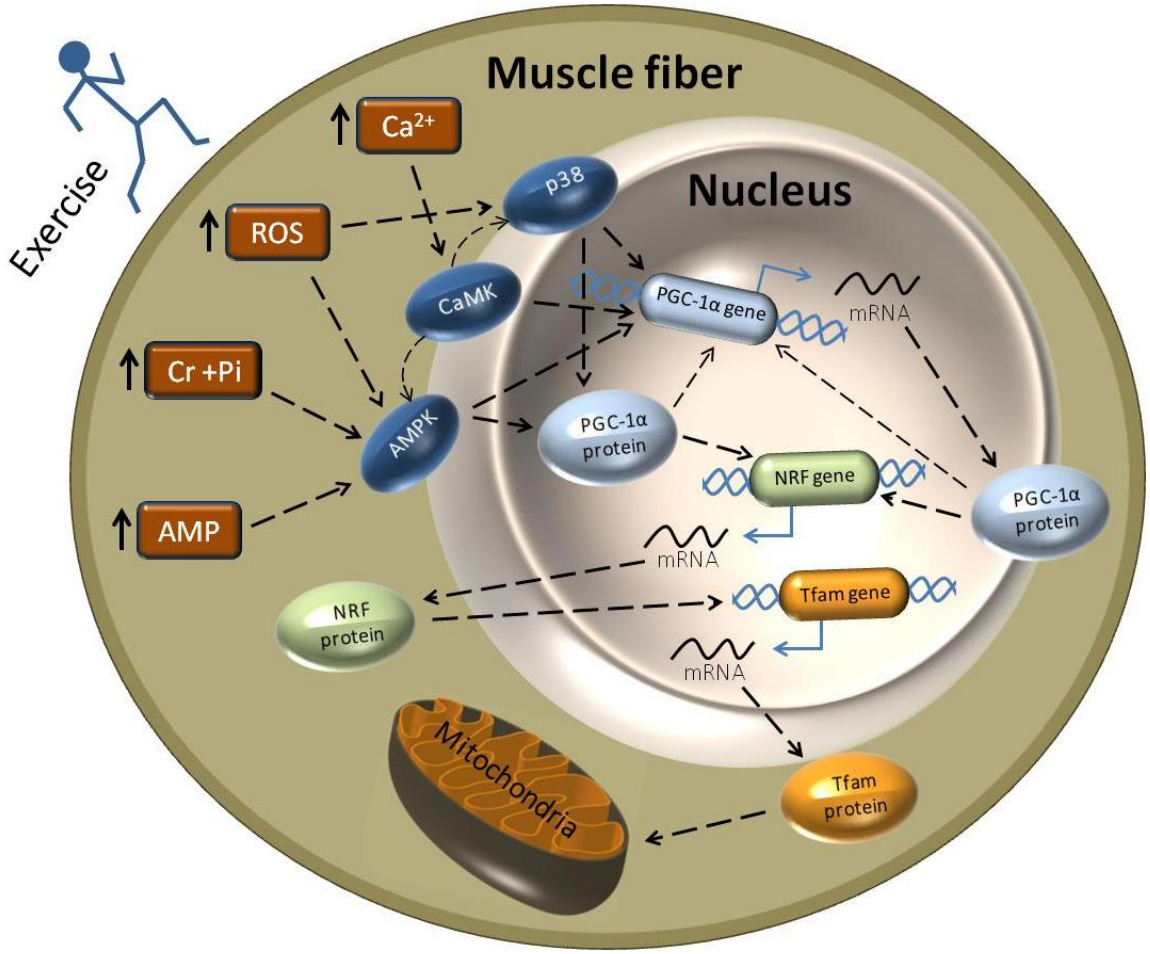

Figure 2. Simplified overview of exercise induced signaling pathways that regulate PGC-1α expression and activity, as well as the subsequent PGC-1α induced mitochondrial biogenesis. ROS, reactive oxygen species; Cr, creatine; Pi, unbound phosphate group; AMP, adenosine monophosphate; p38, p38 mitogen-activated kinase; CaMK, calcium/calmodulin-dependent protein kinases; AMPK, AMP-activated protein kinase; PGC-1α, peroxisome proliferative–activated receptor-γ coactivator 1α; NRF, nuclear respiratory factors; Tfam, mitochondrial transcription factor A.

Even though up-stream regulators and down-stream targets of PGC-1α are still under intense investigation, it is likely that PGC-1α has a key role in mitochondrial biogenesis. By measuring the acute effects of exercise on PGC-1α expression, it is possible to take a "shortcut" and to get information regarding how different exercise regimens affect mitochondrial biogenesis without doing long-term training studies with measurement of mitochondrial content.

Training strategies to promote mitochondrial biogenesis

While there is agreement that exercise is a powerful stimulus to increase mitochondrial content, the optimal training stimulus to induce mitochondrial biogenesis is under dispute (Bishop et al., 2014). A common view has been that traditional endurance training promotes muscle aerobic capacity and mitochondrial biogenesis, whereas sprint training promotes strength and anaerobic capacity (Kubukeli et al., 2002). However, recent studies have challenged this point of view. High intensity interval training (HIIT), in particular sprint interval training (SIT), has been shown to be a time efficient strategy to promote mitochondrial biogenesis (Barnett et al., 2004; Burgomaster et al., 2008; Gibala et al., 2006; MacDougall et al., 1998). SIT is usually performed as very short sprints (20-30 s) at maximal intensity ("all out" effort) repeated 4-8 times. Even though the energy production during a single sprint is mainly anaerobic, repeated sprints will gradually increase the aerobic contribution and a high muscular oxidative capacity becomes important. As few as 4-6 sprints have been shown to increase mitochondrial biogenesis to a similar extent as long duration cycling (90-120 min at 65% of VO_2max) in untrained subjects (Gibala et al., 2006). However, untrained subjects respond well to most types of training and it is possible that the intensity and/or duration of exercise are of minor importance for this group, as long as the training is undertaken regularly. In paper I, the effect of different training intensities on mitochondrial biogenesis in untrained subjects was evaluated.

Compared with untrained subjects, muscles of well-trained individuals are highly adapted and the response to exercise is therefore significantly reduced (Evertsen et al., 1999; Perry et al., 2010). A relevant question is whether SIT can be an efficient training strategy for well trained individuals even though the training duration is only a few minutes and members of this group often train 10-20 hours per week? This question is addressed in paper II where we studied the acute effects of SIT in elite cyclists.

Resistance training has traditionally been something that very few endurance athletes have included in their training program. One reason for this is probably that many athletes are afraid of gaining muscle mass and thereby body weight, which can have a negative impact on performance in weight bearing endurance events. One other explanation might be that some early studies showed that muscle hypertrophy dilutes muscle mitochondria content (Tesch, 1988). Today the majority of endurance athletes include resistance exercise in their training programs. The reason for this is that several recent studies show that adding resistance to an endurance training program can improve endurance performance, especially if heavy, explosive resistance training is incorporated (Losnegard et al., 2011; Paavolainen et al., 1999; Ronnestad et al., 2011; Storen et al., 2008). The mechanism behind this is not well understood. It has been suggested that increased strength and power can result in attenuated blood flow restriction, faster force development in each movement cycle or enhanced neuromuscular function (Ronnestad and Mujika, 2013).

The effects of concurrent resistance and endurance training on mitochondrial biogenesis have not been well studied. However, if resistance and endurance exercise are performed on separate training sessions, the mechanism behind improved endurance performance does not seem to be related to changes in mitochondrial content (Bell et al., 2000; Bishop et al., 1999; Hickson et al., 1988). This might not be the case when resistance and endurance exercise are combined within the same training session. Sale and colleagues observed enhanced CS-activity, a marker of mitochondrial biogenesis, when resistance training was performed immediately after endurance training (Sale et al., 1990). A possible explanation for this might be that the molecular signals induced by endurance exercise are enhanced when resistance exercise is performed in close proximity. This was investigated in paper III of this thesis. Further, the study by Sale et al. only examined the effects of concurrent exercise on physiological adaptations, and did not study at the effect of training on performance. Also, that study examined untrained subjects and the effect of concurrent training on more trained subjects is unknown. In paper V, the effects of endurance followed by resistance training on both mitochondrial biogenesis and performance in trained cyclists were evaluated.

In the last 20-30 years, experts have recommended that endurance athletes consume large quantities of carbohydrates before, during and after training and competition (Burke, 2010). This was based on the perceived benefit of promoting daily muscle glycogen recovery so that every training session could be performed under optimal conditions. Maximizing muscle glycogen is a wise strategy before competition (Karlsson and Saltin, 1971), but there is little evidence that training adaptations are enhanced by performing endurance training in a "high" glycogen state. Instead, recent research in this area show that it is more beneficial (Hansen et al., 2005), or at least not disadvantageous (Morton et al., 2009; Yeo et al., 2008), to undertake most training in a "low" glycogen state. None of these studies have examined the acute effect of low muscle glycogen on genes regulating mitochondrial biogenesis. Also, it is unclear if the observed metabolic adaptations are related to exercise with low muscle glycogen or to other factors related to the training programs, such as timing and intensity of exercise sessions. Therefore, in paper IV, the acute effects of low glycogen exercise on PGC-1α and other genes involved in mitochondrial biogenesis and CHO metabolism were studied.

AIMS

The overall objective of this thesis was to identify efficient training strategies to promote mitochondrial biogenesis and endurance performance

The specific aims were to evaluate how major markers of mitochondrial biogenesis are influenced by:

- High intensity interval exercise
- Muscle glycogen status during exercise
- Concurrent strength and endurance training

METHODS

Subjects

Table 1. Summary of subject characteristics (mean ± SE)

Study	Sex	Number	Age (yr)	Height (cm)	Weight (kg)	VO_2max (L min^{-1})
I	M/F	9(7/2)	26 ± 1	174 ± 3	72 ± 5	3.0 ± 0,2
II	M	10	25 ± 1	179 ± 2	70 ± 2	4.8 ± 0,1
III	M/F	10(7/3)	26 ± 1	177 ± 3	72 ± 4	3.6 ± 0,1
IV	M	10	28 ± 2	183 ± 2	75 ± 2	4.9 ± 0,1
V	M	19	35 ± 2	185 ± 2	80 ± 2	4.5 ± 0,1

A total of 58 subjects were enrolled in the studies. Subjects were informed about the experimental protocols and associated risks before written consent was given. Each study was approved by the Regional Ethical Review Board in Stockholm and performed in accordance with the principles outlined in the Declaration of Helsinki. Subjects in study II and IV were highly trained cyclists that competed at a national elite level or had been competing at this level during the preceding years in road or mountain biking. Subjects in Study V were moderately trained cyclists some of whom competed or had been competing at the national level. Subjects in study I and III were relatively untrained and had not been engaged in programmed endurance or strength training during the 6-month period before the studies were conducted.

Intervention protocols

Table 2. Summary of intervention protocols. FFA, free fatty acids; CS, citrate synthase; MHC, myosin heavy chain, ROS, reactive oxygen species; LT4, work rate corresponding to 4 mmol lactate per L blood; TT40, 40 min time trial; CE, cycling economy; 1RM, one repetition maximum

Study	Type	Protocol	Biopsies	Analysis
I	Acute, cross-over	Continuous vs. interval exercise (matched for total work)	Before and 3 h after exercise	In muscle: gene expression and glycogen. In blood: lactate, glucose and FFA
II	Acute, cross-over	Long interval (3 x 20 min) vs. sprint interval (7 x 30 s) exercise	Before and 3 h after exercise	In muscle: gene expression, glycogen, CS-activity and MHC content. In blood: lactate
III	Acute, cross-over	Endurance vs. concurrent exercise (cycling only or cycling + leg press)	Before, 1 and 3 h after exercise	In muscle: gene expression, protein phosphorylation and glycogen. In blood: lactate, glucose and FFA
IV	Acute, cross-over	Low vs. normal muscle glycogen exercise	Before and 3 h after exercise	In muscle: gene expression, protein phosphorylation, respiration, ROS and glycogen. In blood: lactate, glucose, FFA and insulin
V	8 weeks, two groups	Endurance vs. concurrent exercise (cycling only or cycling + leg press)	Before and after the training period	In muscle: enzyme activity and protein content. In blood: hormones and lactate. Performance tests: VO_2max, LT4, TT40, CE, Wingate and IRM leg press

Study I

Each subject participated in two experimental sessions, in randomized order, separated by an interval of at least 2 weeks for men and 4 weeks in women (to minimize the possible influence of the menstrual cycle) (Fig. 3). In one of these sessions, the subject cycled for 90 min at a constant work rate corresponding to 60% of VO_2peak (CE) and in the other session he/she performed IE involving cycling alternating for 12 s at 120% of VO_2peak and for 18 s at 20% of VO_2peak. The work rate during the second exercise session was adjusted to match the duration and the total work performed during the first session. The participants were instructed to eat normally but to refrain from the consumption of alcohol, caffeine, and tea during the 24-h period preceding the test, and not to do any strenuous exercise for 48 h before the tests. Venous blood samples were drawn before exercise, during the final minutes of exercise and 3 h after completion of cycling. Muscle biopsies were taken before beginning the exercise (Pre) and 3 h after completion (3 h Post).

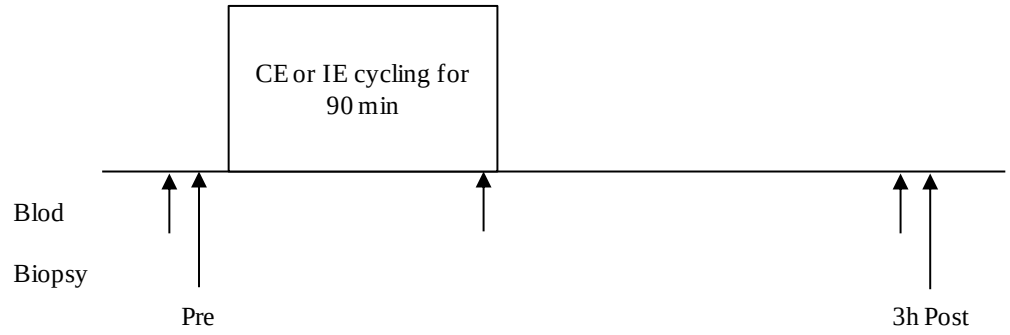

Figure 3. Schematic overview of the intervention protocol in study I.

