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To symbiotic life

Abstract

Regular physical activity has many positive implications on health and performance, such as lowered risk for premature death, improved glucose metabolism and cardiovascular function as well as endurance capacity. Even a single bout of exercise is enough stimulus to change expression of many skeletal muscle genes. When exercise is performed regularly, the added effects of many bouts lead to changes of muscle phenotype. One of the most profound changes in skeletal muscle is an increased mitochondrial density. This contributes to an improved oxidative capacity, enhanced aerobic and endurance performance as well as to the positive health effects associated with regular endurance training. The exercise-induced skeletal muscle adaptations have been suggested to be influenced by many stimuli, e.g. changes in calcium concentration, metabolic alterations and oxygen tension. The understanding of the regulatory pathways *in vivo*, especially in humans, is largely lacking.

This thesis aimed to further explore the exercise-regulation of selected mitochondrial biogenesis factors in human skeletal muscle. The influence of a single bout of exercise, endurance training and activity level on the co-activators PGC-1α and -1β and the mitochondrial transcription factors TFAM, TFB1M and TFB2M was studied. Additional aims were to explore the relative importance of signaling pathways suggested to regulate PGC-1 in exercising skeletal muscle and the role of enhanced metabolic perturbation. A one-legged knee extension model was used where blood flow is restricted and metabolic perturbation induced in a controlled fashion. Also, a comparison was made between elite athletes, moderately active and spinal cord injured.

A single bout of exercise with enhanced metabolic perturbation induced both PGC-1α mRNA expression and AMPK activation (phosphorylation) more than exercise with normal blood flow. Even though the calcineurin and p38 signaling pathways were activated with exercise, there was no difference in the increase between exercise conditions why they do not seem to have dominant roles in the regulation of exercise-induced PGC-1α expression. PGC-1β mRNA increased with normal blood flow exercise, suggesting differential regulation of the two coactivators. TFAM protein, but not mRNA, levels were increased after 4 weeks of endurance training. Also, elite athletes had a higher TFAM protein level compared to moderately active. TFB1M and TFB2M mRNA, but not protein, levels were higher in elite athletes than in moderately active, and increased with endurance training in the leg that exercised with restricted blood flow.

It is concluded that exercise transcriptionally activates PGC-1α and -1β but their regulation seems to be different. It is indicated that AMPK has a greater influence on exercise-induced PGC-1α mRNA compared to calcineurin and p38. The involvement of TFAM in exercise-induced mitochondrial biogenesis is supported and TFAM is most likely regulated through protein stabilization. Conversely, pre-translational changes occur for the TFB factors. Exercise-induced expression of PGC-1α most likely contributes to the increase in TFAM protein which in turn drives mitochondrial biogenesis. In the future, studies are needed to better establish the regulatory links between PGC-1α and mitochondrial regulatory factors, and to evaluate the temporal patterns between these factors. Such knowledge would further explain how exercise leads to mitochondrial biogenesis.

List of publications

The thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I **Norrbom J**, Sundberg C.J, Ameln H, Kraus W.E, Jansson E & Gustafsson, T
PGC-1 mRNA expression is influenced by metabolic perturbation in exercising human skeletal muscle
Journal of Applied Physiology, 96: 189-194, 2004

II **Norrbom J**, Rundqvist H, Österlund T, Sundberg C.J & Gustafsson T
PGC-1α and upstream regulators in human skeletal muscle in response to exercise
Submitted, 2008

III Krämer D.K, Ahlsén M, **Norrbom J**, Jansson E, Hjeltnes N, Gustafsson T & Krook A
Human skeletal muscle fibre type variations correlate with PPARa, PPARd and PGC-1a mRNA
Acta Physiol (Oxf), 188: 207-16, 2006

IV **Bengtsson J**, Gustafsson T, Widegren U, Jansson E & Sundberg C.J
Mitochondrial transcription factor A and respiratory complex IV increase in response to exercise training in humans
Pflugers Archive, 443: 61-6, 2001

V **Norrbom J**, Gustafsson T, Rundqvist H, Wallman S, Jansson E & Sundberg C.J
Expression of mitochondrial transcription factors TFAM, TFB1M and TFB2M in response to training in human skeletal muscle
Submitted, 2008

Contents

List of abbreviations

ADP	Adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AR	Adrenergic receptor
ATF	Activated transcription factor
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BAT	Brown adipose tissue
BSA	Bovine serum albumin
CaM	Calmodulin
CaMK	Calmodulin-dependent kinase
cAMP	Cyclic adenosine monophosphate
CK	Creatin kinase
COX	Cytochrome oxidase
CREB	cAMP responsive element binding protein
Cyt c	Cytochrome c
EA	Elite athletes
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT	Glucose transport protein
HMG	High mobility group
HSP	Heavy strand promoter
IMF	Intermyofibrillar
LSP	Light strand promoter
MCIP1	Myocyte-enriched calcineurin interacting protein 1
MA	Moderately active
MAPK	Mitogen-activated protein kinase
MEF	Myocyte enhancer factor
MHC	Myosin heavy chain
mtDNA	Mitochondrial DNA
NADH	Nicotinamide adenine dinucleotide
N	Number of subjects
NFAT	Nuclear factor of activated T cells
NR	Non-restricted
NRF	Nuclear respiratory factor
PGC-1	Peroxisome proliferator-activated receptor γ coactivator-1

POLRMT	Mitochondrial DNA-directed RNA polymerase
PPAR	Peroxisome proliferator-activated receptor
PRC	PGC-1-related coactivator
R	Restricted
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
Sp1	Specificity protein 1
SS	Subsarcolemmal
T3	3,5,3'-triiodo-L-thyronine
TFAM	Mitochondrial transcription factor A
TFB1M	Mitochondrial transcription factor B1
TFB2M	Mitochondrial transcription factor B2
TIM	Translocase of inner membrane
TOM	Translocase of outer membrane
tRNA	Transfer RNA
VO_2 max	Maximal oxygen uptake
YY1	YingYang 1

Introduction

Physical activity has always been a part of human life. Regular exercise has many positive implications on health and performance, such as improved cardiovascular function and endurance capacity (Morris *et al.*, 1953; Åstrand, 1968; Blomqvist & Saltin, 1983; Paffenbarger, 1988; Blair *et al.*, 1992). As more people become less active in everyday life, the significance of inactivity on health have become obvious, resulting in increased prevalence of patients with e.g. impaired glucose tolerance, overweight and cardiovascular disease (Booth *et al.*, 2002; Booth & Shanely, 2004).

Physical training has been described as the ability of an animal to adapt to regular physical activity over a period of weeks so that oxidative capacity is improved (Booth & Thomason, 1991). Most tissues are positively influenced by regular physical activity. In skeletal muscle tissue, a variety of adaptations occur. One of the most profound changes is an increase in mitochondrial density (Holloszy, 1967; Gollnick & King, 1969; Hoppeler et al., 1973; Holloszy & Booth, 1976; Henriksson & Reitman, 1977; Hoppeler et al., 1985; Freyssenet et al., 1996), leading to an improvement of oxidative capacity and thus enhanced aerobic and endurance performance as well as a shift in the metabolic preference toward fat oxidation (Coggan et al., 1990a; van Loon et al., 1999; Tarnopolsky et al., 2007). The biological importance of mitochondria is also demonstrated by the reduced muscle function in patients with mitochondrial myopathies and the association to e.g. diabetes, heart disease and neurodegenerative diseases (Heddi et al., 1999; Wallace, 2000; Trifunovic et al., 2004).

Background

Skeletal muscle structure and function

The principal functions of skeletal muscle are to produce force, generate power, act as a brake, stabilize joints, protect underlying soft tissue and to be a reserve protein source during starvation. Skeletal muscle is made up of elongated cells, called muscle fibers, that each contains many nuclei. Muscle fibers are organized parallel to each other and are arranged in bundles, or fascicles, that are surrounded by connective tissue. A network of capillaries that supply the cell with oxygen and nutrients encompasses the muscle fibers. Each fiber is innervated by a motor neuron which at the motor end plate releases acetylcholine that binds to nicotinic acetylcholine receptors. When activated, an action potential is induced which releases calcium, subsequently leading to a muscle contraction (Peachy *et al.*, 1983).

Muscle fibers can be classified according to their functional, biochemical and morphological properties, and those with similar properties can be grouped into fiber types. Depending on the properties analyzed, different classification systems have been established. A common classification is to group fibers into type I and type II fibers depending on their expression of myosin heavy chain (MHC) isoforms. MHC type I fibers are characterized by slow-contractile velocity and MHC type II by fast-contractile velocity. Type I fibers are more oxidative and fatigue resistant whereas type II fibers are more glycolytic and easier to fatigue (Barany, 1967; Essén *et al.*, 1975). Type II fibers can be further divided into type IIA, IIB and IIC (Brooke & Kaiser, 1970a, b). However, skeletal muscle fibers have the ability to alter their properties due to changes in contractile activity. Even though change from type I to type II or *vice versa* is uncommon in humans, the metabolic properties and oxidative capacity can change dramatically within each MHC fiber type. The fiber type composition is largely genetically determined, but it is also related to training status as shown by both cross-sectional and longitudinal studies in humans (Saltin & Gollnick, 1983). In untrained individuals the proportion of type I fibers in the vastus lateralis muscle is typically around 55 % (Saltin *et al.,* 1977*).* The amount of mitochondria is strongly correlated to the oxidative capacity of the tissue. Among muscle tissues, mitochondrial density is highest in the heart, followed by the diaphragm, soleus, quadriceps and triceps in a decremental order. In untrained individuals, the total volume density of mitochondria in the vastus lateralis muscle is reported to be between 3.5–5.7 %, with the largest volume in type I fibers. In highly trained individuals the volume density of mitochondria have been reported to be around 8-10 % or in extreme cases up to 12 % (Hoppeler, 1986).

Skeletal muscle plasticity

The structural changes that occur in response to exercise training are very well recognized and data from transgenic animals and functional genomics have added numerous possible regulatory pathways. Even so, an understanding of the relative importance of these pathways *in vivo*, and especially in humans, is largely lacking. Every time even a single bout of exercise is performed, this stimulus is enough to change the expression of many skeletal muscle genes. When exercise is performed regularly, the added effects of such changes in gene expression (Williams & Neufer, 1996; Timmons *et al.*, 2005b) result in changes of the muscle phenotype. Numerous stimuli have been suggested to contribute to the exercise-induced skeletal muscle adaptations. These include (see Figure 1) changes in shear stress, calcium concentration in the muscle fiber, metabolic alterations, pH, reactive oxygen species (ROS), substrate levels and flux, as well as oxygen tension (Booth & Thomason, 1991; Williams & Neufer, 1996).

The different stimuli activate a variety of intracellular signaling pathways, leading to changes in gene expression and ultimately contributing to adaptational processes in skeletal muscle. The adaptations in response to increased contractile activity are specific

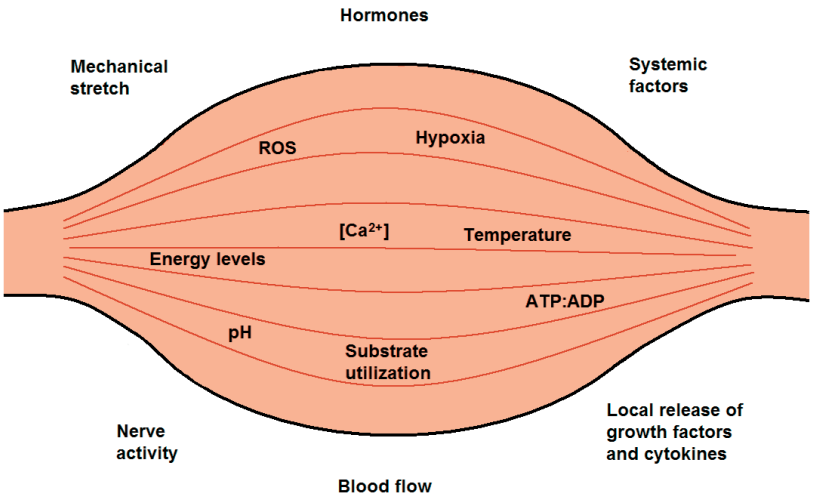

Figure 1. Factors involved in exercise-induced adaptations in skeletal muscle. Exercise responses are probably due to a combination of several of these and other factors. ROS; reactive oxygen species.

and highly dependent on the type of exercise, its duration, intensity and frequency, but also largely due to genetic predisposition that influences the individual variability (Holloszy, 1967; Booth & Vyas, 2001; Timmons *et al.*, 2005a). Even though systemic factors such as maximal cardiac output are dominant in determining the aerobic capacity, peripheral characteristics such as oxidative capacity and fiber type composition may partly contribute to the variation seen between individuals and after a period of changed activity level (Coyle, 1995; Hawley & Stepto, 2001).

In the mitochondrial adaptation to exercise, communication between the cell nucleus and mitochondria is essential. Transcription factors and coactivators that regulate both nuclear and mitochondrial gene expression are involved to induce synthesis and recruitment of mitochondrial proteins (Hood, 2001).

Mitochondria and mitochondrial biogenesis

Mitochondrial structure and function

Mitochondria are abundantly present in mammalian cells. Their fraction varies from tissue to tissue, ranging from <1 % (volume) in white blood cells to 35 % in heart muscle cells (Wiesner, 1997). However, mitochondria should not be thought of as single entities, but rather a dynamic network that continuously undergoes fission and fusion processes (Kirkwood *et al.*, 1986).

