Exercise-induced mitochondrial biogenesis in human skeletal muscle

With special reference to mitochondrial transcription factors and lipin-1

Susanna E Wallman Appel
In all science, error precedes the truth, and it is better it should go first than last.

Sir Hugh Walpole
Mitochondrial biogenesis is one prominent adaptation to endurance training in skeletal muscle tissue. An increased mitochondrial density of the muscle fibres contributes to an enhanced aerobic capacity and thereby to improved fatigue-resistance. Multiple signalling pathways and transcriptional networks are involved in controlling mitochondrial biogenesis. The transcriptional co-regulator lipin-1 is one factor proposed to contribute, based on its ability to interact with PGC-1α and co-active transcription of metabolic genes. The mitochondrial transcription factors TFAM, TFB1M, TFB2M and mTERF have also been put forward as candidates. They are transcribed in the nucleus and post-translationally translocated to the mitochondria in order to govern the stability and use of the mitochondrial genome.

Firstly, this thesis explores the presence of lipin-1 splice variants in human skeletal muscle. Secondly, the importance of lipin-1 and mitochondrial transcription factors in exercise-induced mitochondrial biogenesis was assessed by investigating the influence of acute exercise and long term training on their expression in human skeletal muscle. Biopsies were obtained from the vastus lateralis of untrained healthy voluntary subjects before and after one bout of endurance exercise as well as after regular endurance training. In addition, biopsies were taken from well-adapted endurance athletes and moderately active individuals.

Two lipin-1 mRNA isoforms were identified in human skeletal muscle, corresponding to the previously described murine Lpin1α and Lpin1β. There were, however, no significant increases of total human LPIN1 or LPIN1α mRNA levels for up to 24 hours after a single bout of exercise or in response to 12 weeks of endurance training. This might imply LPIN1β to be more involved than LPIN1α in exercise-induced mitochondrial biogenesis in humans.

Furthermore, TFAM mRNA was more abundant after 10 days of training and elite athletes had higher levels of TFAM protein compared to moderately active. This indicates that TFAM may be important for maintenance of muscle mitochondrial
mass and mainly regulated by protein stabilisation. The mRNA, but not protein, levels of *TFB1M* and *TFB2M* were higher in elite athletes compared to individuals with a moderate level of physical activity. Both factors were also elevated in response to 10 days of training with reduced blood flow to the working leg. This might suggest that TFB1M and TFB2M are altered pre-translationally in response to training.

The mitochondrial termination factor *mTERF* mRNA levels were higher in elite athletes with enhanced oxidative capacity, but did not change in response to endurance training. This implies that mTERF is not inhibitory for mitochondrial biogenesis, that long-term endurance adaptation increases its transcription and that it thus rather supports mitochondrial adaptations.

Further studies are needed to better understand the potential roles of lipin-1 isoforms and mitochondrial transcription factors in skeletal muscle adaptation to exercise training, and possibly also in some of the health benefits that accompanies an active lifestyle.
LIST OF PUBLICATIONS

This thesis is based on the following papers, referred to in the text by their Roman numerals:

   Jessica Norrbom, SUSANNA E WALLMAN, Thomas Gustafsson, Helene Rundqvist, Eva Jansson, Carl Johan Sundberg.
   *Acta Physiol (Oxf.*) 2010; 198: 71–79.*

II. Lipin-1 expression in skeletal muscle cells after exercise or AICAR stimulation.
    SUSANNA E WALLMAN APPEL, Heléne Fischer, Maléne Lindholm, Eva-karin Sällstedt, Jessica Norrbom, Carl Johan Sundberg.
    *Manuscript.*
# CONTENTS

1 BACKGROUND ........................................................................................................ 1

1.1 HUMAN ENERGY REQUIREMENTS ......................................................... 1

1.2 THE MITOCHONDRION ............................................................................. 1
   1.2.1 Mitochondrial structure and function .............................................. 2
   1.2.2 The proteome and genome of mitochondria .................................. 3
   1.2.3 Mitochondrial DNA transcription .................................................. 4
   1.2.4 TFAM ............................................................................................ 5
   1.2.5 TFB1M and TFB2M ....................................................................... 5
   1.2.6 MTERFs ....................................................................................... 6
   1.2.7 Mitochondrial DNA replication ...................................................... 7

1.3 HUMAN SKELETAL MUSCLE ...................................................................... 7
   1.3.1 Skeletal muscle structure and function ........................................ 8

1.4 ADAPTABILITY OF SKELETAL MUSCLE ................................................ 9
   1.4.1 Exercise as a stimulus for adaptation in skeletal muscle .......... 10

1.5 MITOCHONDRIAL BIOGENESIS .............................................................. 11
   1.5.1 Exercise-induced mitochondrial biogenesis in skeletal muscle ... 11
   1.5.2 Mitochondrial transcription factors ................................................. 12
   1.5.3 PGC-1α ....................................................................................... 12
   1.5.4 Lipin-1 ......................................................................................... 14

2 AIMS ............................................................................................................... 17

3 METHODOLOGY ......................................................................................... 18

3.1 EXPERIMENTAL MODELS ....................................................................... 18
   3.1.1 Acute exercise study ................................................................. 18
   3.1.2 10-day training study ................................................................. 19
   3.1.3 12-week training study ............................................................... 21
   3.1.4 Cross-sectional study ................................................................. 22
   3.1.5 Stimulation of primary human myocytes ..................................... 23
   3.1.6 Stimulation of mouse myocyte cell line ..................................... 23

3.2 SKELETAL MUSCLE BIOPSIES .............................................................. 23
3.3 LABORATORY ANALYSES ................................................................. 25
  3.3.1 Citrate synthase activity ...................................................... 25
  3.3.2 Protein extraction and Western Blotting ............................... 26
  3.3.3 Primer design and isoform detection .................................. 26
  3.3.4 RNA extraction and Reverse transcribed real-time PCR ......... 28

3.4 STATISTICAL ANALYSES ............................................................ 28

4 RESULTS AND DISCUSSION .......................................................... 30
  4.1 LIPIN-1 ................................................................................... 30
    4.1.1 Lipin-1 transcript isoforms in human skeletal muscle .......... 30
    4.1.2 Lipin-1 expression in response to a single endurance exercise
      bout .......................................................................................... 31
    4.1.3 Basal expression of lipin-1 following endurance training ...... 32
    4.1.4 Effect of AMPK and adrenoreceptor activation on the response
      of lipin-1 and its target genes .................................................. 34
    4.1.5 Further considerations on lipin-1 ....................................... 35
  4.2 MITOCHONDRIAL TRANSCRIPTION FACTORS ............................. 36
    4.2.1 TFAM levels in response to acute exercise and endurance
      training .................................................................................... 37
    4.2.2 TFB1M and TFB2M levels in response to endurance training ... 38
    4.2.3 Expression of mTERF in response to endurance training ...... 39
    4.2.4 Further considerations on mitochondrial transcription factors .... 40

4.3 GENERAL DISCUSSION ............................................................... 41

5 CONCLUSIONS .............................................................................. 43

6 FINAL REMARKS .......................................................................... 44

7 POPULÄRVETENSKAPLIG SAMMANFATTNING ................................. 45

8 ACKNOWLEDGEMENTS .............................................................. 48

9 REFERENCES ................................................................................ 50
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Mitochondrial transcription factor A</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-aminoimidazole-4-carboxamide ribonucleoside</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>5-aminoimidazole-4-carboxamide ribonucleoside</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine-5’-diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>CaMK</td>
<td>Calmodulin-dependent kinase</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome oxidase</td>
</tr>
<tr>
<td>CPT1</td>
<td>Carnitine palmitoyltransferase 1</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP responsive element binding protein</td>
</tr>
<tr>
<td>CS</td>
<td>Citrate synthase</td>
</tr>
<tr>
<td>Cyt</td>
<td>Cytochrome</td>
</tr>
<tr>
<td>D-loop</td>
<td>Displacement loop</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglyceride</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EA</td>
<td>Elite athletes</td>
</tr>
<tr>
<td>ERR</td>
<td>Estrogen-related receptor</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HPRT1</td>
<td>Hypoxanthine phosphoribosyltransferase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>LPIN</td>
<td>Lipin</td>
</tr>
<tr>
<td>LSP</td>
<td>Light strand promoter</td>
</tr>
<tr>
<td>MA</td>
<td>Moderately active</td>
</tr>
<tr>
<td>MHC</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>MTERF</td>
<td>Mitochondrial termination factor</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide, oxidised</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide, reduced</td>
</tr>
<tr>
<td>NR</td>
<td>Non-restricted</td>
</tr>
<tr>
<td>NRF</td>
<td>Nuclear respiratory factor</td>
</tr>
<tr>
<td>O&lt;sub&gt;H&lt;/sub&gt;</td>
<td>Origin of replication for heavy strand</td>
</tr>
<tr>
<td>O&lt;sub&gt;L&lt;/sub&gt;</td>
<td>Origin of replication for light strand</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PAP</td>
<td>Phosphatidate phosphatase</td>
</tr>
<tr>
<td>PGC-1</td>
<td>Peroxisome proliferator-activated receptor γ co-activator-1</td>
</tr>
<tr>
<td>POLRMT</td>
<td>Mitochondrial DNA-directed RNA polymerase</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PRC</td>
<td>PGC-1-related co-activator</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>R</td>
<td>Restricted</td>
</tr>
<tr>
<td>RIP-140</td>
<td>Receptor-interacting protein 140</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SIRT1</td>
<td>NAD-dependent deacetylase sirtuin-1</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglyceride</td>
</tr>
<tr>
<td>TFAM</td>
<td>Mitochondrial transcription factor A</td>
</tr>
<tr>
<td>TFB1M</td>
<td>Mitochondrial transcription factor B1</td>
</tr>
<tr>
<td>TFB2M</td>
<td>Mitochondrial transcription factor B2</td>
</tr>
<tr>
<td>TIM</td>
<td>Translocase of inner membrane</td>
</tr>
<tr>
<td>TOM</td>
<td>Translocase of outer membrane</td>
</tr>
<tr>
<td>TORC2</td>
<td>CREB-regulated transcriptional co-activator</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>VO&lt;sub&gt;2max&lt;/sub&gt;</td>
<td>Maximal oxygen uptake</td>
</tr>
</tbody>
</table>
1 BACKGROUND

1.1 HUMAN ENERGY REQUIREMENTS

The human body is in constant need of energy - to keep us alive, as well as to facilitate movement and interaction with our surroundings. The adenosine-5’-triphosphate (ATP) molecule drives virtually all energy-requiring processes in our cells, from the secretion of hydrochloric acid to the gastric ventricle to the rapid electric signalling of neurons. One process that consumes very large amounts of energy is muscle contraction. Every time we voluntarily move parts of our bodies, groups of skeletal muscle fibres undergo simultaneous shortening to enable for example walking, talking or swimming. When a person advances from standing to running, the muscle fibre demand for energy can increase by more than 100-fold (Sahlin et al., 1998). The muscle cells first use a combination of existing ATP-stores, energy stored in creatine kinase and ATP formed in oxygen-independent (anaerobic) breakdown of glucose, whereafter oxygen-dependent (aerobic) catabolism of glucose and fatty acids is imperative. The principal intracellular organelles that carry out the latter type of metabolic reactions are the mitochondria.