Study II

The study was executed for 3 weeks in the middle of May, 6 weeks into the competition season. All testing was performed 48–72 h after the weekends' racing and the subjects were only allowed to do recovery type exercises (heart rate < 120 bpm and duration < 1 h) during this period. After a standardized warm-up ending at a power output corresponding to RER 1.0, the subjects performed two different interval exercise sessions, 1 week apart in a counterbalanced fashion (Fig. 4). The SIE consisted of 7 x 30-s "all-out" Wingate type intervals (separated by 4 min of active rest at 50 W) performed at ~110 rpm on the ergometer. The IE consisted of 3 x 20-min intervals (separated by 4 min of active rest at 50 W), where the subjects were instructed to ride as hard as possible (i.e., time trial effort). Capillary blood lactate sampled from a fingertip directly after each interval, peak power and mean power were measured during both SIE and IE. A total of four muscle biopsies were obtained over the course of the experiment, two at each exercise trial. The first biopsy was obtained 15 min prior to the exercise sessions and the second biopsy was obtained 3 h after completion. All subjects kept a dietary record during the 48 h preceding the first trial, and were instructed to duplicate this during the second trial.

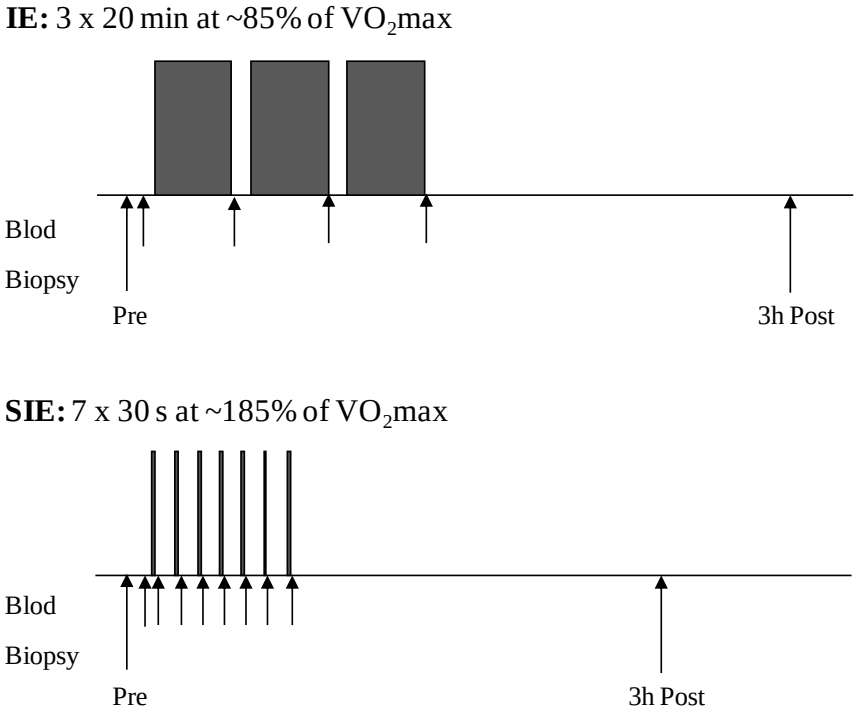

Figure 4. Schematic overview of the intervention protocol in study II.

Study III

An overview of the exercise protocol is shown in figure 5. On the morning of the experimental day, subjects reported to the laboratory around 8 AM. After a 10-min rest in the supine position, a blood sample was taken from an arm vein, and a muscle biopsy sample was obtained from the middle portion of the vastus lateralis muscle of one leg. After a 5-min warm-up on the cycle ergometer, the subjects exercised (60–70 rpm) for 60 min at a work rate corresponding to 65% of VO_2peak (3-min rest was allowed after 30 min of cycling; E), or performed the same cycling exercise followed by one bout of resistance exercise after 15-min rest (ER). The resistance exercise was performed in a leg press machine (243 Leg Press 45°, Gymleco, Stockholm, Sweden) and consisted of six sets of leg presses at workloads corresponding to 70, 75, 80, 80, 75, and 70% of the individual 1 RM, with 3-min rest between each set. During each set, subjects were encouraged to do as many repetitions as possible up to 15. One hour after the cycling exercise (i.e., 20 min following resistance exercise in the ER experiment), a second muscle biopsy and blood sample was taken [1 h post exercise (Post)]. A third muscle and blood sample was taken 3 h after the cycling exercise (3 h Post) (i.e., 2 h and 20 min following resistance exercise in the ER experiment). The pre exercise (Pre) biopsy was taken from one leg, and both Post biopsies (1 h and 3 h Post) were taken from the other leg. The leg chosen for the first biopsy sample was randomly assigned. The food intake in the evening before the first experiment was recorded and then duplicated during the evening before the second experiment. In addition, subjects were instructed to fast for 12 h and refrain from heavy physical exercise for at least 2 days before each trial. Subjects were fasted throughout the experiment.

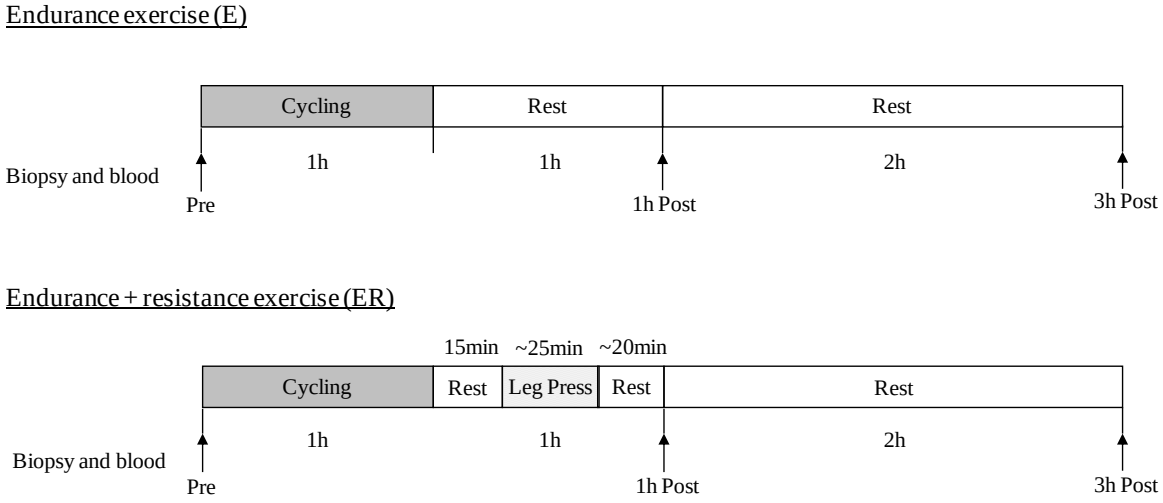

Figure 5. Schematic overview of the intervention protocol in study III.

Study IV

Subjects participated in two experimental sessions separated by at least 1 week in a crossover design with randomized order (Fig. 6). In one of the sessions, subjects had a high CHO (NG) diet and in the other a low CHO (LG) diet (see below for details). Both sessions included two exercise tests separated by about 14 h. The purpose of the first exercise was to deplete muscle glycogen (depletion exercise) and the second exercise to test the influence of low muscle glycogen on the signaling response (test exercise). Subjects were instructed to refrain from exhaustive exercise and alcohol during the 2 days prior to the experiment. Subjects arrived to the laboratory in the afternoon. A blood sample was taken from an arm vein and a muscle biopsy sample was obtained from the middle portion of the vastus lateralis muscle of one leg. The depletion exercise was approx. 2.5 h long and included both continuous and interval cycling. The test exercise was performed the following morning, about 14 h after the depletion exercise, and included six intervals of 10 min cycling with 4 min active rest (100 W) between intervals. Capillary blood samples were collected from fingertips before test exercise and during the last seconds of intervals 2, 4, and 6 and were analyzed for lactate and glucose. Venous blood samples and muscle biopsies were obtained approximately 15 min before and 3 h after the test exercise.

Subjects recorded their food intake during the 24-h period preceding the first experiment and were instructed to duplicate this prior to the second experiment. During and after the glycogen depletion exercise on day 1, and the following the test exercise on day 2, subjects either consumed a high CHO (NG) or low CHO (LG) diet as shown in figure 4. The NG diet included two high CHO meals and eight high CHO beverages plus a banana. The LG diet included two low CHO meals. NG provided 88% of total energy intake from CHO, 6% from protein and 6% from fat, while LG provided less than 1% of total energy intake from CHO, 22% from protein, and 77% from fat.

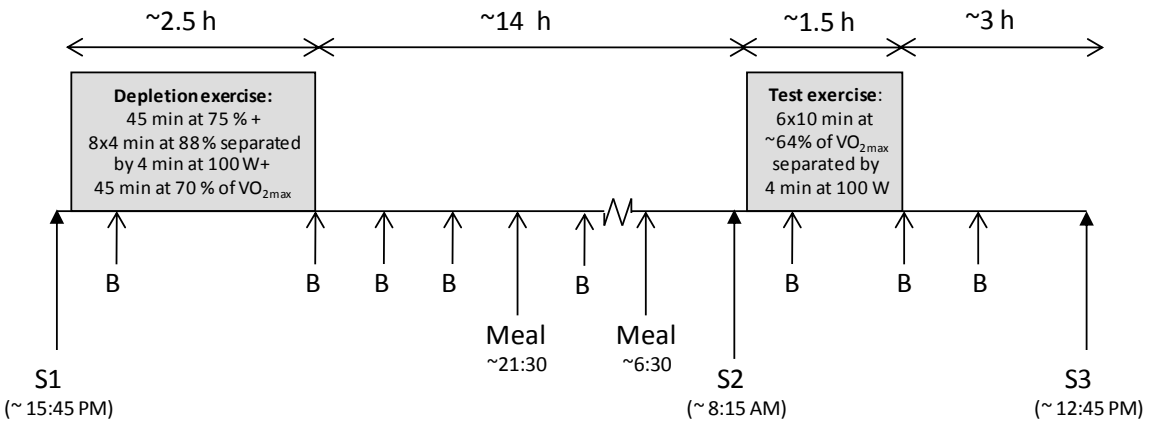

Figure 6. Schematic overview of the intervention protocol in study IV. B beverages containing CHO (NG normal glycogen) or only water (LG low glycogen). Meals contained either high (NG) or low CHO (LG). Muscle biopsies and venous blood samples were obtained approximately 15 min before the depletion (S1) and test exercise (S2), as well as 3 h after the test

Study V

The subjects were instructed to continue their habitual cycle training but to exchange two ordinary training sessions per week with supervised laboratory training. The first 8 supervised training sessions consisted of 60 min of continuous cycling at a work rate corresponding to 90% of the mean power output during the 40 min time trail in the pre-test. The work rate was then raised to 92.5% (sessions 9-12), and to 95% (sessions 13-16). Following 10-15 min rest the subjects in the ES group performed strength training in a leg press machine (243 Leg Press 45°, Gymleco, Stockholm, Sweden). The session started with a warm up set with 10 repetitions at 50% of 1 RM (determined in pre-test) followed by sets at 65, 70, 75, 75, 70, and 65% of 1 RM. The subjects were instructed to do as many repetitions as possible and if they were able to complete 15 reps, the load was increased by 5% the next set. Instead of resistance exercise, the E group cycled for 2.5-4 min corresponding to an equal amount of energy expenditure.

The subjects were instructed to keep a balanced diet throughout the intervention and to avoid diets with a low amount of calories or carbohydrates. The subjects recorded food intake during the 36 h preceding pretests and were instructed to duplicate the diet prior to posttests. Pre- and posttests were performed three hours after the last meal, at the same time of the day for each subject.

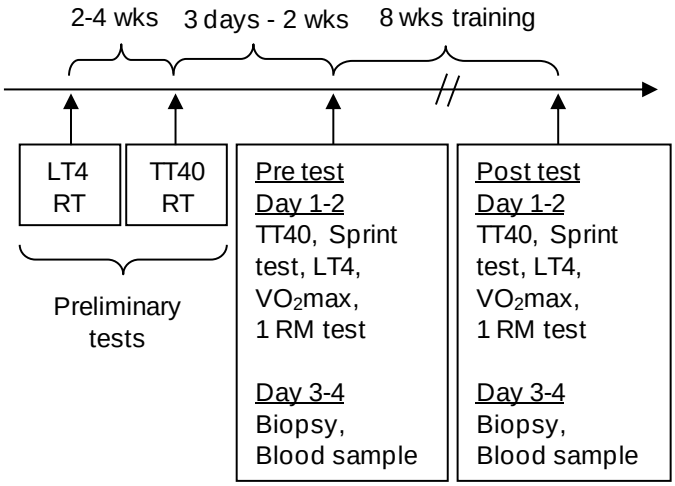

Figure 7. Schematic overview of the intervention protocol in study V. LT4, work rate corresponding to 4 mmol lactate per l blood; TT40, 40 min time trial; RT, resistance training; 1 RM, one repetition maximum.

Exercise tests

VO_2max

The testing of VO_2max was performed on an ergometer (Monark 839E, Monark Exercise, Varberg, Sweden or SRM, Konigskamp, Germany) with a two stage incremental exercise protocol. The first part (3-5 min cycling at 5-6 submaximal intensities) was used to establish the relation between VO_2 and work rate (W), and to get a rough estimate of the work rate corresponding to VO_2max. After 3-5 min of active rest, the work rate was increased rapidly until voluntary exhaustion, with a protocol designed to elicit VO_2max after 7–8 min. Oxygen uptake was measured using an online system (Oxycon Pro, Erich Jaeger, Hoechberg, Germany or AMIS 2001; Inovision A/S Odense, Denmark) and VO_2max was defined as the highest recorded oxygen uptake during 40-60 consecutive seconds. The following criteria were used for attaining VO_2max: RPE > 18, RER > 1.1, and a plateau of VO_2 with increased workload.

Lactate threshold

Lactate threshold was determined during incremental, submaximal ergometer exercise (the first part of the above described VO_2max protocol). Blood was sampled from a finger capillary at the end of each work period and analyzed for lactate. LT4 was defined as the interpolated workload corresponding to a blood lactate concentration of 4 mmol l^{-1} blood.

1RM

Maximal strength was measured in a leg press machine (243 Leg Press 45°, Gymleco, Stockholm, Sweden). After warm up, the load was progressively increased until the subjects could not perform more than one single repetition (turning point between the eccentric and concentric phase was set at a knee angle of 90° degree). Most subjects reached their 1 RM within five to seven trials. There was a 3-5 min rest between trials to avoid muscle fatigue.