The mitochondria in skeletal muscle are divided into two morphologically different sub-fractions that are regionally distinct in the muscle fiber. These are the subsarcolemmal (SS) mitochondria that lie directly beneath the sarcolemmal membrane and the intermyofibrillar (IMF) mitochondria that are located in close contact with the myofibrils (Hoppeler, 1986). Their different properties are likely to influence their capacity for adaptation. SS mitochondria account for 10-15 % of the mitochondrial volume and this population has been shown to be more susceptible to adaptation than the IMF mitochondria (Hood, 2001). However, the IMF mitochondria were found to have higher rates of protein synthesis, enzyme activities and respiration (Cogswell *et al.*, 1993; Takahashi & Hood, 1996).

The mitochondria are equipped with double membranes, creating the intermembrane space between the outer and inner membranes as well as the inner matrix compartment, where most of the metabolic processes take place. The inner membrane is highly folded, forming so-called cristae, to accommodate its large surface area. Embedded in the inner mitochondrial membrane are the five complexes that make up the respiratory chain where oxidative phosphorylation takes

place. In this process, a proton gradient across the inner membrane is coupled to ATP synthesis at complex V (Hatefi, 1985; Lowell & Spiegelman, 2000). In addition to producing ATP essential for cell survival, the mitochondria are a source for free radical or reactive oxygen species, ROS, production. ROS are small, highly reactive molecules that can be generated by mitochondrial respiration and in active skeletal muscle (Murrant & Reid, 2001; Pattwell *et al.*, 2004).

Mitochondria are unique organelles in that they contain their own DNA (mitochondrial DNA, mtDNA), which consists of a circular DNA molecule of about 16.6 kb in humans and 16.3 kb in mice. It encodes 13 of the around 90 proteins that make up the respiratory chain. In addition, mtDNA also encodes 2 ribosomal RNAs (rRNA) and 22 transfer RNAs (tRNA) (Falkenberg *et al.*, 2007). The presence of mtDNA is explained by the evolutionary origin of the mitochondrion as a free-living prokaryotic organism (Gray, 1989). During the course of time, genes have been transferred to the nuclear genome (Andersson *et al.*, 2003), and mitochondrial function is highly dependent on close coordination between the nuclear and mitochondrial genomes. In mammals, mtDNA is maternally inherited, the paternal mtDNA being destroyed during the first embryonic cell divisions (Sutovsky *et al.*, 1999; Shoubridge, 2000). The individual strands of mtDNA are termed heavy (H) and light (L) strand. Introns are lacking, but there is a long non-coding region, the D loop, which contains control elements for transcription and replication of mtDNA (Shadel & Clayton, 1997).

The mitochondria are often referred to as the powerhouses of the cell. In mitochondria, glucose, free fatty acids and amino acids are oxidized to yield ATP, carbon dioxide and water. The maintenance of mtDNA is essential for normal function of the respiratory chain that controls aerobic ATP production. Deficient respiratory chain function is lethal during embryonic development (Larsson *et al.*, 1998). The mitochondria are also involved in processes such as ROS production, programmed cell death (apoptosis), and ageing (Murrant & Reid, 2001; Chomyn & Attardi, 2003; Dufour & Larsson, 2004; Trifunovic *et al.*, 2005).

Mitochondrial biogenesis

The mitochondria are very dynamic and plastic structures. They continuously fuse and divide and their density changes with changes in energy demand. As previously mentioned, two mitochondrial subpopulations exist (SS and IMF), where SS mitochondria are reported to respond more to exercise. The increase in mitochondrial content that results from regular exercise or other stimuli is generally referred to as mitochondrial biogenesis (Hood, 2001). Mitochondrial adaptations lead to a change in the metabolic preference of the muscle,

with a greater reliance on lipid rather than carbohydrate metabolism at submaximal exercise intensities (Coggan *et al.*, 1990b; van Loon *et al.*, 1999; Tarnopolsky *et al.*, 2007). A higher mitochondrial content as an adaptation to exercise has a protective effect against ROS-mediated damage due to lower respiration per mitochondrion and thereby attenuated ROS production at a given workload (Sen, 1995). As stated previously, endurance training induces a higher steady-state mitochondrial content, the increase being dependent on the duration, frequency and intensity of the performed exercise (Hickson, 1981; Dudley *et al.*, 1982; Hoppeler, 1986; Hood *et al.*, 2000), as well as genetic factors. Increases in volume density of mitochondria after 6-8 weeks of training have been reported to be between 38-43 % (Hoppeler *et al.*, 1985; Rosler *et al.*, 1985). Common for the conditions that stimulate mitochondrial biogenesis is a disturbed energy balance, i.e. an increased energy demand in relation to supply (Wiesner 1997). Apart from endurance exercise, mitochondrial biogenesis can also be induced by other conditions, such as thyroid hormone treatment (Weitzel *et al.*, 2003; Sheehan *et al.*, 2004). Thyroid hormone increases the metabolic rate by increasing oxygen consumption accompanied by upregulation of mitochondrial genes (Wiesner *et al.*, 1992).

Contrary to replication of the nuclear genome, mitochondria are continuously turned over and replicated, independent of the cell cycle (Bogenhagen & Clayton, 1977). In human cells, each mtDNA strand contains a single promoter region for transcription initiation, the light strand promoter (LSP) and the heavy strand promoter (HSP) (Montoya *et al.*, 1982), see Figure 2. Transcription from the mitochondrial promoters results in polycistronic precursor RNA molecules, that are processed to yield individual mRNA, rRNA and tRNA molecules (Falkenberg *et al.*, 2007). Replication and transcription of mtDNA are tightly coupled, with LSP transcription producing RNA primers for mtDNA replication initiation (Clayton, 1991). The transcriptional activity of mitochondria is in turn reliant on nuclear-encoded proteins, which are imported to the organelle. For example, the mitochondrial RNA polymerase (POLRMT) and regulatory transcription factors are brought in from the cytoplasm to facilitate expression of the mitochondrial genome.

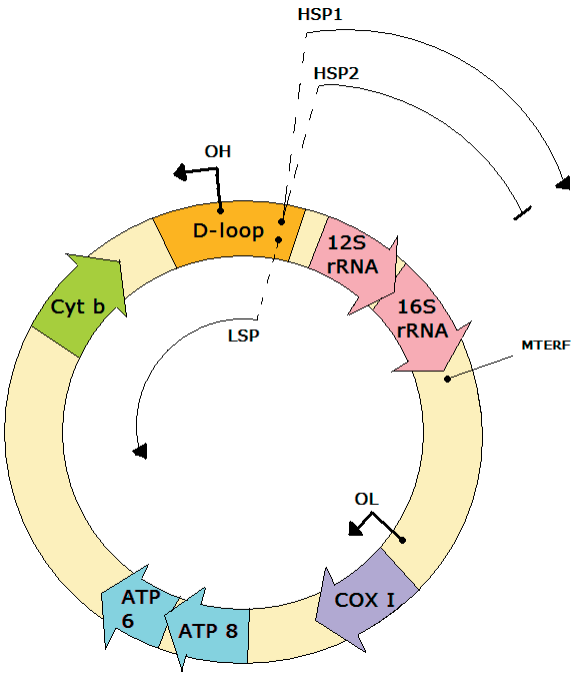

Figure 2. Schematic illustration of the mitochondrial genome. ATP; ATP synthase subunits, Cyt b; cytochrome b, COX I; cytochrome oxidase subunit I, O; Origin of replication, HSP; heavy strand promoter, LSP; light strand promoter, MTERF; mitochondrial transcription terminating factor.

As described above, the formation of new mitochondria is a unique process in that it requires the controlled coordination of two separate genomes. Even though the mitochondrial genome is small, it is essential for proper function of the respiratory chain (Hood *et al.*, 2000). The mitochondrial proteome consists of around 1000 proteins, thus the majority of the gene products for mitochondria are derived from the nucleus (Goffart & Wiesner, 2003). From this it is understood that many cellular processes must act in concert in response to an exercise stimulus, which ultimately leads to formation of new mitochondria. These processes include activation signals, transcription and import of nuclear gene products into the mitochondria, as well as replication and transcription of mtDNA, translation to protein and finally the correct assembly of functional protein complexes. In the present thesis, several of the processes in this continuum have been investigated and their importance further highlighted. Since the majority of mitochondrial proteins are derived from the nuclear genome, they must somehow be targeted for the mitochondria and subjected to the mitochondrial protein import machinery. This involves the import protein complexes translocase of

the outer membrane (TOM) and translocase of the inner membrane (TIM) (Hood, 2001). These complexes also adapt to contractile activity and protein import constitutes an important post transcriptional mechanism in regulation of the mitochondrial phenotype (Takahashi *et al.*, 1998; Gordon *et al.*, 2001).

Factors of importance for mitochondrial transcription and replication

During the last decade, the advancing technologies in using tissue knock-out models and bioinformatics have created the possibility to identify, characterize as well as measure the individual importance of different regulatory factors involved in mitochondrial biogenesis (Larsson *et al.*, 1998; Falkenberg *et al.*, 2002). The transcriptional machinery in mitochondria is very small compared to that of the nucleus. The components required for efficient transcription of mtDNA are mitochondrial DNA-directed RNA polymerase (POLRMT), mitochondrial transcription factor A (TFAM, also known as mtTFA), and one of the two mitochondrial transcription factor B paralogues (TFB1M and TFB2M, also called mtTFB1 and mtTFB2), with TFAM levels determining the activity of mtDNA transcription *in vitro* (Falkenberg *et al.*, 2002). Little is known about the changes of TFB1M and TFB2M in physiological conditions that activate mitochondrial biogenesis in humans.

MtDNA-directed RNA polymerase (POLRMT)

POLRMT cannot interact with promoter sequences and initiate transcription on its own, but requires the assistance of TFAM and one of either TFB1M or TFB2M (Falkenberg *et al.*, 2002; McCulloch *et al.*, 2002). The function of POLRMT is demonstrated by N-terminal deletion of its sequence which resulted in decreased stability and loss of the mitochondrial genome in *Saccharomyces cerevisiae* (Wang & Shadel, 1999).

Mitochondrial transcription factor A (TFAM)

The first mitochondrial transcription factor described was TFAM (Fisher & Clayton, 1988; Parisi *et al.*, 1993), a nuclear encoded protein that is translocated to the mitochondria where it exerts its effects by binding to mtDNA. TFAM is a member of the high-mobility group (HMG) of proteins. It has the ability to bind, unwind and bend DNA without sequence specificity, but shows higher affinity for LSP and HSP (Fisher *et al.*, 1992; Dairaghi *et al.*, 1995). C-terminal deletion of TFAM resulted in loss of specific DNA-binding and transcription activation of the protein (Dairaghi *et al.*, 1995). The vital role of TFAM during development and maintenance of the organism is shown by the lethality of homozygous TFAM knock-outs in both mouse (Larsson

et al., 1998) and chicken (Matsushima *et al.*, 2003). Homozygous TFAM knock-out mice die between embryonic days 8.5-10.5. The mutant embryos lack mtDNA, and display severe respiratory chain deficiency with massive accumulation of morphologically abnormal mitochondria, as well as delayed neural development and defective hearts (Wang *et al.*, 1999). The biological regulatory function is further demonstrated by that TFAM level varies with the level of mtDNA in human cells (Larsson *et al.*, 1994; Poulton *et al.*, 1994).

Mitochondrial transcription factor B1 (TFB1M) and B2 (TFB2M)

Recently, through the use of a pure recombinant *in vitro* system, two novel factors, mitochondrial transcription factor B1 and B2 (TFB1M and TFB2M) in close association to TFAM were shown to activate human mtDNA (Falkenberg *et al.*, 2002). Of these novel factors, TFB2M had a higher transcriptional activity. This has led to the suggestion that TFB2M is a specialized transcription factor in mammalian mitochondria (Cotney & Shadel, 2006), whereas RNAi knock down of TFB1M in *Drosophila melanogaster* suggests a role for TFB1M in translation modification (Matsushima *et al.*, 2005). The TFBs show sequence similarity to a family of rRNA methyltransferases, RNA modifying enzymes, that dimethylates the small rRNA subunit during ribosome biogenesis (Falkenberg *et al.*, 2002; McCulloch *et al.*, 2002). It has been demonstrated that both TFB1M and TFB2M have rRNA methyltransferase activity, but that TFB1M is a more efficient enzyme (Cotney & Shadel, 2006). However, the exact molecular roles of the TFBs are largely unknown.

PGC-1 – suggested master regulator of mitochondrial biogenesis

During the 90's, several orphan nuclear receptors were identified, e.g. the peroxisome proliferator-activated receptors (PPARs) (Mangelsdorf & Evans, 1995). A coactivator of the PPARs, PPAR γ coactivator-1α (PGC-1α) was also identified and shown to regulate several of these nuclear receptors (Puigserver *et al.*, 1998a; Puigserver & Spiegelman, 2003). The PGC-1 family is a group of transcriptional coactivators that coordinate the expression of numerous nuclear-encoded mitochondrial transcription factors. The protein family includes PGC-1α, PGC-1β and PGC-1-related coactivator (PRC) (Wu *et al.*, 1999; Andersson & Scarpulla, 2001; St-Pierre *et al.*, 2003). PGC-1α and PGC-1β are preferentially expressed in tissues with high oxidative capacity, such as heart, skeletal muscle and brown adipose tissue (BAT), where they are important for mitochondrial capacity and energy metabolism (Puigserver *et al.*, 1998a; Wu *et al.*, 1999; Lin *et al.*, 2002a; Kamei *et al.*, 2003; St-Pierre *et al.*, 2003). They are suggested to exert overlapping, but at the same time different, effects

on skeletal muscle phenotype (Mortensen *et al.*, 2006). Further, PGC-1α and PGC-1β have been demonstrated to have crucial roles in different aspects of metabolic regulation (Lin *et al.*, 2005; Finck & Kelly, 2006) and are differentially regulated in response to changes in nutritional status (Lin *et al.*, 2002a; Kamei *et al.*, 2003; Lin *et al.*, 2003).

