GENE EXPRESSION IN HUMAN SKELETAL MUSCLE

Effects of activity, fibre type and inheritance for diabetes

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To Jonas, Lisa and Felix
ABSTRACT

One fundamental underlying risk factor for the development of non-insulin dependent (type 2) diabetes mellitus is insulin resistance, which 80-90% of all type 2 diabetic patients suffer from. Risk factors for insulin resistance are for example obesity, surgery, severe illness, spinal cord injury, and pregnancy. Too high energy intake and physical inactivity are direct causes of the increasing prevalence of overweight and obesity. Exercise prevents or delays the onset of type 2 diabetes. A series of beneficial effects of exercise are seen e.g. increased muscle mass, increased oxygen uptake, increased insulin sensitivity and glucose transport. Skeletal muscle represents 55% of the total body weight and is the major organ for insulin-stimulated glucose uptake. The increased uptake of glucose during exercise is mediated via alternative, insulin-independent mechanisms e.g. through activated protein kinase (AMPK).

A cohort of healthy glucose tolerant first-degree relatives of type 2 diabetic patients was studied to determine whether early metabolic and molecular defects contribute to insulin resistance in skeletal muscle. Skeletal muscle strips from relatives and control subjects were incubated in vitro in the absence or presence of increasing concentrations of insulin. Glucose transport, Akt phosphorylation, AS160 phosphorylation, and GLUT4 expression were assessed. Insulin-stimulated glucose transport at a maximal insulin concentration was reduced in the relatives. Insulin increased Akt and AS160 phosphorylation in a dose-dependent manner, with similar responses between the groups. Gene expression of some of the key transcriptional factors and co-regulators of mitochondrial biogenesis PGC-1α, PGC-1β, PPARδ, NRF-1 and UCP-3 was assessed. All these genes showed similar expression when corrected to either β-actin or GAPDH, indicating that disturbances in mitochondrial function do not precede development of insulin resistance and type 2 diabetes. PPARα, PPARδ, PGC-1α, PGC-1β, calcineurin Aα and calcineurin Aβ have also been implicated in the regulation of skeletal muscle fibre type distribution, as well as mitochondrial biogenesis. mRNA levels of these genes was determined in skeletal muscle biopsies from elite athletes with a significantly (P<0.05) higher portion of type I fibres compared to sedentary controls. mRNA expression of these genes was also assessed in spinal cord injured subjects who have lost almost all of their type I fibres. PPARα, PPARδ, PGC-1α, PGC-1β mRNA correlated to fibre types in skeletal muscle, whereas calcineurin mRNA expression was unrelated.

Finally, gene expression pattern of AMPK α, β and γ isoforms in skeletal muscle from able-bodied subjects and spinal cord injured individuals was established. The effect of an eight week training study was determined on gene expression in spinal cord injured subjects after cycling seven days a week. Expression of AMPK also differed depending on time since injury.

In summary, this thesis work has shown that exercise promotes a more oxidative phenotype in skeletal muscle, with increased glucose uptake. This is also seen in skeletal muscle that has been unused for several years. Exercised muscle, with a more oxidative phenotype has increased expression of several genes important for mitochondrial biogenesis and function. No alteration in mRNA expression of these genes is noted in skeletal muscle from subjects with a genetic predisposition for metabolic disease prior to onset of clinical symptoms.
LIST OF PUBLICATIONS

I. **Insulin signaling and glucose transport in skeletal muscle from first-degree relatives of type 2 diabetic patients.**
   *In press, Diabetes 2006*

II. **Transcription profiling of AMP-activated protein kinase isoforms in skeletal muscle of spinal cord injured human pre- and post-exercise training.**
    Maria Ahlsén, Nils Hjeltnes, Yun Chau Long, Anna Krook, Ulrika Widegren
    *Manuscript submitted*

III. **Human skeletal muscle fibre type variations correlate with PPARα, PPARδ, PGC-1α, and PGC-1β mRNA.**
     David Kitz Krämer, Maria Ahlsén, Jessica Norrbom, Eva Jansson, Nils Hjeltnes, Thomas Gustafsson, Anna Krook
     *Manuscript submitted*
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LIST OF ABBREVIATIONS

ACC  acetyl CoA carboxylase
AICAR  5-aminoimidazole-4-carboxamide ribonucleoside
AMP  Adenosine monophosphate
AMPK  AMP-activated protein kinase
AS160  Akt substrate 160 kDa
ATP  Adenosine triphosphate
BMI  Body mass index
CaMK  Calcium/calmodulin-dependent protein kinase
CBS  Cystathionine-β-synthase
cDNA  Complementary DNA
CPT  Carnitine palmitoyltransferase
DNA  2’-deoxy-5’-ribonucleic acid
ESLC  Electrically-stimulated leg cycling
GAPDH  Glyceraldehyde phosphate dehydrogenase
GLUT  Glucose transporter
GSK-3  Glycogen synthase kinase-3
GTP  Guanosine triphosphate
HOMA  Homeostasis model assessment
IGF  Insulin-like growth factor
IR  Insulin receptor
IRS  Insulin receptor substrates
MAPK  Mitogen activated protein kinase
MCD  Malonyl CoA decarboxylase
MHC  Myosine heavy chain
MODY  Mature onset diabetes of the young
NRF  Nuclear respiratory factor
OGTT  Oral glucose tolerance test
PCR  Polymerase chain reaction
PDK  Phosphoinositide-dependent protein kinase
PGC  Peroxisome proliferators-activated receptor γ coactivator
PI3-kinase  Phosphatidylinositol 3-kinase
PIP  Phosphatidylinositol phosphate
PKC  Protein kinase C
PPAR  Peroxisome proliferators-activated receptor
RNA  Ribonucleic acid
SCI  Spinal cord injury
SH2  Src-homology 2
UCP  Uncoupling protein
WHO  World Health Organisation
1 INTRODUCTION
Skeletal muscle accounts for about 55% of the total body mass, and plays an important role in whole body metabolism. It is the main tissue that contribute to systemic glucose clearance after a meal, in the postprandial state, in order to maintain normal glycaemia. During insulin-stimulated conditions \textit{in vivo}, skeletal muscle accounts for 80-90% of glucose uptake (DeFronzo \textit{et al}., 1981). Skeletal muscle is a highly adaptive tissue and responds to environmental changes such as use and disuse.

1.1 REGULATION OF GLUCOSE UPTAKE
To maintain health, blood glucose levels need to be tightly regulated around 5 mmol/l. Glucose homeostasis is a regulated balance between glucose uptake and clearance. In the postprandial state, glucose enters the circulation from the gastrointestinal tract. Between meals, in the post-absorptive state, glucose is released from the liver (and kidneys) by glycogenolysis and glucogenesis. Skeletal muscle does not break down glycogen and release glucose back to the blood stream, since the enzyme Glucose 6-phosphatase is not present in muscle cells. Glucose is cleared from the circulation mainly by skeletal muscle, but also in the splanchnic circulation and the adipose tissue.

In order for glucose to be metabolised, it 1) must be delivered to the organ, 2) transported through the plasma membrane, and 3) be phosphorylated to glucose 6-phosphate (Wasserman & Ayala, 2005). The process of glucose transport is the most studied of these three, and defects in glucose transport have been reported for example in skeletal muscle from people with type 2 diabetes (Cline \textit{et al}., 1999; Krook \textit{et al}., 2000).

1.1.1 Insulin and the insulin receptor
The hormone insulin regulates blood glucose by increasing tissue glucose uptake. Insulin is produced and secreted by the pancreas. The pancreas is a gland which is 1% endocrine, and 99% exocrine. The endocrine tissue, islets of Langerhans, contains four types of hormone-secreting cells. The \( \alpha \)-cells secrete glucagon, the \( \beta \)-cells secrete insulin, the \( \delta \)-cells secrete somatostatin, and the F-cells secrete pancreatic polypeptide.

The insulin receptor (IR) is present in all vertebrate tissues. The receptor concentration varies from as few as 40 receptors on erythrocytes in the circulation to more than 200,000 receptors on hepatocytes and adipocytes. The IR belongs to the superfamily of transmembrane receptor tyrosine kinases and is a heterotetrameric glycoprotein composed of two \( \alpha \)- and two \( \beta \)-subunits that are linked together by disulfide bonds (Kasuga \textit{et al}., 1982). The receptor is encoded by a gene with 22 exons and 21 introns, and occurs in two different isoforms due to alternative splicing of exon 11. The two isoforms of the IR differ slightly in affinity for insulin (De Meyts, 2004). Insulin-like growth factor (IGF) –I and –II also bind the IR, but with an affinity that is at least 100 times lower than that of insulin.