Biopsy sampling and preparation

Muscle samples were obtained from the middle portion of the vastus lateralis muscle at a depth of 2-3 cm, about one-third of the distance from the upper margin of the patella to the anterior superior iliac spine. After local anaesthesia (2-4 ml Carbocaine 20 mg ml-1; Astra Zeneca, Södertälje, Sweden) an incision (0.5-1 cm) was made through the skin and fascia and a muscle sample (50-100 mg) was obtained with the percutaneous needle biopsy technique utilizing suction (Bergstrom, 1975). If more than one biopsy was obtained from the same muscle, a new incision was created approximately 2-4 cm proximal to the previous one. The samples were rapidly frozen in liquid nitrogen and stored at -80°C. The frozen samples were thereafter freeze-dried, dissected free of blood and connective tissue, pulverized with forceps, extensively mixed and finally divided into aliquots for subsequent analysis.

Blood analysis

Capillary blood samples

Capillary blood samples were collected from fingertips, and lactate and glucose were analyzed using an automated analyzer (Biosen 5140, EKF Diagnostics, Barleben, Germany).

Venous plasma samples

To obtain plasma, blood (4 ml) from an antecubital vein was collected into heparinized tubes and centrifuged at 1,500 g at 4 °C for 10 min. A commercially available colorimetric enzymatic procedure (NEFA C test kit; Wako Chemicals GmbH, Neuss, Germany) was used for determining free fatty acid concentrations. Lactate and glucose were analyzed by enzymatic methods according to Bergmeyer and Bernt (Bergmeyer and Bernt, 1974). Insulin, cortisol and testosterone were analyzed with ELISA kits (Mercodia, Uppsala, Sweden and Calbiotech, CA, USA) using a plate reader (Tecan infinite F200 pro, Männedorf, Switzerland).

Muscle analysis

RNA extraction and real-time PCR analysis

Total RNA was extracted from 2–5 mg freeze-dried muscle tissue using a glass (study I) or Polytron PT 1600 E homogenizer (Kinematica, Lucerne, Switzerland) and a PureZOL RNA isolation kit according to the manufacturer's instructions (Bio-Rad Laboratories AB, Sundbyberg, Sweden). The yield and quality of extracted RNA were estimated by spectrometry and micro-gel electrophoresis (Experion, Bio-Rad, study II-IV). The 260/280 absorbance ratios were between 1.6 and 2.1 depending on the buffer used. The RNA quality indicator values (RQI) from the micro-gel electrophoresis were greater than 0.7, verifying high quality RNA samples. RNA (1 µg) was reverse transcribed to cDNA (20 µl) using the iScript cDNA synthesis kit (Bio-Rad). Real-time RT-PCR was performed with an iCycler (Bio-Rad) in a mixture containing 12.5 µl 2x SYBR Green Supermix (Bio-Rad), 0.5 µl of both the forward and reverse primers (final concentrations 10 µM), and 11.5 µl template cDNA. All reactions were performed in triplicate with GAPDH as the reference gene. β-actin and cyclophilin were also used as reference genes in study I, but they were excluded because their expression levels tended to increase with exercise whereas GAPDH was stable. This finding that GAPDH is unaffected by exercise has been confirmed by others (Jemiolo and Trappe, 2004). The melting curves of the PCR product showed only one peak, demonstrating specificity of the primers and absence of contamination. The cDNA concentration, annealing temperature and thermocycling conditions were optimized for each primer pair, and assay sensitivity was high for all PCR products (RSq > 0.99, and efficiency > 90%). The comparative critical threshold (CT) method could therefore be used to calculate changes in mRNA levels (Livak and Schmittgen, 2001).

Enzyme activity

Muscle samples were homogenized using a bullet blender in a buffer (150 µl mg^{-1}) with the following composition (in mM): 50 K_2HPO_4, 1 EDTA and 0.05% Triton X-100 adjusted to pH 7.4. The homogenate was centrifuged at 10 000 rpm for 10 min and the supernatant was collected and diluted x3. Citrate synthase (CS) activity was measured in a reagent solution (in mM): 50 Tris-HCl, 0.2 DTNB and 0.1 acetyl-CoA. The reaction was initiated by adding oxaloacetate (7 mM) and the change in absorbance at 412 nm was measured spectrophotometrically at 25°C. Hydroxyacyl-CoA dehydrogenase (HAD) activity was measured in a reagent solution (in mM): 65 Triethanolamine HCL, 0.3 EDTA and 0.3 NADH adjusted to pH 7.0. The reaction was initiated by adding acetoacetyl coenzyme A (4 mM) and the change in absorbance at 340 nm was measured spectrophotometrically at 25°C.

Western blot

The samples were homogenized using a bullet blender (Bullet Blender 1.5, Next Advance, NY, USA) in ice-cold buffer (80 µl mg^{-1}) with the following composition (in mM): 2 HEPES, 1 EDTA, 5 EGTA, 10 $MgCl_2$, 50 β-glycerophosphate, 1% TritonX-100, 1 Na_3VO_4,

2 Dithiothreitol, 20 ìg ml^{-1} Leupeptin, 50 ìg ml^{-1} Aprotinin, 1% Phosphatase inhibitor cocktail (Sigma P-2850, St Louis, MO, USA), 40 ìg il^{-1} PMSF. The homogenates were centrifuged to pellet the insoluble debris. Protein concentration was determined with the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL, USA) by measuring the absorbance at 560 nm with a plate reader (Tecan infinite F200 pro, Männedorf, Switzerland). The samples were diluted with Laemmli sample buffer (Bio-Rad Laboratories, Richmond, CA, USA) and homogenizing buffer (1:1) to a final protein concentration of 1.5 µg $µl^{-1}$ and heated to 95°C for 5 min to denature proteins. The diluted samples were stored at -20°C prior to analysis. The proteins of the diluted samples were separated by SDS-PAGE (Criterion cell gradient gels, Bio-Rad Laboratories) for 45 min at 300 V on ice and then transferred to polyvinylidine fluoride membranes (Bio-Rad Laboratories) for 3 h at 300 mA on ice. The amount of protein loaded to the membranes was kept constant for all samples and was verified by staining with MemCodeTM Reversible Protein Stain Kit (Pierce Biotechnology). After blocking for 1 h at room temperature in 5% non-fat milk, the membranes were incubated over-night with primary antibodies. This was followed by 1 h incubation with anti-rabbit or anti-mouse HRP (1:10 000) as the secondary antibody. The antibodies were visualized by chemiluminescent detection on a Molecular Imager ChemiDocTM XRS system and the bands were analyzed using Quantity One® version 4.6.3 software (Bio-Rad Laboratories).

Muscle glycogen content

Glycogen was analyzed in 1-2 mg of freeze-dried muscle according to the method previously described by Harris et al. (Harris et al., 1974), which includes enzymatic hydrolysis of glycogen followed by enzymatic analysis of glucose.

Fiber type composition

MHC composition was analyzed as described previously (Talmadge and Roy 1993), with some minor modifications. Briefly, approximately 1 mg of freeze-dried muscle was homogenized in a buffer containing 2 mM HEPES (pH 7.4), 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 1 mM N_3VO_4, 2 mM DTT, 1% Triton X-100, 20 µg leupeptin and 50 µg aprotinin and 40 µg PMSF per mL, and 1% of a phosphatase inhibitor cocktail (Sigma P-2850). The protein concentration was determined using a BCA protein assay (Thermo Fisher ScientiWc Inc., Rockford, IL, USA), after which the muscle homogenates were dissolved in a sample buffer (Laemmli, containing 5% β-Mercaptoethanol; Bio-Rad Laboratories AB, Sundbyberg, Sweden) and brought to a final protein concentration of 0.3 µg $µl^{-1}$ by heating them for 10 min at 60°C. 20 µl of each sample was heated for 5 min at 95°C and allowed to cool before loading onto a SDS–PAGE separation and concentration gel containing 8 and 4% acrylamide, respectively. β-Mercaptoethanol (at a final concentration of 0.16%) was added to the top running buffer, to improve the sharpness of the bands. Electrophoresis was done on ice in a 4°C room for 24 h at 145 V/12 mA and the bands on the gel were visualized and quantified by silver staining (PlusOne Silver Staining Kit, Protein; Amersham Bioscience, GE Healthcare, Little Chalfont, UK) and scanned with a Gel Doc 2000 apparatus. Finally, the relative amounts of the

different isozymes of MHC were determined utilizing the Quantity One software, version 4.6.3 (Bio-Rad Laboratories AB, Sundbyberg, Sweden).

Mitochondrial respiration and ROS production

Muscle samples (10-25 mg wet weight) were stored in an ice cold medium with the following composition (in mM): 2.8 CaK_2EGTA, 7.2 K_2EGTA, 5.8 Na_2ATP, 6.6 $MgCl_2$, 20 Taurine, 15 Na_2Phosphocreatine, 20 Imidazole, 0.5 Dithiothreitol and 50 MES adjusted to pH 7.1. The specimen was split into 2-5 mg fiber bundles and each bundle was mechanically separated using surgical needles into a network formation to expose fiber membranes to the surrounding medium. The bundles were incubated with saponin (50 µg ml^{-1}), washed twice and stored in a medium with the following composition (in mM): 0.5 EGTA, 3 $MgCl_2$, 60 K-lactobionate, 20 Taurine, 10 KH_2PO_4, 20 Hepes, 110 Sucrose and 1 g l^{-1} BSA adjusted to pH 7.1.

Mitochondrial respiration was measured in a Clark-type electrode (Hansatech instruments, Kings Lynn, England) in a water-jacketed glass chamber at 25°C. Permeabilized muscle fiber bundles (n = 8) were added to the storage medium supplemented with benzyltoluene sulfonamide (45 µM) to prevent fiber contraction. The oxygen consumption was measured after sequential additions of: octanoyl-carnitine (1.5 mM), ADP (5 mM), pyruvate (20 mM), glutamate (5 mM), succinate (5 mM), and cytochrome c (10 µM).

The rate of mitochondrial H_2O_2 production was measured with Amplex red (Invitrogen, Eugene, OR, USA), which, in the presence of peroxidase enzyme, reacts with H_2O_2 and produces the red fluorescent compound Resorufin. Permeabilized fiber bundles (n = 8) were added to the measuring medium (in mM): mannitol (225), sucrose (75), Tris-base (10), K_2HPO_4 (10), EDTA (0.1), $MgCl_2$ (0.08), BSA (2 g/l), horseradish peroxidase (13.5 U/ml), benzyltoluene sulfonamide (45 µM) and superoxide dismutase (SOD; 45 U/ml) adjusted to pH 7.1 and kept at 30°C. The change in fluorescence was recorded on a Hitachi f-2500 fluorescence spectrophotometer equipped with a magnetic stirrer (Tokyo, Japan) after subsequent additions of: octanoyl-carnitine (1.5 mM), pyruvate (5 mM), succinate (5 mM) and rotenone (0.5 µM).

Statistical analysis

Values were expressed as mean ± SE and the threshold for significance was set at $P < 0.05$. A paired t-test was used for normally distributed data to compare changes in dependent variables within the same group at different conditions or at two time points. A nonparametric Wilcoxon signed rank test replaced the t-test if the data was not normally distributed. A one-way repeated-measure ANOVA, followed by a Newman Keul's post-hoc test, was used to compare within group changes over time when more than two time points were analyzed. A two-way repeated-measure ANOVA, followed by a Fishers LSD or paired t-test, was used to identify differences over time for two different conditions (e.g., E/ER, NG/LG, E/ES). A Spearman's correlation test was used to analyze correlations between variables.

METHODOLOGICAL CONSIDERATIONS

Wet vs. freeze-dry muscle

Most studies use frozen muscle samples ("wet" muscle), freeze-dried muscle has been used in studies performed by our laboratory. The reason for this is that the biopsies become less sensitive to temperature and it is possible to remove non-muscle tissue. However, freeze-drying might influence the yield and quality of the RNA extracted. To examine this, eight biopsies were obtained. Each biopsy was divided into four pieces, of which two were freeze-dried. RNA was extracted, and the yield and quality tested in a spectrophotometer (absorbance at 280 and 260 nm) and micro-gel electrophoresis station (Experion, BioRad). The yield was ~7% lower (Fig. 8) when the samples were freeze-dried, but the quality was unaffected. Real-Time RT-PCR was performed but no difference in gene expression (PGC-1α) was observed between the wet and freeze-dried samples. We therefore concluded that freeze-dried muscle samples would not compromise RNA quality or PCR quantity and could therefore be used for gene expression analysis.

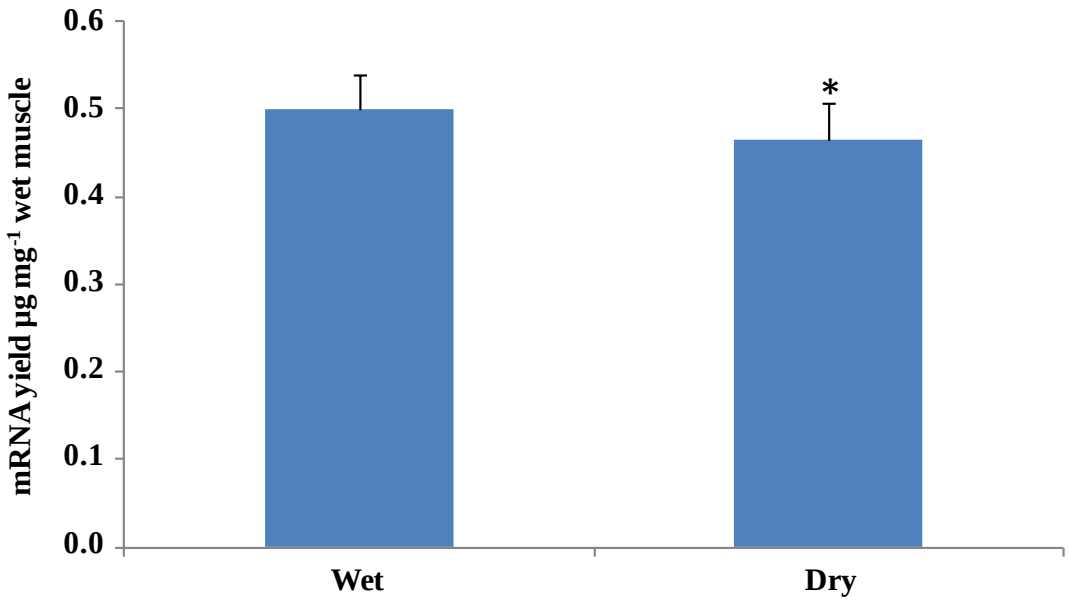

Figure 8. mRNA yield in frozen (wet) and freeze-dried (dry) muscle samples obtained from the same biopsies. Values are reported as means ± SE. *P < 0.01 vs. wet.