PGC-1α is now recognized as a master regulator of mitochondrial biogenesis. It binds to and coactivates many nuclear receptors and transcription factors, such as the PPARs, the nuclear respiratory factors (NRFs) and myocyte enhancer factor 2 (MEF2), resulting in transcriptional activity of their target genes (Wu *et al.*, 1999; Vega *et al.*, 2000; Wang *et al.*, 2003; Lin *et al.*, 2005). Overexpression of PGC-1α in myotubes strongly induces mitochondrial biogenesis (Wu *et al.*, 1999; Lin *et al.*, 2002b; St-Pierre *et al.*, 2003). Its role in maintaining metabolic function is illustrated by that PGC-1α knock-out mice have reduced mRNA levels of a number of metabolic genes (Lin *et al.*, 2004). Furthermore, PGC-1α deficient skeletal muscle fatigues faster and the knock-out mice show exercise intolerance (Leone *et al.*, 2005). Since PGC-1α is strongly connected to mitochondrial biogenesis, numerous attempts have been made to evaluate its upstream signaling pathways, aiming to better understand the regulation of mitochondrial biogenesis. Several signaling networks have been shown to influence PGC-1α at both the transcriptional and post translational level, e.g. calcium signaling through calcineurin and CaMK pathways, p38 MAPK and AMPK activity. These results are mainly from cell and animal studies, integrated analysis of these pathways in humans are still needed.

Coordination of nuclear and mitochondrial gene expression

Mitochondrial biogenesis most likely involves integration of multiple transcriptional regulatory pathways, coordinating the expression of nuclear and mitochondrial genes. Highlighted candidate coordinators of the two genomes are the Nuclear Respiratory Factors 1 and 2 (NRF-1 and -2) (Evans & Scarpulla, 1990; Wu *et al.*, 1999), assumed to be major regulators of PGC-1 effects on mitochondrial biogenesis. Many of the NRF target genes are linked to mitochondrial function and biogenesis, among them subunits of the five respiratory complexes (Virbasius *et al.*, 1993). Since the NRFs have been shown to activate TFAM expression (Virbasius & Scarpulla, 1994; Wu *et al.*, 1999), they have been put forward as integrative factors coupling the cross talk between the nucleus and mitochondria. Both NRF-1 and TFAM mRNA seem to be responsive to oxidative stress, their levels increase in mtDNA depleted cells, and in response to oxidative damage to the mitochondria (Miranda *et al.*, 1999; Suliman *et al.*,

2003). The importance of NRF-1 in mitochondrial function is further demonstrated by disruption of the NRF-1 gene in mice which leads to severely reduced mtDNA levels, defective mitochondrial membrane potential and embryonic lethality before day 7 (Huo & Scarpulla, 2001).

Other transcription factors that control the expression of mitochondrial proteins have been identified and seem to be relatively common in gene promoters of nuclear encoded mitochondrial proteins. Binding sites for the general transcription factor Sp1 (Specificity protein 1) have been found in several promoters encoding mitochondrial genes (Zaid *et al.*, 1999). Another general transcription regulator is Ying Yang 1 (YY1) with binding sites in promoters for several cytochrome c oxidase subunits (Lenka *et al.*, 1998). The cyclic AMP-responsive element binding protein (CREB) is a common nuclear target for intracellular signaling. CREB has been shown to be a target of calmodulin-dependent kinase (CaMK) (Dash *et al.*, 1991; Sheng *et al.*, 1991), indicating a role in CaMK-induced mitochondrial biogenesis (Wu *et al.*, 2002). Myocyte-specific enhancer factor 2 (MEF2) is highly expressed in muscle and dimerizes with transcription factors of the MyoD family which potentiates expression of many muscle-specific genes, e.g. glucose transport protein GLUT4 (Ojuka *et al.*, 2002). Induction of PGC-1α has also been shown through Activated transcription factor 2 (ATF2) and MEF2 (Akimoto *et al., 2005).* However, many mitochondrial genes are regulated by yet other factors and the mitochondrial biogenesis involves integration of multiple transcriptional regulatory pathways, coordinating expression of nuclear and mitochondrial genes, specific to the stimuli in question.

Suggested exercise-regulated stimuli for mitochondrial biogenesis

During exercise, the mean oxygen tension in the muscle decreases to 3-4 mmHg at intensities above 50 % of VO_2 max (maximal oxygen uptake) (Richardson *et al.*, 1995). Numerous animal and human studies support hypoxia as a stimulus for the training response, and it has been shown that reduced oxygen delivery during exercise leads to an augmented increase in exercise adaptation (Kaijser *et al.*, 1990; Terrados *et al.*, 1990; Sundberg *et al.*, 1993; Melissa *et al.*, 1997; Hoppeler & Vogt, 2001; Vogt *et al.*, 2001). For example, one-legged exercise training with reduced oxygen delivery augmented the increase in capillarization and oxidative capacity (Esbjornsson *et al.*, 1993; Sundberg, 1994) compared to the contralateral leg trained at the same absolute workload during normal conditions. Metabolic perturbation as a stimulus has been suggested to be important for the increased oxidative capacity observed with hypoxic and ischemic

training (Desplanches *et al.*, 1993; Sundberg, 1994). As has been mentioned before, a disturbed energy balance is a common feature in conditions with mitochondrial biogenesis and several signaling pathways have been suggested to be involved in these processes.

Calcium signaling

Sarcoplasmic Ca^{2+} increases in skeletal muscle during contractile activity. Exercise with restricted blood flow leads to a higher calcium accumulation in the muscle, due to the fact that lower pH requires a higher sarcoplasmic calcium level to sustain the same muscle tension (Westerblad *et al.*, 1991; Sundberg, 1994). Calcium can activate both protein kinases and protein phosphatases to alter the state of phosphorylation of various molecules, such as transcription factors (Berridge *et al.*, 2003). Calmodulin (CaM) is a Ca^{2+} receptor protein that binds to and alters the activity of downstream targets, e.g. calcium/calmodulin-dependent protein kinases (CaMK) and the phosphatase calcineurin (Crabtree, 2001; Hook & Means, 2001).

It has been suggested that both CaMK and calcineurin signaling pathways are involved in PGC-1α regulation (Wu *et al.*, 2002; Handschin *et al.*, 2003; Irrcher *et al.*, 2003a; Ojuka *et al.*, 2003; Guerfali *et al.*, 2007; Kusuhara *et al.*, 2007). These signaling pathways result in subsequent activation of transcription factors such as MEF2, CREB and nuclear factor of activated T cells (NFAT) that bind to respective target gene promoters, such as PGC-1α (Olson & Williams, 2000; Handschin *et al.*, 2003).

p38 Mitogen-Activated Protein Kinase (MAPK) signaling

The mitogen-activated protein kinases (MAPK) are ubiquitous kinases that transduce extracellular signals and induce a variety of cellular responses, e.g. differentiation, adaptation to environmental stress and apoptosis (Ichijo *et al.*, 1997; Leppa *et al.*, 1998; Kyriakis, 1999). p38 MAPK is activated by phosphorylation and in turn activates downstream targets including ATF2 and MEF2 (Zhao *et al.*, 1999). As mentioned previously, they bind to the CREB binding site on the PGC-1α promoter, thereby inducing transcription (Cao *et al.*, 2004; Akimoto *et al.*, 2005). p38 MAPK has been shown to regulate the expression of PGC-1α in cultured muscle cells and *in vivo* (Knutti *et al.*, 2001; Puigserver *et al.*, 2001; Akimoto *et al.*, 2005; Wright *et al.*, 2007a).

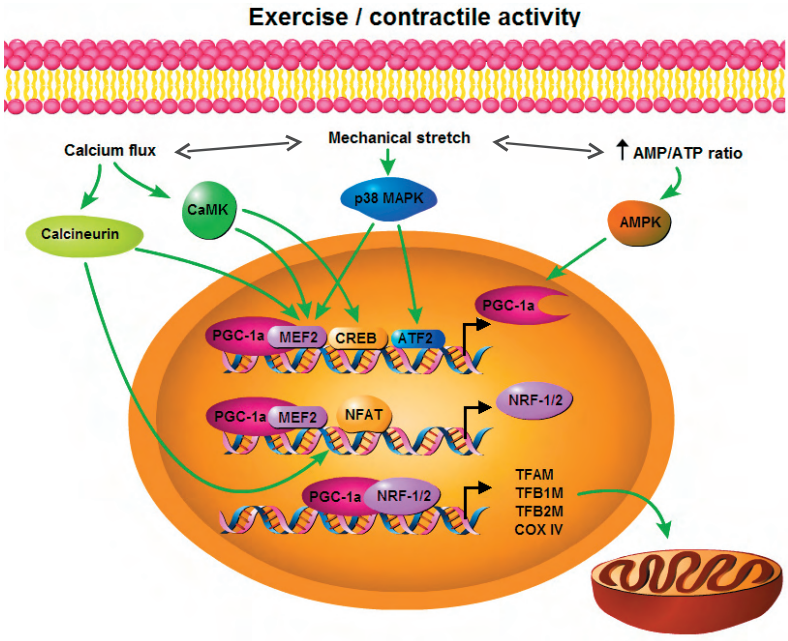

Figure 3. Schematic overview of contractile activity-induced signaling pathways influencing the expression of mitochondrial transcription factors through PGC-1α coactivation. Exercise has been suggested to stimulate induction or activation of PGC-1α through either calcium signaling via calcineurin and CaMK, mechanical stretch via p38 MAPK and/or increased AMP/ATP ratio via AMPK.

AMP-activated protein kinase (AMPK) signaling

AMP-activated protein kinase (AMPK) is a heterotrimeric protein that consists of α (catalytic), β (regulatory) and γ subunits. When cells are exposed to a stressful situation, such as exercise in skeletal muscle cells, the AMP:ATP ratio increases. AMP has the ability to allosterically activate AMPK, but activation can also occur through phosphorylation of the kinase domain (Winder & Hardie, 1996; Carling, 2004). AMPK is generally stimulated by processes that either limit ATP production, e.g. hypoxia and glucose shortage, or increase ATP consumption, e.g. muscle contractions (Carling, 2004; Kahn *et al.*, 2005; Hardie *et al.*, 2006). Activation of AMPK in skeletal muscle induces metabolic changes through either phosphorylation, or through control of gene expression (Hardie & Sakamoto, 2006; Winder *et al.*, 2006). The chemical compound 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) has in several *in vivo* studies been used to chronically activate AMPK. These studies have shown that activation of AMPK is associated with induction of mitochondrial enzymes and proteins involved in glucose uptake in skeletal muscle (Holmes *et al.*, 1999; Winder *et al.*, 2000; Zhou *et al.*, 2000; Bergeron *et al.*, 2001; Jorgensen *et al.,* 2007*).*

Aims

During the past decade, advancing technologies such as the use of transgenic models, whole genome gene expression and bioinformatics have been introduced and refined. They have created the possibility to identify, characterize as well as measure the importance of several signaling pathways and networks, as well as specific factors in the regulation of numerous biological processes. Some genes have been identified as being central to the skeletal muscle remodeling that takes place with increased physical activity. This thesis aimed to further explore the exercise regulation of selected mitochondrial biogenesis factors in human skeletal muscle.

More specifically *the aims* were to study, in human skeletal muscle:

The influence of a single bout of exercise, endurance exercise training and physical activity level on expression of

- the mitochondrial transcription co-activators PGC-1α and PGC-1β
- the mitochondrial transcription factors TFAM, TFB1M and TFB2M

Additional aims were to explore:

- exercise-activation of regulatory pathways previously suggested to influence PGC-1
- the influence of enhanced metabolic perturbation on signaling pathways, coactivators and transcription factors
- PPARα and PPARδ expression levels in subjects with markedly different physical activity levels

Methods and Methodological considerations

Experimental protocols

Before each study, the experimental protocol was explained to all subjects and informed consent was obtained. Each study was approved by the Ethics Committee of Karolinska Institutet and conformed to the Declaration of Helsinki.

Subjects

The subjects included in Papers I-V were all healthy men, apart from a group included in Paper III that consisted of six spinal cord injured individuals. In Paper I, nine healthy men were subjected to a single bout of exercise, also described as acute exercise, and ten additional healthy men were recruited as control subjects that did not exercise. In Papers II and V, twelve healthy men were studied after a single bout of exercise and after ten days and five weeks of repeated bouts of exercise, i.e. endurance training. In Papers III and V, ten elite athletes (EA) and ten moderately active (MA) men were included. Additionally, six men with complete chronic lesion of the cervical spinal cord were included in Paper III, and in Paper IV, six healthy men performed four weeks of endurance training. The age, height and weight of the subjects are summarized in Table 1.

Table 1. Summary of subject characteristics in Papers I-V. Values are expressed as mean (range). SCI; spinal cord injured, MA; moderately active, EA; elite athletes.

Publication	Number of subjects	Age (yrs)	Height (cm)	Weight (kg)
Paper I (acute exercise)	9	23 (18-26)	180 (170-188)	74 (65-84)
Paper I (control)	10	24 (20-29)	180 (174-190)	74 (65-92)
Papers II + V (5 w training)	12	24 (20-27)	181 (173-191)	75 (57-95)
Papers III + V (cross-sectional)	EA:10, MA:10	23 (18-27)	181 (174-190)	73 (65-92)
Paper III (SCI)	6	37 (28-44)	186 (184-187)	77 (64-91)
Paper IV (4 w training)	6	23 (20-26)	177 (174-182)	78 (74-83)

Exercise protocols

The subjects in Paper I, II, IV and V performed one-legged knee extension exercise using a pressure chamber model to induce blood flow restriction, that was first described by Eiken & Bjurstedt (Eiken & Bjurstedt, 1987). Local application of external pressure over the working leg was used to restrict blood flow during exercise in a controlled fashion. The subject was positioned supine in the opening of a large pressure chamber with both legs inside the chamber and one leg strapped to a pad. The pad was connected to a crank-arm of an electrically braked cycle ergometer with locked flywheel. The chamber opening was sealed off at the level of the crotch by a rubber diaphragm. Shoulder supports were used to prevent craniad displacement of the body with increased chamber pressure. For exercise with restricted (R) blood flow, the chamber pressure was elevated to 50 mmHg above atmospheric pressure. This has been shown to restrict leg blood flow during one-legged cycle exercise by 15-20 %, reduce oxygen saturation by 10-12 percentage units (Sundberg & Kaijser, 1992), cause a greater depletion of ATP and creatine phosphate and increase lactate concentrations in the exercising leg (Sundberg, 1994). Exercise with non-restricted (NR) blood flow was performed using the same experimental set-up but with normal atmospheric pressure.