The \( \alpha \)-subunits, which contain the insulin binding sites, of the IR are located entirely outside the cell. The \( \beta \)-subunits, on the other hand, are transmembrane proteins and they contain the insulin-regulated tyrosine protein kinase. When insulin binds one of
the α-subunits, the β-subunits autophosphorylate and by that allow transmission to downstream events, including the insulin receptor substrates.

Besides signal transduction, the IR also mediates internalisation of insulin. Endocytosis of the insulin-receptor complex leads to insulin degradation by a specific protease, while most of the unoccupied receptors recycle to the plasma membrane. After prolonged insulin stimulation, the receptor itself is degraded, resulting in receptor down-regulation and a decreased insulin signal (Backer et al., 1990).

1.1.2 Insulin receptor signalling

There are several substrates to the insulin receptor. Insulin receptor substrates (IRS) 1 and 2 are the most predominant, and are involved in metabolic regulation in human skeletal muscle. Tyrosine-phosphorylated IRS-1 mediates the insulin signalling to downstream enzymes by binding to a number of src-homology 2 (SH2) domain-containing signalling proteins (White, 2003) (Figure 1).

Phosphatidylinositol 3 (PI3)-kinase is one important signalling intermediate in the insulin cascade (Shepherd et al., 1998). In skeletal muscle, the majority of insulin-stimulated PI 3-kinase activity is associated with IRS-1 (Henry et al., 1995; Jackson et al., 2000). PI 3-kinase is composed of one regulatory subunit, that binds IRS-1 (p85), and one catalytic subunit (p110) which phosphorylates phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2 or PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P_3 or PIP_3) (van der Kaay et al., 1997).

![Figure 1. Regulation of glucose uptake in skeletal muscle.](image-url)
One of the major ways the lipid products of PI 3-kinase act in insulin signalling is by binding to pleckstrin homology (PH) domains of phosphoinositide-dependent protein kinase (PDK) (Alessi et al., 1997). PDK activates protein kinase B (PKB/Akt) and atypical protein kinase C isoforms PKCζ and PKCλ (Bandyopadhyay et al., 1997).

The serine/threonine kinase Akt/PKB appears in three isoforms Akt 1,2, and 3 (PKBα, β, and γ). Akt 1/PKBα is the major isoform activated by insulin in skeletal muscle and liver (Walker et al., 1998). Akt is activated upon insulin-stimulation by phosphorylation on Thr^{308} and Ser^{473} (Alessi et al., 1996).

Activated Akt phosphorylates and inactivates the constitutively active serine/threonine kinase glycogen synthase kinase (GSK)-3 (Cross et al., 1995). The inactivation of GSK-3 will lead to an active form of the enzyme glycogen synthase, which acts in the cascade of glycogen synthesis.

Another downstream target of Akt is the Akt substrate AS160. It was found by the use of the phosho-Akt-substrate antibody. AS160 has been proposed to connect insulin signalling to membrane trafficking (Kane et al., 2002), and to be necessary for insulin-stimulated glucose uptake in adipocytes (Sano et al., 2003). AS160 contains a GTPase-activating domain for Rabs, which are small G proteins required for membrane trafficking (Kane et al., 2002).

### 1.1.3 Glucose transport

The initial rate-limiting step for glucose clearance in skeletal muscle and adipose tissues is the transport of glucose across the plasma membrane. This process is affected by facilitated diffusion of glucose through a family of specific glucose transporters (GLUT) that are either constitutively present in the plasma membrane, or actively translocated to the plasma membrane in response to various stimuli. There are 13 members of the GLUT-family (Joost et al., 2002). Skeletal muscle expresses GLUT1 and GLUT4 glucose transporters. GLUT4 is the main glucose carrier expressed in skeletal muscle, whereas GLUT1 accounts for only 5-10% of total glucose carriers (Marette et al., 1992). Under basal conditions, most of GLUT4 is found in intracellular membranes. Insulin or exercise can induce a rapid increase in glucose uptake by translocation of pre-existing GLUT4 from endosomal compartment(s) to surface membranes.

### 1.1.4 Insulin-independent glucose uptake

Glucose can be taken up by the skeletal muscle cell through GLUT4 translocation independently of insulin. This happens during exercise due to contraction of the muscle cell. Possible mechanisms of the exercise-mediated glucose uptake are through calcium and/or AMPK signalling.

It was suggested about 40 years ago that the enhanced permeability to glucose may be related to changes in the intracellular concentration of calcium (Holloszy & Narahara, 1967). The action potential generated from the motor nerve leads to increased calcium levels in the sarcoplasm. The increased calcium concentration triggers the contraction. The downstream events of the calcium signal have not been identified with certainty, but calcium/calmodulin-dependent protein kinase II, CaMKII, and αPKC have been suggested to play important roles (Richter et al., 2004).

AMPK is a protein kinase that has been suggested to play an important role in contraction-induced glucose uptake.
1.2 AMPK

AMPK is a heterotrimeric serine/threonine protein kinase that regulates cellular metabolism in response to alterations in the energy status of the cell. It is activated by AMP, as the name indicates, and also by pharmacologic agents such as 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), metformin, and the PPARγ agonist rosiglitazone.

AMPK is activated during states when the energy supply of the cell is diminished, therefore the activation will result in increased ATP production and decreased ATP consumption. Thus, AMPK is activated in skeletal muscle by exercise.

1.2.1 Structure

AMPK consists of a catalytic (α) subunit and two regulatory subunits (β and γ), all of which are necessary for full kinase activity. There are two isoforms of the α- (α1 and α2) and the β-subunits (β1 and β2), and three of the γ-subunit (γ1, γ2, and γ3) (Hardie et al., 1998; Kemp et al., 1999). The isoforms of all subunits are encoded by distinct genes, and give rise to 12 possible heterotrimeric combinations. Homologues of all three subunits have been found in every eukaryotic species.

The α subunits contain a protein kinase catalytic domain at the N-terminus and a C-terminal regulatory domain that is involved in binding to the β and γ subunits and also contains an auto-inhibitory region (Crute et al., 1998). The β subunits contain a glycogen-binding domain that is found in enzymes that metabolise starch and glycogen (Hudson et al., 2003; Polekhina et al., 2003), but the physiological role of this domain within the β subunits remains unclear. The β subunits also contain a C-terminal domain that is sufficient to bind to, and form complexes with, the α and γ subunits (Hudson et al., 2003). The γ subunits contain four domains known as cystathionine-β-synthase (CBS) domains. These domains usually occur in pairs, and it has been suggested that these tandem repeats of CBS domains be termed Bateman domains (Kemp, 2004).

Naturally occurring mutations in human γ2, and pig γ3 have been identified and these occur in or close to the Bateman domains (Carling, 2004). Mutations in γ2 cause cardiac hypertrophy associated with an increase in glycogen content, together with an arrhythmia, similar to the Wolff-Parkinson-White syndrome (Blair et al., 2001; Gollob et al., 2001; Arad et al., 2002). A missense mutation in pig γ3, altering an arginine at residue 225 to a glutamine (R225Q), causes a marked accumulation of glycogen in skeletal muscles (Estrade et al., 1993; Milan et al., 2000) and the activities of citrate synthase (CS) and hydroxyacyl-CoA dehydrogenase are increased (Estrade et al., 1994; Lebret et al., 1999), suggesting that the γ3 subunit can regulate metabolic properties of skeletal muscle. A transgenic mouse model with the same mutation, R225Q, as the pig has allowed to further investigate the effects of this activating mutation on skeletal muscle (Barnes et al., 2004). Glycogen resynthesis after exercise was markedly enhanced in the transgenic mutant mice model. When placed on a high fat diet, the mutant mice were protected against excessive triglyceride accumulation and insulin resistance in skeletal muscle. The mutation was also shown to be associated with higher basal AMPK activity and diminished AMP dependence.
The mRNA expression of the γ3 isoform is predominantly expressed in skeletal muscle, whereas γ1 and γ2 show a broader tissue distribution (Mahlapuu et al., 2004). In rat and mouse, γ3 is the predominant isoform in glycolytic skeletal muscle such as the gastrocnemius in mouse and white gastrocnemius and white quadriceps in rat. These skeletal muscle types consist of type II (glycolytic) fibres that depend on the anaerobic metabolism of glucose when there is a high demand for energy. The γ1 subunit was expressed to a higher extent than γ2 in human skeletal muscle. The highest human γ2 mRNA expression was found in heart. After precipitation with γ3 antibody, the γ3 isoform in skeletal muscle preferentially forms a ternary complex with α2 and β2 (Mahlapuu et al., 2004). This α2β2γ3 complex was found in human skeletal muscle as well, but α2β2γ1 was reported to be the most dominant AMPK complex (Wojtaszewski et al., 2005). The tissue distribution of the α and β subunits has not yet been reported.