1.2 THE MITOCHONDRION

Mitochondria are abundantly present in mammalian cells and generally accepted to be an evolutionary product from a prokaryotic ancestor (Andersson et al., 2003; Gray, 2012). According to the endosymbiosis theory, aerobic bacteria managed to survive the endocytotic engulfment of a eukaryotic cell and became incorporated into its cytoplasm (Gray, 1999). This occurred about 2300 million years ago (Karlberg & Andersson, 2003), around the same time as oxygen tension in the earth’s atmosphere began to rise (Farquhar, 2000). The developed symbiosis between the two species thus provided a survival benefit for the eukaryote, as an oxygen-dependent metabolic system became available (Gray, 1999). The mitochondrial organelles still contain their own genome and modern-day DNA sequencing have established high homology with the α-proteobacteria class.
(Karlberg et al., 2000), something that provides firm evidence for the postulation above.

1.2.1 Mitochondrial structure and function

The mitochondria are surrounded by dual phospholipid membranes, which create two aqueous compartments separated from the cytosol; the mitochondrial matrix and the intermembrane space (Ryan & Hoogenraad, 2007) (Figure 1). These structural characteristics are a prerequisite for mitochondrial functionality.

Mitochondria are highly involved in the oxidative metabolic pathways that extracts energy from nutrients. Enzymes responsible for β-oxidation of fatty acids and the tricarboxylic acid cycle operate in the matrix and feed the respiratory chain with reduced co-enzymes (Hatefi, 1985). There are five mammalian respiratory complexes located at the inner mitochondrial membrane; NADH:ubiquinone oxidoreductase (I), succinate:ubiquinone reductase (II), cytochrome bc₁ (III), cytochrome c oxidase (IV) and ATP synthase (V). This OXPHOS machinery transfers electrons to molecular oxygen whilst building up a

Figure 1. Schematic illustration of a mitochondrion. The outer and inner phospholipid membranes create two separate compartments; the mitochondrial matrix and the intermembrane space. Mitochondria make up a dynamic network that is continuously undergoing growth, fusion, fission and autophagy in response to cellular demands. Illustration: Elina Anttila.
proton gradient across the membrane, which is then used to drive the oxidative phosphorylation of ADP to ATP (Saraste, 1999).

In addition to its participation in aerobic metabolism, mitochondria serve a critical role in thermoregulation (Silva, 2006), apoptosis (Ott et al., 2007), Ca$^{2+}$ signalling (Glancy & Balaban, 2012) and the production of reactive oxygen species (ROS) (Turrens, 2003).

1.2.2 The proteome and genome of mitochondria

Human mitochondria contain more than 1500 different proteins, the majority of which are located in the matrix (Calvo et al., 2006). Therein, the genetic material of the organelle (mtDNA), originating from the prehistoric endosymbiont, also resides (Falkenberg et al., 2007). In a somatic cell, between 1000 and 10 000 copies of the circular mitochondrial chromosome can be found (Smeitink et al., 2001). The ∼16 500 bp human mitochondrial genome is small compared to its nuclear counterpart and encodes merely 13 polypeptide subunits of the respiratory chain, along with the 22 tRNAs and 2 rRNAs required for its translation (Falkenberg et al., 2007) (Figure 2). Hence, the mitochondrial genomic apparatus does not single-handedly control the organelle’s proteome.

Through evolution, most of the prokaryotic genes were lost, retaining no more than core metabolic functions. However, some genes were transferred to the host’s genome and subsequently, their protein products were post-translationally imported back into the mitochondria (Andersson et al., 2003). Furthermore, a subset of genes, now coding for mitochondrial proteins, came about and evolved independently in the nuclear genome of the eukaryote (Karlberg et al., 2000). After translation, these are targeted for mitochondrial translocation. As a consequence, maintenance or enhancement of mammalian mitochondrial function relies on the coordinated expression of genes from two separate genomes.

Precursors of nuclear-encoded mitochondrial proteins enter the mitochondria via specific transport complexes; the translocase of the outer membrane (TOM) and the translocase of the inner membrane (TIM) (Dudek et al., 2012). The most
frequent mitochondrial import signal is an amphipathic N-terminal extension, which is proteolytically removed after import (Mossmann et al., 2012).

1.2.3 Mitochondrial DNA transcription

Transcription of the mitochondrial genome is vital to the function and continuation of the organelle. Although the mitochondria are self-sufficient when it comes to the production of ribosomal subunits and tRNA molecules, enzymes and other factors required for transcription of mtDNA are encoded in the cell nucleus and subsequently imported to the mitochondrial matrix.

The mitochondrial chromosome contains no introns. There is, however, a non-coding regulatory region known as the displacement loop (D-loop) (Figure 2) (Clayton, 2000). In this area, the promoters for transcription of both the heavy strand (HSP1 and HSP2) and the light strand (LSP) are located. Once initiated at these sites, the bidirectional mitochondrial gene expression generates polycistronic transcripts that are processed to produce the final RNA molecules (Montoya et al., 1981; Ojala et al., 1981; Topper & Clayton, 1989). Thus far, a

![Figure 2. Schematic illustration of the mitochondrial genome.](image)

D-loop, displacement loop; Cyt b, cytochrome b; NADH 4, NADH dehydrogenase subunit 4; COXI, cytochrome oxidase subunit 1; LSP, light strand promoter; HSP1/2, heavy strand promoter 1/2; OL/H, origin of replication of light/heavy strand; MTERF, mitochondrial transcription termination factor.
handful of proteins have been shown to contribute to mammalian mitochondrial transcription. For this process to be reconstituted in vitro, mitochondrial RNA polymerase (POLRMT), mitochondrial transcription factor A (TFAM) and either mitochondrial transcription factor B1 or B2 (TFB1M, TFB2M) are required (Falkenberg et al., 2002; Fisher & Clayton, 1988). Moreover, mitochondrial transcription termination factors (MTERFs) have been described as additional regulators of mitochondrial transcription (Roberti et al., 2009).

1.2.4 TFAM
TFAM was the first mitochondrial transcription factor to be discovered (Fisher & Clayton, 1988). By means of its tandem high-mobility group domains, this molecule has the ability to interact directly with mtDNA. It has been demonstrated that TFAM stimulates transcription via specific binding to recognition sites upstream from LSP and HSP, as well as through unwinding promoter regions (Fisher & Clayton, 1988; Fisher et al., 1992). Recently, it was established that TFAM is absolutely required for recruitment of the transcription machinery during initiation of mtDNA transcription (Shi et al., 2012). Evidently, the TFAM protein is abundantly present in human mitochondria and has been described to fully coat the mitochondrial genome (Alam, 2003). In this capacity, TFAM seems to greatly contribute to mtDNA stability and maintenance. Transgenic mice over-expressing TFAM display increased mtDNA copy numbers (Ekstrand, 2004). The importance of TFAM for cellular function is reflected in the fact that global ablation of the of the mouse Tfam gene causes mtDNA depletion, respiratory chain dysfunction and embryonic death (Larsson et al., 1998).

1.2.5 TFB1M and TFB2M
The other described stimulating mitochondrial transcription factors are TFB1M and TFB2M, two seemingly very similar proteins. In vitro, both factors have the individual capacity to form a heterodimeric complex with POLRMT to initiate transcription of mtDNA, together with TFAM (Falkenberg et al., 2002). Yet, recent studies suggest that TFB1M, although still holding the function previously reported, mainly act as an rRNA dimethyltransferase and thereby promoting the
maturation of mitochondrial ribosomal subunits (Metodiev et al., 2009). It has become evident that TFB2M is considerably more active as a transcription factor than TFB1M in vitro, which supports the idea that this is the main role for TFB2M in mammalian mitochondrial gene expression (Falkenberg et al., 2002; Litonin et al., 2010; Rantanen et al., 2003). Adding to this hypothesis is the fact that RNAi of TFB2M in a Drosophila melanogaster markedly reduces the abundance of certain mitochondrial RNA transcripts (Matsushima, 2004), while RNAi of TFB1M instead of altering the abundance of these transcripts rather decreases mitochondrial translation (Matsushima, 2005).

### 1.2.6 MTERFs

The mammalian mitochondrial transcription termination factor (MTERF) protein family comprises four members; MTERF1 to MTERF4. The latter three were identified based on their homology with MTERF1 (also known as mTERF) (Linder et al., 2005). In vitro, MTERF1 binds a termination site located downstream of the 16S rRNA gene on the mitochondrial chromosome heavy strand (Figure 2) and thereby mediates arrest of mitochondrial transcription (Kruse et al., 1989). This mechanism is suggested to regulate mitochondrial gene expression and explain the observed high rate of rRNA synthesis relative to mRNA (Roberti et al., 2009). MTERF1 is also reported to interact with a site in close proximity to the HSP1 promoter to create a loop structure that facilitates re-initiation of transcription from this promoter (Martin et al., 2005). This would add further support to the hypothesis that MTERF1 acts as an indirect positive regulator of mitochondrial translation. Furthermore, there are reports that MTERF1 terminates in vitro transcription in a bidirectional way, also halting light strand expression (Asin-Cayuela, 2005).

MTERF3 is established to be present in mouse mitochondria and negatively regulate steady-state mtDNA transcripts in vivo (Park et al., 2007). Also, homozygotic loss of MTERF3 causes severe mitochondrial dysfunction and is embryonically lethal (Park et al., 2007). Apart from their roles in mtDNA transcription regulation, both MTERF1 and MTERF3 have been described as modulators of human mtDNA replication in vitro (Hyvärinen et al., 2010).
MTERF2 binds mtDNA in a non sequence-specific manner in vitro and is demonstrated to operate in close proximity to the mitochondrial chromosome (Pellegrini et al., 2009). The least studied member of the mitochondrial termination factor family is MTERF4. In general, there are few investigations of MTERFs in vivo and further research is required to elucidate their roles in mammalian mitochondria.

1.2.7 Mitochondrial DNA replication

Copying of mtDNA is necessary for maintenance of the organelle and human mitochondria replicate their genome independently of the cell cycle (Bogenhagen & Clayton, 1977). Nevertheless, it is highly dependent on nuclear events. Just as for the transcription of mtDNA, the factors needed for replication of mitochondrial genetic material are nuclear-encoded and hence imported across the mitochondrial membranes. The proteins known to be of importance for this process are; DNA polymerase γ, mitochondrial single-stranded DNA-binding protein (mtSSB) and the TWINKLE helicase (Wanrooij & Falkenberg, 2010). In addition, POLRMT and the transcriptional apparatus are greatly influential on the mtDNA replication. The RNA primers used to initiate mtDNA synthesis at the origin of replication for the heavy strand (O\textsubscript{H}) are generated from mitochondrial RNA transcripts (Figure 2) (Chang & Clayton, 1985). Copying of the heavy strand later facilitates priming of replication of the light strand from O\textsubscript{L} (Wanrooij et al., 2008). This illustrates that mitochondrial replication and transcription are tightly coupled (Wanrooij & Falkenberg, 2010).