Biopsy sampling

In all studies we used the percutaneous needle biopsy technique with suction (Fig. 9A, right) (Bergstrom, 1975). This technique requires the cooperation of two people. One that takes the biopsy and one that assists by handling the syringe and the suction. One problem with this method is obtaining the correct suction pressure. If too much pressure is applied, blood and muscle can be sucked up into the tube connected to the syringe. If too little pressure is applied, the muscle might not be fully sucked into the biopsy needle. Another disadvantage with this method is that the biopsy depth is difficult to standardize due to the sharp tip of the needle and the location of the opening. The biopsy procedures performed during our trials

were completed by experienced professors and technicians, and the biopsy size and quality, as well as the depth and location, were standardized.

There is also another biopsy technique available, the Weil–Blakesley chonchotome. Instead of a biopsy needle, a special forceps is used and only one person is needed for the biopsy procedure (Fig. 9A, left). The Weil–Blakesley chonchotome technique is easier and has a higher success rate in some cases, with repeated attempts rarely needed. The disadvantage to this method is that the maximal sample size is smaller compared with the percutaneous needle biopsy technique (~100 mg vs. >200 mg), however the average sample size is usually similar.

Observation and instruction in the Weil–Blakesley chonchotome technique occurred during 2012-2014 and the author obtained a total of ~30 biopsies during this period (Fig 9B). Future studies will incorporate this method once the technique is fully mastered.

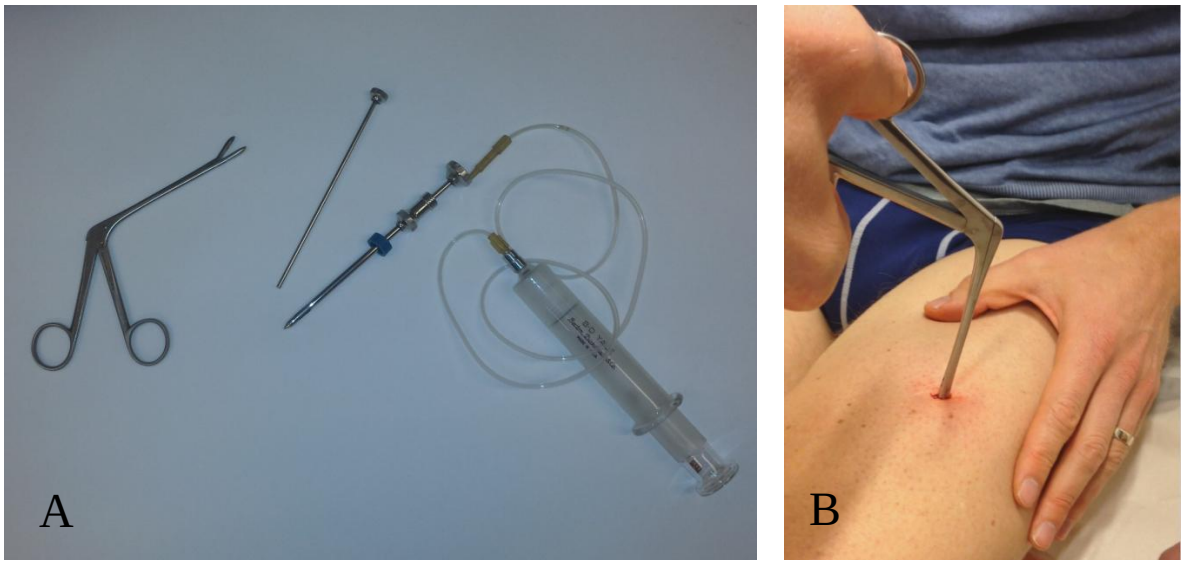

Figure 9. A. Percutaneous needle (right) and Weil–Blakesley chonchotome forceps (left). B. Muscle biopsy obtained with Weil–Blakesley chonchotome technique from the Vastus Lateralis muscle (performed by the author on the author).

RESULTS

High Intensity Interval Exercise (study I and II)

In Study I and II we investigated the acute effects of interval exercise on expression of genes regulating mitochondrial biogenesis and metabolism. In study I we used untrained subjects and compared interval exercise (90 min alternating between 120 and 20% of VO_2max) with continuous exercise (90 min at 60% of VO_2max). The duration and work performed were identical in the two trials. In study II, we used highly trained cyclists and compared low-volume sprint interval exercise (7x30s at ~184% of VO_2max) with high-volume interval exercise (3x20 min at ~87% of VO_2max).

Study I: interval (IE) vs. continuous (CE) exercise in untrained subjects

VO_2 increased approximately six-fold during both CE and IE, and corresponded to 67 ± 2% and 66 ± 2% of VO_2max, respectively. Changes in lactate, glucose and free fatty acids were also highly similar between the two exercise types (table 3). Muscle glycogen decreased 35% during CE and 40% during IE, but no significant difference was observed between conditions. There was also no difference in gene expression between the two exercise types. Both IE and CE induced a marked increase in mRNA of key regulators of mitochondrial biogenesis (Fig. 10).

Table 3. Concentration of blood lactate, blood glucose and plasma FFA

Variable	Group	Pre	Post	3 h Post
Lactate (mmol L^{-1})	CE	1.11 ± 0.24	2.32 ± 0.28*	0.72 ± 0.06
	IE	0.78 ± 0.13	2.17 ± 0.33*	0.70 ± 0.06
Glucose (mmol L^{-1})	CE	4.41 ± 0.11	3.70 ± 0.06*	4.07 ± 0.07*
	IE	4.40 ± 0.10	3.85 ± 0.09*	3.99 ± 0.10**
FFA (mmol L^{-1})	CE	0.18 ± 0.03	0.60 ± 0.08*	0.59 ± 0.05*
	IE	0.29 ± 0.05	0.78 ± 0.12*	0.78 ± 0.18*

*P < 0.05 and **P < 0.01 vs. Pre-exercise

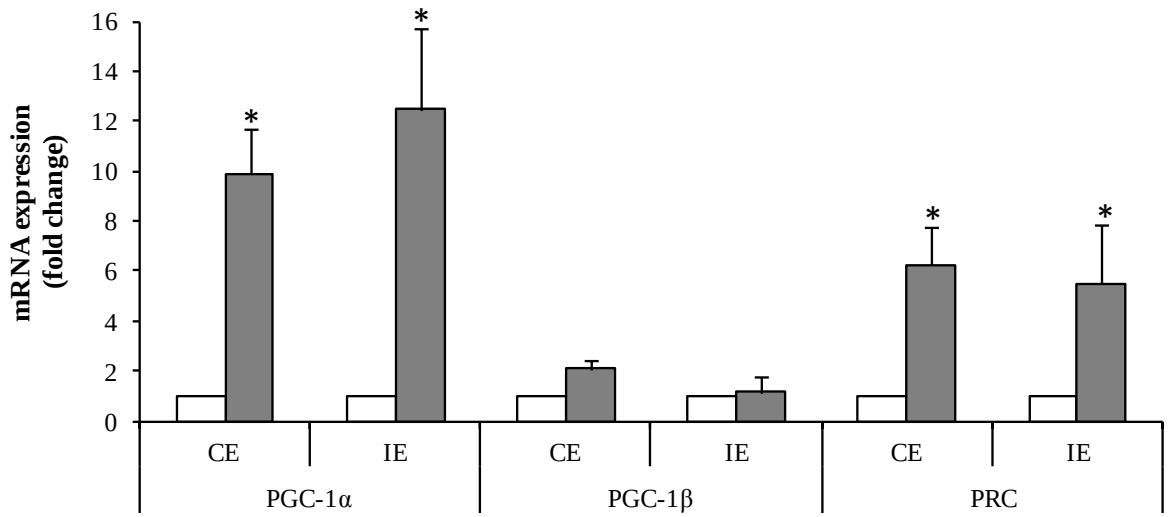

Figure 10. Effect of continuous (CE) and interval exercise (IE) on mRNA levels of proteins regulating mitochondrial biogenesis. White bars, pre-exercise; gray bars, 3 h post exercise; PGC-1α, peroxisome proliferative–activated receptor-γ coactivator 1α; PGC-1β, peroxisome proliferative–activated receptor-γ coactivator 1 β; PRC, PGC-1-related coactivator. Values are reported as means ± SE. *P < 0.05 vs. pre exercise.

Study II: Low-volume sprint interval exercise (SIE) vs. high-volume interval exercise (IE) in well trained subjects

The total work during IE was eightfold higher than that during SIE, and the total exercise duration 17-fold longer (table 4). As expected, the metabolic response was very different between the two protocols, with very high lactate levels during SIE compared with IE (14.9 vs. 4.4 mmol L^{-1}). Even though the anaerobic energy production was high during SIE, there was a strong correlation between the accumulated work produced during SIE and the subjects VO_2max (r = 0.87, P < 0.01). Already after the second bout of SIE the subjects, with a high VO_2max performed better. Muscle fiber composition did not correlate with any of the measured physiological or metabolic parameters. Gene expression of major regulators of mitochondrial biogenesis were increased to a similar extent after SIE and IE, with the exception of Tfam which was more increased after SIE (Fig. 11).

Table 4 Summary of low-volume sprint interval exercise (SIE) and high-volume interval exercise (IE) protocols

	SIE (7 x 30 s)	IE (3 x 20 min)
Exercise intensity (mean power)	643 ± 22 W (~ 184% of VO_2peak)	304 ± 12 W (~ 87% of VO_2peak)
Time commitment including rest	27.5 min	68 min
Time commitment excluding rest	3.5 min	60 min
Total work	135 ± 5 kJ	1094 ± 43 kJ

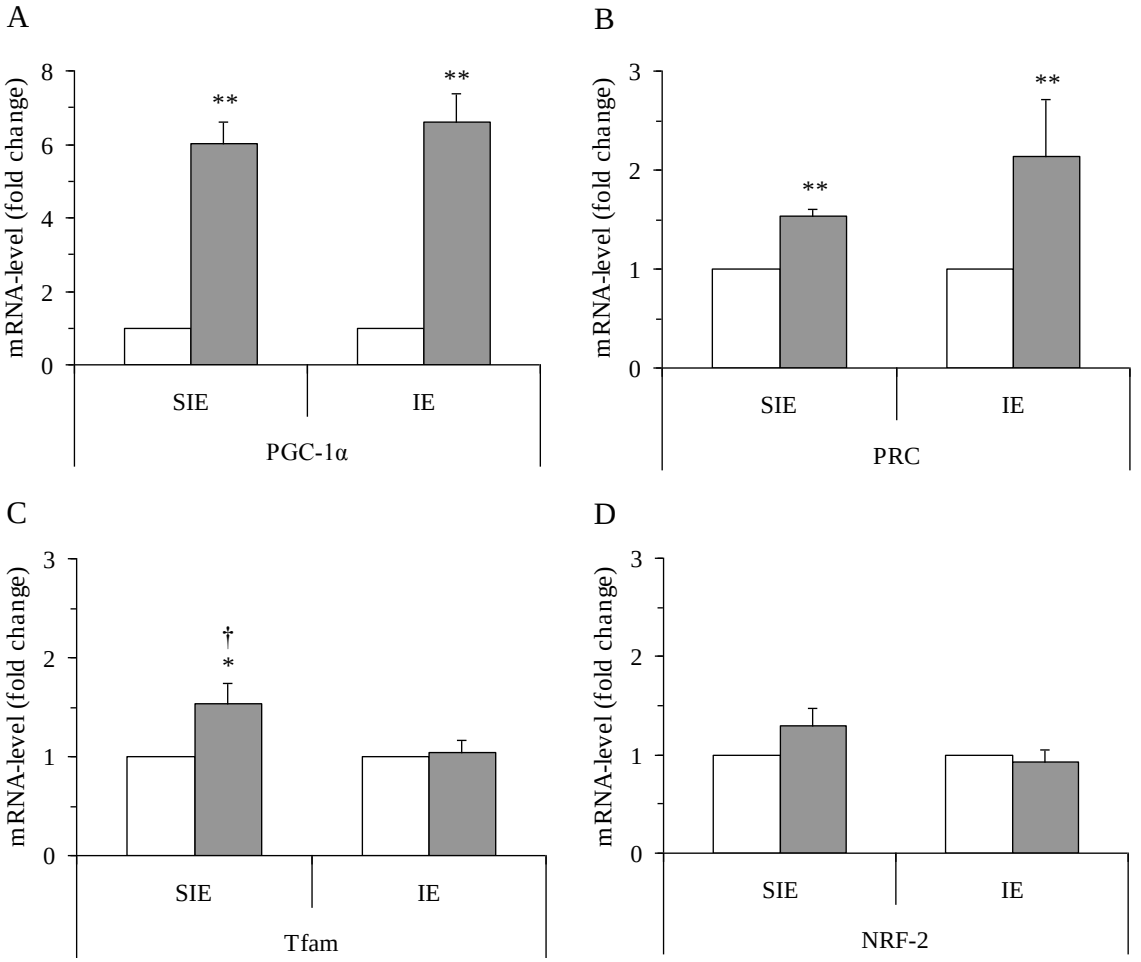

Figure 11. Effect of low-volume sprint interval exercise (SIE) and high-volume interval exercise (IE) on mRNA levels of genes regulating mitochondrial biogenesis. White bars, pre exercise; gray bars, 3 h post exercise; PGC-1α, peroxisome proliferative–activated receptor-γ coactivator 1α; PRC, PGC-1-related coactivator. Tfam, mitochondrial transcription factor A; NRF-2, nuclear respiratory factor 2. Values are reported as means ± SE. *P < 0.05 vs. pre exercise; †P < 0.05 vs. IE.

Concurrent exercise/training (study III and V)

In study III, we investigated the acute effects of concurrent endurance and resistance exercise on genes and proteins regulating mitochondrial biogenesis. In study V, we investigated the long-term effects of concurrent training on muscle oxidative capacity and endurance performance. Both studies used a similar exercise protocol where cycling was immediately followed by resistance exercise. The main difference between the studies, besides acute vs. chronic adaptation, was that the subjects in study III were untrained, whereas trained cyclists were evaluated in study V.

Study III: concurrent exercise in untrained subjects

The mRNA of key marker genes of mitochondrial biogenesis (PGC-1α, PRC) and substrate regulation (PDK4) increased significantly after both models of exercise. After the endurance only session (E), the mRNA of PGC-1α, PRC, and PDK4 was increased 10-, 2-, and 14-fold, respectively. The adaptive response was, however, more pronounced after the endurance + resistance session (ER), and 3 h post-exercise mRNA was about twofold higher after ER than after E (Fig. 12).