The physiological factors that regulate the relative expression of AMPK isoforms in human skeletal muscle are currently undefined. Regular physical activity in the form of endurance training has been associated with reduced protein expression of the γ3 isoform and increased protein expression of α1, β2 and γ1 (Langfort et al., 2003; Nielsen et al., 2003; Frosig et al., 2004). Strength training results in similar changes; a higher protein expression of α1, β2 and γ1, whereas γ3 is reduced (Wojtaszewski et al., 2005).

1.2.2 Activation

AMPK is activated by increased intracellular concentrations of AMP. The AMP:ATP ratio is increased during contraction, ischemia, hypoxia, pharmacological inhibition of glycolysis and oxidative phosphorylation, and heat shock (Hardie et al., 1998).

AMP has three additional effects to activate the system, i.e. 1) activation of the upstream kinase. AMPK complexes are activated by phosphorylation at a specific threonine residue (Thr 172) on the α-subunit, catalyzed by an upstream kinase, LKB1. 2) binding of AMP alters AMPK configuration, making it a better substrate for LKB1, and finally 3) at the same time AMPK becomes a poor substrate for the protein phosphatase (Hardie, 2005).

Several studies in humans and rodents have shown that AMPK in skeletal muscle is activated during exercise in vivo (Winder & Hardie, 1996; Hutter et al., 1997; Fujii et al., 2000; Wojtaszewski et al., 2000), and by contraction in vitro (Hayashi et al., 1998; Ihlemann et al., 1999; Derave et al., 2000; Hayashi et al., 2000; Ihlemann et al., 2000). The different mechanisms by which AMPK has been shown to be activated by are decreased ATP/AMP and PCr/Cr ratios (Ponticos et al., 1998; Hardie et al., 1999), decreased pH (Ponticos et al., 1998), and reduction of muscle glycogen content (Derave et al., 2000; Wojtaszewski et al., 2002a) and substrate delivery (Moore et al., 1991; Itani et al., 2003; Kimura et al., 2003).

Apart from AMP, AMPK has also been shown to be activated by AICAR, which is taken up by the cell and is phosphorylated to ZMP (5-aminomidazole-4-carboxamide-1-D-ribofuranosyl-5'-monophosphate), which mimics the activating effect of AMP on AMPK (Corton et al., 1995; Hardie et al., 1998). This has been shown in rat, mouse, and human skeletal muscle in vitro (Merrill et al., 1997; Hayashi et al., 1998; Wojtaszewski et al., 2002a; Al-Khalili et al., 2003) and in vivo in conscious rats (Bergeron et al., 1999). Treatment with AICAR increases glucose uptake by an insulin-independent mechanism (Hayashi et al., 1998) that seems to
depend on translocation of GLUT4 to the skeletal muscle surface membrane (Kurth-Kraczek et al., 1999). AICAR stimulation of skeletal muscle is often used as a contraction model since it activates AMPK, but *in vitro* studies of isolated mouse skeletal muscle overexpressing a kinase dead AMPK construct showed that AICAR and hypoxia-stimulated glucose uptake was totally abolished, whereas contraction-stimulated glucose uptake was lowered by 30–40% (Mu et al., 2001). This indicates that there might be different pathways involved in the AICAR/hypoxia- and contraction-stimulated glucose transport, and that AMPK is only partially responsible for contraction-stimulated glucose transport in muscle. Apart from AMP and AICAR some pharmacological agents used for treating type 2 diabetes, such as metformin and rosiglitazone, have also been suggested to activate AMPK (Zhou et al., 2001; Fryer et al., 2002; Hawley et al., 2002; Musi et al., 2002).

Finally, Topiramate, a substance used for medicating epilepsy and migraine, has also recently been shown to stimulate glucose transport through AMPK in rat skeletal muscle, L6-cells (Ha et al., 2006).

### 1.2.3 Action

Activation of AMPK protects skeletal muscle against an energy deficiency by promoting increased glucose transport and fatty acid oxidation (Ihlemann et al., 1999; Hayashi et al., 2000; Bergeron et al., 2001). The increased glucose transport is achieved by translocation of GLUT4 to the cell surface (Kurth-Kraczek et al., 1999). The increased fatty acid oxidation is mediated through phosphorylation and inhibition of the downstream target of AMPK, acetyl CoA carboxylase (ACC) (Merrill et al., 1997). When ACC is inhibited, the intracellular concentrations of malonyl-CoA will decrease (Figure 2). AMPK may also stimulate malonyl CoA decarboxylase (MCD), the enzyme responsible for malonyl CoA degradation (Saha et al., 2000), and this would also decrease the malonyl-CoA concentrations. Malonyl CoA is an allosteric inhibitor of carnitine palmitoyltransferase (CPT)-1, the enzyme that controls the transfer of long-chain fatty acyl CoA into the mitochondria. The decreased in malonyl CoA will therefore lead to an increased activity of CPT-1, and subsequently an increased fatty acid transport into the mitochondria where they are oxidised.

![Figure 2. AMPK simulates fatty acid uptake into the mitochondria by inhibiting ACC, which causes decreased levels of Malonyl-CoA and decreased inhibition of CPT-1.](image)

With prolonged exercise, activation of AMPK is sustained, whereas ACC activation goes back to resting values (Wojtaszewski et al., 2002b). Consequently, the decreased malonyl-CoA concentrations can not fully explain the increased fatty acid oxidation that is seen upon AMPK activation. Long-term stimulation of fatty acid oxidation that occur in skeletal muscle in response to AMPK activation could be due to an up-
regulated levels of peroxisome proliferators-activated receptor (PPAR) α, as well as its coactivator PPARγ coactivator 1 (PGC-1), upon AICAR stimulation (Lee et al., 2006). This may explain the long-term stimulation of fatty acid oxidation that occurs in skeletal muscle in response to AMPK activation (Lee et al., 2006). It was also just recently suggested that the diminished concentration of malonyl CoA in skeletal muscle after physical training is most likely due to PGC1α mediated increases in MCD expression and activity. These changes persist after the increases in AMPK activity (Kuhl et al., 2006).

In addition to these metabolic actions, AMPK has recently been implicated in the control of transcription and gene expression. AICAR causes an increase in uncoupling protein 3 (UCP-3) mRNA and protein compared with untreated skeletal muscle (Zhou et al., 2000). UCP-3 is a member of the mitochondrial transporter superfamily, and expressed primarily in skeletal muscle, where it may play a role in altering metabolic function under conditions of fuel depletion caused, for example, by fasting and exercise. AICAR has also been shown to increase GLUT-4 mRNA levels in skeletal muscle (Zheng et al., 2001). Activation of AMPK has also been demonstrated to increase the expression of PGC-1α and CaMK IV (Zong et al., 2002). AMPK is involved in modulating key genes regulating lipid and glucose metabolism in skeletal muscle (Long et al., 2005).

1.3 MITOCHONDRIAL BIOGENESIS AND FUNCTION

Mitochondria are the main sites for generation of ATP by the cellular respiration. Active cells, such as skeletal muscle, have a large number of mitochondria. Mitochondria have their own DNA encoding 13 subunits of the oxidative phosphorylation system, the remaining subunits, as well as other mitochondrial proteins being encoded by the nucleus. For mitochondrial biogenesis in skeletal muscle to be induced, it is essential that genes from the nuclear and mitochondrial genomes be expressed in a coordinated fashion (Hood et al., 2000). Contractile activity-induced metabolic changes have been suggested as an important condition for induction of mitochondrial biogenesis through up-regulation of the co-activators PGC-1α and its downstream transcription factors nuclear respiratory factor (NRF) 1, NRF-2 and mitochondrial transcription factor A (TFAM), (reviewed by (Koulmann & Bigard, 2006). Factors affecting the skeletal muscle oxidative capacity will be further described in section 1.6.2.

Skeletal muscle is generally categorised into three classes of fibres (see section 1.5); slow-twitch type I fibres and two types of fast-twitch type II fibres. Each of these fibre types has varying mitochondrial content. In humans, the type I fibres have the largest fraction of mitochondria, which contributes to the oxidative capacity of these fibres (Spangenberg & Booth, 2003). Under conditions of skeletal muscle disuse (e.g. microgravity, denervation, immobilisation, sedentary lifestyle and aging), mitochondrial content is diminished (Ircher et al., 2003). Skeletal muscle is highly plastic and alters its gene expression profile and phenotype in response to changes in functional demand imposed by muscle use, disuse, and to developmental or neural influences.