1.3 HUMAN SKELETAL MUSCLE

Skeletal muscle tissue is essential for voluntary movements of the human body. The coordinated action of muscle fibres propels us forward, on land and in water, and enables us to lift, pull and push object in our surrounding environment. The skeletal muscle cells execute direct orders from the nervous system. This neural signalling might be reflexive or voluntary, but the the endpoints are the same; to
activate the contractile machinery within the muscle and thereby produce mechanical force.

1.3.1 Skeletal muscle structure and function

Skeletal muscle is made up of long muscle cells, the individual muscle fibres (Figure 3). They are bundled together in fascicle structures, which are girdled by connective tissue. The skeletal muscle fibres are multi-nucleated, with their nuclei located just beneath the plasma membrane (sarcolemma). Interspersed in the fascicles is a large network of capillaries that supply the cells with necessary oxygen and nutrients, as well as transporting carbon dioxide and other metabolic by-products away from the tissue (Peachey et al., 1983).

Muscle contractions arise from myosin and actin filament interfacial pulling within myofibrils of individual muscle fibres (Figure 3). Necessary for this is the

Figure 3. Schematic illustration human skeletal muscle structure. Each muscle contains several bundles (fascicles, light grey) of individual skeletal muscle cells (fibres, coral). The individual muscle fibres are surrounded by capillaries (red) to ensure proper blood supply. An organised pattern of contractile myofibrils (dark grey) can be found inside the fibres, along with ATP-producing mitochondria (green), several nuclei (protruding under the sarcolemma) and the Ca\(^{2+}\)-containing sarcoplasmic reticulum (yellow). Illustration: Elina Anttila.
ATP-consuming activity of the myosin heads following Ca\textsuperscript{2+}-mediated de-inhibition of filament interaction. The calcium ions are released from the sarcoplasmic reticulum (SR) upon motor neuron stimulation at the neuromuscular junctions (Peachey et al., 1983). Energy-producing mitochondria are distributed between the myofibrils as well as directly under the sarcolemma (Cogswell et al., 1993; Picard et al., 2012).

To cope with the varying demands of human life, skeletal muscle cells with considerable differences in metabolic profile, contractile speed and fatiguability are present throughout our bodies (Westerblad et al., 2010). The predominating system for classifying human skeletal muscle fibres is based on isoforms of myosin heavy chain (MHC); type I, IIa and IIx (Spangenberg & Booth, 2003). In terms of filament cross-bridge cycling speed, type I MHC is the slowest, type IIa intermediate and IIx the fastest. The metabolic characteristics are closely related to the fatiguability of the muscle cells. Type I fibres are more oxidative and fatigue-resistant, whilst the second types are more glycolytic and easier to fatigue (Bárány, 1967; Brooke & Kaiser, 1970; Essén et al., 1975).

1.4 ADAPTABILITY OF SKELETAL MUSCLE

The human body is a greatly adaptable system and different circumstances can bring about short- or long-term changes in several tissues. Physical training is one of the most potent stimuli for multi-tissue adaptation in humans and the events taking place are of major importance for the health benefits of an active lifestyle. Regular exercise positively influences the cardiovascular system, adipose tissue, nervous system and endocrine system (Bowles et al., 2000; Enevoldsen et al., 2001; Henriksson, 1995; van Praag, 2009; Winder et al., 1978). It also contributes to improved mental health (Fox, 2007). In addition, the working skeletal muscle will to a large degree undergo acclimatisation in order to meet the new demands.

Different types of training, ranging from heavy resistance exercise to repetitive aerobic bouts, give rise to distinctive physiological effects in the skeletal muscle tissue. Each mode of exercise entails specific changes that ultimately touch down somewhere in the adaptation continuum (Coffey & Hawley, 2007). The outcome
is dependent on factors like duration, intensity, frequency and the amount of muscle mass involved (Coffey & Hawley, 2007). Muscle hypertrophy and enhanced maximal contractile force output are seen in response to resistance exercise (Braith & Beck, 2007; Knuttgen, 2007), which brings about circumstances for a more powerful muscle contraction. At the other end, two prominent alterations resulting from endurance training are increases in the mitochondrial mass within the muscle fibres and in the capillary density around them (Bloor, 2005; Holloszy, 1967). Both these features contribute to a higher capacity of aerobic ATP production and thereby to a fundamental adaptation to regular endurance exercise, namely improved fatigue-resistance. Along with this, endurance training compels the muscle fibres to shift their metabolic substrate preference to fatty acids, which are more abundant and generate more energy than does glucose (Kiens et al., 1993). The fibre type composition of a certain muscle is in part dependent on its anatomical location and general activity pattern, but is also related to the genetic make-up and training status of the individual (Gollnick et al., 1972; Gollnick et al., 1985). The latter since skeletal muscle adaptations to some degree may transform existing fibres towards more oxidative or glycolytic phenotypes, depending on the type of exercise performed.

1.4.1 Exercise as a stimulus for adaptation in skeletal muscle

There are several aspects of contractile activity that can induce adaptation signalling in skeletal muscle. During exercise, the internal and external milieu of the muscle fibres is drastically changed. The temperature rises, the hormone and cytokine profile of the blood is altered and pH- and oxygen levels decrease. Furthermore, within the muscle cells, released Ca$^{2+}$, the ATP/ADP-ratio, ROS and mechanical stretch may act as modulators of skeletal muscle adaptation. These and other variables are potent triggers of intracellular protein signalling pathways in skeletal muscle. For example, the magnitude of phosphorylation of protein kinases is affected, which directly or via secondary molecules can adjust gene expression of muscle fibre components (Saltin & Gollnick, 2010). It has been concluded that the cumulative effect of repeated acute exercise bouts is what eventually builds adaptation in a gradual manner (Hawley, 2002). Transient bursts
of signalling events and gene expression occur after each exercise occasion when remodelling of the tissue is initiated. Once adapted to the training intensity, duration and frequency, subjects’ intracellular response to the same biomechanical stimuli plateaus as the muscle fibres enter a state of maintenance (Pilegaard et al., 2003). Hence, when studying exercise-induced signalling or adaptations, deliberate selection of time-points for tissue sampling is essential to obtain a desired mechanistic window or steady-state condition.

1.5 MITOCHONDRIAL BIOGENESIS

The great disparity in mitochondrial density that exists between different cell types reflects that proper adjustments of metabolic systems occur during differentiation. Nevertheless, this distribution pattern is not static. Mitochondria are plastic structures and should be thought of as a dynamic intracellular network, constantly undergoing fusion or fission in response to internal or external stimuli (Ogata & Yamasaki, 1997; Stetler et al., 2012). As for other cellular events, the balance between production and break-down of mitochondrial components highly influences the mitochondrial density and function. Beyond the classical proteolytic degradation of mitochondrial components, mitochondrial units may also be subjected to mitophagy (Youle & Narendra, 2011). This process comprises the selective recruitment of mitochondria into isolation membranes, which seal to become autophagosomes and then fuse with lysosomes to eliminate their content. Mitophagy regulates mitochondrial number to match the metabolic demand and is also proposed to be a form of quality control to remove damaged organelles. As mitochondria cannot be made de novo, they instead recruit new proteins that are added to preexisting sub-compartments. By controlling the mitochondrial growth, i.e. biogenesis, cells in our bodies are able to fine-tune their current need for e.g. aerobic energy production.

1.5.1 Exercise-induced mitochondrial biogenesis in skeletal muscle

Fifty-five years ago, it was unveiled for the first time that endurance exercise training leads to mitochondrial biogenesis in rat skeletal muscle (Holloszy, 1967).
Later, it was further established in humans that a only a few weeks of endurance training leads to a up to a 50 percent increase in mitochondrial volume in previously untrained subjects (Fernstrom, 2003; Hoppeler & Fluck, 2003). Notably, regular endurance exercise induces mitochondrial biogenesis in adipose tissue and has been suggested to do so also in liver, brain and kidney (Little et al., 2011). Furthermore, recent studies demonstrates that resistance and interval training regimens can also bring about skeletal muscle mitochondrial biogenesis in humans (Balakrishnan et al., 2010; Burgomaster et al., 2007). Progress has been made continuously in terms of charting the mechanistic pathways involved in mitochondrial biogenesis and several possible regulatory factors have been identified, a few of which are investigated in the present thesis.

1.5.2 Mitochondrial transcription factors

Mitochondrial biogenesis, in any context, implies the transcription and replication of mtDNA and is thus theorised to involve the mitochondrial transcription factors TFAM, TFB1M, TFB2M and MTERFs in its regulation. Our group has previously demonstrated that TFAM protein levels increase following four weeks of endurance training in humans (Bengtsson et al., 2001), but that a single 45 minute bout of one-legged exercise does not influence TFAM mRNA levels up to six hours after terminating the exercise (Norrbom, 2003). In contrast to this observation, it has been reported that TFAM mRNA increased six hours after the end of a three hour one-legged exercise bout (Pilegaard et al., 2003) and in response to a single 45 minute running bout (Bori et al., 2012). Also, acute and repeated high intensity interval training seem to elicit a response of TFAM mRNA, but not of TFAM protein levels (Perry et al., 2010). Our group have published a study observing no differences in mRNA levels of TFB1M or TFB2M for up to six hours following a single exercise bout (Norrbom, 2003). No data on protein levels or the effect of exercise training on the TFB factors have been reported.

1.5.3 PGC-1α

Peroxisome proliferator-activated receptor γ co-activator 1-alpha (PGC-1α) is one proposed regulator of exercise-induced mitochondrial biogenesis in skeletal
muscle and belongs to a family of co-activators, including also PGC-1β and PGC-1 related co-activator (PRC) (Puigserver, 2003). They all harbour the ability to bind transcription factors and thereby stimulate gene expression. PGC-1α acts to govern transcription of nuclear-encoded mitochondrial proteins. Through its physical interaction with peroxisome proliferator-activated receptors (PPARs), a group of fatty acid-activated nuclear receptors, PGC-1α is conducive to e.g. fatty acid transport into the mitochondria and β-oxidation of fatty acids (Gilde & Van Bilsen, 2003). When instead being teamed up with nuclear respiratory factors (NRFs), PGC-1α influences the expression of TFAM, TFB1M, TFB2M and components of the respiratory chain (Gleyzer et al., 2005; Scarpulla, 2002; Z. Wu et al., 1999). Furthermore, the estrogen-related receptor alpha (ERRα) is a more recently discovered liaison of PGC-1α (Schreiber, 2003). Together, they activate promoters of several genes that are vital for aerobic metabolism (Arany et al., 2008; Huss et al., 2004).

By means of these mechanisms, PGC-1α co-ordinates the expression of mtDNA with that of nuclear-encoded mitochondrial proteins. Hence, it plays an important role in increasing or maintaining mitochondrial mass, which requires a concurrent transcription of genes from two genomes. To illustrate its pivotal position, muscle-specific Pgc-1α knockout mice suffer from impaired muscle function, reduced oxidative capacity of skeletal muscles and attenuated running-induced expression of cytochrome c and COXIV (Geng et al., 2010; Handschin et al., 2007).