Approximately 3-years after the study was completed, we did some complementary mRNA measurements on the remaining cDNA samples. This analysis showed that the truncated isoforms of PGC-1α were increased in a similar fashion as total PGC-1α 3 h post exercise (Fig. 12).

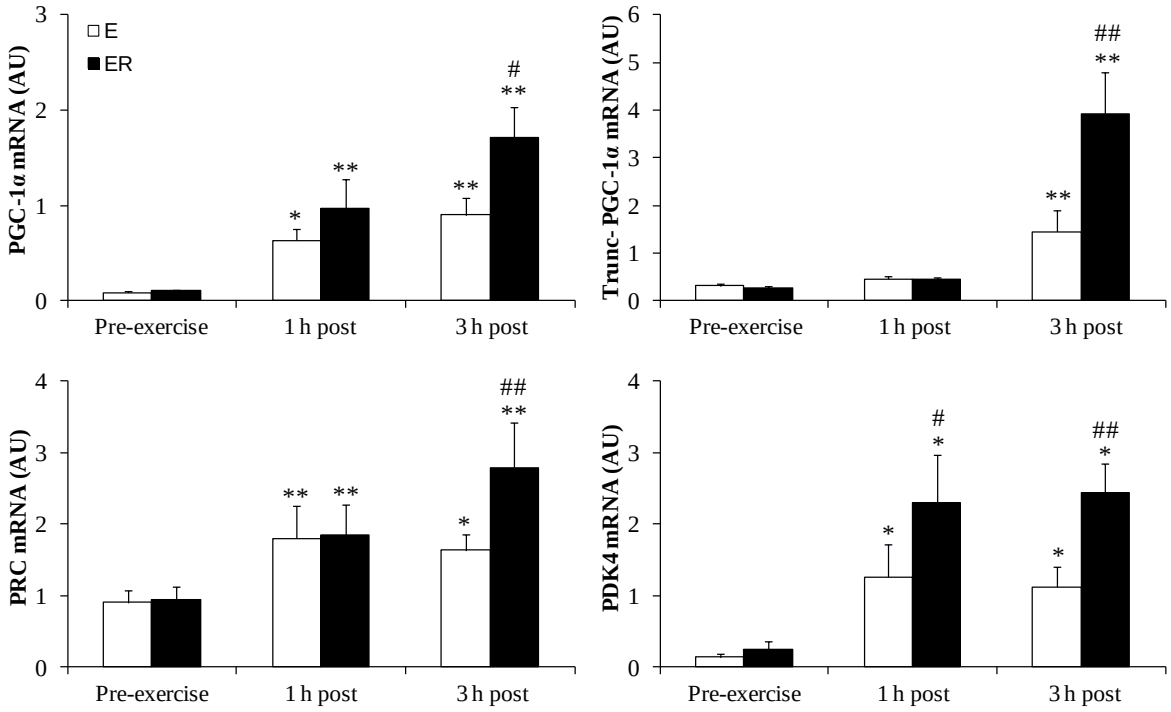

Figure 12. Effects of concurrent endurance and resistance exercise (ER) and endurance exercise only (E) on mRNA levels of genes regulating mitochondrial biogenesis and metabolism. Muscle samples were taken Pre, 1 h and 3 h Post exercise. Values of the target genes were expressed in relation to the reference gene (GAPDH). Values were mean ± SE for 10 subjects. PGC-1α, peroxisome proliferative–activated receptor-γ coactivator 1α; PRC, PGC-1-related coactivator; PDK4, pyruvate dehydrogenase kinase isozyme 4. *P < 0.05 and **P < 0.01 vs. Pre. $^{\#}$P < 0.05 and $^{\#\#}$P < 0.01, ER vs. E.

Muscle glycogen content was reduced by 58% (E) and 66% (ER) 1 h post exercise and remained reduced 3 h post. There was no significant difference between exercise models in muscle glycogen depletion. However, in both E and ER, there was a negative correlation between muscle glycogen content and gene expression of PGC-1α (Fig. 13).

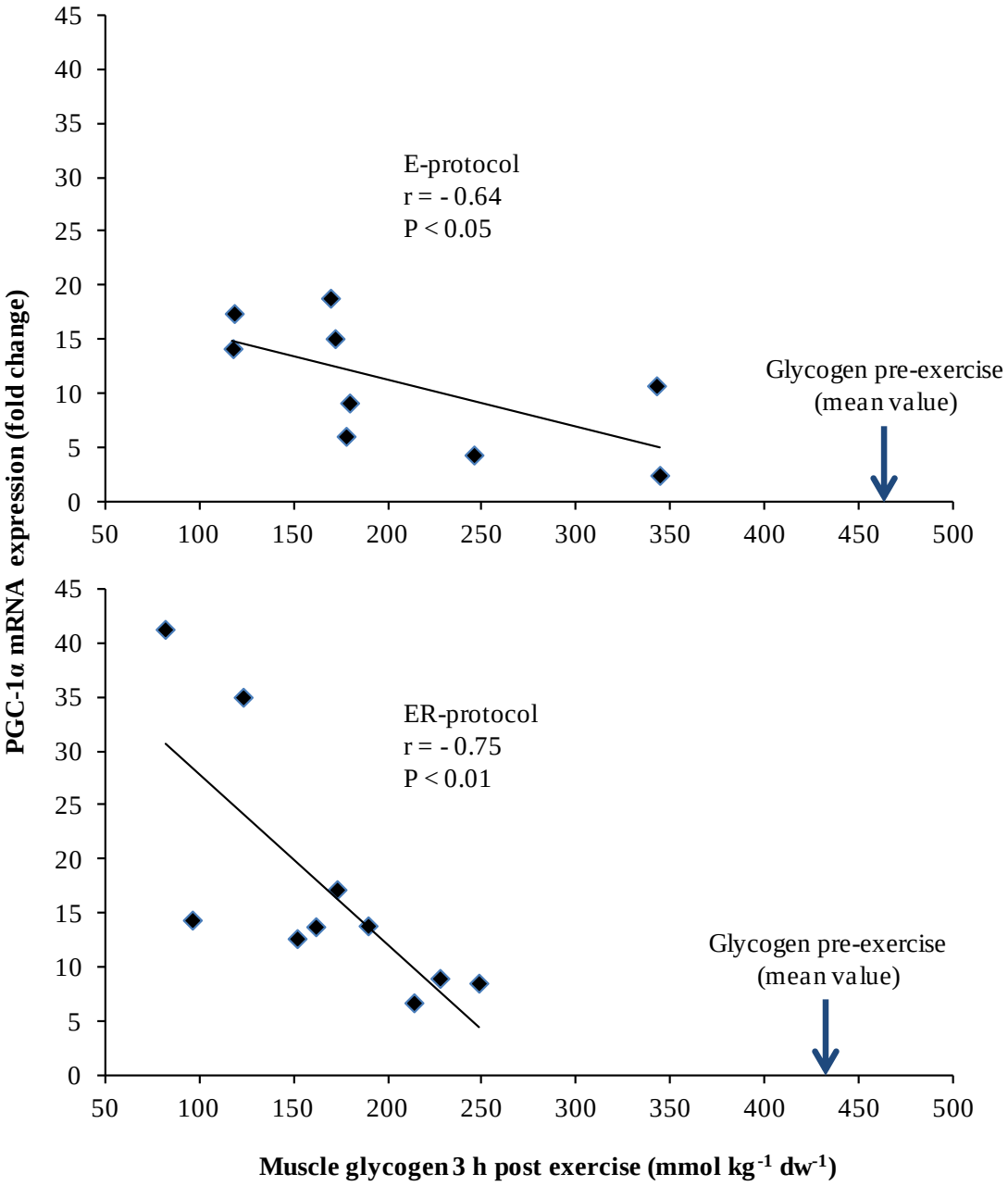

Figure 13. Correlation between exercise induced mRNA expression and muscle glycogen levels post exercise. E, endurance exercise only (n = 9); ER, concurrent endurance and resistance exercise (n = 10). PGC-1α, peroxisome proliferative–activated receptor-γ coactivator 1α.

Phosphorylation of the upstream regulator of mitochondrial biogenesis ($AMPK^{Thr172}$) increased similarly after E (56%) and ER (69%) at 1 h Post ($P < 0.01$), but was reversed at 3 h without difference between E and ER. Phosphorylation of p38 MAPK showed a heterogeneous response with a large variability between subjects. There was a tendency (main effect of time $P = 0.051$) for increased phosphorylation of p38 MAPK at 1 h in ER (Fig. 14). p-CaMKII was not affected by exercise.

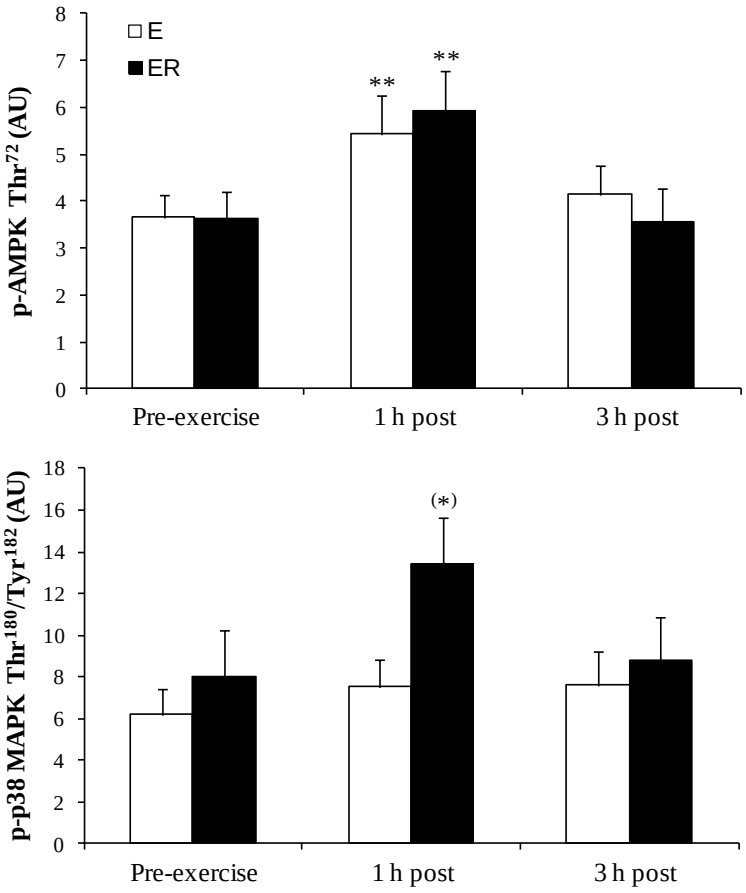

Figure 14. Effect of concurrent endurance and resistance exercise (ER) and endurance exercise only (E) on phosphorylation levels of upstream modulators of mitochondrial biogenesis. Muscle samples were taken Pre, 1 h and 3 h Post exercise. p-AMPK, AMP-activated protein kinase; p-p38 MAPK, mitogen-activated protein kinase. Values are mean ± SE for 10 subjects. **P < 0.01 and $^{(*)}$P = 0.054 vs. Pre.

Study V: Concurrent training in well trained subjects

Muscle strength, measured as 1 RM during leg press, increased to a larger extent after concurrent strength and endurance training (ES) (19 ± 2%), than after endurance training alone (E) (3 ± 2%). Peak power during a 30 s Wingate test increased in ES (5 ± 2%) but not in E (1 ± 2%).

VO_2max increased similarly in both groups but time to exhaustion during the VO_2max test only increased in ES. Also, LT4 and mean power output during the TT40 only increased in E (table 5). A trend towards increased CS activity was observed in E, but not in ES. HAD was unaffected by training in both groups (Fig. 15)

Table 5. Effect of training on performance and performance related variables

	E		ES	
	Pre	Post	Pre	Post
VO_2max (ml min^{-1} kg^{-1})	55 ± 1	58 ± 1**	57 ± 1	58 ± 1*
TTE- VO_2max (s)	402 ± 14	423 ± 15	407 ± 22	443 ± 21**
LT4 (W)	287 ± 11	295 ± 11*	283 ± 9	286 ± 8
TT40 (W)	284 ± 11	294 ± 11*	282 ± 10	290 ± 10

E, endurance training only (n = 10); ES, endurance + strength training (n = 9). TTE, time to exhaustion during the VO_2max test; LT4, intensity at the 4 mmol lactate threshold; TT40, mean power output during the 40 min time trial. Values are reported as means ± SE. *P < 0.05 and **P < 0.01 vs. Pre.

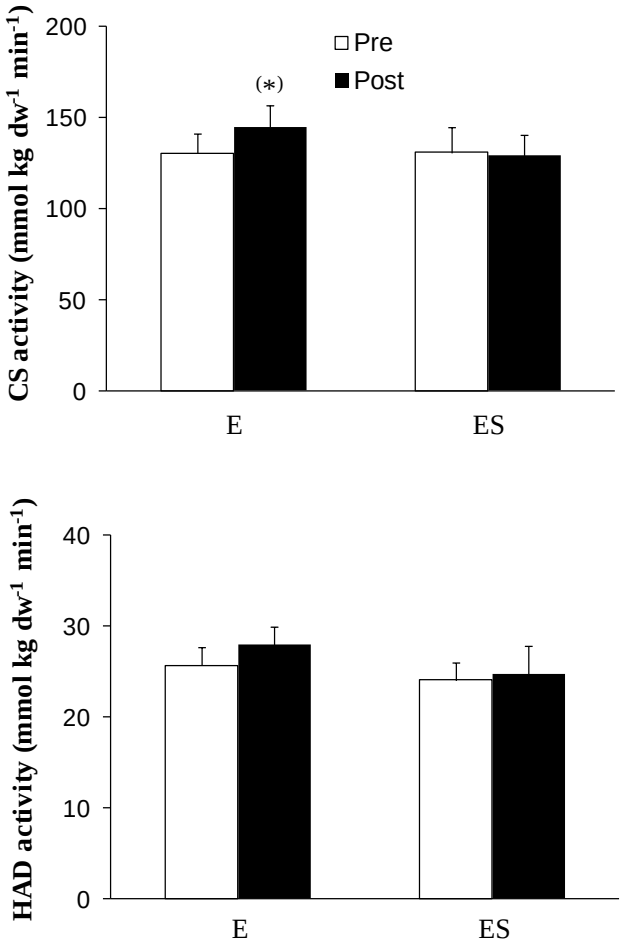

Figure 15. Effect of 8 weeks of training on mitochondrial enzyme activities. E, endurance training only (n = 10); ES, endurance + strength training (n = 9). CS, citrate synthase; HAD, 3-hydroxyacyl-CoA dehydrogenase. Values are reported as means ± SE. $^{(*)}$P = 0.06 vs. Pre.