Thus mitochondrial function is a key determinant of skeletal muscle metabolism, and therefore also affects whole body metabolism and substrate utilisation.
1.4 **INSULIN RESISTANCE AND DIABETES**

1.4.1 **Insulin resistance**

Insulin resistance is present when the biological effects of insulin are less than expected for glucose disposal in skeletal muscle and suppression of endogenous glucose production primarily in the liver (Dinneen *et al.*, 1992). The mechanism by which skeletal muscle becomes insulin resistant is unclear, but there is a strong correlation between increased levels of plasma free fatty acids and intra-muscular fatty acid metabolites (long-chain acyl-CoA, diacylglycerol, and triglycerides) and insulin sensitivity (McGarry, 2002). Factors related to signalling events downstream the insulin receptor include tumour necrosis factor \(\alpha\) (TNF\(\alpha\), which has been suggested to reduce the protein expression of the insulin receptor, IRS-1 and GLUT4. Dysregulation of PKCs has been linked to IRS-1 phosphorylation of Ser 612 which also inhibits the signalling (reviewed by (Bjornholm & Zierath, 2005).

There are different models to assess insulin sensitivity in humans, e.g. the homeostasis model assessment (HOMA), oral glucose tolerance test (OGTT), and *in vivo* euglycemic-hyperinsulinemic clamp methods.

- The homeostasis model assessment (HOMA) technique is a method for assessing \(\beta\)-cell function and insulin resistance from basal glucose and insulin or C-peptide concentrations. It was first described in 1985 (Matthews *et al.*, 1985), and has been widely used since then.
- In the oral glucose tolerance test (OGTT) the patient, after fasting for 15 hours, drinks 75g of glucose in 300 ml of water, over 5 minutes. Blood glucose is measured before the drink and after 30, 60, 90 and 120 minutes.
- Euglycaemic hyperinsulinemic clamp measures the tissue sensitivity to glucose. The technique was described in 1979 (DeFronzo *et al.*, 1979).

1.4.2 **Diabetes**

According to the Swedish National Board of Health and Welfare 3-4%, i.e. about 300,000 individuals of the Swedish population have diabetes. The life time risk of getting the disease at some point is about 15%. Around 20% of men and women over the age of 80 have diabetes.

There are four different kinds of diabetes;

- Type 1, autoimmune and non-autoimmune, with beta-cell destruction
- Type 2 with varying degrees of insulin resistance and insulin hyposecretion, 85-90% of all diabetes
- Gestational Diabetes Mellitus (GDM)
- Other Types where the cause is known (e.g. mature onset diabetes of the young (MODY), endocrinopathies)
Diabetes is a life-long condition. If left untreated, diabetes can lead to severe medical complications, such as heart disease, stroke, kidney disease, blindness, and nerve damage. Maintaining glucose homeostasis prevents the development of these complications.

The diagnosis diabetes mellitus as defined by the Swedish National Board of Health and Welfare is based on WHO’s outline (Alberti & Zimmet, 1998), and is evident by a fasting blood glucose $\geq 6.1$ mmol/l measured at two different occasions, or one randomly taken test showing a blood glucose $\geq 11$ mmol/l (Table 1). The latter test combined with symptoms of hyperglycaemia is sufficient for diagnosis.

<table>
<thead>
<tr>
<th>Blood/Plasma-glucose (mmol/l)</th>
<th>Diabetes mellitus</th>
<th>Decreased glucose tolerance</th>
</tr>
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<tbody>
<tr>
<td><strong>Fasting</strong></td>
<td></td>
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<tr>
<td>Capillary whole blood</td>
<td>$\geq 6.1$</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>$\geq 7.0$</td>
<td></td>
</tr>
<tr>
<td>Venous whole blood</td>
<td>$\geq 6.1$</td>
<td></td>
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<tr>
<td><strong>2-hour value, OGTT</strong></td>
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<td></td>
</tr>
<tr>
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<td>$\geq 11.1$</td>
<td>7.8-11.0</td>
</tr>
<tr>
<td>Plasma</td>
<td>$\geq 11.1$</td>
<td>7.8-11.0</td>
</tr>
<tr>
<td>Venous whole blood</td>
<td>$\geq 10.0$</td>
<td>6.7-9.9</td>
</tr>
</tbody>
</table>

Table 1. Blood/Plasma-glucose levels for diabetes diagnosis in Sweden
(http://www.sos.se/fulltext/9900-061/9900-061.htm#Diabetes)

1.4.3 Type 2 diabetes

Non-insulin-dependent (type 2) diabetes mellitus in humans is characterized by defects in insulin action and insulin secretion. It has been difficult to identify a single molecular abnormality underlying these features. In a pre-diabetic state, insulin resistance often exists in the insulin responsive peripheral tissues, namely skeletal muscle and adipocytes. This leads to an impaired glucose tolerance. In an initial period of pre-diabetes, the pancreatic $\beta$-cells have the ability to compensate for peripheral insulin resistance by secreting more insulin, which results in hyperinsulinaemia. In a later stage of the disease, hepatic insulin resistance will be followed by hyperglycaemia since the liver will release glucose despite the high insulin levels. The constant increased blood glucose levels will by time lead to $\beta$-cell failure, because of glucose toxicity (Stumvoll et al., 2005).

Skeletal muscle is quantitatively the most important tissue involved in maintaining glucose homeostasis under insulin-stimulated conditions. Identification of the molecular mechanisms in skeletal muscle that contribute to type 2 diabetes
pathogenesis may prevent the development of the complications that occur in uncontrolled glucose homeostasis.

A number of signalling defects have been observed in skeletal muscle from type 2 diabetes patients (Leng et al., 2004). One example is postreceptor signal transduction via IRS-1/PI 3-kinase, which has been shown to be markedly impaired, as well as a reduced glucose transport, whereas phosphorylation of the insulin receptor and MAP kinase was intact (Krook et al., 2000). Impaired insulin action on AS160 was associated with reduced Akt Thr\textsuperscript{308} phosphorylation, but unchanged Ser\textsuperscript{473}, suggesting that AS160 and Akt phosphorylation on Thr\textsuperscript{308} are linked (Karlsson et al., 2005). Activation of atypical PKCs is defective in skeletal muscle from Type 2 diabetic patients (Beeson et al., 2003). These signalling defects are correlated with decreased glucose transport and cell-surface GLUT4 content (Koistinen et al., 2003). However, whether these defects are causing type 2 diabetes, or are a result of the disease, is unknown.

The activity of AMPK and protein expression of AMPK isoforms do not differ between patients with type 2 diabetes and controls, which might exclude skeletal muscle AMPK as a cause of type 2 diabetes (Koistinen et al., 2003; Wojtaszewski et al., 2005).

### 1.4.4 First-degree relatives to subjects with type 2 diabetes

First-degree relatives of patients with type 2 diabetes are one group of subjects who have an increased risk of developing the disease. The genetic definition of a first degree relative is that 50% of the genetic material is shared (i.e. a parent or sibling is a first degree relative). Dependent on the genetic burden of type 2 diabetic relatives, the lifetime risk of developing type 2 diabetes is 40-80% (Martin et al., 1992). Healthy and normal glucose tolerant first-degree relatives of people with type 2 diabetes show a range of metabolic abnormalities (Vaag et al., 1992; Rothman et al., 1995; Perseghin et al., 1997). As metabolic disturbances due to glucose toxicity are not apparent in these subjects, potential primary steps in the development of type 2 diabetes can be identified through investigations on these participants.

Alterations in insulin signalling at the level of IRS-1, PI 3-kinase, and Akt have been observed in skeletal muscle from glucose intolerant first-degree relatives of type 2 diabetic patients, concomitant with impaired whole body glucose uptake and metabolism (Storgaard et al., 2001).

### 1.5 SKELETAL MUSCLE

As mentioned in section 1.3, skeletal muscle consists of different fibre types that have been classified depending on functional, biochemical, and morphological properties. Skeletal muscle fibres have a high degree of plasticity and can alter their properties in response to changed conditions and demands, for example denervation or exercise.

Muscle fibres can be divided into groups and classified in several different ways (Table 2). The first classifications were made upon the colour of the muscle fibres, red and white.

Another conventional and commonly used way to divide skeletal muscle fibres between fast and slow twitch properties is by histochemical stains indicating myosin ATPase (mATPase). The activity of mATPase is correlated to the contractile
properties of a muscle fibre (Barany, 1967). Based on the mATPase staining, skeletal muscle fibre types were divided into type I and II fibres, where the type II fibres had a 2-3 fold higher activity than the type I fibres. This indicated a faster contraction speed in type II fibres. The type II fibres were later subdivided into IIA and IIB, and the intermediate IIC. These subgroups are identified upon their acid and alkali stability. The nomenclature I, IIC, IIA, and IIB directly corresponds to the more physiological classification ST (slow twitch), FT (fast twitch)c, FTa, and FTb.

Humans have a very small proportion of the IIC fibres.

A second commonly used system for classifying skeletal muscle fibres is through the identification of myosin heavy chain (MHC) isoforms by immunohistochemistry or electrophoretic separation. Ten different MHC isoforms have been identified in human skeletal muscle, but only three pure forms: I, IIA, IIX, also called IID. IIB which are found in small adult animals are not expressed in human skeletal muscle (Pette et al., 1999).