Under a number of conditions and using different exercise modes, PGC-1α expression has been shown to increase in human skeletal muscle after an acute bout of exercise (Norrbom, 2003; Perry et al., 2010; Pilegaard et al., 2003; Russell, 2005) as well as after a period of training (Perry et al., 2010). In addition, PGC-1α mRNA is down-regulated after five weeks of local muscle inactivity (Timmons et al., 2006).

There are several upstream routes for increasing PGC-1α gene expression in response to exercise, including 5' adenosine monophosphate-activated protein kinase (AMPK) activation, ROS signalling, β-adrenergic receptor signalling and Ca²⁺-mediated pathways involving calmodulin-dependent protein kinase (CaMK).
(Akimoto, 2005; Irrcher et al., 2003; Irrcher et al., 2008a; Irrcher et al., 2008b; Miura et al., 2007; Pogozelski et al., 2009). The canonical PGC-1α promoter contains response elements for several transcription factors, which become activated via these pathways.

PGC-1α abundance is not the only factor of importance for cellular PGC-1α activity. Post-translational modifications and the presence of interacting molecules are two established alternatives. For example, phosphorylation by AMPK, de-acetylation by NAD-dependent deacetylase sirtuin-1 (SIRT1) and interaction with antagonist RIP-140 have been demonstrated to modulate the activity of PGC-1α (Hallberg et al., 2008; Jäger et al., 2007; Nemoto, 2005; Rodgers et al., 2005). Moreover, the function of PGC-1α has been described to be enhanced upon binding of transcriptional co-activator lipin-1 (LPIN1) (Finck et al., 2006).

### 1.5.4 Lipin-1

The mechanistic connection between lipin-1 and PGC-1α with reference to metabolic regulation was first established in mouse hepatocytes, in which lipin-1 was found to bind PGC-1α and histone acetylase p300 to activate transcription of the Ppara gene (Finck et al., 2006). In the same study, it was also learned that lipin-1 physically interacts with the PPARα and PPARγ proteins. Induction of the lipin-1 gene is partly managed by PGC-1α (Finck et al., 2006). Supporting these findings are the original characterisations of the Lpin1 gene, made on account of the fact that mice carrying a mutation in the Lpin1 sequence suffer from fatty liver dystrophy including hyperlipidaemia, insulin resistance and diminished rates of hepatic fatty acid oxidation (Reue & Zhang, 2008; Reue et al., 2000). Furthermore, a study on non-diabetic humans demonstrated that LPIN1 mRNA levels in abdominal visceral adipose tissue negatively correlated with body mass index, body fat percentage and plasma triacylglycerol (TAG) levels (Y.-C. Chang et al., 2009). In addition to being a transcriptional co-regulator, the translated lipin-1 functions as a phosphatidylic acid phosphatase (PAP) enzyme converting phosphatidate to diacylglycerol (DAG), the immediate precursor of TAG and phospholipids (Finck et al., 2006; Reue & Zhang, 2008). The latter activity is
carried out in the cytoplasm, whilst direct modulation of gene expression by definition takes place in the nucleus (Sugden et al., 2010).

However, conflicting data exists, suggesting lipin-1 to be involved in the development of insulin resistance when being induced via the CREB-regulated transcriptional co-activator (TORC2) pathway (Koo et al., 2005; Ryu et al., 2009). It has not yet been clarified how expression of lipin-1 is controlled in response to different physiological conditions. One piece of the puzzle, or a further layer of complexity, may be the existence of Lipin1 mRNA isoforms.

Two Lipin1 splice variants, Lipin1α and Lipin1β, have been documented in mouse. They are set apart by a 99 bp segment within exon 7 that is exclusive to Lipin1β. The translated lipin-1 isoform polypeptides have differences in tissue expression levels, subcellular localisation as well as in cellular function (Péterfy et al., 2005). Lipin-1α is predominantly a nuclear protein, whilst lipin-1β is mainly found in the

![Figure 4](image)

**Figure 4. Proposed model for the role of lipin-1 in exercise-induced mitochondrial biogenesis in skeletal muscle.** PGC-1α, peroxisome proliferator-activated receptor gamma co-activator 1-alpha; TF, specific transcription factors, e.g. nuclear respiratory factors; TFAM, mitochondrial transcription factor A; TFB1/2M, mitochondrial transcription factor B1/B2. Consult text for details.
cytoplasm. If these features are directly representative of the two lipin-1 functions, as a regulatory factor for gene expression and as a metabolic enzyme, remains to be determined. Notably, alternative splicing of LPIN1 transcripts also occurs in humans (Croce et al., 2007). Four human mRNA species have been described (GenBank data), of which two are homologues of the mouse isoforms and designated as LPIN1α and LPIN1β (Croce et al., 2007; Han & Carman, 2010).

One study investigating the influence of exercise on lipin-1 and its possible role in exercised-induced mitochondrial biogenesis in rat skeletal muscle has been published (Higashida et al., 2008). The experiments showed an increase of lipin-1 mRNA levels in rat triceps muscle immediately after three and six hours of swimming, respectively. Furthermore, they report that administration of AMPK stimulator 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) and β2-adrenergic receptor agonist clenbuterol lead to higher levels of Lpin1 mRNA in vivo and that overexpressing lipin-1 in rat myotubes triggered transcription of the mitochondrial fatty acid transporter muscle carnitine palmitoyl transferase-1 (Cpt1b).
2 AIMS

The overall aim of this thesis was to further elucidate the mechanisms of mitochondrial biogenesis induced by exercise in human skeletal muscle. More specifically, the performed studies aimed at investigating:

Lipin-1

- The presence of lipin-1 isoforms and the influence of a single bout of exercise and regular endurance training on the expression of lipin-1 in human skeletal muscle

- The influence of AMPK and adrenoreceptor signalling on the expression of lipin-1 and its target genes in skeletal muscle cells

Mitochondrial transcription factors

- The influence of a single bout of exercise, regular endurance training and physical activity level on the expression of mitochondrial transcription factors TFAM, TFB1M, TFB2M and mTERF in human skeletal muscle
3 METHODOLOGY

Some aspects of the experimental models and laboratory analyses used in this thesis will be discussed in the following section. Consult the individual papers for more in-detail descriptions of study designs, included subjects and applied protocols.

3.1 EXPERIMENTAL MODELS

Four studies using healthy human voluntary men and women were conducted to address the aims of this thesis. An overview of the study designs, subjects, interventions and analyse is presented in Table 1. As the possibility of detecting significant training effects might in part depend on pre-training aerobic fitness status (Saltin et al., 1968), a VO$_2$ max upper limit was used as an inclusion criterion in the acute exercise study, as well as in the 10-day and 12-week training studies.

Moreover, stimulation of cell cultures was performed to complement the in vivo models with a more mechanistic approach.

3.1.1 Acute exercise study (Paper II)

To gain insight into the temporal resolution of gene expression after a single exercise bout, an acute study was performed. Nineteen subjects (12 men and 7 women) were recruited and randomly assigned to the resting control (n = 5) or exercise (n = 14) group. All participants had a measured VO$_2$ max below 60 ml min$^{-1}$ kg$^{-1}$ (males) or 50 ml min$^{-1}$ kg$^{-1}$ (females).

All subjects received standardised meals before and during participation in the study, to minimise effects of dietary variations. The intervention performed by the exercise group consisted of 60 minutes of supervised ergometer cycling at a work intensity corresponding to 70% of individual VO$_2$ max. Muscle biopsies were obtained from both legs in an alternate fashion, in all individuals just before the exercise/resting as well as 30 minutes, 2 hours, 6 hours and 24 hours after the end.
of the session. To reduce the risk of circadian influences, all subjects began their participation around the same time of day.

### 3.1.2 10-day training study (Paper I)

Ten healthy males with a VO\textsubscript{2} max below 60 ml min\textsuperscript{-1} kg\textsuperscript{-1} were included in the study and performed one-legged knee extension exercise four times per week, over a 10-day period. It was hypothesised that metabolic perturbation in the form of restricted blood flow (ischaemic) exercise with ensuing tissue hypoxia could elicit a stronger or different intracellular response in skeletal muscle than exercise under normal blood flow conditions. Hence, one leg was trained using local application of external pressure over the working leg to reduce blood flow (R-leg), whilst the other leg was trained with non-restricted blood flow (NR-leg). A pressure chamber technique originally described by Eiken & Bjurstedt was employed (Eiken & Bjurstedt, 1987) (Figure 5). To induce ischaemia, the pressure acting on the exercising leg was elevated to 50 mmHg above atmospheric pressure. This has been shown to reduce leg blood flow during one-legged cycling.

![Figure 5. Exercise set-up for lower body restriction of blood flow.](image)

The model allows modulation of pressure during one-legged knee extension exercise, facilitating a reduction of blood flow to the working leg. Modified from Eiken (1987).
Table 1. Summary of human studies in Papers I and II. In order of hypothesised level of skeletal muscle adaptation; study design, intervention, biopsy times and factors analysed. Consult text for explanations concerning abbreviations.
by 15–20% and reduce oxygen saturation by 10-12 percentage units (Sundberg & Kaijser, 1992). In addition, a larger depletion of ATP occurs and muscle pH is lower (Sundberg, 1994). The other leg was subjected to exercise under normal atmospheric pressure.

At random, the subjects were assigned to exercise either their right or left leg with restricted blood flow. The effective duration of each training session was 90 minutes, always starting with the R-leg as the ischaemic exercise is perceived as much more strenuous, and therefore work load limiting. Study participants were instructed to kick at their highest tolerable workload for 45 minutes, taking into account that the whole session must be completed. After 10 minutes of rest, the exact same workload protocol was performed with the other leg, now with normal atmospheric pressure. Consequently, both legs developed the same power and amount of work in each session.

Resting muscle biopsies were obtained from both legs before the first exercise bout and 24 h after the 10th day exercise bout. Prior to the study, all participants were familiarised twice with the experimental model.

The average cumulative workload for each leg over the first 5 days of the study compared to the last 5 days were 1975 and 2461 Watts, respectively, which corresponds to a 1.3-fold increase in tolerated workload.

3.1.3 12-week training study (Paper II)

Twenty-three healthy men (n = 12) and women (n = 11) were recruited to the study based on their VO$_2$ max values; < 48 ml min$^{-1}$ kg$^{-1}$ and < 46 ml min$^{-1}$ kg$^{-1}$ for males and females, respectively. The subjects performed a 12-week (4 times/week) supervised one-legged training regimen, using a modified cycle ergometer. Each session involved a 45-minute training bout. The leg that was subjected to training was randomised in all subjects. The other leg served as an internal resting control for each individual.

Prior to, and after, participation, all subjects underwent a 15-minute time trial performance test, using both legs in a sequential manner. Before the test, the subject warmed up at very low work intensity for two minutes. Thereafter, the
workload was kept as high as possible for the subject to keep the pace at 60 rpm over the entire 15-minute period. Each minute, the heart rate was measured and the subject estimated the perceived exertion of the working leg (Borg-scale, RPE 6-20).

The first week of the study training regimen, i.e. the first four sessions, were completed as follows; minute 1-15 at 50%, minute 16-30 at 70% and minute 31-45 at 80% of the 15-minute time trial test average workload. Over the course of the training period, the workload was adjusted weekly. For the final week of the study, the work load during minute 1-15, minute 16-30 and minute 31-45 corresponded to 80%, 100% and 110% of the average workload for the 15 minute time trial test.