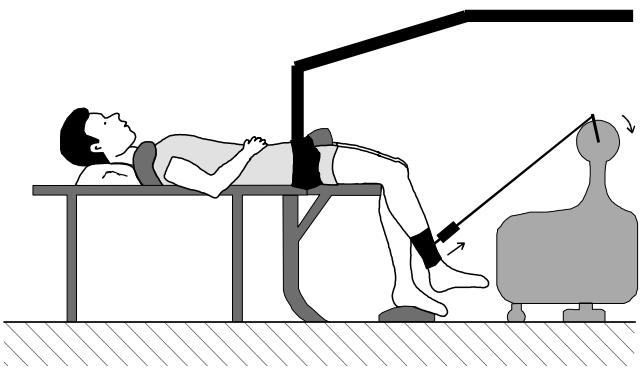

Figure 4. Exercise set-up for one-legged knee-extension with restricted and non-restricted leg blood flow. Modified from Eiken (1987).

Maximal oxygen uptake (VO_2 max) was determined on a cycle ergometer, where the workload was increased stepwise until exhaustion and respiratory gases were continuously analyzed (Sensor Medics Vmax 229; Intra Medic AB, Bålsta, Sweden). Prior to the start of each study, the subjects were familiarized twice with the experimental models and procedures.

Exercise and timing of muscle biopsies

Biopsies were obtained from the vastus lateralis muscle using the percutaneous needle biopsy technique (Bergström, 1962). All biopsy samples were frozen within 30 seconds in liquid nitrogen and stored at -80˚ C until further analysis.

In Paper I, the nine subjects performed a single, 45-min exercise bout with each leg. The condition with which they performed the first exercise bout was randomly assigned to the subjects. Five subjects performed the NR-exercise first, the other four began with R-exercise. Each visit was separated by ten days. The mean (SD) relative workload subsequently used in the experiments was 10 (1) W or 26 (4) % of the one-legged peak load in the NR-condition. Skeletal muscle biopsy samples were obtained at rest from the leg not about to exercise, and at 0 min, 30 min, 2 hours and 6 hours after the end of exercise in the exercised leg. The control group was subjected to biopsy sampling at the same four time points as for the exercised leg in the other group. The non-exercised/control group displayed a small increase in PGC-1α mRNA 6 hours after the first biopsy which illustrates the importance of methodological controls when repeated biopsy samples are performed. It is not known, however, if the biopsy procedure itself or other changes over time induced this increase.

In Papers II and V, the exercise was performed 4 times per week during 5 weeks, giving a total of 20 sessions. Each exercise session began with 45 min of R-condition exercise, followed by 45 min of NR-condition exercise. The subjects were randomized to either exercise their right leg or their left leg in the R-condition. The workload was gradually increased throughout the training period according to the R-leg, to maximize the training response of the subjects. The restricted (R), exercise, being ischemic, was experienced as very strenuous whereas the exercise performed by the contralateral NR-leg was considered very light. Skeletal muscle biopsy samples were obtained at rest before the training period started, after 10 days and 5 weeks of endurance training, in both legs. In addition, biopsy samples were obtained directly after and 2 hours after the first exercise bout, in both legs.

In Paper IV, the subjects performed totally 15 exercise sessions (4 times/week during 4 weeks). The exercise sessions were performed as described above for the study in Papers II and V. Skeletal muscle biopsy samples were obtained at rest before the first training session and 24 hours after the last training session, in both legs.

In Papers III and V, skeletal muscle biopsy samples were obtained at rest from elite athletes and normally active individuals, as well as from spinal cord injured subjects. Following spinal cord injury,

alterations in neuronal input are associated with marked changes in muscle phenotype, e.g. fiber area is reduced, percentage of type IIB fibers are increased and type I fibers are dramatically reduced (Grimby *et al.*, 1976; Aksnes *et al.*, 1996).

Table 2. Summary of interventions, biopsy sampling time and factors analyzed in Papers I-V. XS; cross-sectional. R; restricted, NR; non-restricted.

Publication	Intervention	Biopsy time	Factors
Paper I	45' single bout, one-legged exercise. R- and NR-blood flow	Pre, 0', 30', 2h, 6 h post	PGC-1α, MCIP1, NRF-1, TFAM, TFB1M, TFB2M
Paper II	45' single bout and 5 w training, one-legged exercise. R- and NR-blood flow	Pre, 0', 2h post 1st session, and at rest 5 w	PGC-1α, PGC-1β, MCIP1, AMPK, pAMPK, p38, Pp38
Paper III	XS-study; athletes vs. moderately active, and spinal cord injured individuals	At rest, 24 h after any previous exercise	Calcineurin Aα & Aβ, PGC-1α, PGC-1β, MEF2C, PPARα, PPARγ
Paper IV	4 w training, one-legged exercise. R- and NR-blood flow	Pre and post	TFAM, COX I, COX IV
Paper V	5 w training, one-legged exercise. R- and NR-blood flow, and XS - study; athletes vs. moderately active.	Pre and post 5 w, and at rest 24 h after previous exercise in XS-study	TFAM, TFB1M, TFB2M, COX IV

Performance tests

In the studies included in Papers II, IV and V, one-legged step-wise incremental exercise tests were performed before and after the training period. Each leg was tested during NR-conditions. The subject was instructed to maintain a pedaling rate of 60 rpm, starting with 4 min at 20 W. The workload was then increased by 10 W every minute until 60 rpm could no longer be maintained. When the pedaling rate fell below 55 rpm for more than 5 seconds, the time and peak load were recorded and the test completed.

In Paper IV, the work performed during one training session had increased by 100 % after 4 weeks of training. Independent of training condition, the relative increase in time to fatigue was 13 % and peak load increased by 9 %. In Papers II and V, the average cumulative workload for each leg increased by 30, 50, 80, and 100 %, respectively over the last 4 weeks compared to the first week of training.

Fiber typing

In Paper I, a small portion of the pre exercise and 2 hours post exercise biopsy samples from the R-condition was freeze-dried, and around 400 single fibers were dissected out from each sample. They were histochemically classified as either fiber type I or II (Essen *et al.*, 1975) and thereafter divided into two separate pools of 200 type I or II fibers, respectively.

In paper III, serial transverse sections (10 μm) from mounted biopsies were cut with a microtome at -20° C and stained for myofibrillar adenosine triphosphatase (ATPase) activity. The sections were preincubated at different pH values in acid (pH 4.3 and 4.6) and alkaline (pH 10.3) buffers. Muscle fiber types were classified by the mATPase isoforms (I, IIA, IIB, IIC) based on the myofibrillar ATPase staining characteristics (Brooke & Kaiser, 1970a).

NADH dehydrogenase staining

Serial transverse sections from biopsies (Paper III) were cut, and cross-sectional fiber area was measured morphologically by planimetry from an NADH dehydrogenase stain (Novikoff *et al.,* 1961) and the relative area of the different fiber types (%) were calculated. After extreme endurance training in humans only red or oxidative fibers could be identified by the NADH staining in gastrocnemius muscle, although classification by another system, the myofibrillar ATPase, clearly revealed two distinct fiber types (Jansson & Kaijser, 1987).

Table 3. Fiber type percentage (SEM) distribution in individuals participating in the study in Paper III. Values are mean (SE), SCI; spinal cord injured. * denotes significant (P<0.05) difference vs Normally (moderately) active and # denotes significant P<0.05 difference vs Cyclists.

	mATPase fiber types (%)			
Subjects	**Type I**	**Type IIC**	**Type IIA**	**Type IIB**
Normally active (n=9)	57 (4)	2 (1.0)	23 (3)	18 (2)
Cyclists (n=11)	71 (3)*	0.4 (0.1)	21 (3)	8 (2)
SCI (n=7)	1 (0.5)*#	0 (0.0)	38 (7)	61 (7)*#

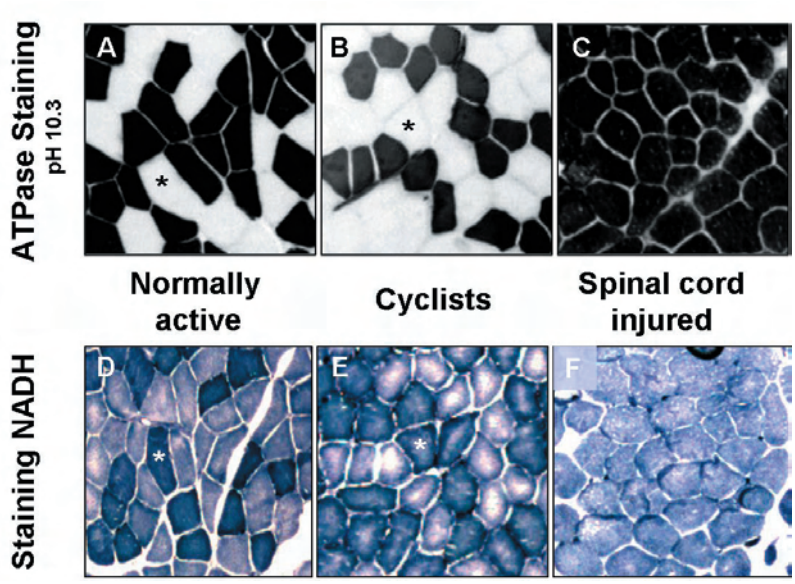

Figure 5. mATPase fiber type staining (A-C) and NADH dehydrogenase staining (D-F) in human skeletal muscle biopsies from normally active individuals, cyclists and spinal cord injured individuals. * denotes type I oxidative, slow fibers.

Citrate synthase activity

In Papers III, IV and V, the activity of the oxidative enzyme citrate synthase was determined. Briefly, a part of a biopsy was freeze-dried and homogenized in 0.1 M phosphate buffer (pH 7.7) with 0.5 % BSA. The citrate synthase activity was analyzed by a fluorometric method according to the principles of Lowry and Passonneau as described by Lin et al (Lin *et al.*, 1988).

In Paper IV, the relative increase in citrate synthase activity was 24 % after 4 weeks of training, independent of exercise condition. In Papers III and V, the elite athletes had a more than two-fold higher average (± SEM) citrate synthase activity than the moderately active (0.56 (± 0.03) and 0.22 (± 0.03) μkat/g dry muscle).

Gene and protein expression

RNA extraction and mRNA quantification

Total RNA from biopsy samples and fiber pools (Papers I and III) was extracted using the acid phenol method. The extracted RNA was quantified spectrophotometrically by absorbance at 260 nm and the integrity of total RNA was determined by 1 % agarose

gel electrophoresis. Two micrograms of total RNA were reverse transcribed by Superscript reverse transcriptase (Life Technologies) using random hexamer primers (Roche Diagnostics) in a total volume of 20 µl. Real-time PCR (TaqMan®) was used to measure mRNA expression (ABI-PRISM® 7700 Sequence Detector, Perkin-Elmer Applied Biosystems Inc.). Oligonucleotide primers and TaqMan® probes were either designed using Primer Express version 1.5 (Perkin-Elmer Applied Biosystems Inc.) or supplied as TaqMan® gene expression assays (see the respective papers for additional information and sequences).

To control for potential variation in RNA loading and quantification, endogenous controls (or housekeeping genes) were used. The validity of housekeeping genes have been determined for each study and control experiments revealed approximately equal efficiencies over different starting template concentrations for target and housekeeping genes. All samples from an individual subject or group were analyzed simultaneously in one assay run. In Paper I, β-actin was chosen as endogenous control, in Papers II and V, 18S was chosen as the endogenous control and in Paper III, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used. In Paper III, there was a non-significant trend for an increase in β-actin mRNA expression with training status, similar to that previously reported (Mahoney *et al.*, 2004), whereas β-actin appeared to be more stable following acute exercise, also in line with our findings.

In Papers I and II, cycle threshold (C_T) was converted to a relative amount by using dilution curves for each target and housekeeping gene from a representative RT sample. The gene expression of the target gene was then normalized to the housekeeping gene for each sample. In Papers III and V, the comparative C_T method was used; a C_T value was obtained by subtracting the control C_T values from respective target C_T values. The expression of each target was then evaluated by 2^{-DDCT} (DeltaDelta C_T method) (Winer *et al.*, 1999).

Protein extraction and Western blot

Muscle samples were homogenized using glass homogenizers in ice-cold homogenization buffer, to suit the proteins of interest. The different buffers are described in detail in each paper. Consideration had to be taken to factors such as shortage of material etc. Protein concentrations were determined using Bradford Assay (BioRad). The protein samples were mixed with 4 X loading buffer (Laemmli; pH 6.8, 8 % SDS, 40 % Glycerol, 250 mM Tris-HCl and Brompehnol blue) and boiled before use.