Low glycogen exercise (study IV)

Low glycogen exercise in well-trained subjects

In study IV, we investigated the acute effect of exercise, with low (LG) and normal (NG) muscle glycogen, on genes and proteins regulating mitochondrial biogenesis. Muscle glycogen content was reduced 14 h after the depletion exercise by 73% in LG and 26% in NG, but was not further reduced by the subsequent test exercise (Fig. 16).

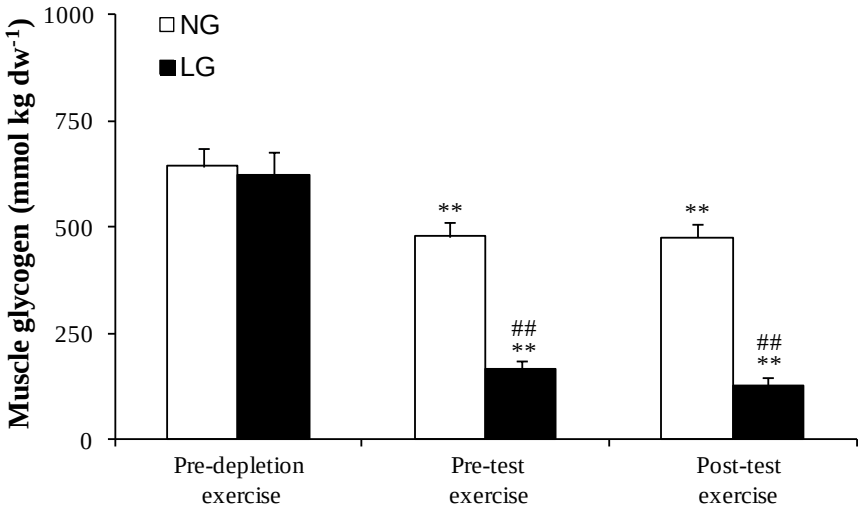

Figure 16. Muscle glycogen levels. Muscle samples were obtained in the afternoon before the depletion exercise session on day 1 (pre-depletion exercise), before the test exercise the following morning on day 2 (pre-test exercise) and 3 h after the test exercise on day 2 (post-test exercise). NG normal glycogen, LG low glycogen. Values reported as the mean ± SE, n = 10. *P < 0.05 and **P < 0.01 vs. pre-depletion exercise, $^{\#\#}$P < 0.01 LG versus NG.

Free fatty acids, measured in blood samples obtained approximately 15 min before the muscle biopsies, were unchanged in NG but increased in LG (table 6). In the same samples, blood glucose was slightly reduced in both NG and LG, with no difference between conditions (table 6).

Table 6. Effect of exercise and diet on plasma FFA and glucose

		Pre-depletion exercise	Pre-test exercise	Post-test exercise
FFA	LG	0.31 ± 0.07	0.66 ± 0.05$^{**\#\#}$	1.21 ± 0.18$^{**\#\#\dagger\dagger}$
	NG	0.23 ± 0.04	0.10 ± 0.02	0.03 ± 0.01
Glu	LG	6.0 ± 0.4	5.0 ± 0.1**	4.3 ± 0.2**
	NG	5.8 ± 0.3	5.1 ± 0.1	5.0 ± 0.3*

Muscle and blood samples were obtained before the depletion exercise on day 1 (pre-depletion exercise), before the test exercise on day 2 (pretest exercise) and 3 h after the test exercise on day 2 (post-test exercise). FFA venous plasma free fatty acids (mmol L^{-1}), Glu venous plasma glucose (mmol L^{-1}), LG low glycogen, NG normal glycogen. Values are reported as mean ± SE from 10 subjects. * P < 0.05 and ** P < 0.01 vs. pre-depletion exercise, ††P < 0.01 vs. pre-test exercise and $^{\#\#}$ P < 0.01 LG vs. NG.

The mRNA content of major regulators of mitochondrial biogenesis and lipid metabolism was increased to a greater extent after LG compared with NG (Fig. 17). However, phosphorylation of proteins involved in the upstream signaling pathways of mitochondrial biogenesis (AMPK and p38MAPK), as well as mitochondrial ROS-production, did not change significantly 3 h after test exercise in any of the conditions.

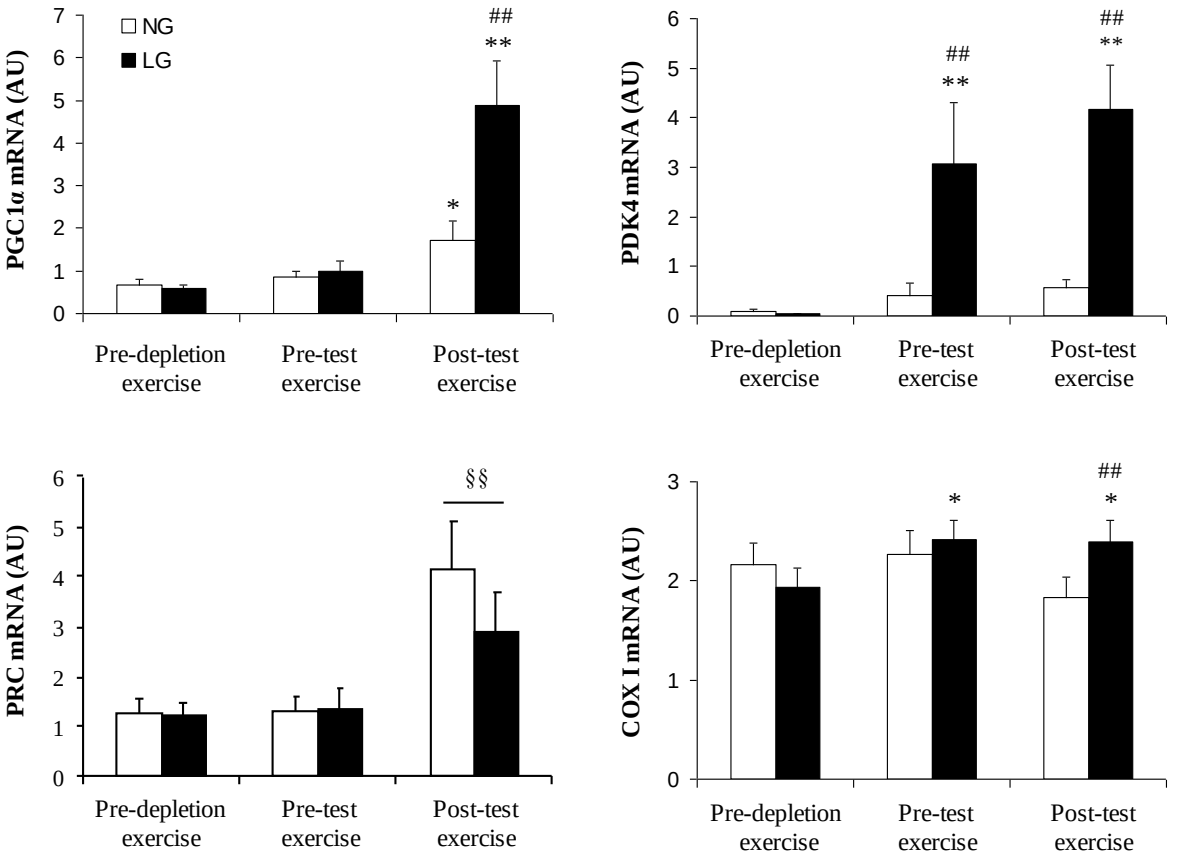

Figure 17. Effect of low glycogen exercise (LG) and normal glycogen exercise (NG) on mRNA levels of proteins regulating mitochondrial biogenesis and metabolism. Muscle samples were obtained before the depletion exercise on day 1 (pre-depletion exercise), before the test exercise on day 2 (pre-test exercise) and 3 h after the test exercise on day 2 (post-test exercise). Values were expressed in arbitrary units (AU) related to the reference gene GAPDH, and are reported as mean ± SE, n = 10. PGC-1α, peroxisome proliferative–activated receptor-γ coactivator 1α; PRC, PGC-1-related coactivator; PDK4 pyruvate dehydrogenase kinase isozyme 4; COX I cytochrome c oxidase subunit I. *P < 0.05 and **P < 0.01 vs. pre-depletion exercise; $^{\#\#}$P < 0.01 LG vs. NG; §§P < 0.01 vs. pre-depletion exercise (time-dependent effect)

DISCUSSION

High intensity interval training (HIIT)

In studies I and II, we examined the effects of high intensity interval training on genes regulating mitochondrial biogenesis and metabolism. In study I, the subjects were untrained, whereas in study II they were highly trained cyclists. The major finding in study I was that continuous (CE) and interval (IE) cycling exercise of the same duration and work increased the expression of genes regulating mitochondrial biogenesis to a similar extent. The major finding in study II was that low-volume sprint interval exercise (SIT) and high-volume interval exercise (IE) had a similar effect on genes regulating mitochondrial biogenesis, despite a large difference in time commitment and work.

In study I, we hypothesized that IE would increase the expression of genes regulating mitochondrial biogenesis to a greater extent than CE, due to a more pronounced energetic stress and type II fiber recruitment. This was however not the case. Both IE and CE induced a ~10-fold increase in PGC-1α expression. There were also no differences in mRNA levels of the other PGC-family members (PRC and PGC-1β). It is possible that the exercise protocols were too similar and did not induce a large enough difference in fiber recruitment pattern and metabolic perturbation. Our data indirectly support this because changes in blood lactate and glycogen did not differ between CE and IE. Also, in a follow-up study from our laboratory, it was shown that PGC-1α expression increased to the same extent in type I and II muscle fibers after CE and IE (Wang and Sahlin, 2012).

A similar observation was included in a recent study by Bartlett and colleagues (Bartlett et al., 2012). Similarly, they compared the effect of work-matched exercise (6x3 min at 90% of VO_2max vs. 50 min at 70% of VO_2max) and observed no difference in glycogen depletion or PGC-1α expression. They also could not detect any difference in AMPK activation. Even though they used an interval protocol that was more metabolically demanding than ours, the end result was similar. This indicates that the work performed, rather than the intensity of exercise, is important for mitochondrial biogenesis in untrained subjects. However, this seems only to be true for exercise intensities that are relatively high (> 65-70% of VO_2max). When low intensity exercise (40% of VO_2max) was compared with work matched high intensity exercise (80% of VO_2max), both a larger glycogen reduction and PGC-1α expression was observed after the high intensity exercise session (Egan et al., 2010). A possible explanation for this might be that 40% of VO_2max is too low intensity to induce a large energetic stress, especially in type II fibers. This idea is supported by the large difference in glycogen utilization observed between the 40 and 80% trail (1.3 vs. 3.1 mmol $(kg\ dw)^{-1}\ min^{-1}$).

Training prescriptions to induce mitochondrial biogenesis in untrained individuals therefore seem to be relatively straight forward: if exercise duration is relatively long, there is no point

in exercising harder than 65-70% of VO_2max. However, several studies show that if time is a limiting factor, HIIT protocols, especially SIT, are the most time efficient training strategies to promote mitochondrial biogenesis.

In study II we hypothesized that SIT would increase the expression of genes regulating mitochondrial biogenesis to a similar extent as IE, despite much less time commitment and work done. We based this hypothesis on previous studies where SIT has been shown to be a time efficient training strategy to promote mitochondrial biogenesis and performance in untrained subjects (Barnett et al., 2004; Burgomaster et al., 2008; Gibala et al., 2006; MacDougall et al., 1998). Our results confirm and extend these findings by showing that SIT is also a powerful inducer of PGC-1α in highly trained subjects, even when compared with a very demanding exercise protocol such as IE. But how is it possible that 3.5 min of exercise can induce such a pronounced increase in PGC-1α expression in elite cyclists that usually train 2-3 h/day and have metabolic adaptations that are almost maximized? One explanation could be that even highly trained athletes have a pool of fast type II fibers (e.g., type IIx) that are rarely activated during conventional endurance training, e.g., the IE protocol, but becomes activated during SIE. These fibers are very anaerobic in nature (low mitochondrial content) and therefore have a high aerobic adaptation potential. The marked increase in PGC-1α levels observed after SIE might therefore be reflected, in part, by enhanced transcription in these fast fibers. This idea is supported by the observations by Henriksson and Reitman, that mitochondrial content (SDH-activity) is increased to a larger extent in fast type II muscle fibers compared to slow type I fibers after 8 weeks of HIIT (Henriksson and Reitman, 1976).

Concurrent training

In study III and V, we examined the effect of concurrent endurance and resistance training on regulators and markers of mitochondrial biogenesis. In study III, the subjects were untrained whereas in study V, they were trained cyclists. The major finding in study III was that concurrent exercise increased the expression of key regulators of mitochondrial biogenesis to a much greater extent than endurance training alone. The major finding in study V was that eight weeks of concurrent training did not increase CS-activity or improve cycle performance more than endurance training alone.

In study III we hypothesized that adding resistance exercise directly after endurance exercise would blunt the signaling pathways regulating mitochondrial biogenesis. This was based on earlier findings that resistance training might interfere with the signaling pathways regulating mitochondrial biogenesis (Atherton et al., 2005; Baar, 2006). We examined PGC-1α, as well as upstream regulators (AMPK, p38MAPK, CaMK), downstream targets (PDK4 and SDH) and key regulatory factors of protein synthesis (Akt, mTOR and S6K). In contrast to our hypothesis, PGC-1α and PRC expression was enhanced almost 2-fold after the concurrent exercise session (ER) compared with endurance only (E). However, the upstream regulators of PGC-1α did not differ between conditions, and the exact mechanism responsible for the enhanced expression of PGC-1α could therefore not be clarified. Nonetheless, the powerful

effect of ER on PGC-1α and PRC suggests that this might be an efficient training strategy to promote mitochondrial biogenesis and muscle oxidative capacity in untrained individuals.

Study V was a follow-up on study III, where we wanted to investigate if long-term training with a similar exercise model as in study III, e.g., endurance immediately followed by strength, would enhance mitochondrial biogenesis and performance. But instead of using untrained subjects, we recruited trained cyclists. The reason for this was that we wanted to increase the impact of the study by making the results more useful to athletes and coaches. We hypothesized that endurance + strength (ES) would be more efficient than endurance only (E), based on our previous acute exercise study. However, eight weeks of ES training did not improve mitochondrial biogenesis and performance more than E. In fact, only the E-group had increased CS-activity, lactate threshold and time trial performance. However, these changes were not significantly different from the ES-group.