Skeletal muscle tissue samples were collected at rest, before the training period was begun as well as 24 hours after the last training session.

The average (± SD) increase in performance in terms of the highest mean workload (in Watts) during the 15-minute time trial, was 40.5% (± 12.7) in the trained leg.

### 3.1.4 Cross-sectional study (Paper I)

To evaluate the influence of endurance training and elite level aerobic conditioning on the factors of interest for mitochondrial biogenesis, a cross-sectional study was performed. The comparison of steady-state conditions was made between ten well-adapted elite level endurance athletes (EA) and eight moderately active individuals (MA), all males. Their level of physical activity was assessed by the subjects’ leisure-time physical activity. Their mean (range) values were 22 (17–27) hours of endurance training per week for the EA group and 3 (0–6) hours of moderate intensity physical activity per week for the MA group. The mean (range) VO\textsubscript{2} max values for subjects of the EA and MA groups were 73 (64–77) ml min\textsuperscript{-1} kg\textsuperscript{-1} and 46 (36–56) ml min\textsuperscript{-1} kg\textsuperscript{-1}, respectively.

Skeletal muscle biopsies were obtained at rest from all subjects, at least 24 hours following the last bout of physical activity.
3.1.5 Stimulation of primary human myocytes (Paper II)

From human skeletal muscle biopsies taken at rest, satellite cells were extracted. To ensure minimal infiltration of fibroblasts, cells were transiently seeded in plates during passage splitting.

Initially, the resulting myoblasts were cultured for proliferation, but when 80% confluence was reached, they were differentiated into myotubes by changing to a different culturing medium. Once differentiated, a 24-hour stimulation using 1 mM of the known AMPK-activator AICAR (Sun et al., 2007) and/or 5 µM of norepinephrine was conducted to explore the potential involvement of the AMPK-PGC-1α and/or adrenoreceptor-PGC-1α axes in the regulation of the factors of interest. In addition, untreated control cultures were included in the experiment.

3.1.6 Stimulation of mouse myocyte cell line (Paper II)

The cell line C2C12 is a subclone of an immortal line of mouse skeletal muscle myoblasts (Blau et al., 1985). Originally, it was derived from satellite cells from the thigh muscle of a female belonging to the C3H mouse strain (Yaffe & Saxel, 1977).

As the myoblasts had proliferated to 80% confluence, they were subsequently differentiated into myotubes using an alternative culturing medium. The myotubes were subjected to a 24-hour stimulation using 1 mM of the known AMPK-activator AICAR (Sun et al., 2007) and/or 5 µM of norepinephrine. These substances were used in order to investigate the roles of the AMPK-PGC-1α and/or adrenoreceptor-PGC-1α axes in the regulation of the factors of interest. Untreated control cultures were also included in the experiment.

3.2 Skeletal muscle biopsies

All human studies included in this thesis involved biopsies from the outer thigh muscle, the vastus lateralis (Figure 6). It is part of the quadriceps femoris muscle group and has an advantageous localisation for obtaining tissue samples since no large vessels perforate the area. The interventions used in the acute exercise and
training studies were selected or designed with the vastus lateralis in mind, as it would be used for the molecular analyses.

The tissue sampling was performed by a medical doctor using the percutaneous needle biopsy technique (Bergström, 1962). After locally anaesthetising the area by subcutaneous injections of carbocaine, a small incision in the skin was made. Through this, a Bergström 5-mm biopsy needle was used to penetrate the subcutaneous tissue, including adipose tissue and the fascia, to reach the muscle (Fig 6). By connecting a suction device to the top of the needle, the tissue exchange could be increased as the guillotine motion of the needle completed the biopsy sampling. Generally, the pea-sized muscle biopsies carried a weight of between 50 and 150 mg.

Immediately after being collected, the tissue was frozen in either pure liquid nitrogen or isopentane cooled in liquid nitrogen. The samples were then stored at −80°C until further analysis.

Figure 6. Skeletal muscle biopsy sampling using a Bergström needle. The percutaneous needle biopsy technique is performed under local anaesthesia to obtain a section of tissue from the vastus lateralis muscle.
3.3 LABORATORY ANALYSES

3.3.1 Citrate synthase activity

Citrate synthase (CS) is a rate-limiting enzyme in the tricarboxylic acid cycle. It is nuclear-encoded but the protein is transported into the mitochondrial matrix. Based on this, CS is commonly used as a quantitative marker enzyme for the content of intact mitochondria (Holloszy et al., 1970; Hood et al., 1989; Williams et al., 1986). The enzyme catalyses the chemical merging of 2-carbon acetyl-CoA with 4-carbon oxalacetate to form 6-carbon citrate, which may then proceed in the citric acid cycle. Coenzyme A is regenerated in the CS reaction.

It has been clearly established that CS activity in skeletal muscle tissue is increased in response to endurance-type training (Duscha et al., 2012; Holloszy et al., 1970; Siu et al., 2003). Hence, it is used as a tool for assessing the metabolic adaptation status of an individual.

The experimental procedure was done according to the fluorometric principles of Lowry & Passonneau from 1972 (Lin et al., 1988). In brief, a section of a biopsy was freeze-dried and homogenised in 0.1 M phosphate buffer (pH 7.7) with 0.5% BSA. The tissue lysates were added to reagent mixes containing 0.1 M Tris-HCl, 2.5 mM EDTA, 0.5 mM L-malate, 512.5 nM NAD$^+$ and 399 µg malate dehydrogenase. 50 µg acetyl-CoA was added to each mix to start the citrate synthase reaction and a fluorometer were used to register the velocity of the increased fluorescence that occur when NAD$^+$ is reduced to NADH. A standard curve computed from the fluorescence of known amounts of NADH was subsequently used to determine citrate synthase activity of the muscle samples.

In Paper I, the elite athletes displayed a more than two-fold higher average (± SEM) skeletal muscle citrate synthase activity than the moderately active individuals; [0.56 (± 0.03) µmol s$^{-1}$ g$^{-1}$ dry muscle for EA compared to 0.22 (± 0.03) µmol s$^{-1}$ g$^{-1}$ dry muscle for MA] (p < 0.01). In Paper II, the average (± SD) citrate synthase activity was 1.5-fold higher [31.5 (± 7.1) µmol s$^{-1}$ g$^{-1}$ dry muscle] in the working leg after 12 weeks of endurance training compared to before [21 (± 6.2) µmol s$^{-1}$ g$^{-1}$ dry muscle] (p < 0.001).
3.3.2 Protein extraction and Western blotting

The obtained skeletal muscle biopsies were decomposed in appropriate ice-cold buffer using hand-operated glass homogenisers. By Bradford Assay (BioRad), the protein concentrations of the homogenates were determined. The samples were then boiled in SDS-containing Laemmli loading buffer and subjected to SDS-PAGE. After separation, the proteins were transferred to nitrocellulose membranes and uniform conveyance was verified by Ponceau S red staining. After blocking the membranes in 5% dry non-fat milk, they were incubated in primary antibody solutions (for TFAM, TFB1M, TFB2M and COXIV) with concentrations, durations and temperatures according to previous optimisation of protocols. After being exposed to secondary HRP-linked antibodies and chemiluminescent substrate reagents, the membranes were developed on films. By scanning and densitometric quantification, the protein amounts in each sample, relative to background levels, were determined.

To generate loading controls for normalisation of values, the membranes were stripped from antibodies, re-blocked and probed with an antibody against α-actin.

In order to confirm that the blotting system utilising TFB1M and TFB2M antibodies held the ability to detect different protein amounts, lysates with increasing protein concentrations were loaded on the same gel. The blotting procedure was performed as described above. Resulting data demonstrate that both TFB1M and TFB2M antibodies could indeed detect varying antigen amounts in skeletal muscle homogenates.

3.3.3 Primer design and isoform detection

Based on the first publication that described \textit{Lpin1} splice variants in the mouse (Péterfy et al., 2005), together with the results of sequence homology analyses, primers for recognition of human LPIN1 transcripts were designed (Figure 7). Alignment of mouse and human sequences from the NCBI genome database facilitated identification of the position of the hypothesised isoform divergence in the human exon 10. The location corresponded to the murine equivalent revealed in mouse exon 7. Oligonucleotides were then created to capture specifically
PCR amplification of the cDNA fragments was performed. The amplicons were run on agarose gels with ethidium bromide and subsequently visualised in a UV transilluminator. The amplified PCR product from pF$^h$ and pR$^h_{α}$ was purified and verified by sequencing.

**Figure 7. Primers for detection of lipin-1 mRNA isoforms.** Schematic depiction of exons of the lipin-1 transcript splice variants in mouse (A) and human (B). Arrows indicate the sense (pF) and antisense (pR) primers. In mouse, the *Lpin1α* transcript is lacking exon 7 and is detected using a forward primer spanning the exon 6-exon 8 boundary. In humans, the *LPIN1α* splice variant does not include exon 10 and is detected using a reverse primer covering the exon 9-exon 11 junction. Both *LPIN1α* and *LPIN1β* were detected simultaneously by sense and antisense primers complementary to sequences in exon 8 and exon 11, respectively. Representations were made according to GenBank sequences.
3.3.4 RNA extraction and reverse transcribed real-time PCR

Total RNA was isolated from the cells using Trizol® reagent. For muscle tissue, the extraction was made with either the Trizol® method (Paper II) or the acid phenol method (Chomczynski & Sacchi, 1987) (Paper I). The prepared RNA was quantified by measuring absorbance at 260 nm. From each sample, cDNA was synthesised by reverse transcription.

Real-time RT-PCR was used for measuring gene expression levels. For the lipin-1 splice variants, qPCR was performed using SYBR green reactions. The specificity of the quantification reactions were monitored using a melting curve and primer pair efficiencies was tested by standard titration curves. The mRNA levels of mitochondrial transcription factors TFAM, TFB1M and TFB2M were quantified using custom primers and probes. Consult Papers I for sequence details. All other transcripts were quantified using TaqMan gene expression assays (Applied Biosystems). For each of the qPCR reactions, non-template control samples were included.

Endogenous controls were utilised to correct for possible variations in RNA loading or in efficiency of the amplification. In Paper I, the 18S human rRNA was used, whilst the human glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) and the murine hypoxanthine phosphoribosyltransferase 1 gene (Hprt1) were selected in Paper II.

3.4 STATISTICAL ANALYSES

In the acute exercise study (Paper II), two-way analyses of variance (ANOVAs) for repeated measures were used to test for differences in expression of target/reference gene ratios over the 24-hour period and between the exercise and control groups.

In the 10-day training study (Paper I), the statistical analyses on mRNA data were conducted on logarithmic transformed ratios of target/reference genes. Two-way ANOVAs were applied to assess the effects of exercise condition (restricted or non-restricted blood flow) and time. A one-way ANOVA was applied to investigate the effect of time for each condition independently.
Performance responses and gene expression levels in the 12-week training study (Paper II), as well as gene expression differences between cell culture treatments, were evaluated with student’s t-tests on target/reference gene ratios, whilst correlations were calculated using the Pearson’s Product Moment correlation test.