Muscle homogenates were separated by 10 or 15 % SDS-PAGE and transferred to nitrocellulose membranes. After transfer, membranes were briefly washed in Tris-buffered saline (TBS) with 0.1 % Tween-20 (TBS-T) and stained with Ponceau S to provide visual evidence of uniform loading and transfer of proteins. Membranes were then incubated in blocking solution (5 % dry non-fat milk) for 60 min at RT and then probed with primary antibodies over night at 4˚ C. After being washed in TBS-T, membranes were incubated with appropriate HRP-linked secondary antibodies for 45-60 min at room temperature and thereafter washed with TBS-T. Bound antibodies were detected using either LumiGLO Chemiluminescent Substrate (BioLabs), Super Signal® West Femto Sensitivity Substrate (Pierce) or Immun-Star™ Western C™ Kit (BioRad) and developed on films (Agfa or Amersham). The films were scanned and quantified densitometrically using Quantity One software (BioRad).

The membranes were stripped and probed with antibodies against alpha-actin (Sigma) as a control for loading differences (Papers IV and V). To test the ability of the TFB1M and TFB2M antibodies to detect increasing protein amounts, increasing concentrations of protein lysates were subjected to blotting and antibody detection against TFB1M and TFB2M, respectively. The result showed that the antibodies could detect differing protein amounts.

Statistical analysis

Values are expressed as means ± SD (Paper IV) or means ± SEM (Papers I, II, III, and V), unless otherwise stated. P-values < 0.05 were considered significant.

Hemodynamics, lactate, workload and time to fatigue

Paper I: A two-way (time and exercise condition) parametric analysis of variance (ANOVA) was used to analyze the hemodynamic parameters, venous lactate, L-RPE. **Paper IV:** A two-way (time and exercise condition) parametric ANOVA was used to analyze the absolute changes in time to fatigue, peak load and citrate synthase activity, as well as the effects of training. A two-way parametric ANOVA was applied to evaluate the effects of exercise condition and time in the training study. A one-way ANOVA was applied to evaluate the effect of time separately for each condition.

mRNA levels

In **Papers I and II,** the mRNA data were analyzed using logarithmic-transformed ratios (target:endogenous control). A two-way (time and exercise condition) parametric ANOVA was used to evaluate the

mRNA response to a single bout of exercise (pre exercise and 2 h post exercise) or in response to training (before and after 5 weeks) in the two exercise conditions (R and NR). Planned comparison was used (i.e. *post hoc* test) to locate significant interactions, or when no interaction was found, to locate differences corresponding to significant main effects in the ANOVA model. In **Paper III**, groups were compared using one-way ANOVA with Fischer´s as *post hoc* analysis. Correlations were calculated by simple linear regression. In **Paper V**, a non-parametric test for independent levels, Mann-Whitney U-test, was applied to test for differences in mRNA (pre and 2h post) on ratios of target:endogenous control.

Protein levels

In **Paper II,** a non-parametric Friedman Analysis of Variance by Ranks for multiple dependent samples was used to evaluate the effects of time and exercise condition (R and NR) on protein phosphorylation. Wilcoxon paired-samples test was used (i.e. *post hoc* test) to locate differences corresponding to significant interactions. In **Paper IV,** to evaluate the effects of training-induced changes within and between conditions, a non-parametric test for dependent values, Wilcoxon matched pairs test, was applied. In **Paper V,** a non-parametric test for independent levels, Mann-Whitney U-test, was applied to test for differences in protein phosphorylation (pre and 2h post) between elite athletes and moderately active individuals.

Results and discussion

Mitochondrial biogenesis in skeletal muscle is affected by various stimuli, e.g. changes in contractile activity and energy state. During the last decade, advancing technologies such as tissue knock-out models and bioinformatics have created the possibility to identify, characterize as well as measure the individual importance of different regulatory factors involved in mitochondrial biogenesis. The present thesis aimed to investigate central components suggested for mitochondrial biogenesis following e.g. a single bout of exercise, endurance training or long periods of inactivity.

Key mitochondrial coactivators – influence of physical activity

Exercise- and training-induced expression of PGC-1α

The present thesis demonstrates that a single exercise bout induces a pre translational increase in PGC-1α (Papers I and II), Figure 6. This finding is supported by other reports in human and rodent skeletal muscle (Baar *et al.*, 2002; Terada *et al.*, 2002; Pilegaard *et al.*, 2003; Russell *et al.*, 2005; Mortensen *et al.*, 2007). The findings in Papers I and II lend further support to the involvement of the PGC-1α pathway in the initial process of exercise adaptation in human skeletal muscle.

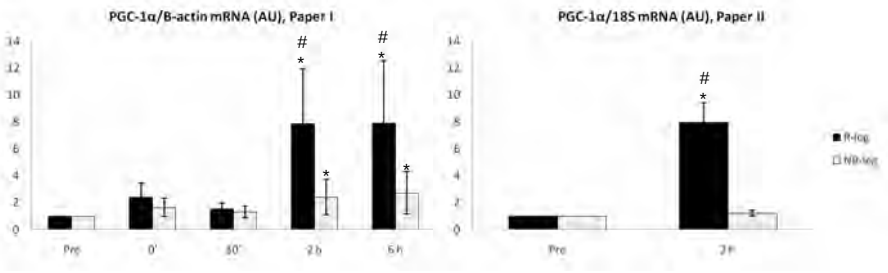

Figure 6. Peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) mRNA expression in response to a single bout of exercise in human skeletal muscle. Values are mean ± SEM. Graphical data represents arbitrary units (AU) corrected to housekeeping genes β-actin and 18S, respectively. R-leg and NR-leg; leg exercised under restricted and non-restricted blood flow condition, respectively. * denotes significant difference (P<0.05) relative to Pre, # denotes interaction between R- and NR-conditions. N=9 (Paper I), N=12 (Paper II).

In response to five weeks of endurance training, there was a small but significant increase in basal PGC-1α mRNA levels in the R-leg (Paper II). In addition, in Paper III it was shown that resting PGC-1α mRNA levels were higher in elite athletes than in moderately active individuals, and even lower in spinal cord injured subjects (Figure 7). This supports physical activity and/or training status-related influence on basal PGC-1α mRNA levels. Other human studies have also demonstrated increased basal PGC-1α expression by endurance exercise training in skeletal muscle (Russell *et al.*, 2003; Short *et al.*, 2003; Kuhl *et al.*, 2006). However, one study showed that four weeks of one-legged exercise did not affect basal PGC-1α mRNA levels (Pilegaard *et al.*, 2003). Thus, the majority of studies show that there seems to be a positive relation between training states with high oxidative capacity and PGC-1α mRNA levels, the data supporting a role for PGC-1α in mitochondrial biogenesis. That inactivity down-regulates PGC-1α has also been shown in a previous longitudinal human study (Timmons *et al.*, 2006). Furthermore, denervation in rats has been shown to result in reduced PGC-1α levels (Adhihetty *et al.*, 2007a). Taken together, the majority of the long-term training studies show a clear up-regulation of basal PGC-1α mRNA levels by between 50-300 %, indicating an importance of this coactivator for maintenance of adaptation.

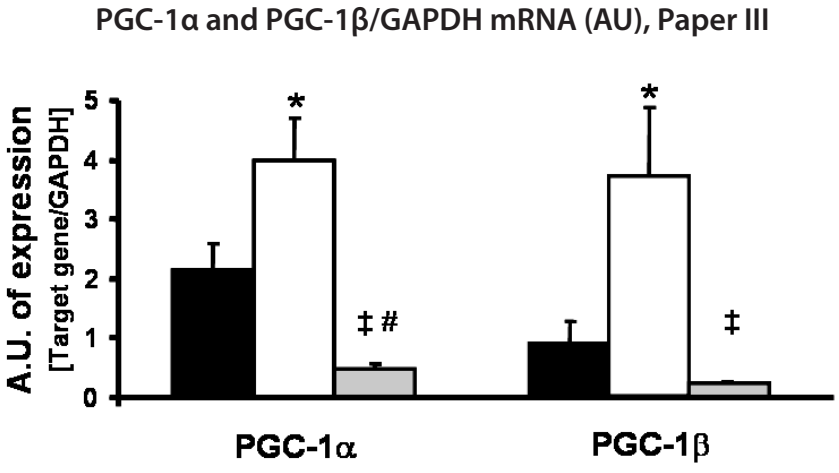

Figure 7. Peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) and 1β (PGC-1β) mRNA expression in human skeletal muscle from normally active individuals (black bars), cyclists (white bars) and spinal cord-injured subjects (grey bars). Values are mean ± SEM. Graphical data represents arbitrary units (AU) corrected to GAPDH. * and # denote significance ($P<0.05$) vs normally active individuals, ‡ denotes significance ($P<0.01$) vs cyclists.

Skeletal muscle fiber types I and II showed similar basal PGC-1α mRNA levels (Paper I). This can be considered as somewhat surprising since PGC-1α has been shown to be preferentially expressed in muscles with a high percentage of type I muscle fibers in mice (Lin *et al.*, 2002b; Russell *et al.*, 2003; Koves *et al.*, 2005). Overexpression of PGC-1α in skeletal muscle cell cultures has been shown to enhance the mRNA expression of the slow oxidative MHC I isoform, whereas MHC IIX and IIB expression was downregulated (Mortensen *et al.*, 2006). However, another human study reports, in line with the results in Paper I, no difference in PGC-1α mRNA levels in different fiber types (Plomgaard *et al.*, 2006). Altogether, these findings show that it is of importance to consider species differences in fiber-type specific expression and to be cautious in extending rodent findings to humans. Important to remember is that changes in oxidative capacity are directly physiologically relevant, more so than the markedly smaller changes observed in fiber type transition (Henriksson & Reitman, 1977; Lin *et al.*, 2002b). The physiological consequences of altered PGC-1α expression have been investigated by different approaches. Studies in PGC-1α deficient mice revealed that although PGC-1α is dispensable for mitochondrial biogenesis *per se*, it is absolutely required for the normal expression of a large number of mitochondrial genes (Lin *et al.*, 2004; Leone *et al.*, 2005). Also, overexpression of PGC-1α in muscle has been shown to render a more endurance-type trained muscle with higher expression of oxidative genes (Lin *et al.*, 2002b). Furthermore, the functional importance of PGC-1α is demonstrated by the finding that muscle specific overexpression of PGC-1α in mice improved exercise capacity with both better running performance and higher peak oxygen uptake than the control littermates (Calvo *et al.*, 2008).

PGC-1α has the ability to coactivate the peroxisome proliferator-activated receptor (PPAR) isoforms. In Paper III, mRNA levels of PPARα and PPARδ were higher in athletes compared to moderately active subjects, with the expression being even lower in spinal cord injured subjects. Furthermore, there was a positive correlation between PPARα and PPARδ and oxidative fiber content. The PPARs have been suggested to be involved in regulation of skeletal muscle fiber type (Luquet *et al.*, 2003; Wang *et al.*, 2004) and PPARα appears to regulate expression of muscle specific markers such as MEF2 (Finck *et al.*, 2005). A single bout of exercise has previously been associated with increased mRNA expression of PPARα and PPARδ in skeletal muscle (Watt *et al.*, 2004). However, endurance training has previously only been coupled to increased PPARα, not PPARδ (Horowitz *et al.*, 2000; Russell *et al.*, 2003). In another study, PPARδ tended to be negatively

related to the occurrence of type I fibers (Plomgaard *et al.*, 2006). In that study, different muscle types from the same individuals were investigated, whereas in Paper III more extreme fiber type differences in the same muscle type have been studied. Paper III provides further evidence that PPARα and PPARδ mRNA are related to muscle activity and/or fiber type-specific gene expression in humans.

Exercise regulation of PGC-1α transcript

PGC-1α is likely to be regulated both at the transcriptional and post translational levels and may thus be affected by numerous regulatory mechanisms not studied in the present thesis. In fact, the initial phase of the exercise-induced changes ultimately leading to an increase in mitochondrial density has been suggested to be mediated by activation, rather than increased expression of PGC-1α (Wright *et al.*, 2007b). To elicit a response in PGC-1α protein in skeletal muscle, it has been shown in animal and *in vitro* studies that repeated bouts of contractile activity are needed (Irrcher *et al.*, 2003b; Akimoto *et al.*, 2004b; Terada & Tabata, 2004). Short-term nerve stimulation does not cause any detectable change in PGC-1α protein levels (Akimoto *et al.*, 2004b). During the present thesis work, extensive attempts were made to measure PGC-1α protein levels, using several commercial antibodies as well as a non-commercial antibody. However, no convincing signal at the right molecular size could be detected, or with other antibodies, no signal was detected at all. Therefore, no protein data on PGC-1α is presented in this thesis.

The regulating mechanisms behind pre-translational activation of PGC-1α are not fully elucidated but several pathways have been suggested from various experimental settings in cell and animal models. In Paper I, two hours after a single bout of exercise, PGC-1α mRNA was increased significantly more in the restricted leg (R-leg) than in the non-restricted leg (NR-leg). Six hours after completion of exercise, the mRNA levels were still elevated in both conditions. Similarly, in Paper II, PGC-1α mRNA increased significantly two hours after a single exercise bout in the R-leg, but not in the NR-leg. Following five weeks of endurance training, only the R-leg showed increased basal PGC-1α mRNA levels (Paper II), coherent with the findings from the single bout experiments (Papers I and II). That PGC-1α mRNA increases more after R-exercise clearly demonstrates that factors related to metabolic perturbation are of importance. This could be attributed to enhanced activation of signaling pathways associated with altered metabolism, such as increased AMP:ATP ratio as wel as reduced phosphocreatine and/or glycogen levels. When the metabolic perturbation is enhanced, the degree of fatigue is increased which requires a higher degree of muscle activation

and higher intracellular calcium levels for a given force output (Westerblad *et al.*, 1991). Therefore, as has been suggested earlier, the regulating role of calcium-activated kinases and phosphatases, as well as posttranslational regulation through AMPK and p38 MAPK should be considered (Winder *et al.*, 2000; Wu *et al.*, 2002).