Type I fibres have a high oxidative capacity, including high volume of mitochondria, high levels of myoglobin, and high capillary densities, compared to the more glycolytic fast-twitch type IIB fibres, which fatigue faster during repetitive contraction.

Metabolic and functional properties (i.e. speed of contraction) are closely coupled to the skeletal muscle fibre type profile (Schiaffino & Serrano, 2002; Spangenburg & Booth, 2003). Insulin-stimulated glucose transport is greater in slow-twitch compared to fast-twitch skeletal muscle fibres in humans (Henriksen et al., 1990; Daugaard et al., 2000) and rats (Song et al., 1999). Moreover, insulin sensitivity is positively correlated with the proportion of slow twitch oxidative fibres in humans (Lillioja et al., 1987).

In humans, most muscles have a fairly equal occurrence of the two main fibre types, slow type I fibres and fast type II fibres. This is in contrast to for example rodents that show whole muscles consisting of one fibre type. The human individual variation is known to be related to genetic predisposition, level and type of physical activity, age and gender (Komi et al., 1977a; Komi et al., 1977b; Bouchard et al., 1986; Simoneau

<table>
<thead>
<tr>
<th>Fibre type</th>
<th>Slow-twitch</th>
<th>Fast-twitch</th>
<th>Fast-twitch</th>
<th>Fast-twitch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contractile speed</td>
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<td>Fast-twitch</td>
<td>Fast-twitch</td>
<td>Fast-twitch</td>
</tr>
<tr>
<td>mATPase</td>
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<td>IIA</td>
<td>IIB</td>
<td>IIB</td>
</tr>
<tr>
<td>Myosin heavy chain</td>
<td>I</td>
<td>IIA</td>
<td>IIX/IID</td>
<td>IIB</td>
</tr>
<tr>
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<td>Oxidative</td>
<td>Oxidative</td>
<td>Glycolytic</td>
<td>Glycolytic</td>
</tr>
<tr>
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<td>Red</td>
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<td>White</td>
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<tr>
<td>Fibre cross sec. area</td>
<td>Small</td>
<td>Medium</td>
<td>Large</td>
<td>Large</td>
</tr>
</tbody>
</table>

**Table 2. Classification of human skeletal muscle fibre types.** Modified from Spangenburg and Booth (Spangenburg & Booth, 2003)
& Bouchard, 1989; Monemi et al., 1999). Insulin resistant type 2 diabetic patients, morbidly obese insulin resistant subjects, and first-degree relatives of patients with type 2 diabetes have a reduced percentage of type I and an elevated percentage of type II fibres, particularly type IIB-fibres, compared to insulin sensitive subjects (Marin et al., 1994; Nyholm et al., 1997). However, it is difficult to distinguish whether the differences in fibre types in the first-degree relatives are effects of reduced physical activity level and fitness in the studied subjects, since a newer study has not shown the same result (Ostergard et al., 2005).

1.6 MUSCLE DISUSE AND EXERCISE

Since skeletal muscle constitutes such a big portion of the total body mass, inactivity as a consequence of spinal cord injury, will result in great metabolic changes. Conversely, exercise, results in a series of beneficial effects on the whole-body level and directly within the skeletal muscle such as changes in gene expression.

1.6.1 Spinal cord injury

Spinal cord injury is associated with reduced skeletal muscle mass and metabolic capacity. This leads to impaired glucose homeostasis and increased the risk for type 2 diabetes mellitus and cardiovascular diseases (Yekutiel et al., 1989). Spinal cord injury leads to varying degrees of physical inactivity depending on the medullar level and extension lesion (Van Loan et al., 1987). The immobilization associated with spinal cord injury causes profound changes in skeletal muscle metabolism and morphology below injury level including insulin resistance, reductions in fibre size and capillary density, increased connective tissue (Kannus P., 1992; Aksnes et al., 1996) and decreased oxidative type I muscle fibres (Grimby et al., 1976; Lotta et al., 1991; Aksnes et al., 1996). After a short term paraplegia (1-6 months), atrophy of IIA fibres were observed and after a longer period of time, 8-10 months, atrophy and reduction of type I fibres were seen (Lotta et al., 1991). Reduced fibre area (i.e. fibre size), increased percentage of type IIB fibres, and a dramatically reduced or even undetectable portion of type I fibres has also been observed (Aksnes et al., 1996).

1.6.2 Effects of exercise

Regular physical activity and consequently exercise training is considered to lead to marked improvement in whole-body insulin sensitivity (Lindgarde et al., 1983; Dela et al., 1994). In addition, it is suggested that strength training leads to improved insulin sensitivity and insulin action, most likely because of an increase in skeletal muscle mass (Castaneda et al., 2002; Holten et al., 2004). These improvements have been observed in healthy subjects and type 2 diabetic patients, and they are associated with changes in protein expression of members in the insulin signalling cascade, as well as proteins involved in the process of glucose uptake and storage in skeletal muscle (Dela et al., 1993; Hughes et al., 1993; Dela et al., 1994; Vukovich et al., 1996).

The effects of exercise have also been studied in skeletal muscle from spinal cord injured subjects. After electrically-stimulated leg cycling, skeletal muscle mass of the exercised legs is increased (Hjeltnes et al., 1997), as is whole body insulin-stimulated glucose uptake, and expression of proteins involved in regulation of glucose
metabolism such as GLUT4, glycogen synthase and hexokinase II (Hjeltnes et al., 1998).

AMPK has been suggested to be involved in the molecular mechanisms behind many of the beneficial effects of exercise. Apart from activation of AMPK, endurance training is associated with increases in PPARα, and PGC-1 expression (Baar, 2004). PGC-1 is expressed in several tissues, including skeletal muscle and brown adipose tissue. It has been reported to increase oxidative metabolism, mitochondria biogenesis, and basal and insulin-stimulated glucose uptake (Puigserver et al., 1998; Wu et al., 1999; Vega et al., 2000; Michael et al., 2001; Hara et al., 2002). PGC-1 consists of two isoforms, α and β, which are coactivators involved in the regulation of mitochondrial metabolism, maintenance of glucose and lipid metabolism, and energy homeostasis (Lin et al., 2005). Because of the important role in the control of energy metabolism and insulin sensitivity, PGC-1 and PPAR isoforms are candidate factors in the cause of type 2 diabetes (Hara et al., 2002; Muoio et al., 2002). PGC-1 mRNA and protein levels have been reported to increase after a single bout of exercise in rats (Baar et al., 2002; Terada et al., 2002), and also after several days of training (Goto et al., 2000). In humans, an increase in PGC-1 mRNA levels have been observed a short time after a single bout of exercise in humans (Pilegaard et al., 2003). After 4 weeks of leg-extension training, when steady-state was reached, no significant differences were shown. Furthermore PGC-1 mRNA levels were unaltered after 9 days of cycling training in human (Tunstall et al., 2002).

PGC-1α and β are coactivators of the NRF-1 and -2. NRFs are transcription factors that act on nuclear genes and regulate the transcription and replication of mitochondrial DNA. Similar to PGC-1, NRF expression is reduced in humans with type 2 diabetes (Patti et al., 2003), and a defect in these genes encoding proteins important for oxidative phosphorylation may contribute to the development of insulin resistance and type 2 diabetes.

The PPARs are members of the large family of nuclear hormone receptors, and consists of three isoforms, α, δ, and γ. The biological activity of each PPAR isoform is regulated by the availability of natural ligands, but also by the recruitment of coactivators and repressors, and phosphorylation. PPAR isoforms are implicated in the transcriptional regulation of genes essential for lipid homeostasis (Chawla et al., 2001; Berger & Moller, 2002). PPAR α and δ have been reported to increase the expression of genes involved in lipid uptake, inter-organ lipid transport and fatty acid metabolism, while PPARγ has been mainly implicated in the control of genes involved in glucose metabolism.

Apart from positive metabolic changes, exercise has also been shown to influence the skeletal muscle phenotype. These phenotypic changes include enhanced mitochondrial biogenesis and enhanced angiogenesis. The mitochondria content and oxidative capacity are the highest in the slow oxidative type I, lower in the fast oxidative-glycolytic type IIA, and lowest in the fast glycolytic type IIX fibres. The opposite is observed for the glycolytic capacity of these fibres. In mouse skeletal muscle, PGC-1 protein content is higher in type I–rich, as compared with type II–rich fibres (Lin et al., 2002). PGC-1 has also been implicated in the formation of oxidative skeletal muscle type I fibres in mice (Lin et al., 2002). In engineered mice with targeted expression of an activated form of PPARδ an increased number of type I fibres were observed (Wang et al., 2004). These mice could run up to twice the distance of a wild-type littermate. Another mouse model that showed an increase in type I fibres are transgenic mice expressing an activated calcineurin (Naya et al.,
2000), suggesting that also this protein is important for fibre-type regulation in rodents.
2 AIMS

The overall aim of this thesis is to investigate the mechanism by which expression of key genes involved in mitochondrial function in human skeletal muscle are influenced by changes in activity (extreme training or spinal cord injury), and inheritance for type 2 diabetes. Whether defects in insulin action and gene expression precede the development of metabolic disease was also investigated.