The statistical calculations on gene expression data in the cross-sectional study (Paper I) were made on ratios of target/reference transcripts. The nonparametric Mann–Whitney U-test was applied to assess any differences in mRNA and protein levels between EA and MA individuals.

P-values ≤ 0.05 were considered statistically significant throughout all studies.
4 RESULTS AND DISCUSSION

In four different settings, this thesis has explored factors proposed to be of importance for exercise-induced mitochondrial biogenesis in human skeletal muscle. The abundance of a selection of mitochondrial transcription factors and the co-regulator lipin-1 have been analysed in skeletal muscle biopsies from i) before and up to 24 hours following a single exercise bout, ii) before and after 10 days of endurance training with and without restricted blood flow to the working leg, iii) before and after 12 weeks of endurance training and iv) elite endurance trained athletes and moderately active individuals.

In addition, the present thesis has investigated the presence of lipin-1 mRNA isoforms in human skeletal muscle, as well as the influence of AICAR and/or norepinephrine on the expression of lipin-1 and potential down-stream genes in cultured myotubes.

4.1 LIPIN-1

Lipin-1 first gained my interest when it was revealed to physically interact with PGC-1α to promote gene expression in murine hepatocytes (Finck et al., 2006). Hence, lipin-1-mediated modulation of PGC-1α transcriptional co-factor activity may be one way of regulating mitochondrial biogenesis.

4.1.1 Lipin-1 transcript isoforms in human skeletal muscle

In the mouse, it has been described that mRNA splicing occurs and that the resulting protein isoforms lipin-1α and lipin-1β display differences in intracellular localisation, tissue expression pattern and function (Péterfy et al., 2005). In Paper II, it is shown that at least two mRNA isoforms are present in human skeletal muscle.

As predicted from sequence homology investigations comparing human lipin-1 isoforms with their murine counterparts, the human transcript variants differ by a 108 bp segment, making up exon 10, which is included only in LPIN1/β. The
results are in line with current GenBank information. However, in a publication investigating human liver and adipose tissue samples, it is claimed that the isoform pattern instead is directly corresponding to that in the mouse, where \textit{Lpin1α} and \textit{Lipin1β} display a 99 bp dissimilarity, equivalent to the entire exon 7 (Croce et al., 2007). It remains to be determined if the splicing events of lipin-1 mRNA are in fact tissue-specific, or if the experimental design employed in the study by Croce \textit{et al.} does not enable proper detection of human lipin-1 transcripts.

Translated lipin-1 is known to function as both a phosphatidic acid phosphatase and a transcriptional co-activator (Finck et al., 2006; Han et al., 2006). To note, both lipin-1\textit{α} and -\textit{β} variants contain the nuclear localisation signal, a DIDGT motif for enzymatic activity as well as the LXXIL motif for transcriptional co-activation (Finck et al., 2006; Han & Carman, 2010; Han et al., 2006). Still, it may be so that the splice variants are directly related to the functional disparity observed for the lipin-1 protein and consequently differ in their importance for metabolic adaptation in skeletal muscle.

\textbf{4.1.2 Lipin-1 expression in response to a single endurance exercise bout}

It has been reported that lipin-1 mRNA expression increases in rat skeletal muscle after a single endurance exercise bout (Higashida et al., 2008). In Paper II, one hour of aerobic cycling did not lead to any differences in total \textit{LPIN1} mRNA levels at 30 minutes, 2 hours, 6 hours or 24 hours post exercise compared to resting controls.

As previous studies indicate that lipin-1\textit{α} is more abundant in the nucleus than lipin-1\textit{β} (Khalil et al., 2008; Péterfy et al., 2005), the \textit{LPIN1α} transcript was selected for separate quantification. However, no significant differences compared to the control group could be established up to 24 hours after the cycling bout.

An evaluation of the primers used by Higashida \textit{et. al}, unveils that the PCR reaction described in their article amplified either total \textit{Lpin1}, or only \textit{Lpin1β}, depending on if the shorter \textit{α} isoform is in fact present in rat skeletal muscle.
From the published gel images in that paper, it is not possible to exclude the presence of an additional splice variant of a different size.

One group have published data that describe differing lipin-1 mRNA expressions in rat hepatocytes when utilising two different primer sets, an indication that there is more than one splice variant (Manmontri et al., 2008). However, this finding by itself does not prove the expression of a lipin-1α-equivalent isoform in the rat.

The fact that the same pattern of lipin-1 mRNA expression was not observed in humans after a cycling bout, as was described for acute endurance exercise in rats (Higashida et al., 2008), might be related to differences in exercise stimuli. Although swimming belongs to a rat’s natural behaviour, the neuroendocrine response to six hours of forced, likely exhaustive and highly stressful swimming in rodents is most probably very different from that seen with voluntary cycling in humans.

4.1.3 Basal expression of lipin-1 following endurance training

In Paper II, neither LPIN1 nor LPIN1α resting mRNA levels were significantly altered in response to 12 weeks of regular endurance exercise. Despite this, it was noticeable that very few individuals displayed a stable expression pattern of

![Figure 8. Skeletal muscle gene expression in response to 12 weeks of one-legged endurance training.](image)

Levels of mRNA (in arbitrary units, A.U., normalised to GAPDH) for (A) both LPIN1 isoforms, (B) TFAM. (n = 23)
lipin-1 mRNA over the training period (Figure 8A). As high and low responders in terms of the 15-minute time trial performance and citrate synthase activity were classified for this study, it was hypothesised that the lipin-1 mRNA expression would harmonise with these response findings. However, it could not be established that individuals with an increase in the adaptation variables in general also displayed a higher expression of lipin-1.

On the other hand, a correlation between the change in $LPIN1$, but not $LPIN1\alpha$, levels and the change in TFAM expression (Figure 8B) was discovered, which may reflect that lipin-1$\beta$ is more involved in mitochondrial biogenesis signalling than previously thought. It may also be a sign of that in some individuals, an increase in lipin-1 expression occurs with endurance training and that lipin-1 then acts an upstream regulator of TFAM, possibly working in tandem with PGC-1$\alpha$.

The relationship between TFAM and total $LPIN1$ might also merely be symptomatic of the notion they are both downstream target genes regulated by PGC-1$\alpha$ (Finck et al., 2006; Wu et al., 1999). The mRNA levels of $PGC-1\alpha$, expressed from its canonical promoter (Norr bom et al., 2011), was not increased after 12 weeks of regular endurance exercise (Figure 9) (unpublished data). It has previously been shown that the mRNA levels of this factor are augmented in human skeletal muscle after a period of training (Perry et al., 2010).

![Figure 9. Skeletal muscle gene expression of PGC-1α in response to 12 weeks of one-legged endurance training.](image)

Levels of $PGC-1\alpha$ mRNA (in arbitrary units, A.U., normalised to $GAPDH$). Values are reported as mean ± SD. ($n = 23$)
As a matter of course, this does not exclude that an alteration of PGC-1α abundance or activity occurred in response to the training in this study. First, the isoform PGC-1α-b has been shown to be strongly induced by exercise in human skeletal muscle (Norrborom et al., 2011). It has not been established whether this variant, once translated, has similar or different functions compared to the regular PGC-1α protein. Second, post-translational modifications, for instance phosphorylation or acetylation switches, have been established as a way of modulating the activity of existing PGC-1α and may be part of metabolic adaptations in skeletal muscle (Cantó et al., 2009; Jäger et al., 2007).

4.1.4 Effect of AMPK and adrenoreceptor activation on the expression of lipin-1 and its target genes

Activation of the AMPK enzyme through ROS signalling as well as activation of adrenoreceptors through circulating hormones has been suggested as two of the major pathways triggering exercise-induced metabolic adjustments in skeletal muscle, e.g. via PGC-1α expression (Miura et al., 2007; Tadaishi et al., 2011; Winder et al., 2006). In Paper II, it was shown that neither 1 mM AICAR nor 5 µM norepinephrine influenced the mRNA levels of both lipin-1α and lipin-1β isoforms or specifically lipin-1α in cultured myotubes that had been differentiated from primary human satellite cells or from a mouse myoblast cell line. In contrast to this data, Higashida et al concluded that AICAR and clenbuterol (a β2 sympathomimetic) administration in vivo led to higher levels of the Lpin1 transcript in rats (Higashida et al., 2008). The concentrations of AICAR and norepinephrine that were chosen for the experiments in the present thesis have previously been shown to elicit mRNA responses of PGC-1α in human cultured myocytes (Norrborom et al., 2011). Hence, the lack of effect on lipin-1 mRNA species does not seem to be explained by too low amounts of the stimulatory compounds.

The influence of AICAR and/or norepinephrine treatment on CPT1B, PPARα and TFAM was also investigated. It has been shown that over-expression of human lipin-1 in the L6 rat skeletal muscle cell line stimulates transcription of muscle carnitine palmitoyl transferase-1 (Cpt1b) - a mitochondrial enzyme shuttling long-
chain fatty acids across the outer mitochondrial membrane (Higashida et al., 2008). Together with PPARα, this enzyme has been demonstrated to be a target gene of lipin-1 (Donkor et al., 2007; Finck et al., 2006). In addition, based on the relationship between PGC-1α and lipin-1, I hypothesised that TFAM might be a directly or indirectly lipin-1-regulated gene.

The results show that AICAR and/or norepinephrine do not cause a general up-regulation of CPT1B, PPARα or TFAM expression in primary human myotubes. In the mouse myotubes, significantly higher Tfam mRNA levels were detected in the cells stimulated with AICAR and norepinephrine compared to untreated cultures. However, the data are inconclusive as the combination of both compounds did not lead to higher Tfam expression compared to the untreated controls. At the same time, no differences in mRNA levels of Ppara or Cpt1b were observed with the used treatments. This may indicate that, under these circumstances, Tfam transcription was induced by the classical PGC-1α model and not via increased expression of lipin-1. Furthermore, one study shows TFAM as up-regulated upon ectopic expression of lipin-1β in mice liver (Finck et al., 2006). Again, this might point to the longer LPIN1 isoform being more important for the mitochondrial biogenesis process.

4.1.5 Further considerations on lipin-1

Lipin-1 is probably not only regulated by abundance. For example, post-translational modifications might affect the stability or activity of the lipin-1 protein in skeletal muscle fibres. In nerve cells, it has been established that sumoylation of lipin-1α facilitates nuclear translocation in cultured neuronal cells. This, in turn, enables lipin-1 to execute actions related to transcriptional regulation.

It has also been demonstrated that murine lipin-1 expression in liver follows a circadian rhythm, partly ascribed to glucocorticoid fluctuations (Oishi et al., 2005; Panda et al., 2002). Accordingly, the skeletal muscle biopsies for the acute study and the 12-week training study presented in Paper II were obtained at approximately the same time of day from all participating subjects.
As lipin-1 is expressed in several tissues, it could potentially be differentially regulated or have contrasting roles in different cell types. In addition, it is still unclear exactly how it affects metabolic status in general, or muscle metabolism in particular. It has been incontestably established that lipin-1 is highly implicated in mammalian energy balance. Lipin deficiency in mice caused a profound metabolic disturbance including whole-body insulin resistance as well as an inability for substrate utilisation shifts during fasting-fed cycles and in response to a changed diet (J. Xu et al., 2006). In both healthy humans and mice, adipose tissue lipin-1 mRNA levels were correlated with a favourable metabolic profile and expression of fatty acid oxidation genes (Donkor et al., 2007). Additionally, human expression of lipin in adipose tissue was inversely correlated to measures of insulin resistance (Suviolahti, 2005; Yao-Borengasser, 2006).