Calcium signaling through Calcineurin

Rodent studies have suggested that calcineurin activity, rather than its total expression, may shift fiber type in the direction of slow twitch, more mitochondria-rich fibers (Ryder *et al.*, 2003; Talmadge *et al.*, 2004). Rodent skeletal muscle has been shown to display increased mRNA expression of the phosphatase calcineurin Aα and Aβ with exercise (Miyazaki *et al.*, 2004). However, in humans performing one-legged exercise a transient increase was observed only in the non-exercising leg (Pilegaard *et al.*, 2003). In Paper III, calcineurin Aα and calcineurin Aβ mRNA expression were similar between groups, i.e. elite athletes, normally active and spinal cord injured, showing that basal calcineurin mRNA expression is not related to training status in human skeletal muscle. Myocyte-enriched calcineurin interacting protein 1 (MCIP1) reflects the activated state of the calcineurin system and has therefore been used as a marker for activated calcineurin *in vivo* (Yang *et al.*, 2000; Rothermel *et al.*, 2003; Garnier *et al.*, 2005). In both Papers I and II, skeletal muscle levels of MCIP1 mRNA were increased two hours after a single bout of exercise (Figure 8). However, basal MCIP1 mRNA levels were found to be elevated after five weeks of endurance training in the R-leg only (Paper II). This finding, which is in contrast to that seen after a single bout of exercise, could be due to an "accumulation" of repeated responses. It could contribute to the

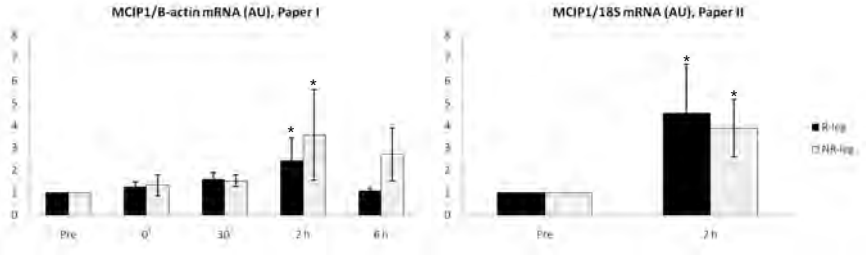

Figure 8. Myocyte-enriched calcineurin interacting protein 1 (MCIP1) mRNA expression in response to a single bout of exercise in human skeletal muscle. Values are mean ± SEM. Graphical data represents arbitrary units (AU) corrected to β-actin and 18S, respectively. R-leg and NR-leg; leg exercised under restricted and non-restricted blood flow condition, respectively. * denotes significant difference ($P<0.05$) relative to Pre in respective exercise condition. N=9 (Paper I), N=12 (Paper II).

upregulation of basal PGC-1α mRNA and to generally greater skeletal muscle remodeling following training with R-blood flow as previously reported (Sundberg, 1994).

Intermittent calcium increases in L6 myotubes have been shown to result in increased PGC-1α protein levels (Ojuka *et al.*, 2003), and overexpression of constitutively active calcineurin in skeletal muscle of transgenic mice resulted in increased expression of PGC-1α (Ojuka *et al.*, 2003; Ryder *et al.*, 2003). There is also support for involvement of both the calcineurin and the CaMK pathways in regulation of PGC-1α expression (Handschin *et al.*, 2003; Kusuhara *et al.*, 2007). The increase in MCIP1 mRNA in both exercise conditions in Paper I and II suggests that calcineurin may be activated by exercise and could play a role in PGC-1α transcription activation in human skeletal muscle. However, since PGC-1α but not MCIP1 increased more in the R-condition, other signaling pathways or factors may be important for this regulation as well. Other studies have shown that calcineurin does not seem to mediate exercise-induced PGC-1α expression in adult muscle (Ojuka *et al.*, 2003; Garcia-Roves *et al.*, 2006; Vaarmann *et al.*, 2008). Inhibition of CaMK completely blocked the increase in mitochondrial biogenesis (Ojuka *et al.*, 2003), suggesting a more potent role for CaMK, rather than for calcineurin. Thus, calcineurin may regulate muscle metabolic profile when activated during development, but available data indicates a less important role in muscle adaptation in the adult.

CaMK levels were not measured in this thesis. The main CaMK isoform that has been demonstrated to induce PGC-1α is CaMK IV (Wu *et al.*, 2002), however it has later been proven not to be expressed in skeletal muscle (Akimoto *et al.*, 2004a). The importance of calcium in the physiological regulation of PGC-1α should not be rejected since other CaMK isoforms than CaMK IV are expressed in skeletal muscle (Rose *et al.*, 2006), and since calcineurin has been demonstrated to influence PGC-1 beyond the pre-translational level (Guerfali *et al.*, 2007). It has been shown that CaMK II is present in skeletal muscle and that this isoform is activated by exercise (Rose & Hargreaves, 2003; Rose *et al.*, 2006). However, it has also been suggested that CaMK II does not play a major role in regulating mitochondrial biogenesis in skeletal muscle in response to chronic energy deficiency (Zong *et al.*, 2002).

p38 Mitogen-activated protein kinase (p38 MAPK)

In Paper II, p38 MAPK phosphorylation increased in both exercise conditions immediately following a single exercise bout, but already two hours after exercise the levels had returned to pre values, Figure 9. That muscle contractile activity can activate p38 has been established previously (Widegren *et al.*, 1998; Boppart *et al.*, 2000; Wretman *et al.*, 2000; Irrcher *et al.*, 2003b).

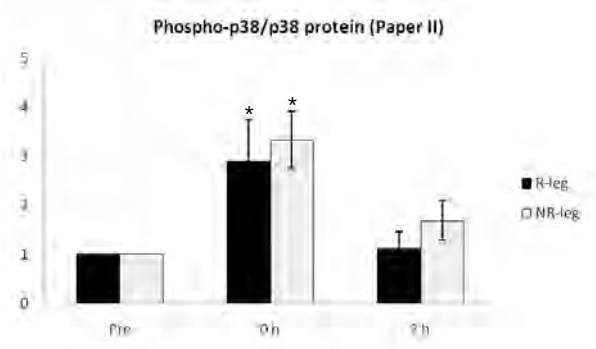

Figure 9. p38 protein phosphorylation in response to a single bout of exercise in human skeletal muscle. Values are expressed as mean ± SEM, relative to Pre in each condition. Graphical data represents arbitrary units (AU) corrected to total p38 protein. R-leg and NR-leg; leg exercised under restricted and non-restricted blood flow condition, respectively. * denotes significant difference ($P<0.01$) relative to Pre in respective exercise condition. N=6.

p38 seems to regulate both the activity and expression of PGC-1α (Knutti *et al.*, 2001; Puigserver *et al.*, 2001; Akimoto *et al.*, 2005). p38 has the ability to phosphorylate and increase the stability of PGC-1α, as well as affect the transcription through phosphorylation of ATF2 (Akimoto *et al.*, 2005; Wright *et al.*, 2007a). Cross talk between the p38 MAPK and Ca^{2+} signaling pathways has been suggested in the activation of PGC-1α. Both pathways activate the transcription factors MEF2 and ATF2 that in turn activates the cAMP-response element binding site in the PGC-1α promoter (Cao *et al.*, 2004; Akimoto *et al.*, 2005). Furthermore, p38 has been suggested to be downstream of CaMK in the Ca^{2+} signaling cascade (Enslen *et al.*, 1996; Takeda *et al.*, 2004; Wright *et al.*, 2007a). This illustrates the complexity in the connections and modifications of different networks and pathways in the tissue remodeling processes.

Taken together, the p38 and the calcineurin system, as indicated by MCIP1 expression, are both activated after a single exercise bout (Papers I and II), but neither could explain the differential PGC-1α response observed between the R- and NR-exercise conditions.

Therefore, any major contribution of these pathways in exercise-induced pre translational increase of PGC-1α was not supported.

AMP-activated protein kinase (AMPK)

The importance of AMPK activity for mitochondrial biogenesis was shown in a recent creatine kinase (CK) deficient mice study (Vaarmann *et al.*, 2008), in which increased mitochondrial biogenesis was related to AMPK activation rather than to calcium-dependent processes. In Paper II, AMPK phosphorylation increased in the R-leg two hours after a single exercise bout, but no change was observed in the NR-leg, Figure 10. This is in accordance with the pattern of PGC-1α mRNA expression.

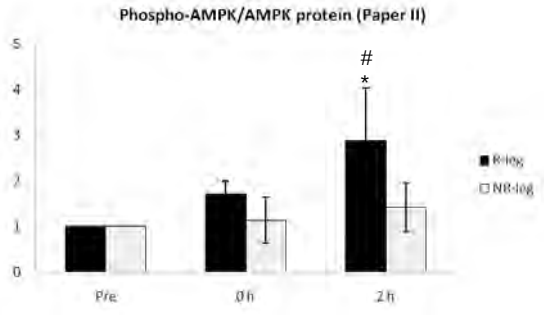

Figure 10. AMPK protein phosphorylation in response to a single bout of exercise in human skeletal muscle. Values are expressed as mean ± SEM, relative to Pre in each condition. Graphical data represents arbitrary units (AU) corrected to total AMPK protein. R-leg and NR-leg; leg exercised under restricted and non-restricted blood flow condition, respectively. * denotes significant difference ($P<0.01$) relative to Pre in the R-condition, # denotes interaction between R- and NR-conditions. N=5.

Numerous reports, including acute exercise studies in rodents and humans, demonstrate activation of AMPK in skeletal muscle (Vavvas *et al.*, 1997; Fujii *et al.*, 2000; Hayashi *et al.*, 2000; Wojtaszewski *et al.*, 2000; Musi *et al.*, 2001; Chen *et al.*, 2003). Furthermore, others have suggested that AMPK signals through PGC-1α to induce mitochondrial biogenesis (Atherton *et al*., 2005; Lee *et al*., 2006; Zong *et al*, 2002). For example, AMPK has been shown to have a critical role for the induced expression of PGC-1α in response to chronic energy deficiency *in vivo* in skeletal muscle (Zong *et al.*, 2002). It was recently shown that AMPK has the ability to bind to and directly phosphorylate, and thereby activate PGC-1α (Jager *et al.*, 2007). It has also been demonstrated that PGC-1α can regulate its own expression in a feed-forward loop in skeletal muscle (Handschin *et al.*, 2003), and involvement of post translational modifications of PGC-1α by AMPK in this induction has been suggested (Jager *et al.*, 2007).

Furthermore, AMPK is constitutively activated in skeletal muscle lacking PGC-1α, possibly caused by a state of energy deficiency (Lin *et al.*, 2004). That metabolic perturbation is a major regulator of AMPK activation was recently elegantly demonstrated in exercising human skeletal muscle (Wadley *et al.*, 2006). They showed that AMPK activation was greater with greater metabolic disturbance, which is coherent with the observation in Paper II of higher phosphorylation of AMPK in the R-condition after a single exercise bout. Further support for metabolic perturbation and AMPK as stimuli in PGC-1α regulation is that exercise with glycogen depletion has been shown to activate AMPK *and* PGC-1α to a greater extent (Wojtaszewski *et al.*, 2003; Mortensen *et al.*, 2006). This fits with the findings in Paper II since greater glycogen degradation has been reported in the R-leg (Sundberg, 1994).

An alternative explanation to metabolic perturbation behind the enhanced activation of AMPK could be stimulation of the adrenergic receptors (AR), preferably β2-AR, the dominant AR type in skeletal muscle (Jensen *et al.*, 1995; Miura *et al.*, 2007). Activation of the adrenergic receptors has been reported to activate AMPK (Minokoshi *et al.*, 2002; Ruderman *et al.*, 2003), as well as increase PGC-1α mRNA expression (Miura *et al.*, 2007). In the one-legged exercise model employed in this thesis, it has been shown that plasma adrenaline and noradrenaline increase markedly more during R- than NR-exercise (Sundberg, 1994), providing an alternative explanation for the enhanced PGC-1α mRNA expression in the R-leg. However, the contralateral NR-leg will also be exposed to the increase in circulating catecholamine levels. This may contribute to the tendency of slightly elevated basal PGC-1α mRNA levels in the NR-leg before the NR-exercise bout, since the NR-leg exercised after the R-leg.

Taken together, the enhanced AMPK activation in the R-leg might constitute an important signal for the differential PGC-1α transcriptional response seen in the two exercise conditions. Thus, AMPK could be the initial signaling step linking the acute metabolic response to energy deficiency to the chronic adaptive response of increased mitochondrial biogenesis.

Exercise-induced expression of PGC-1β

In Paper II, PGC-1β mRNA levels were significantly increased two hours after a single exercise bout in the NR-leg only (Figure 11).

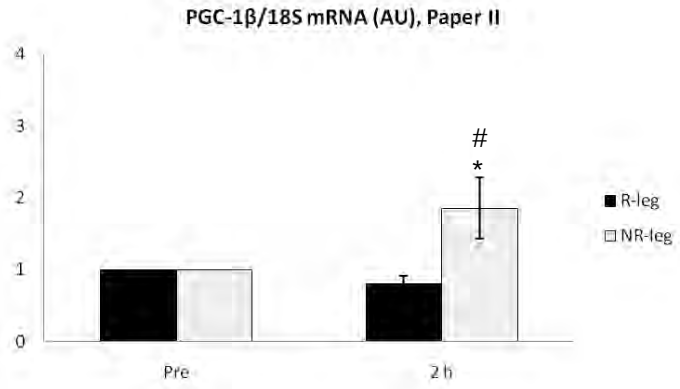

Figure 11. Peroxisome proliferator-activated receptor γ coactivator 1β (PGC-1 β) mRNA expression in response to a single bout of exercise in human skeletal muscle. Values are mean ± SEM. Graphical data represents arbitrary units (AU) corrected to 18S. R-leg and NR-leg; leg exercised under restricted and non-restricted blood flow condition, respectively. * denotes significant difference (P<0.05) relative to Pre, # denotes interaction between R- and NR-conditions. N=12.