There are several possible explanations for these conflicting results. Strength training alone has been shown to induce mitochondrial biogenesis in the untrained, but not in the trained state (Wilkinson et al., 2008). One explanation might be that the untrained subjects in study III got an additive effect from the strength training, but the trained subjects in study V did not. This additive effect could have been induced by the mammalian target of rapamycin (mTOR) signaling pathway because mTOR was more activated in ER compared with E. Even though mTOR is a major regulator of contractile protein synthesis, it has also been shown to indirectly regulate PGC-1α expression (Cunningham et al., 2007).

Another possible explanation for divergent results between studies III and V is that the observed increase in PGC-1α was not solely related to mitochondrial biogenesis. It has recently been shown that PGC-1α exists in several isoforms (at least 4) with different roles in the signaling cascades preceding muscle adaptation. In rat skeletal muscle, one of these isoforms, PGC-1α4, has been shown to be more related to hypertrophy than to mitochondrial biogenesis (Ruas et al., 2012). In study III, we used primers that measured all PGC-1α isoforms and it is therefore possible that the enhanced PGC-1α expression was due to increased PGC-1α4 expression induced by the resistance exercise. To address this possibility, cDNA from study III was reanalyzed and the results showed that the expression of the truncated isoforms of PGC-1α (PGC-1α4 and NT- PGC-1α) was ~3-fold higher after ER compared with E. This suggests that the enhanced expression of total PGC-1α after ER could be the result of increased expression of PGC-1α4 (or NT- PGC-1α). Recent human studies report that up-regulation of PGC-1α4 is not solely related to resistance exercise and hypertrophy (Lundberg et al., 2014; Ydfors et al., 2014). The precise role of PGC-1α4 and other isoforms of PGC-1α in human skeletal muscle remodeling remains to be clarified.

Low glycogen training

In study IV, we examined the effect of exercise with low muscle glycogen on key regulators of mitochondrial biogenesis. The major finding was that exercise with low muscle glycogen

enhanced the expression of genes regulation mitochondrial biogenesis compared with normal glycogen exercise.

In study IV, we hypothesized that exercising with low muscle glycogen levels would enhance the expression of PGC-1α. This was based on earlier studies showing an enhanced activity of both upstream (AMPK) and downstream (CS and SDH) targets of PGC-1α after training with reduced muscle glycogen (Hansen et al., 2005; Morton et al., 2009; Yeo et al., 2010; Yeo et al., 2008). However, several other parameters could have influenced the results in these long-term training studies, especially since the reduction in glycogen was modest in two out of three studies. It is therefore not possible to conclude if the enhanced mitochondrial biogenesis (CS and SDH-activity) observed in these studies is an effect of low glycogen.

In line with our hypothesis, cycling with low muscle glycogen increased PGC-1α expression to a greater extent than normal glycogen exercise (8- vs. 2.5-fold). This shows that exercise with low glycogen is a very potent activator of the major regulator of mitochondrial biogenesis. The previous observations of increased CS and SDH activity after long-term training are therefore probably a result of an up-regulated AMPK- PGC-1α signaling pathway induced by low glycogen exercise.

It has been suggested that a critically low level of glycogen might be needed to induce a strong activation of metabolic genes by exercise (Pilegaard et al., 2002). Broberg and Sahlin showed that very low glycogen levels are associated with reduced levels of high energy phosphates and elevated AMP, which are known inducers of metabolic genes (Broberg and Sahlin, 1989). Further evidence for this theory comes from a study by Cochran and colleagues. They used a similar set-up as ours in study IV, but did not observe enhanced PGC-1α expression (or AMPK activation), which might be explained by the relatively high (200-300 mmol kg dw^{-1}) glycogen levels during their "low" exercise session. Also, in a study by Mathai et al. it was shown that the largest increase in PGC-1α protein abundance was identified in subjects who had the largest reduction in glycogen during exercise (Mathai et al., 2008).

The idea that glycogen needs to be critically low during exercise is also supported by data in the present thesis. When PGC-1α expression was related to glycogen levels post exercise, a strong negative correlation was observed in both the E and ER group (study III). Also, when the results from study I, III and IV were merged into one figure, there seems to be a relationship between glycogen levels and PGC-1α expression (Fig. 18). This was more clearly visualized if these samples were divided into two groups based on their glycogen content (Fig. 19). The low group (< 210 mmol kg^{-1} dw^{-1}, n = 28) had a ~13-fold increase in PGC-1α expression whereas the high group (> 210 mmol kg^{-1} dw^{-1}, n = 29) only had a ~7-fold increase. Taken together these studies and our data indicate that a "lower the better" rule might be true when it comes to exercise, glycogen and PGC-1α expression.

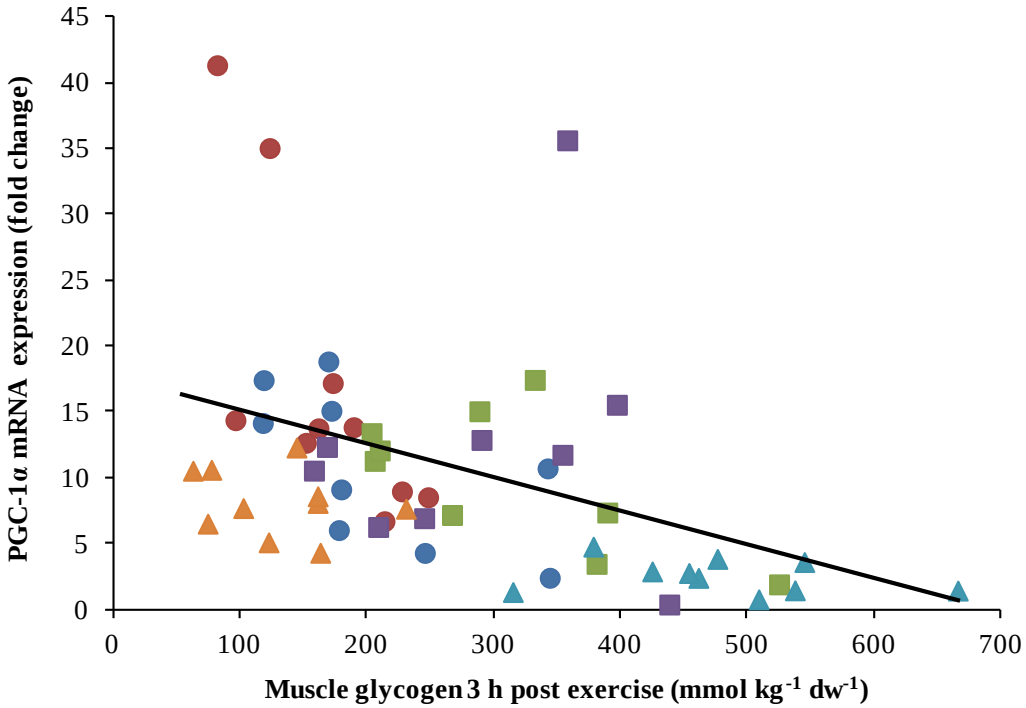

Figure 18. Correlation between exercise induced mRNA expression and muscle glycogen levels post exercise. Rectangles, study I (green: continuous exercise, purple: interval exercise); circles, study III (blue: endurance exercise only, red: concurrent endurance and resistance exercise); triangles, study IV (blue: normal glycogen exercise, orange: low glycogen exercise). PGC-1α, peroxisome proliferative–activated receptor-γ coactivator 1α. Significant correlations for: red circles ($r = -0.75$, $P < 0.01$), blue circles ($r = -0.64$, $P < 0.05$) and green rectangles ($r = -0.65$, $P < 0.05$). There is also a strong correlation for all data points ($r = -0.45$, $P < 0.001$), however it is not statistically correct to do such an analysis and the trend line is only there to visualize a potential relationship.

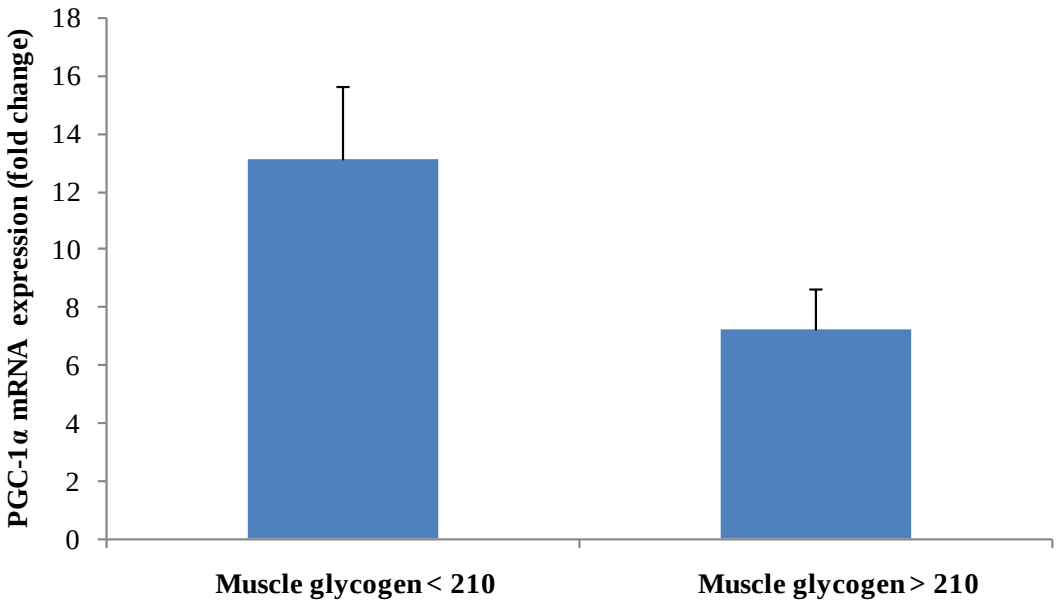

Figure 19. Average PGC-1α expression for samples with a muscle glycogen content < 210 mmol kg^{-1} dw^{-1} (n = 28) and > 210 mmol kg^{-1} dw^{-1} (n = 29). PGC-1α, peroxisome proliferative–activated receptor-γ coactivator 1α.

Even though exercise with low glycogen seems to be important for mitochondrial biogenesis, the long-term effect on performance is equivocal. Hansen and colleagues showed a marked benefit of "training low" compared with "training high" (Hansen et al., 2005), whereas two other studies did not show enhanced performance when training was committed with reduced glycogen levels (Morton et al., 2009; Yeo et al., 2008). One explanation for these conflicting results might be that the exercise protocols and performance tests used were quite different. Also, the variability is much greater in performance tests compared with enzymatic measurements, such as CS-activity. Another explanation could be that training with reduced glycogen levels down-regulated carbohydrate metabolism, and that this had a negative effect on performance in the studies by Morton et al. and Yeo et al. This idea is supported by an investigation showing blunted pyruvate dehydrogenase (PDH) activity when exercise was performed in a carbohydrate restricted state (Stellingwerff et al., 2006). Therefore, a critical factor when designing endurance training programs might be to find an optimal balance between "low" and "high" training sessions.

Low glycogen, a major trigger for mitochondrial biogenesis?

One might speculate that low glycogen is a critical factor for inducing mitochondrial biogenesis during most types of exercise, e.g., also during exercise that is normally not associated with low glycogen, such as HIIT. Is it possible that the type of exercise is secondary and its effect on glycogen primary? Could this also partly explain why subjects accrue blunted responses when becoming more trained? It is well known that increased fitness level is associated with elevated glycogen levels, as well as a glycogen sparing effect during exercise (Burgomaster et al., 2008).

As previously discussed, HIIT is a time efficient training strategy to promote mitochondrial biogenesis. HIIT is also a time efficient strategy to reduce muscle glycogen. A single 30 s all-out sprint can reduce muscle glycogen by as much as 25% (Barnett et al., 2004) and 8x60 s sprints can reduce glycogen by more than 75% (Gollnick et al., 1974). Further, HIIT seemed to reduce glycogen to a greater extent in fast type II fibers than in type I (Thomson et al., 1979). It has also been shown that HIIT increases mitochondrial biogenesis (SDH-activity) more in type-II fibers than in type-I (Henriksson and Reitman, 1976). The efficacy of HIIT training to induce mitochondrial biogenesis might therefore be a consequence of its ability to effectively deplete some muscle fibers of their glycogen.

Elite endurance athletes generally have a very high percentage of type I fibers and elevated glycogen levels compared with untrained subjects. If an individual, for example, has 80% type I and 20% type II fibers, which is common among elite endurance athletes (Costill et al., 1976), it would be wise to focus most training on something that maximizes adaptation in the most abundant fiber types. Long duration training, rather than HIIT, is needed to induce major glycogen depletion in type I muscle fibers (Gollnick et al., 1974; Thomson et al., 1979). Elite endurance athletes do large quantities of LSD training, often two sessions per day and 10-20 hour per week. Therefore, several training sessions will be undertaken with

low muscle glycogen. A large volume of LSD might be a particularly efficient training strategy to promote mitochondrial biogenesis in individuals with a high percentage of type I fibers.

Further support for the important role of glycogen as a regulator of mitochondrial biogenesis comes from cell signaling studies. These studies show that glycogen can regulate AMPK directly, not just indirectly, through fluctuations in ATP/AMP levels. Glycogen does this by binding to the AMPK β subunit (McBride et al., 2009). AMPK cannot be activated when bound to glycogen, but when glycogen is metabolized, as during exercise, AMPK is released and AMP and upstream kinases can bind to and activate the enzyme. It has also been shown in humans that exercise with low muscle glycogen is associated with greater AMPK activity, less AMPK associated with glycogen, and greater AMPK translocation to the nucleus than when muscle glycogen is high (Steinberg et al., 2006).

Altogether, the glycogen-AMPK-PGC-1α signaling pathway seems to be a key regulator of mitochondrial biogenesis. However, the effect of low glycogen exercise needs to be further investigated to clarify to what extent this pathway regulates mitochondrial biogenesis during different exercise modalities.

How do endurance athletes train?