The specific aims of this thesis are:

- To determine glucose uptake, insulin signalling, GLUT4 expression, and gene expression of PGC-1α, PGC-1β, PPARδ, NRF-1 and UCP-3 in first degree relatives to subjects with type 2 diabetes compared to control subjects.

- To determine the mRNA expression of different AMPK isoforms in human skeletal muscle, and to determine whether this is altered in skeletal from spinal cord injured subjects, in order to characterize the influence of nerve activity on AMPK expression. Furthermore, the effect of exercise training on the mRNA expression of AMPK isoforms was assessed in individuals with spinal cord injury.

- To couple the physiological and pathological variations in fibre type in response to activity or inactivity, respectively with genes implicated in signalling network controlling skeletal muscle fibre type transformation and mitochondrial biogenesis.
3 MATERIALS AND METHODS

3.1 SUBJECTS
The study protocols were approved by the Regional Ethical Committee at Karolinska Institutet and the Regional Committee for Medical Ethics at Helseregion East, Norway, and informed consent was received from all subjects before participation. The studies were conducted according to the principles expressed in the Declaration of Helsinki. None of the study participants were tobacco users. None of the able-bodied subjects were taking any medications.

The first-degree relatives were instructed to avoid strenuous exercise for 72 h, and spinal cord injured subjects 48 h, before the experimental day. The elite athletes were studied at least 24 hours after exercise training, in order to avoid acute effects of the last exercise bout. However, as the normal routine for these subjects is daily training, a more prolonged rest may induce changes that would not reflect the skeletal muscle profile actually seen in these individuals.

The relatives and control subjects in Study I were well matched as well as the elite athletes and the controls in Study III. The spinal cord injured subjects and the control subjects in Study II were not matched for age and BMI (Table 4).

One control subject in Study I had a grandfather with type 2 diabetes.

3.2 ELECTRICALLY STIMULATED LEG CYCLING (ESLC)
Electrically stimulated leg cycling (ESLC) is a means of artificial stimulation of denervated skeletal muscle in order to force contraction. Before each exercise session, surface electrodes were placed on the skin over the motor neuron end plates (motor points) of the quadriceps, hamstrings, and gluteal muscle groups of both legs of the subjects. Three electrodes (2 active and 1 reference) were applied over each muscle group. Six separate channels for sequential surface stimulation were used during ergometry with a computer-controlled closed-loop system. Each channel supplied monophasic rectangular pulses lasting 350 μs and delivered at 30 Hz to each of the two active electrodes. Stimulation intensities ranged from the preset threshold level, determined for each individual muscle group to elicit a palpable contraction (28-44 mA in range), up to a maximum of 130 mA. A pedal position sensor, allowing continuous calculation of velocity, was used by the computer to control the instantaneous stimulus amplitude required for each of the six muscle groups to maintain a smooth motion and a constant cranking frequency of 50 revolutions/min (Hjeltnes et al., 1997).

The tetraplegic subjects were placed in a sitting position and secured with a seat belt around the hips to the ergometer. Each bout of exercise started with 1 min of passive warm-up and 1 min of ramping up to the preset target load. Thereafter, full resistance was administered until fatigue or for 4 min during the graded exercise. During the prolonged continuous exercise, full resistance was administered until fatigue or for 30 min. Fatigue was defined as occurring when revolutions per minute fell below 35 in the face of maximal stimulation intensity (130 mA). Electrical stimulation was automatically stopped at fatigue.

During the training sessions, the ergometer power output was set at a level low enough for the subjects to ideally work for 30 minutes in one bout. However, if
fatigue occurred, up to five exercise bouts interspaced with pauses of five minutes each, were performed to achieve a total of 30 minutes of exercise. When 30 minutes of continuous exercise was achieved at 6 watts, subsequent sessions were conducted at a power output (PO) of additional 6.1 watts. When 30 minutes of continuous pedalling at this higher PO was achieved, the PO was further increased by 6.1 watts for subsequent sessions.

The training period consisted of seven exercise sessions per week; one session per day for three days and two sessions per day for two days. The study period included a 2-wk “run-in” period for adaptation to the electro stimulation equipment and procedure and an 8-wk “full” training phase. The training bouts were carried out on a computer-controlled electrical stimulation exercise ergometer (ERGYS-I-Clinical Rehabilitation System, Therapeutic Alliances, Fairborn, OH, USA). All ESLC sessions were supervised by a physician and a physiotherapist. No ESLC bouts were performed 48 h before muscle biopsies were obtained.

3.3 BIOPSIES

3.3.1 Needle biopsies
Muscle biopsies, 100-150 mg, used in study II and III were obtained, as described previously (Zierath et al., 1996; Hjeltnes et al., 1998), under local anaesthesia from the vastus lateralis portion of the quadriceps femoris muscle and immediately frozen in liquid nitrogen. All investigations were undertaken in the morning after an overnight fast.

3.3.2 Open biopsies
Open biopsies of about 1 g, used in Study I were taken from vastus lateralis muscle under local anaesthesia (mepivakain chloride 5 mg/ml). Briefly, a 4 cm incision was made 15 cm above the proximal border of patella, and the muscle fascia was exposed. A smaller muscle biopsy was also removed from the incision site using a Weil-Blakesley conchotome and immediately frozen and stored in liquid nitrogen for subsequent mRNA analysis (Study I). Thereafter 4-5 muscle fibre bundles were excised and placed in oxygenated Krebs-Henseleit buffer (KHB), which contained 5 mM HEPES, 5 mM glucose, 15 mM mannitol and 0.1% bovine serum albumin (BSA) (RIA Grade; Sigma, St. Louis, MO). Smaller skeletal muscle strips were dissected from the muscle biopsy specimen, mounted on Plexiglass clamps, and incubated in vitro in pre-gassed (95% O₂ and 5% CO₂) KHB in shaking water bath at 35°C. The gas phase in the vials was maintained during the incubation procedure. After a 30 min incubation in KHB, skeletal muscle strips were incubated for 30 min at 35°C in KHB without (basal) or with increasing concentrations of insulin (0.6 nM, 1.2 nM, and 120 nM). The concentrations of insulin were maintained throughout the incubation procedures.

3.3.3 Fibre typing
Serial transverse sections (10 mm) were cut with a microtome at -20°C and stained for myofibrillar adenosinetriphosphatase (ATPase) activity. The sections were preincubated at different pH values in acid (pH 4.3. or 4.6) or alkaline (pH 10.3)
buffers. The fibres were typed as type I or type II (subgrouped IIA –IIC) based on the myofibrillar ATPase staining characteristics (Brooke & Kaiser, 1970)

### 3.4 GLUCOSE TRANSPORT

Skeletal muscle strips were transferred to fresh KHB containing 20 mM mannitol and incubated at 35°C for 10 min. Thereafter muscles were incubated 20 min in KHB containing 5 mM 3-O-methyl [3H] glucose (800 μCi/mmol) and 15 mM [14C]mannitol (53 μCi/mmol). Thus, muscle strips were exposed to insulin for a total of 60 min. After the incubation, the muscle strips were blotted of excess fluid, snap frozen in liquid nitrogen and stored at –80°C until analysis. Glucose transport was analysed by the accumulation of intracellular 3-O-methyl [3H] glucose, as previously described (Wallberg-Henriksson et al., 1987).

### 3.5 mRNA AND PROTEIN EXTRACTION

#### 3.5.1 mRNA isolation

Portions of the skeletal muscle biopsies (15-35 mg) were homogenised using a polytron mixer. Total RNA was purified using TRIzol Reagent (Invitrogen, Carlsbad, CA) in Study I, and Tri-Reagent (Sigma, St. Louis, MO, USA) in Study II and III. 2-3 μg of RNA was used as template for subsequent cDNA synthesis in the reverse transcription reaction, using Promega Reverse Transcription System (Promega, Madison, WI) in Study II and III. DNA-free kit (Ambion, Austin, TX) and SuperScript First Strand Synthesis System (Invitrogen, Carlsbad, CA) were used in study I. The reaction volume was 20-μl in all studies. After synthesis was complete, cDNA was diluted and stored in aliquots at –20°C.