The increased PGC-1β mRNA in the NR-leg compared to the increased PGC-1α levels in the R-leg after a single bout of exercise indicates that the regulation of these two coactivators is quite different. None of the studied upstream signaling pathways were activated in a way that could explain the increase in PGC-1β expression in the NR-leg. This picture adds to the findings of an earlier report which suggests that these factors have overlapping, but at the same time different effects on skeletal muscle phenotype (Mortensen *et al.*, 2006). Support for exercise-induced PGC-1β expression is that even though basal PGC-1β mRNA expression was not altered after five weeks of endurance training (Paper II), the levels were higher in elite athletes compared to normally active and spinal cord injured subjects (Paper III).

Free fatty acid oxidation was most likely higher when exercise was performed with non-restricted blood flow since this was performed at a lower relative intensity than restricted blood flow exercise. In light of the findings in the present study and earlier observations of an association between PGC-1β expression and lipid oxidation (Ling *et al.*, 2004; Lin *et al.*, 2005), support that low exercise intensity could stimulate PGC-1β expression more than high intensity exercise. Furthermore, the importance of free fatty acids in the regulation of PPARs and thus the association between expression of PPAR and PGC-1β further support such a hypothesis (Kamei *et al.*, 2003). Also, it has been shown that overexpression of PGC-1β decreased basal glycogen content in skeletal muscle cells (Mortensen *et al.*, 2006). In contrast to the findings in Paper II, another report showed that acute exercise had no effect on PGC-1β mRNA levels in human skeletal

muscle (Mortensen *et al.*, 2007). Those subjects performed a three hour exercise bout at 50 % of maximal intensity, and if free fatty acid utilization is of major importance, the mRNA expression of PGC-1β would be increased. Still, there are other differences between the two study protocols. In the present study (Paper II), one-legged exercise was employed, in contrast to two-legged exercise in the Mortensen study. All together, the present findings support a possible exercise-induced increase in PGC-1β, but the response seems to be more complex compared to PGC-1α.

The physiological consequences of altered PGC-1β expression have been shown in for example transgenic mice that overexpress PGC-1β in skeletal muscle (Arany *et al.*, 2007). These mice demonstrated increased mitochondrial gene expression, a switch to type IIX muscle fibers and an improvement in exercise capacity. Reversely, PGC-1β deficient mice displayed impaired mitochondrial gene expression and reduced mitochondrial volume, but only slight changes in mitochondrial respiration during basal conditions (Lelliott *et al.*, 2006).

Mitochondrial transcription factors

Exercise-induced expression of mitochondrial transcription factors

Induction of PGC-1α mRNA after a single exercise bout was not followed by any transcriptional changes for downstream transcription factors NRF-1, TFAM, TFB1M and TFB2M (Paper I). There may be several reasons for this lack of transcriptional changes. It is not known if there was a concomitant change at the PGC-1α protein level. In a study on swimming rats, PGC-1α protein did not increase until 18 hours after the end of exercise (Baar *et al.*, 2002), i.e. much later than the period studied in Paper I. Thus, it is not possible to exclude that the time between the exercise bout and the final biopsy was too short to detect transcriptional changes for mitochondrial factors downstream of PGC-1α.

In Paper I, exercise did not influence the mRNA levels of NRF-1. In a study by Pilegaard *et al* (2003) it was also shown that NRF-1 mRNA was unchanged six hours after a three-hour exercise bout, even though TFAM mRNA increased three-fold (Pilegaard *et al.*, 2003). The discrepancy between their finding and that in Paper I regarding TFAM may very well depend on the different exercise durations. Support for exercise-induced increases in NRF-1 mRNA has been reported 6 hours after a 90 min bout of treadmill running in rats (Murakami *et al.*, 1998), and a small but significant change in NRF-1 was observed

following 16 weeks of endurance training in humans (Short *et al.*, 2003). Still, based on the findings in Paper I, our own unpublished data and those of Pilegaard *et al* (2003) it may be speculated that regulation of NRF-1 could be determined by other mechanisms, such as interaction with other factors, covalent modification and intracellular localization. Considering its possible role in response to exercise, the regulation of NRF-1 may instead be post transcriptional or that exercise related transcriptional regulation of TFAM is not solely related to NRF-signaling. Support for such post transcriptional regulation comes from the report of an increase in NRF-1 protein binding after a single bout of exercise (Baar *et al.*, 2002).

Basal expression of TFAM

Paper IV was the first to report increased protein levels of TFAM in human tissue in response to a stimulus known to induce mitochondrial biogenesis, i.e. endurance training. The protein levels of TFAM, COX I and COX IV were all higher after four weeks of endurance training (Figure 12), with no difference in response between R- and NR-exercise.

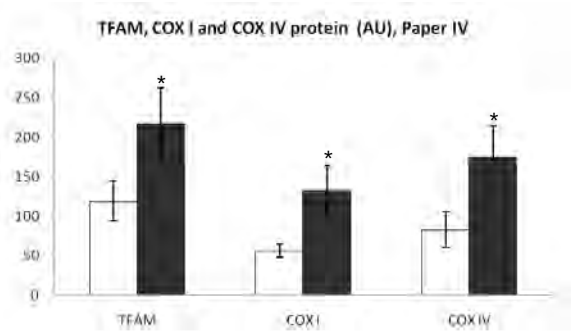

Figure 12. Mitochondrial transcription factor A (TFAM), cytochrome oxidase subunit I (COX I) and subunit IV (COX IV) protein expression in human skeletal muscle before (white bars) and after (black bars) four weeks of endurance training. Values are mean ± SEM. Graphical data represents arbitrary units (AU). * denotes significant difference ($P<0.05$) compared to the pre value. N=6 individuals.

The oxidative capacity was higher in the trained state, reflected by increased protein levels of both COX I and COX IV subunits (Figure 12) (Paper IV) and by increased citrate syntase activity. Since COX I is a mitochondrial encoded gene, the training-induced increase of COX I is most likely mediated by TFAM-induced activation. Furthermore, the training-induced concurrent increases of both COX I and COX IV proteins support activation of both the nuclear and mitochondrial genomes with this exercise model.

The lack of a further increase with restricted blood flow indicates that the stimulus was sufficient already in the NR-condition. However, one cannot exclude the possibility of a crossover effect (e.g. neural or humoral) that may have influenced the contralateral leg. This finding contrasts previous studies with similar models, which have shown that blood flow restriction during one-legged training further augments the oxidative capacity (Sundberg, 1994). One explanation for these differences could be that the immunoblot technique for analyzing protein levels is less sensitive than earlier used methods for assessing oxidative capacity, like enzyme activity assays, and therefore not able to detect possible protein differences between the two exercise conditions. However, in Paper IV, the endurance performance was increased in both the R- and NR-conditions, as shown by increased time to fatigue and peak load. It is therefore suggested that TFAM could be of importance in normal physiological mitochondrial adaptation in healthy individuals. Similar to the findings in Paper IV, TFAM protein levels were significantly higher in the elite athletes (EA) compared to the moderately active (MA) individuals (Paper V), see Figure 13.

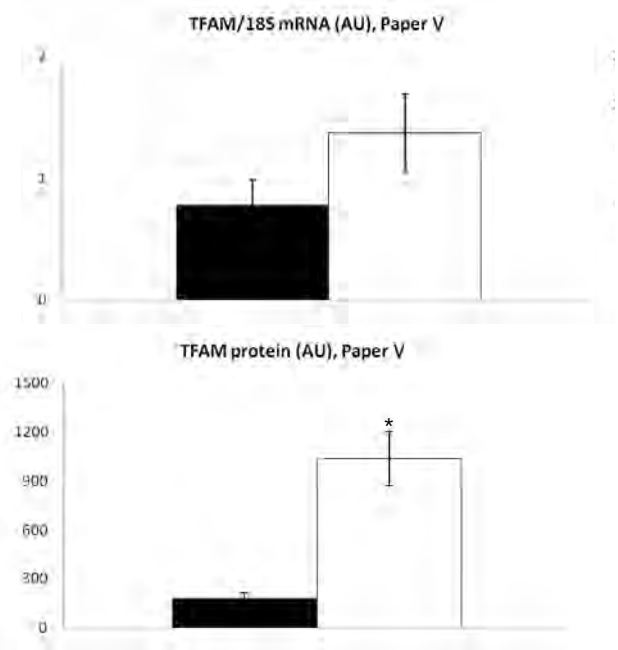

Figure 13. Mitochondrial transcription factor A (TFAM) mRNA (left panel) and protein (right panel) expression in human skeletal muscle from moderately active individuals (black bars) and elite athletes (white bars). Values are mean ± SEM. Graphical data represents arbitrary units (AU), the mRNA data is corrected to 18S. * denotes significant difference (P<0.01) vs moderately active individuals. mRNA; N=8 (moderately active) and N=10 (elite athletes). Protein; N=8 (moderately active) and N=6 (elite athletes).

The EA also had higher COX IV protein levels than the MA individuals. However, basal TFAM mRNA expression was not statistically different in EA compared to MA (P=0.12, Figure 13).

Observations from *in vitro* and knockout models imply that the mitochondrial transcription factor TFAM plays a critical role in regulating both oxidative capacity as well as mitochondrial biogenesis (Fisher & Clayton, 1985; Parisi & Clayton, 1991; Larsson *et al.*, 1994; Dairaghi *et al.*, 1995; Larsson *et al.*, 1998). However, TFAM protein has been shown to be very abundant and to completely wrap the human mtDNA (Alam *et al.*, 2003). This might be considered to be in conflict with the importance of the promoter-recognizing features of TFAM for the mitochondrial transcription machinery. In fact, it has been demonstrated that TFAM protein amount, both when TFAM was overexpressed in human cells and suppressed by RNA interference, correlated to the total mtDNA amount but not to the TFAM mRNA level (Kanki *et al.*, 2004). A recent report failed to show increased TFAM protein levels with training in healthy controls and mitochondrial myopathy patients, despite evidence of increased mitochondrial content (Adhihetty *et al.*, 2007b). However, the findings in Papers IV and V clearly show that TFAM protein is higher in states with enhanced oxidative capacity and increase with training.

A higher protein level does not necessarily require higher mRNA levels. In Paper V, there was a tendency of higher basal TFAM mRNA levels in the elite athletes, possibly due to the extreme amounts of exercise performed over a long period of time. Another human study report increased TFAM mRNA by 85 % after a training period of 16 weeks (Short *et al.*, 2003). In rat muscle, electrically-induced contractile activity has been shown to increase both the mRNA expression and protein function of TFAM (Gordon *et al.*, 2001). TFAM mRNA increased after four days of electrical stimulation, but after 14 days of stimulation TFAM mRNA levels were similar to those of the control tissue, reflecting the importance of sample timing. Similarly, increased TFAM mRNA levels were shown 18 hours after a 5th exercise bout in rats (Garcia-Roves *et al.*, 2006).

Paper IV does not demonstrate which the molecular mechanisms are that increase TFAM expression. However, the nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) have binding sites in the promoter region of the TFAM gene and have shown to be important in regulation of TFAM gene expression *in vitro* (Virbasius & Scarpulla, 1994). In addition, binding sites for NRF-1 and NRF-2 have also been recognized in the promoter region of several other nuclear encoded mitochondrial proteins. One example is the NRF-1 binding site in the promoter region of COX IV (Virbasius *et al.*, 1993). Regardless of the

absence of changes in NRF-1 mRNA, the observation of up-regulation of both TFAM and COX IV in Papers IV and V suggests that NRF-1 could be involved in gene activation in response to exercise.

Basal expression of TFB1M and TFB2M

In Paper V, no differences in basal protein expression for TFB1M and TFB2M could be detected in the EA compared to the MA group. However, the EA group had higher basal mRNA levels of both TFB1M and TFB2M compared to the MA group (Figure 14). The markedly higher VO_2 max levels, citrate synthase activities and COX IV protein

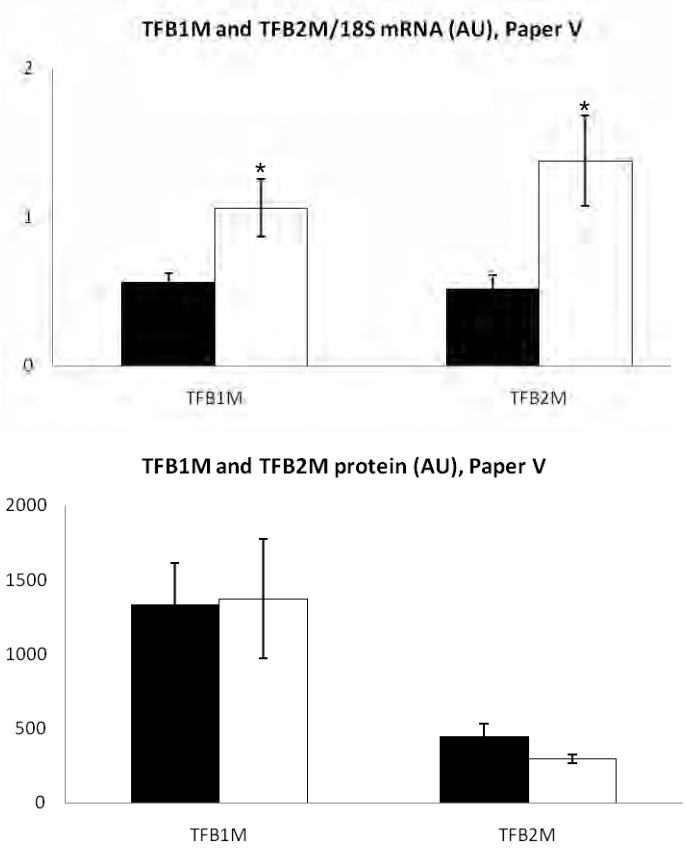

Figure 14. Mitochondrial transcription factor B1 (TFB1M) and B2 (TFB2M) mRNA (left panel) and protein (right panel) expression in human skeletal muscle from moderately active individuals (black bars) and elite athletes (white bars). Values are mean ± SEM. Graphical data represents arbitrary units (AU), the mRNA data is corrected to 18S. * denotes significant difference ($P<0.05$) vs moderately active individuals. mRNA; N=8 (moderately active) and N=10 (elite athletes). Protein; N=8 (moderately active) and N=6 (elite athletes).

levels in the EA verify that they were well adapted to endurance training. In response to endurance training (Paper V), basal TFB1M and TFB2M mRNA expression was increased after ten days in the restricted blood flow condition. This suggests that a transient increase occurs in the time window and exercise condition when the adaptation process could be assumed to be most active. The present observation thereby adds further support to that changes related to "stressed" cell metabolism, i.e. metabolic perturbation, are of importance in the regulation of factors important for mitochondrial biogenesis. However, after five weeks of endurance training, the basal TFB1M and TFB2M mRNA levels had returned close to pre training levels. Using the same exercise model, it was in Paper IV shown that both COX I and COX IV proteins increased after four weeks of endurance training. It is not known whether the absence of a maintained TFB mRNA upregulation after five weeks of training in contrast to the higher basal mRNA level in the EA is due to many years of high intensity training or a need to maintain high mitochondrial densities in the elite group, or if it represents constitutional differences between the groups.