However, the function of lipin-1 seems to be more complex. Intriguingly, lipin-1 deficiency in humans is not associated with lipodystrophy, but instead results in e.g. defective muscle energy metabolism (Zeharia et al., 2008). Transgenic lipin-1 expression in skeletal muscle and white adipose tissue exacerbated high-fat diet-induced obesity and decreased fatty acid catabolism in the muscle (Phan & Reue, 2005). In skeletal muscle, lipin-1 expression is also increased in conditions that cause atrophy, such as diabetes and cachexia (Lecker, 2004).

Data from the present thesis provide knowledge of skeletal muscle lipin-1 expression directly after one bout of cycling and in response to three months of endurance training. If and how exercise affects the intricate connections between lipin-1 and metabolic pathways remains to be determined.

4.2 MITOCHONDRIAL TRANSCRIPTION FACTORS

Furthest downstream in the proposed mechanism of exercise-induced mitochondrial biogenesis are the nuclear-encoded mitochondrial proteins, including the mitochondrial transcription factors TFAM, TFB1M, TFB2M and mTERF that regulate the stability and expression of mtDNA.
4.2.1 TFAM levels in response to acute exercise and endurance training

Previous studies on rats demonstrated that early events in contractile activity-induced mitochondrial biogenesis include increases in Tfam mRNA, followed by boosting of mitochondrial import and increased Tfam content, which in turn is coupled to increased binding to mtDNA (Gordon et al., 2001).

In Paper II, it was not possible to detect any difference in TFAM mRNA expression between the cycling and resting groups after a single 60-minute cycling bout when considering time-points of 30 minutes, two hours, six hours and 24 hours after the end of the exercise. Neither did ten days of regular endurance exercise with and without blood-flow restriction induce changes in the basal TFAM mRNA levels (Paper I). The results from the 12-week endurance training study is consistent with this finding, as no significant basal mRNA changes for TFAM occurred (Paper II). The same was true for the cross-sectional study, where no significant difference between moderately active individuals and elite endurance athletes could be found (Paper I). There was, however, a tendency to higher TFAM mRNA levels in the elite athlete group.

The findings from these studies indicate that TFAM is not mainly regulated on the transcriptional level in response to endurance exercise or training in human skeletal muscle. This endorses previous results from our group, where one 45-minute bout of one-legged exercise did not influence the TFAM mRNA levels up to six hours after the end of the bout (Norrbom, 2003). Nonetheless, another study detected higher TFAM mRNA levels six hours after a three-hour exercise bout (Pilegaard et al., 2003). Exercise modality, intensity, duration and frequency may determine the outcome. For example, it was shown that high intensity interval training using a cycle ergometer gave a rapid increase in TFAM mRNA four hours after one session and remained elevated throughout two weeks of training, inclusive of seven sessions in total (Perry et al., 2010).

In the cross-sectional study in Paper I, protein levels of TFAM and COX IV in the skeletal muscle biopsies were measured. They were both significantly higher in the elite athlete participants compared to the moderately active group. COX IV is a nuclear-encoded mitochondrial protein that is considered a marker for oxidative
capacity and the measurements of this factor, along with citrate synthase activity and \( \text{VO}_2\text{max} \) values, further corroborate that the elite athletes were highly adapted to endurance training. These results also support previous findings from our group, which showed that TFAM protein levels in human skeletal muscle were increased following four weeks of endurance training (Bengtsson et al., 2001). They also add to the hypothesis that TFAM plays a role in sustaining exercise-induced mitochondrial biogenesis. It can therefore be suggested that TFAM is predominantly regulated at the protein level in response to exercise in humans.

Experiments of over-expression and RNA interference in human cells have shown TFAM protein abundance to be correlated with the mtDNA amount, rather than with the level of mtDNA transcription (Kanki et al., 2004). Furthermore, one study presents a strong correlation between \( \text{VO}_2\text{max} \) and TFAM expression, suggesting that TFAM expression levels could impact aerobic endurance (Bori et al., 2012).

To note, contrasting data to the present findings on TFAM protein levels has been published. For example, one study reported that TFAM protein content was essentially unchanged throughout a two-week period of high intensity interval training (Perry et al., 2010).

As for lipin-1, the possibility exists that TFAM protein stability or activity is further regulated by post-translational modifications. In rats, TFAM is acetylated \textit{in vivo} (Dinardo et al., 2003). Experiments on HeLa cells uncover that SIRT1, together with PGC-1\( \alpha \), is present in close proximity to TFAM inside the mitochondria, indicating SIRT1 as a candidate de-acetylator of the TFAM protein (Aquilano et al., 2010).

### 4.2.2 TFB1M and TFB2M levels in response to endurance training

To my knowledge, there is presently only one study where \( TFB1M \) and \( TFB2M \) mRNA have been measured in response to physical activity in healthy humans (Norrbom, 2003). Therein, our group demonstrated that no changes in mRNA levels of these factors could be detected up to 6 hours following a single bout of exercise.
In Paper I, basal mRNA expression of both $TFB1M$ and $TFB2M$ were induced by ten days of regular endurance training with restricted blood flow to the working leg. However, when exercise was performed with normal, unrestricted blood flow, there were no significant changes in these two factors. This adds further support to the notion that metabolic perturbation influences the regulation of factors important for mitochondrial adaptations.

In addition, it was discovered that in well-adapted endurance athletes, the resting mRNA levels of $TFB1M$ as well as $TFB2M$ were different from those in moderately active individuals. The protein levels did not differ between the two groups, which stands in contrast to the transcript abundance in both elite athletes of the cross-sectional study and the observations made after ten days of training. This could be interpreted in various ways. For instance, changes in $TFB$ mRNA levels may not necessarily translate into altered TFB protein levels. Consequently, these factors may not play any major part in mitochondrial biogenesis in exercised human skeletal muscle. The possibility also remains that the Western blotting method for protein detection is not sensitive enough to detect changes in the material, even though the ability of the antibodies to detect varying amounts of protein was confirmed. Nevertheless, smaller changes could still be difficult to distinguish. Furthermore, post-translational modifications of the TFB proteins may negatively influence the antibodies’ affinities for their individual epitopes.

In this thesis, the pattern of TFB expression was found to be similar between the two isoforms in all applied conditions. This does not, however, reflect their molecular functions, which have been shown to differ a great deal. Experiments using Drosophila melanogaster show that TFB2M is more active in regulating mitochondrial transcription and copy number whereas TFB1M is important for regulating mitochondrial translation (Falkenberg et al., 2002; Litonin et al., 2010; Matsushima, 2004; 2005). In mice, higher levels of TFB2M mRNA were observed in organs rich in mtDNA (Rantanen et al., 2003).

4.2.3 Expression of mTERF in response to endurance training

The role of mitochondrial termination factors (MTERFs) in response to exercise has not previously been studied. In Paper I, no changes in mRNA levels of the
termination factor mTERF (also denoted as MTERF1) in human skeletal muscle was observed after ten days of regular endurance training, regardless of blood flow condition. In the cross-sectional study, however, basal mTERF mRNA expression was significantly higher in the elite athlete group compared with the moderately active group.

The endurance-trained individuals expressed higher COXIV protein levels and were therefore expected to have larger mitochondrial densities than the unadapted group. This indicates that mTERF does not inhibit exercise-induced mitochondrial biogenesis in skeletal muscle. Instead, our findings give weight to an earlier study, which demonstrated that mTERF promotes human mitochondrial rRNA synthesis by mtDNA looping that promotes recycling of the transcriptional machinery (Martin et al., 2005). The looping helps to meet the need for a high rate of transcription of mitochondrial ribosomal components. In this way, mTERF acts as an indirect positive regulator of mitochondrial translation.

### 4.2.4 Further considerations on mitochondrial transcription factors

It is worth to note that expression quantification of mitochondrial components is not a definite read-out in terms of mitochondrial adaptation. As previously stated in this thesis, mRNA and protein levels may not be representative of certain molecular events and post-translational modifications may very well be decisive of the half-life and/or activity of a specific factor.

When it comes to quantifications of mitochondrial transcription factors, direct measurements of mitochondrial gene expression or more functional investigations might be suitable complements. For example, citrate synthase activity, featured in both Paper I and II, is one option. Assessment of respiratory chain function in real-time another.

In addition, the mitochondrial structure and function are highly dependent on protein import machineries and scaffolding factors that enable proper assembly of molecular complexes. These aspects may very well boost or hinder effects that are assumed based on mRNA or protein levels of mitochondrial constituents.
4.3 GENERAL DISCUSSION

Skeletal muscle is an attainable tissue, which makes muscle biopsies from vastus lateralis a straightforward option for studying the molecular or histological conditions in a variety of human situations in vivo.

In the acute exercise study, three biopsies were obtained from each leg under a 24-hour period. Also, in the training studies, multiple biopsies from the same muscle were sometimes taken in order to acquire sufficient amounts of material. This raises the question whether tissue reactions to previous biopsies, for example inflammatory responses, influence the results of analyses of later ones (Friedmann-Bette et al., 2012; Vissing, 2004). One study reports that that multiple muscle biopsies obtained from the same muscle do not influence the mRNA response induced by an acute exercise bout for any of the genes examined (Lundby et al., 2005). Also, skeletal muscle is not a fully homogenous tissue with possible differences along the muscle belly’s length and at various depths in fibre type composition and oxidative capacity, as well as in myocyte activation pattern and metabolism during exercise (Lexell et al., 1985).

Furthermore, one of the limitations of studying exercise-induced responses in humans is the inter-individual variability that exists in terms of both acute signalling and long-term adaptations (Timmons, 2011; Timmons et al., 2010). The genetic profile, epigenetic pattern, lifestyle choices and medical status of the subjects may interfere with the phenomena to be investigated, which can make it difficult to draw any general conclusions from the results.