#### 3.5.2 Protein extraction

Muscle strips (10-20 mg) used for glucose transport analysis in Study I, were freeze-dried overnight and subsequently dissected under a microscope to remove visible blood, fat and connective tissue. Muscles were homogenized in ice-cold homogenization buffer (90 μl/μg dry weight muscle) (20 mM Tris (pH 7.8), 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1 % Triton X-100, 10 % (w/v) glycerol, 10 mM NaF, 0.5 mM NaVO4, 1 μg/ml leupeptin, 0.2 mM phenylmethyl sulfonyl fluoride, 1 μg/ml aprotinin, and 1 μM microcystin). Homogenates were rotated for 30 min at 4°C. Samples were subjected to centrifugation (12 000 g for 15 min at 4°C), and protein concentration was determined in the supernatant using a BCA protein assay (Pierce, Rockford, IL). An aliquot of the homogenate was mixed with Laemmli buffer containing β-mercaptoethanol and heated (60°C) for 30 min.

After mRNA isolation in Study I, protein was isolated according to manufacturers instructions (TRIzol Reagent, Invitrogen). The isolated protein was mixed with Laemmli buffer treated as above.

### 3.6 WESTERN IMMUNOBLOT ANALYSIS

Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and blocked with Tris-buffered saline with 0.02 % Tween (TBST) containing 5% milk for
Membranes were incubated overnight with either anti-phospho-Akt (Ser473) (catalogue #9271), anti-phospho-Akt (Thr308) (catalogue #9275), anti-phospho-(Ser/Thr) Akt substrate (PAS) (catalogue # 9611, Cell Signaling Technology, Beverly, MA) or anti-PGC-1 (catalogue #AB3242, Chemicon International) at 4°C or for 1.5 h with anti-GLUT4 (Geoffrey D Holman, University of Bath, UK) at room temperature. Membranes were washed in TBS-T and incubated with appropriate secondary HRP-conjugated antibodies (Bio-Rad, Richmond, CA). Immunoreactive proteins were visualised by enhanced chemiluminescence (ECL plus; Amersham, Arlington Heights, IL) and quantified by densitometry using Molecular Analyst Software (Bio-Rad).

3.7 REAL TIME PCR

Real-time Polymerase Chain Reaction (PCR) monitors the progress of the PCR as it occurs i.e. in real time. Data is collected throughout the PCR process, rather than at the end of the PCR.

3.7.1 TaqMan Sequence Chemistry

An oligonucleotide probe is constructed containing a reporter fluorescent dye on the 5’ end and a quencher dye on the 3’ end. While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET) through space.

If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5’ nuclease activity of Taq DNA polymerase as this primer is extended.

This cleavage of the probe separates the reporter dye from the quencher dye, increasing the reporter dye signal. It also removes the probe from the target strand, slowing primer extension to continue to the end of the template strand. Thus, inclusion of the probe does not inhibit the overall PCR process.

Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced.

3.7.2 PCR cycling conditions

Real-time PCR (ABI-PRISMA 7000 Sequence Detector, Perkin-Elmer Applied Biosystems, Foster City, CA) was used for quantification of specific mRNA. Data was collected and analysed by ABI Prism 7000 SDS Software version 1.1. All reactions were performed in 96-well MicroAmp Optical plates with a sample volume of 25 µl. Amplification mixes (25 µl) contained the diluted cDNA sample, 2xTaqMan Universal PCR Mastermix, forward and reverse primers, and probe for the gene of interest. Thermal cycling conditions included 2 min at 50°C and 10 minutes at 95°C before the onset of the PCR cycles, which consisted of 40 cycles at 95°C for 15 seconds and 65°C for 1 min. Assay numbers of primers and probes for the target genes are presented in Table 3.
3.7.3 Endogenous controls

An endogenous control, housekeeping gene, must be used to correct for loading errors, and deviations in concentration measurements. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as housekeeping genes in Study I. Correction against β-actin did not influence the results. In Study II, the expression of the AMPK isoforms was corrected to β-actin. Expression of GAPDH and β-actin was similar. mRNA expression of human β-actin, ribosomal 18s and GAPDH was assessed in Study III. There was a non-significant trend for an increase in β-actin mRNA expression with training, as reported in previous studies (Mahoney et al., 2004). Expression of GAPDH was unchanged with training status and was therefore selected as an endogenous control for Study III. Assay numbers of primers and probes for the endogenous controls are presented in Table 3.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Assay no</th>
<th>dye</th>
<th>quencher</th>
<th>Study</th>
</tr>
</thead>
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<td>MGB</td>
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<td>VIC</td>
<td>MGB</td>
<td>I, II, III</td>
</tr>
</tbody>
</table>

* PGC-1α designed sequences

<table>
<thead>
<tr>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>AGTCACCAATGACCCCAAGGGTTCC (probe)</td>
</tr>
<tr>
<td>CCAAAACAACTTTTATCTCCTTCC (forward)</td>
</tr>
<tr>
<td>CACACTTGAAGGCGTGTTCAATAGTC (reverse)</td>
</tr>
</tbody>
</table>

Table 3. Primers and probes purchased as Assays-on-demand® from Applied Biosystems, and sequences of designed primers and probes for PGC-1α.

3.7.4 Calculations

In Study II, target gene and β-actin were amplified in multiplex reactions. Multiplex is when e.g. a target gene and a housekeeping gene is analysed at the same time in one well. The primers are in this case labelled with different fluorescent dyes. The multiplex reaction is used in order to save cDNA material. Before running the samples in a multiplex reaction it must be elucidated that there is no cross-reactivity
between the primers and probes for the different genes. Singleplex reactions were used in Study I and III, in duplicates or triplicates. Singleplex is when the expression of only one gene is analysed in each well.

The quantity of cDNA/mRNA was calculated using the comparative C_{T} method (Winer et al., 1999) where the relative quantities of different mRNA transcripts were calculated after normalisation of the data against a chosen housekeeping gene. This method demands similar efficiencies of amplification for the genes compared.

The efficiency for one reaction is calculated by plotting the Ct values of standard curve against the log concentration. The negative value of that slope gives the efficiency of the reaction. A value of -3.3 is seen in a reaction with 100% efficiency, calculated by: efficiency = 10^{(-1/slope)} -1.

Comparison of two different efficiencies was performed by plotting the ΔCt (Ct value for target gene minus Ct value for housekeeping gene) for each concentration in a standard curve against the log concentration. The slope of the trend line between these observations should not exceed 0.1, according to the manufacturer (Appliedbiosystems, 1997).

If the efficiencies of the genes under analysis differ too much, the Standard Curve Method must be used. The concentration of cDNA in the unknown samples is calculated using the equation of the standard curve: y=kx+m; where y is the Ct value of the sample, k is the slope of the curve, x is the concentration you desire, and m is the intercept. X=(y-m)/k gives the log concentration of the sample. 10^{log ((y-m)/k)} gives the concentration of the unknown sample according to the standard curve used. When comparing different samples the value of the target gene should be divided by the value of the housekeeping gene.
4 STATISTICS

Study I
Data are presented as means ± SEM. Differences within and between groups were determined by ANOVA. Fisher’s least significant difference post hoc analysis was used to identify significant differences. Pearson correlation analysis was applied to determine the existence of possible relationships between average glucose transport and average AS160 phosphorylation for each condition. Differences were considered significant at $P<0.05$.

Study II
Data are presented as means ± SEM of AMPK isoform normalised to $\beta$-actin, as well as all individual values. Mann-Whitneys U-test was used to analyse differences in expression of AMPK isoforms between able-bodied and spinal cord injured subjects. Individual values (n = 4) of AMPK isoform expression before and after exercise (ESLC) training are illustrated in scatter diagrams.

Study III
Spinal cord injured subjects and athletes were independently compared to control-subjects using ANOVA and Fischers post hoc analysis. Statistical significance was accepted when $p<0.05$. Correlations were calculated by simple linear regression.
5 RESULTS AND DISCUSSION

This thesis has explored gene expression in skeletal muscle from first-degree relatives to subjects with type 2 diabetes, in spinal cord injured individuals, elite trained athletes, and control subjects. Genes involved in mitochondrial biogenesis and function, metabolic status, and fibre type regulation have been assessed. Whether abnormalities in insulin signalling precede the development of type 2 diabetes has been explored in first degree relatives to subjects with type 2 diabetes.

Spinal cord injury is an extreme form of skeletal muscle inactivity, and is therefore a very interesting model to use when studying gene expression and glucose metabolism. We also have had the opportunity to study muscle from some spinal cord subjects after an eight week training program. It is unique to have material to study human gene expression in skeletal muscle that has been partly denervated and completely disused for between 6 and 23 years and then exercised seven sessions per week for eight weeks.

Type 2 diabetes, a disease with disturbed glucose metabolism, is increasing alarmingly due to changed life styles and overweight. We have studied muscle biopsies from glucose tolerant first-degree relatives to type 2 diabetes patients. These individuals are genetically predisposed to develop the disease in the future, and to get more information about possible changes in the skeletal muscle when they still are healthy might provide greater insight into the course of the disease.