Interestingly, the mean (SEM) TFB2M:TFB1M mRNA ratio was higher in the EA than in the MA group (1.26 (0.15) and 0.82 (0.09), respectively) (Paper V). It has previously been suggested that the ratio between TFB2M and TFB1M constitutes an additional level of regulation of mitochondrial gene expression and mtDNA copy number in mammalian cells (Rantanen *et al.*, 2003). The higher B2:B1 ratio in elite athletes suggests a potential preference for TFB2M in states with high oxidative capacity. It has been shown *in vitro* that TFB2M was more transcriptionally active than TFB1M (Falkenberg *et al.*, 2002).

There was a discrepancy between TFB1M and TFB2M mRNA and protein levels. This could be interpreted in several ways. The results might indicate that the TFBs are transcriptionally regulated in response to chronic endurance training. A more conservative interpretation is that changes in mRNA levels do not always translate to changes in TFB protein levels and consequently that these factors do not play any major part in mitochondrial biogenesis in exercised skeletal muscle. It should be noted that the Western blot method may be too insensitive to detect changes even though the ability of the antibodies to detect varying amounts of protein was confirmed. Moreover, the translational efficiency between mRNA and protein changes or the protein levels needed to induce a physiological effect are not known. Also, post translational changes may diminish the antibodies' affinity for the proteins. The physiological roles of TFB1M and TFB2M in response to exercise have not previously been well characterized. Based on the findings concerning TFB1M and TFB2M in this thesis, it

does not seem as if they are transcriptionally regulated after a single exercise bout, or that the timing of the biopsies failed to capture their changes. The higher TFB1M and TFB2M mRNA levels in elite athletes compared to moderately active and the increase with endurance training might suggest long-term transcriptional regulation.

Conclusions

From the work conducted in this thesis it is concluded that:

- The exercise-induction of PGC-1α mRNA in human skeletal muscle is enhanced by metabolic perturbation.
- PGC-1α, PGC-1β, PPARα and PPARδ mRNA are higher in elite athletes than in moderately active, and even lower in spinal cord injured subjects, clearly indicating that these factors play a role for the maintenance of a "trained" skeletal muscle phenotype.
- After a single bout of exercise, PGC-1α and PGC-1β increase with restricted and non-restricted blood flow, respectively, suggesting regulation through different pathways.
- The importance of exercise-induced AMPK activation for PGC-1α mRNA expression is suggested.
- Calcineurin and p38 signaling pathways are activated to a similar degree in both exercise conditions, why they do not seem to have dominant roles in the regulation of PGC-1α expression.
- TFAM protein is higher in states with enhanced oxidative capacity, increases with training and is most likely regulated through protein stabilization.
- TFB1M and TFB2M mRNA, but not protein, are higher in elite athletes than in moderately active, and increase with endurance training, suggesting transcriptional regulation of these factors.
- Exercise-induced expression of PGC-1α most likely contributes to the increase in TFAM protein which in turn drives mitochondrial biogenesis.

Future perspectives

Physical exercise is an ideal intervention and skeletal muscle is a very good model tissue for studying mechanisms important for physiological adaptation processes such as mitochondrial biogenesis, also in humans. Skeletal muscle is highly adaptive, accessible in sufficient amounts for analysis and can be tested functionally.

The observations made in this thesis encourage future human, mouse and cell studies. To further clarify central mechanisms, a protocol with varying exercise intensities and biopsies taken at several time points over the first 24 hours would be appropriate. In such a study, pre translational differences for the two PGC-1 coactivators and their upstream regulatory pathways e.g. the CaMK pathway, including CAMKII, could be investigated. Such knowledge would further clarify the relations between the temporal expression and activity patterns of these and other factors and possibly explain how exercise leads to increased mitochondrial biogenesis. Since PGC-1α is known to be regulated both at the transcriptional and protein levels, it would be valuable to further explore the exercise-induced changes in PGC-1α protein levels, as well as changes in phosphorylation state in human skeletal muscle. Chromatin immuno precipitation (ChIP) experiments have been initiated on fresh biopsy material and this method is under development in the lab. This method is valuable to for example couple NRF-1 activation and target gene promoter binding in human skeletal muscle in response to exercise. Other methods such as chip-on-ChIP might also be used. Also, the physiological roles of TFB1M and TFB2M and their regulation need to be further clarified, especially at the protein levels.

A deeper understanding of how the adaptive mechanisms are mediated and regulated in human physical activity might lead to the development of new strategies against several common diseases. One such possibility is a combination of pharmaceutical treatment and tailor-made exercise therapy to influence the expression and activity of factors that drive the adaptational processes optimally for improved and health. The mitochondria have unique structural and functional characteristics which have raised the ideas of selective targeting of drugs directly to the mitochondria. These ideas still need to be much more developed, but this approach might provide an attractive mode of treatment for the future. Obese and diabetic patients are characterized by impaired mitochondrial function and skeletal muscle PGC-1α mRNA expression is reduced in diabetics. Since PGC-1α is important in the control of energy metabolism and insulin sensitivity, it is seen as an interesting candidate drug target. Drugs that activate PGC-1α with the aim of developing more mitochondria should be beneficial for people with diabetes since more mitochondria means more efficient energy metabolism. PGC-1α has recently also been implicated in a novel angiogenic pathway, and may thus provide a novel therapeutic target for treatment of ischemic diseases. Future developments in the area of mitochondrial medicine will be very important to progress health and disease management.

Populärvetenskaplig sammanfattning

Fysisk aktivitet har alltid utgjort en del i människans liv. Det är tydligt visat att regelbunden träning ger positiva följder för hälsa och prestation, såsom minskad risk för hjärt- och kärlsjukdomar, övervikt och diabetes, men även förbättrad uthållighet i musklerna. De viktigaste funktionerna för våra muskler är att producera kraft och rörelse, stabilisera leder och skydda underliggande vävnad, samt utgöra en proteinkälla vid svält. Skelettmuskler är uppbyggda av långa fibrer, muskelceller, som är arrangerade i ett antal muskelbuntar. Som svar på träning sker en mängd anpassningar i musklerna för att tillgodose de ökade kraven som ställs. En av de tydligaste förändringarna är en ökning av mängden mitokondrier i muskelcellerna. Mitokondrier är en typ av strukturer, organeller, i cellerna. De benämns ofta ”cellens kraftstationer” eftersom de överför energi från socker och fett till en energirik molekyl som kallas ATP. Med fler mitokondrier kan musklerna producera mer ATP och blir därmed mer uthålliga och kan arbeta på en högre belastning utan att bli lika trötta.

Det som styr biologiska processer i muskelceller och i resten av kroppen är ofta olika proteiner. När en proteinmolekyl ska tillverkas hämtas byggbeskrivningen från DNA, som fungerar som ett referensbibliotek fyllt med ritningar av proteiner. En arbetskopia (s.k. mRNA) tas med till stationer där ritningen läses av och proteinets komponenter sätt ihop. Ritningen i DNA för ett specifikt protein kallas en *gen* eller arvsanlag. När en gen är *aktiv* kopieras ritningen som mRNA och det aktuella proteinet tillverkas. Olika signaler kan påverka celler och påverkal aktivering av specifika gener som leder till dess proteiner produceras och kan utföra sina uppgifter. Mitokondrien är speciell eftersom det är den enda organellen som, förutom cellkärnan, innehåller DNA. Mitokondriellt DNA (mtDNA) innehåller få gener, men dessa är nödvändiga för att bilda funktionella mitokondrier. De proteiner som aktiverar generna i mtDNA kommer från kärnans DNA och därför krävs en samordning mellan kärnan och mitokondrierna.

Ett stort antal gener (både i kärnan och i mitokondrien) i de arbetande muskelcellerna blir aktiverade när man tränar. Vissa gener har beskrivits vara centrala för de förändringar som sker i musklerna när man tränar regelbundet. För att ytterligare studera några av de gener som är viktiga för den nybildning av mitokondrier som sker i samband med träning, samt vad som styr dessa, har muskelprover (biopsier) tagits från frivilliga friska män före och efter ett enstaka fysiskt arbete samt före och efter några veckors regelbunden träning. I flera av studierna har en träningsmodell använts där försökspersonerna placeras liggande med underkroppen i en tryckkammare. Ett ben i taget utför bensparksträning, medan det andra benet vilar. Då det första benet arbetar ökas trycket inne i kammaren vilket leder till att blodflödet i den arbetande muskeln minskar och arbetet upplevs som mycket tyngre. Denna träningsmodell används för att studera om ett minskat blodflöde, och dess effekter, påverkar gener och proteiner som är viktiga för muskelns anpassning till träning annorlunda jämfört med träning med normalt blodflöde.

Den här avhandlingen visar att ett enstaka arbetspass är tillräckligt för att öka mRNA-nivåerna av PGC-1α, en faktor som tidigare visats vara väldigt

viktig för bildningen av nya mitokondrier. Även efter fem veckors träning var nivåerna av PGC-1α högre än innan träningsperioden startade. Det betyder sannolikt att PGC-1α är viktig för att driva och upprätthålla den ökade mitokondriemängd som regelbunden träning ger. PGC-1α styr ett flertal faktorer som har förmågan att aktivera gener vars proteiner deltar i uppbyggnaden av nya mitokondrier. Några av dessa faktorer, TFAM, TFB1M och TFB2M, har förmågan att förflytta sig in i mitokondrierna och där aktivera mitokondriernas egna gener. Försök på möss har visat att TFAM är nödvändig för bildandet av mitokondrier, och därmed för överlevnad. TFB1M och TFB2M upptäcktes ganska nyligen, och deras roller är inte helt klarlagda. Hos försökspersoner som tränade under fyra veckor ökade mängden av TFAM proteinet jämfört med innan de började träna. Dessutom fanns det mer TFAM protein hos elitcyklister jämfört med hos normalaktiva personer. Det visar att personer som är tränar hårt har mer TFAM protein, sannolikt nödvändigt för att bibehålla fler och större mitokondrier i sina muskler. I samband med fyra veckors träning fann jag också bevis för att proteiner från både kärnan och mitokondrien ökade i mängd, vilket tyder på en bra samordning mellan kärnan och mitokondrien. För faktorerna TFB1M och TFB2M var mängden mRNA större efter 10 dagars träning och större hos elitcyklister jämfört med normalaktiva. Det var dock ingen skillnad i proteininnehåll mellan de två grupperna Det kan tyda på att regelbunden träning kan reglera dessa faktorer på mRNA-nivå, snarare än på proteinnivå. För att säkerställa dessa resultat behövs ytterligare studier som närmare undersöker TFB1M och TFB2M i samband med träning.

När man tränar påverkas miljön i och utanför muskelcellen. Dessa miljöförändringar stimulerar ett flertal olika signaleringsvägar i muskelcellerna till att aktivera faktorer som styr vilka gener som ska slås på eller av. Vid regelbunden träning leder dessa förändringar i genaktviering slutligen till att muskeln blir starkare eller mer uthållig beroende på vilken typ av träning man utför. Förbättringen är också beroende av hur länge man tränar och hur ofta, men även genetiska faktorer påverkar hur en individ svarar på träning. Det är ganska väl känt vilka signaleringsvägar som stimuleras av träning, men det är inte helt klarlagt om olika typer av träning påverkar dessa signaleringsvägar på olika sätt. Ett delmål var därför att studera signalmolekyler som påverkar PGC-1α när träning utförs med minskat samt med normalt blodflöde. När träning utfördes med minskat blodflöde sågs en större ökning av PGC-1α mRNA. Om någon av de intressanta signaleringsvägarna uppvisar samma mönster kan det tyda på en direkt betydelse för aktivering av PGC-1α vid träning. Samma mönster sågs för aktivering av AMPK, en signalmolekyl som känner av när energinivåerna i cellen sjunker, vilket de gör vid muskelkontraktion.

En ökad förståelse om de mekanismer som optimerar effekterna av träning är av stor betydelse för många idrotter och skulle även kunna leda till utvecklandet av nya behandlingsmetoder mot flera vanliga sjukdomar. En sådan möjlighet är en kombination av läkemedel och individanpassade träningsråd för att tillsammans påverka de faktorer som är viktiga för ökad funktion och hälsa. En ökad kunskap om olika faktorers betydelse för anpassning är också värdefullt för att öka förståelsen för individuella variationer i träningssvaret och kan möjliggöra en mer skräddarsydd träning för den enskilde idrottsutövaren.

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