Nevertheless, there are obvious advantages of studying humans as opposed to using animals or in vitro models. Results from animal experimentations are often inaccurately extrapolated to the human situation. For instance, in the present thesis, we could not demonstrate the same lipin-1 mRNA surge in humans that previously had been shown to occur in rats (Higashida et al., 2008). In addition, animal studies in the molecular exercise field do not always apply voluntary exercise as an intervention stimulus. This adds another level of complexity to physiological studies, as the animal subjects may experience a ”life or death” type of stress that highly influences e.g. the metabolic pathways to be studied.
However, as animals and cell cultures are particularly suitable for more mechanistic approaches, both primary human satellite cell cultures and a murine myoblast cell line were used in the present thesis. Primary cultures may consist of a mix of different cell types. In the case of satellite cells, fibroblast contamination is an eminent risk. It is without doubt easier to achieve homogenous cultures with the C2C12 cells, but this alternative might also diminish an important scientific perspective as the variability is reduced.
5 CONCLUSIONS

The main findings from the studies presented in this thesis were:

**Lipin-1**

- At least two isoforms of lipin-1 are present in human skeletal muscle

- The mRNA levels of total lipin-1 and the lipin-1α isoform are unaltered in response to a single exercise bout and after a period of endurance training, suggesting that their possible involvement in mitochondrial biogenesis is not transcriptionally regulated

- Neither AMPK nor adrenoreceptor signalling influences the mRNA expression of lipin-1 or its established target genes

**Mitochondrial transcription factors**

- TFAM protein levels are higher in conditions with enhanced oxidative capacity, increases with endurance training and is likely regulated by protein stabilisation

- TFB1M and TFB2M mRNA levels, but not protein, are influenced by endurance training with restricted blood flow to the working leg and by long term endurance training, suggesting transcriptional control of these factors in regulation of exercise-induced mitochondrial biogenesis

- The mRNA levels of mTERF are higher in well-adapted endurance athletes than moderately active individuals but does not change in response to endurance training, indicating that mTERF is not inhibitory for mitochondrial biogenesis but may rather support a high oxidative capacity
6 FINAL REMARKS

Considering the factors and intracellular events investigated in the present thesis, further studies are required to elucidate the mechanisms behind exercise-induced mitochondrial biogenesis. Pertinent analyses could include; the effect of the different exercise stimuli on i) the lipin-1β isoform in human skeletal muscle, ii) post-translational modifications of lipin-1 and mitochondrial transcription factors, iii) protein-complex interactions between lipin-1, NRF-1 and PGC-1α on the promoters of nuclear-encoded mitochondrial proteins, and iv) epigenetic regulation of suggested up- and downstream mitochondrial biogenesis factors.

The exploration of mitochondrial biogenesis and other metabolic adaptations to exercise training in human skeletal muscle is valuable from several perspectives. It can lead to the development of new treatment strategies, e.g. for type-II-diabetics or patients that are incapable of exerting physical activity due to paralysis or severe painful joint diseases. In addition, preventative actions for reducing risks of cardiovascular disease or cancer may be optimised. Knowledge of how mitochondrial biogenesis is initiated can also be advantageous for athletes on all levels when designing training programs for maximal endurance performance.
7 POPULÄRVETENSKAPLIG SAMMANFATTNING


Muskler anpassar sig till träning
Effekterna av fysisk aktivitet är inte omedelbara, utan byggs upp gradvis när kroppen anpassar sig till upprepade träningsspass. Bland annat kommer hjärtat klara av att pumpa blodet mer effektivt och mängden kroppsfett kommer att minska. Syftet med denna avhandling var att på djupet studera de förändringar som sker inuti de muskler som används vid träning.

Vid regelbunden motion blir muskulaturen starkare eller mer uthållig, beroende på vilken typ av träning man utför. Många av anpassningarna till uthållighetsträning syftar till att förändra ämnesomsättningen i muskelfibrerna. Målet är att utvinna energi från näringsämnen på ett så effektivt sätt som möjligt.

Mitokondrier viktiga för ämnesomsättningen
En omställning som sker i muskelfibrerna efter regelbunden uthållighetsträning är att det tillverkas fler mitokondrier. Dessa är små strukturer inuti muskelfibern som bryter ned socker och fett med hjälp av inandat syre för att bilda användbar energi*. Av den anledningen brukar mitokondrierna kallas för ”cellens kraftverk”. Med fler mitokondrier förbättras muskelfibrernas ämnesomsättning och du kommer att orka arbeta längre vid nästa träningsspass.

* För illustrationer av mitokondriers utseende och deras placering i muskelfibrer, se Figur 1 respektive Figur 3 i denna avhandling.
DNA och proteiner i huvudrollerna

När nya mitokondrier ska bildas, eller befintliga byggs ut, krävs en mängd olika komponenter. De flesta är proteiner som sätts ihop enligt ritningar som finns i våra cellers DNA - ämnet som bygger upp arvsmassan. Var och en av de proteiner som finns i människans kropp har en särskild funktion. Olika proteiner ligger bakom exempelvis hormonsignalering, muskelsammandragning, blodets röda färgämne, nedbrytning av mat i magtarm-kanalen, mitokondriernas ämnesomsättning och mycket mer.

Varje protein har sin egen byggbeskrivning, sin egen gen, som utgörs av ett avsnitt i DNA-spiralen. När ett protein ska bildas, kopieras genen för att kunna användas vid de specialiserade tillverkningsstationerna som ligger på avstånd från arvsmassan. Likt analog fotografering tas en negativbild, ett mRNA, av genen och med denna som förlaga ”framkallas” därefter det färdiga proteinet.


Muskelprover från försökspersoner


Före och efter träningen togs muskelprover, biopsier, från larmuskulaturen hos deltagarna. Biopsierna, som var små som ärter, utforskades med en rad
laboratorieanalyser för att mäta vilka variabler som hade förändrats till följd av träningen. Bland annat tog jag reda på vi hur mycket av olika mRNA eller protein som bildats.

**Resultaten av studierna**

Jag upptäckte flera sorters mRNA för lipin-1 i muskelbiopsierna. Ingen av de varianter jag granskade verkade dock öka som svar på de träningstyper jag studerat. Mina resultat ligger inte i linje med vad som tidigare observerats i djurförsök, men mätningarna utsluter inte att lipin-1 kan vara en viktig del i anpassningen till regelbunden motion - och därmed för de hälsoeffekter en aktiv livsstil för med sig. Förekomsten av en särskild sorts mRNA indikerar att motsvarande protein bildas i lika stor utsträckning. Fler variabler påverkar emellertid hur mycket av mRNA som faktiskt översätts till protein, vilket ibland gör det svårt att dra slutsatser av endast mRNA-mätningar.

När det gäller de mitokondriella transkriptionsfaktorerna så fann jag att mRNA-nivåerna för faktorn TFAM ökade efter tio dagars träning. Elitidrottarna hade också större mängder TFAM-protein jämfört med normalt aktiva studiedeltagare. Det tyder på att TFAM sannolikt är viktig för att man ska kunna bibehålla fler och större mitokondrier i sina muskler.

För de övriga mitokondriella transkriptionsfaktorerna TFB1M och TFB2M var mängden mRNA större efter tio dagars träning samt högre hos elitidrottarna än de normalaktiva. Det var dock ingen skillnad i proteininnehåll mellan de två grupperna. Mer forskning behövs för att utröna vilken roll dessa faktorer spelar för den ökning av mitokondrier som triggas av träning.

**Nya behandlingar och träningsprogram**

I förlängningen kan kunskap från denna forskning bidra till utveckling av nya behandlingsmetoder för exempelvis typ 2-diabetiker och patienter som inte kan motionera till följd av rörelsehinder. Den vara också vara värdefull för idrottsutövare på alla nivåer då träningsprogram kan optimeras för att ge maximala resultat.
8 ACKNOWLEDGEMENTS

I wish to express my warm and sincere gratitude to all who have directly or indirectly contributed to this thesis. Especially, I would like to thank:

**Carl Johan Sundberg**, for all the encouragement, for generously sharing both your network and great knowledge of physiology, and for inspiring me to pursue my dreams. **Jessica Norrbom**, for taking me in as a master student, introducing me to mitochondrial biogenesis and for consistently giving me sound advice - both in the lab and in life. **Annika Wallberg**, for conveying your knowledge of molecular biology and giving me the opportunity to spend time in your lab.

All co-authors and collaborators for their essential contributions to the papers included in this thesis. All subjects for bravely participating in our studies.

The Molecular Exercise Physiology group - you make it a pleasure to come to work every day! My fellow ”angels”; **Maléne Lindholm** for keeping me sane and always being up for a good party, **Eva-karin Sällstedt** for your contagious determination and for reminding me of what is truly important in life, and **Mia Ydfors** for your support and amazing sense of humour. **Charlotte Schönbeck**, for being a good listener and for great assistance in the lab.

Past and present members of the ”Muscle Laboratory” for contributing to a fun and stimulating work environment; **Eva Jansson** and **Thomas Gustafsson** for sharing your vast knowledge in exercise physiology; **Heléne Fischer** for excellent laboratory guidance and for caring; **Anna Strömberg** my cochon d’Inde for supporting and trusting me; **Helene Rundqvist** for posing challenging questions; **Tommy Lundberg, Karl Olsson, Eric Rullman, Håkan Rundqvist, Rodrigo Fernandez Gonzalo, Mats Lilja, Barbara Norman, Helena Wallin, Maria Westerståhl, Anna Wiik, Seher Alam, Elisabeth Berg, Eva Brimark, Mona Esbjörnsson, Märit Karls, Majken Lidén, Andreas Montelius**.

Everyone at the Department of Physiology and Pharmacology, in particular; **Lars Karlsson** for adding extraordinary excitement; my unofficial mentor **Maria**
Ahlsén for inspiring me to believe that everything is possible; Ellinor Kenne for being a role model in science and teaching; the lovely senior scientists Lennart Lindbom, Bo Rydqvist, Håkan Westerblad, Mats Rundgren, Jan Henriksson, Lars Gustafsson, Abram Katz, Dag Linnarsson and Jan Lännergren for sharing your stories and knowledge, and for being so sweet; Johanna Christensen, Sofia Pettersson and Peter Wolf for magnificent teaching administration and many laughs; Marie-Louise Sundin for all your help with our courses; Stefan Eriksson for providing good working conditions; Iréne Lund for always lending me an ear; Robert Frithiof, Joel Rasmuson, Anne van der Does, Gustaf Wigerblad, Björn Schilström, Åsa Konradsson Geuken, Peter Lindholm, Lena Norrbrand, Micke Elm, Pierre Rotzius, Niklas Ivarsson, Johanna Lanner, Erik Lundeberg, Lubna Al-Khalili, Cecilia Lövdahl.

The IMM girls; Astrid for sharing your hopes and doubts and for being so vivacious, Rebecca for bioinformatic help and for cheering me on, and Erika for top notch fika. My biomedicine buddies; Elisabet and Emma, for sympathy during the rough bits of under- and postgraduate education, Pontus for embracing my silliness. My long-time friends; Anna and Lisa for making me want to reach my full potential, Simon for all your love and the stomach-cramp-laughs we have.

All members of the Lifvenforth, Wallman, Magnusson, Bergh, Strand and Appel families for great encouragement, especially; my sisters Pia and Jessica for support and for making me keep it real, and Kenth for being my biggest fan and for giving me pep-talks. Pappa, I miss you immensely. Thank you for helping me gain gumption, and for reading ahead in my books just to be involved in my schoolwork. Mamma, thank you for always being on my side and for your great love and care. I could not have achieved any of this without you.

Daniel, for never giving up on me, for your unconditional love and the life we share. Thank you for the consolatory braiding and for always making me smile❤

This work was financially supported by the Swedish National Centre for Research in Sports, Trygg-Hansa Foundation, Ake Wiberg Foundation, Magnus Bergvall Foundation, the Swedish Research Council and Karolinska Institutet.
9 REFERENCES