Several studies show that HIIT efficiently improves and maintains performance in trained subjects (Esfarjani and Laursen, 2007; Iaia et al., 2009; Laursen et al., 2002; Laursen et al., 2005). But if HIIT is such a time efficient training strategy in both trained and untrained, why do elite athletes train so little HIIT? Studies show that most elite endurance athletes train more than 10 times per week and typically have an intensity distribution where 80% of their training sessions are performed at low intensity (≤ 2 mM blood lactate) and 20% at higher intensities (Seiler, 2010). This normally means that 2-3 sessions per week are performed at high intensity and of these, only 1-2 are performed at a very high intensity, e.g., HIIT (Laursen, 2010; Seiler, 2010). A possible explanation for this might be that athletes self-organize their training towards a low intensity, high-volume approach to balances the adaptive signaling and potentially maladaptive stress-inducing components appropriately (Seiler, 2010). It has been shown that increased training intensity is associated with a nonlinear increase in sympathetic stress. Highly trained subjects have rapid autonomic nervous system recovery after long, slow distance sessions but a marked delay after short, high-intensity sessions (Chwalbinska-Moneta et al., 1998; Seiler et al., 2007). Studies also show that HIIT, in particular SIT, increase the risk of knee and hamstring injuries (Gabbett and Ullah, 2012). The explanation for why elite athletes do not incorporate more HIIT might be that they simply cannot tolerate more than a few such sessions per week without risking an overtraining syndrome or injury.

An alternative explanation for the highly polarized training strategy among elite athletes could be that an 80/20 distribution is particularly effective to induce training adaptations in

these individuals. As previously mentioned, elite athletes have a high percentage of type I muscle fibers. It is possible that a large volume of LSD training is required to induce homeostatic disturbance and adaptation in these fatigue resistance fibers. It is also possible that a large volume of training is needed to optimize economy of movement and fiber recruitment patterns (Santalla et al., 2009). However, overall endurance performance will not be optimized solely by LSD and some high intensity training sessions are needed to maximize neuronal and fast fiber adaptations, as well as VO_2max. A recently published study supports the use of a polarized training approach in well trained individuals (Stoggl and Sperlich, 2014). In this study, four different training models were compared; HIIT, LSD, lactate threshold training, and polarized training (POL). After 9 weeks of training the largest increase in endurance performance (+ 17%) was observed in the POL group (68% LSD and 26% HIIT).

Even though an 80/20 distribution seems to be optimal for most endurance athletes, it might not be for other less trained individuals. It is possible that a different intensity distribution is more efficient for recreationally active persons that only train 2-3 times per week. However, well controlled, long term training studies comparing POL with other training strategies in this group of subjects have not yet been done.

PGC-1α mRNA, a valid marker of mitochondrial biogenesis?

Gene expression, especially PGC-1α, has been used in the present thesis to evaluate the effect of different training models on mitochondrial biogenesis. An important question is therefore, how valid is PGC-1α expression as a marker for mitochondrial biogenesis? Although there is no doubt about the importance of PGC-1α expression for mitochondrial biogenesis and muscle oxidative capacity in cell and animal studies (Calvo et al., 2008; Leone et al., 2005; Wu et al., 1999), a direct relationship is much more difficult to establish in humans. It has been shown that repeated transient mRNA bursts precede mitochondrial biogenesis during training in human skeletal muscle, and that the magnitude of mRNA expression seems to be related to the magnitude of mitochondrial biogenesis (Perry et al., 2010). It has also been shown that PGC-1α expression is intensity dependent, at least in trained subjects, with a weaker response after exercise at 70% of VO_2max compared with exercise at 85% (Nordsborg et al., 2010). This is in line with long-term training studies showing that intensities above 60-70% of VO_2max normally are needed to induce mitochondrial biogenesis (CS and SDH-activity) in well-trained subjects (Evertsen et al., 1999; Shepley et al., 1992). Further support for a relationship between acute PGC-1α mRNA expression and a chronic increase in mitochondrial content comes from study IV in the present thesis. Here we show that well trained subjects exercising at ~65% of VO_2max only get elevated mRNA levels of PGC-1α when glycogen is low. This is in line with long-term studies showing that mitochondrial content (CS, COX and SDH-activity) only increases after training with reduced muscle glycogen (Hansen et al., 2005; Morton et al., 2009; Yeo et al., 2008).

Even though increased mRNA expression normally is associated with increased transcription and protein content, several other factors will affect this process. Such factors include mRNA stability, mRNA transport, translation efficiency, amino acid availability, protein assembly, processing and stability. Also, more protein does not necessarily mean more active protein. It is the amount of active protein that, in the end, affects the cellular processes. However, the mRNA level of PGC-1α is not only a marker of protein content, but also a marker of protein activity, because the activated protein regulates its own gene transcription (Benton et al., 2008).

Taken together these findings support the validity of PGC-1α mRNA as a marker for mitochondrial biogenesis. However, results from acute exercise studies should always be verified in long-term training studies before a definitive conclusion can be drawn.

CONCLUSIONS

Untrained subjects

- Continuous and interval exercise have a similar effect on PGC-1α, and other genes regulating mitochondrial biogenesis, if the duration and work done are the same (study I)

- Concurrent resistance and endurance exercise enhances the signaling pathway of mitochondrial biogenesis (study III)

Trained subjects

- Sprint interval training is a powerful inducer of PGC-1α, and other genes regulating mitochondrial biogenesis (study II)

- Exercise with low muscle glycogen enhances the expression of PGC-1α, and other genes regulating mitochondrial biogenesis (study IV)

- Eight weeks of concurrent strength and endurance training does not enhance mitochondrial content (CS-activity) or performance (study V)

General finding

- Intracellular glycogen levels during/after exercise might play a pivotal role for the magnitude of the exercise induced increase in mitochondrial biogenesis regardless of exercise mode

FUTURE PERSPECTIVES

The interest in sprint interval training (SIT) has increased considerably since Burgomaster and colleagues published their first studies on the topic approximately eight years ago. A large number of studies now show that SIT can improve both muscle oxidative capacity and performance in untrained and recreationally active subjects. Studies also show that adding SIT to the training program of well-trained subjects improves performance. However, to my knowledge, study II is the only study to date that has examined the effect of SIT on factors related to muscle oxidative capacity in highly trained individuals. A long-term training study examining the effects of SIT on both mitochondrial biogenesis and performance in this group would provide an answer to the important question; is the improvement in performance observed in well trained subjects related to increased mitochondrial content?

PGC-1α mRNA is one of the best markers for evaluating the acute effect of exercise on mitochondrial biogenesis. However, a long term study examining mitochondrial content after training consisting of exercise sessions with a known "weak" vs. "strong" acute effect on PGC-1α expression would provide more information about the validity of this marker. For example, untrained subjects could perform work matched training for 8 weeks where one group train at 40-50 % of VO_2max (known to induce a weak effect on PGC-1α) and the other group train at 70-80 % of VO_2max (known to induce a strong effect on PGC-1α). After the training period, mitochondrial content following the last training session and PGC-1α expression after the first training session are compared and evaluated.

Essential amino acids, especially leucine, are critical for the activation of the signaling pathway leading to muscle hypertrophy. However, the effect of amino acids on the regulation of mitochondrial biogenesis has not been studied. It is possible that this process is also affected by amino acid availability, especially in a fasted state. Therefore, a study examining the acute effect of training on PGC-1α expression with and without amino acids would provide further understanding of the mechanisms behind mitochondrial biogenesis.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Bakgrund

Det är numera välkänt att fysisk aktivitet har en betydande inverkan på livslängd och hälsa. Det finns ett brett vetenskapligt stöd för att regelbunden träning har en positiv effekt på många av våra vanligaste folksjukdomar så som diabetes, hjärt- och kärlåkommor, vissa typer av cancer, Alzeimer's och depression. Den mest hälsofrämjande träningsformen är uthållighetsträning, dvs. träning som aktiverar en stor muskelmassa och ger en kraftigt förhöjd puls. Förutom att denna träningsform effektivt motverkar många folksjukdomar så medför den dessutom en ökad ork (prestation) både i vardagen och idrottsliga sammanhang. En av de mest påtagliga anpassningar som sker i kroppen vid uthållighetsträning är en ökad mängd mitokondrier i muskulaturen. Detta är förknippat med både god hälsa och prestation och vältränade personer kan ha 3-4 gånger så många mitokondrier i sina muskler som otränade.

Förenklat kan man säga att mitokondrierna är musklernas kraftverk där större delen av de näringsämnen som vi får i oss via kosten (framförallt fett och kolhydrater) förbränns och alstrar energi i from av ATP. ATP är den universala energimolekylen i våra celler och används i nästan alla energikrävande processer i kroppen. En sådan mycket energikrävande process är muskelarbete. En stor mängd mitokondrier är därför avgörande för hur hårt och hur länge en muskel kan arbeta utan att bli trött. Man brukar säga att en vältränad muskel med mycket mitokondrier har en hög aerob förmåga och uthållighetskapacitet.

Även om det sedan lång tid är klarlagt att uthållighetsträning stimulerar tillväxten av mitokondrier så är det fortfarande oklart vilken typ av träning som är mest effektiv. En flitigt diskuterad frågeställning är därför om träningen bör fokuseras på kvalitet (hög intensitet under kort tid) eller kvantitet (låg/medelintensiv intensitet under lång tid).

En av anledningarna till att man inte har kommit längre inom detta område är begränsningar i den traditionella metodiken där förändringar i muskulaturen enbart har kunnat mätas på protein- och prestationsnivå. För att kunna detektera dessa förändringar krävs långa, tidskrävande studier som är mycket kostsamma och svårkontrollerade. Med nya molekylärbiologiska metoder finns det nu möjlighet att studera de initiala processerna som styr muskelns anpassning till träning. Genom att analysera mRNA av gener som reglerar tillväxten av mitokondrier kan man undersöka hur effektiv en viss typ av träning är efter endast ett träningspass.

Den viktigaste genen för mitokondriell tillväxt är PGC-1α (transcriptional coactivator peroxisome proliferator–activated receptor-γ coactivator-1α). PGC-1α är en nyckelspelare som reglerar andra gener som behövs för att bygga fler och större mitokondrier. Djurstudier visar att genmanipulerade råttor som har en ökad mängd PGC-1α i sina muskler har ett mycket större antal mitokondrier samt är betydligt uthålligare än råttor med normala PGC-1α

nivåer. Både i djur- och humanstudier har man dessutom kunnat påvisa att uthållighetsarbete ökar nivåerna av PGC-1α (både mRNA och protein). mRNA nivåerna, som är ett mått på genens aktivering, brukar för PGC-1α vara som högst ca 3 h efter ett träningspass. Det är på grund av detta som muskelproverna (biopsier) i denna avhandling oftast har tagits vid just denna tidpunkt. Andra viktiga gener som styr mitokondriell tillväxt och som regleras av PGC-1α är: peroxisome proliferator-activated receptor δ (PPARδ), mitochondrial transcription factor A (Tfam) och nuclear respiratory factors 1 and 2 (NRF-1 and 2).

Målsättning

Målet med projektet som ligger till grund för denna avhandling var att jämföra olika typer av träning för att undersöka vilken träningsform som är mest effektiv för att stimulera tillväxten av mitokondrier i human skelettmuskulatur.

Resultat och konklusion

Otränade försökspersoner som vid ett tillfälle fick cykla 90 min på en konstant belastning och vid ett annat tillfälle fick cykla 90 min i form av intervaller (dock samma totala arbete i båda fallen) hade en likvärdig aktivering av PGC-1α-genen. Detta tyder på att om träningen är långvarig och belastningen åtminstone är måttlig (> 60% av VO_2max) så verkar uthållighetsträningens karaktär vara av liten betydelse för otränade personer, dvs. det spelar ingen roll om träningen utförs i form av intervaller eller på en konstant belastning. Orsaken till detta är troligen att en måttlig belastning räcker för att ge en maximal adaptation hos en otränad person.

Däremot så verkar den mitokondriella tillväxten hos otränade försökspersoner kunna förstärkas då styrketräning läggs i direkt anslutning till ett uthållighetspass. Mängden PGC-1α mRNA var ca dubbelt så hög i lårmuskulaturen efter ett kombinerat uthållighet- och styrkearbete jämfört med ett renodlat uthållighetsarbete. Att träna styrka och kondition under ett och samma träningspass verkar således vara fördelaktigt för att förbättra uthålligheten hos otränade personer.

Resultaten i denna avhandling tyder dock på att tränade försökspersoner påverkas annorlunda än otränade. Åtta veckors kombinerad uthållighet och styrka medförde nämligen inte någon ökning av mängden mitokondrier eller prestationen hos cyklister jämfört med renodlad uthållighetsträning. Orsaken till denna skillnad skulle kunna vara att otränade personer initialt får en uthållighetseffekt av styrketräning på grund av deras låga träningsstatus. Cyklister däremot har redan en så pass tränad muskulatur att styrketräning inte ger någon ytterligare effekt på deras aeroba förmåga.

En träningsform som däremot verkar vara effektiv för vältränade är sprintintervaller. Elitcyklister fick en mycket kraftig aktivering av PGC-1α-genen efter ett sprintintervallpass (7 x 30 s). Ökningen var till och med lika stor som efter ett betydligt längre intervallpass (3 x 20 min) trots att det totala utförda arbetet var hela 17 gånger mindre. Detta skulle kunna bero

på att denna mycket högintensiva träningsform stimulerar delar i muskulaturen som fortfarande har en viss anpassningspotential kvar och därmed kan påverkas relativt mycket av träningen.

En annan träningsform som också hade stor effekt på vältränade var träning med lågt muskelglykogen. En timmes cykelarbete med lågt glykogen medförde nämligen en betydligt större ökning av PGC-1α mRNA hos cyklister än träning med normalt glykogen. Den klassiska synen att uthållighetsträning alltid bör utföras med välfyllda glykogenlager kan därför ifrågasättas. Det skulle kunna vara så att det är fördelaktigt att utföra vissa träningspass "låg" för att maximera mitokondriell tillväxt, fettmetabolism och muskulär aerob förmåga.

Det kanske mest betydelsefulla fyndet i denna avhandling var att muskelglykogen verkar ha en mycket central roll vid regleringen av mitokondriell tillväxt, oberoende av träningsstatus och träningens karaktär. Personer som hade låga nivåer av glykogen i sin lårmuskulatur hade nämligen den starkaste aktiveringen av PGC-1α och detta samband sågs både hos vältränade och otränade försökspersonerna oberoende av om de tränat kontinuerligt, intervallbetonat eller en kombination av uthållighet och styrka.

Det går således inte att säga att en viss intensitet, duration eller typ av träning är optimal för att stimulera tillväxten av mitokondrier utan resultaten i denna avhandling tyder snarare på att det avgörande är att träna så pass hårt eller länge att delar av muskulaturen mer eller mindre töms på glykogen.

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