In stark contrast to unused skeletal muscle is the extremely trained muscle of the elite athlete. Elite athletes were recruited for muscle biopsy studies. Skeletal muscle from elite athletes differ from control subjects in fibre type composition. They are even more different compared to skeletal muscle form spinal cord injured individuals. Subject characteristics of all participants are presented in Table 4.

5.1 STUDY I: FIRST-DEGREE RELATIVES

Gene expression of some of the key transcriptional factors and co-regulators of mitochondrial biogenesis in muscle biopsies taken from healthy insulin sensitive relatives to subjects with type 2 diabetes was determined. In this study insulin-stimulated glucose uptake, GLUT4 protein expression, and phosphorylation of members of the insulin receptor signalling cascade was also assessed. The results were compared to well-matched control subjects (Table 4).

5.1.1 Clinical characteristics

A clinical examination was performed on all subjects to discover possible differences between the two groups. The clinical characteristics were similar between the relatives and the controls, except for the systolic blood pressure, which was significantly (P<0.05) increased in the relatives. The 2-h plasma insulin concentration showed a non-significant increase by 50%. This might indicate a mild insulin resistance.

Worth mentioning is that probably not all first-degree relatives to subjects with type 2 diabetes will develop the disease, whereas some of the control subjects may. The mean age of the selected subjects was 41 years. We reasoned that younger subjects would perhaps not have knowledge of their family history of type 2 diabetes because their parents would still be young, and may not have any symptoms of type 2
diabetes. If we studied older subjects that were relatives, but still insulin sensitive, we might have chosen those who would not inherit the disease, despite being relatives. As further mentioned, our subjects were not insulin resistant, and that may be an important risk factor for type 2 diabetes. Perhaps the results would have been different if we would have selected insulin resistant individuals. To evaluate our selection criteria we would need to recruit the same subjects in 10 and perhaps also 20 years and make a further clinical examination and study of glucose uptake, signalling and mitochondrial function. This would give important information regarding conversion of type 2 diabetes.

<table>
<thead>
<tr>
<th></th>
<th>I Rel</th>
<th>I Con</th>
<th>II &amp; III Rel</th>
<th>II Con</th>
<th>III Con</th>
<th>III Athl</th>
<th>III SCI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>41±3</td>
<td>40±2</td>
<td>24±1</td>
<td>23±1</td>
<td>37±3</td>
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<td></td>
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<tr>
<td>Weight (kg)</td>
<td>78.5±2.5</td>
<td>75.5±3.0</td>
<td>83.4±2.8</td>
<td></td>
<td></td>
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<tr>
<td>Height (m)</td>
<td>1.77±0.02</td>
<td>1.80±0.02</td>
<td>1.78±0.01</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.1±0.8</td>
<td>23.4±0.7</td>
<td>22.5±0.6</td>
<td>22.0±0.4</td>
<td>19.9±1.4</td>
<td>26.3</td>
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<tr>
<td>Waist (cm)</td>
<td>90.0±2.9</td>
<td>87.8±1.9</td>
<td></td>
<td></td>
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<tr>
<td>Hip (cm)</td>
<td>94.2±2.1</td>
<td>91.4±1.7</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.96±0.02</td>
<td>0.96±0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma glucose (mM)</td>
<td>5.4±0.1</td>
<td>5.4±0.1</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>HbA1c (%)</td>
<td>4.5±0.03</td>
<td>4.6±0.06</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Insulin (pM)</td>
<td>39±7</td>
<td>30±3</td>
<td></td>
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<td></td>
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<tr>
<td>Total cholesterol (mM)</td>
<td>4.7±0.3</td>
<td>4.7±0.2</td>
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<td></td>
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<tr>
<td>HDL cholesterol (mM)</td>
<td>1.30±0.09</td>
<td>1.32±0.07</td>
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<tr>
<td>LDL cholesterol (mM)</td>
<td>2.85±0.19</td>
<td>2.95±0.18</td>
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<td></td>
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<tr>
<td>Triglycerides (mM)</td>
<td>1.14±0.13</td>
<td>0.88±0.07</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Syst. bl. pressure (mmHg)</td>
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<td>125±2</td>
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<td></td>
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<tr>
<td>Diast. bl. pressure (mmHg)</td>
<td>82±3</td>
<td>78±2</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO₂max (ml/kg/min)</td>
<td>39.4±3.7</td>
<td>45.1±2.5</td>
<td>46.3±1.9</td>
<td>74.3±2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>20.4±2.2</td>
<td>17.1±1.9</td>
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<td></td>
</tr>
<tr>
<td>Type I fibres (%)</td>
<td>57±4</td>
<td>71±3</td>
<td>0.7±0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type IIA fibres (%)</td>
<td>23±3</td>
<td>21±3</td>
<td>38±7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type IIB fibres (%)</td>
<td>18±2</td>
<td>8±2</td>
<td>61±7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type IIC fibres (%)</td>
<td>1.7±1.0</td>
<td>0.4±0.1</td>
<td>0.0±0.0</td>
<td></td>
<td></td>
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</tbody>
</table>

*Table 4. Subject characteristics for participants in Studies I, II and III. Rel, relatives; Con, control, Athl, athlete; SCI, Spinal cord injured subject*

### 5.1.2 Glucose transport and insulin signalling

Insulin-stimulated glucose transport was measured in isolated skeletal muscle strips from relatives and controls. Basal glucose transport in isolated skeletal muscle strips from relatives and controls was identical. Insulin-stimulated glucose transport in isolated muscle strips was similar between first-degree relatives and control subjects at 0.6 nM and 1.2 nM insulin concentrations. At the highest insulin concentration, (120 nM) glucose transport was significantly reduced by 38% (P<0.05) in skeletal muscle from relatives, compared to the control subjects. Normal levels of circulating insulin are 0.02-1 nM in healthy individuals. The values differ according to the time since the last meal. The insulin levels used in study I were: 0.6, 1.2, and 120 nM. Hence, the two highest concentrations are not within the physiological range. Glucose transport in the skeletal muscle strips was significantly lower (P<0.05) when the
highest insulin concentration was used. This concentration is a 100-fold higher than what a skeletal muscle would normally be exposed to. Despite the supraphysiological insulin concentration, it might indicate an inherent underlying defect in the glucose transport. We also measured skeletal muscle GLUT4 protein expression, which was similar between control subjects and first-degree relatives. Even though the total expression of GLUT4 was unchanged between the groups (Figure 3), there may be disturbances in the trafficking and fusion of GLUT4 with the plasma membrane in the relatives, which may be reflected by the decreased in glucose uptake in the relatives at the highest insulin concentration.

In vitro exposure of isolated skeletal muscle to insulin was associated with a concentration-dependent increase in Akt phosphorylation at Ser473 and Thr308 and AS160, with similar responses noted between first-degree relatives and control subjects.

5.1.3 Gene expression

Gene expression of PGC-1α, PGC-1β, PPARδ, NRF-1 and UCP-3 were comparable between first-degree relatives and control subjects (Figure 4). Protein expression of PGC-1 was also similar between first-degree relatives and control subjects (Figure 5).

The unchanged gene expression in our material with glucose tolerant relatives is consistent with a previous report in young, lean, and insulin resistant relatives showing that PGC-1α, PGC-1β, NRF-1, NRF-2, and mitochondrial transcription factor A (TFAM) are unaltered, despite reduced mitochondrial density (Morino et al., 2005). In an exercise training study of normal glucose tolerant men, the relatives respond normally to endurance exercise in terms of changes in VO2 max and insulin
Further, in the training study, a correlation between the VO2 max and insulin sensitivity in the control group was also demonstrated. This correlation was lacking in the relatives, suggesting that the improvement of insulin sensitivity is dissociated from skeletal muscle mitochondrial function.

5.2 STUDY II: EXPRESSION OF AMPK

Gene expression of all isoforms of AMPK was compared in spinal cord injured individuals with controls. Gene expression in spinal cord injured subjects was also assessed before and after 8 weeks of cycling. AMPK is a fuel sensor in the cell that when activated, turns off ATP consuming processes, and turns on ATP generating processes. Exercise activates AMPK and increases glucose transport, through GLUT4 translocation to the plasma membrane, and glucose uptake into the cell.

5.2.1 cDNA quality check

cDNA synthesis was assessed from biopsies from the spinal cord individuals which had been synthesised about 10 years ago. Even if cDNA is stable if stored correctly we wanted to validate that the quality of the material was sufficient. We therefore synthesised new cDNA from a subset of muscle biopsies that were left. The skeletal muscle biopsies had been stored in liquid nitrogen. Expression of the AMPK γ isoforms and β-actin in the old cDNA and in the newly synthesised was then compared by TaqMan PCR. When correcting the amounts of the gamma isoforms with the amount of β-actin, an identical expression pattern was shown (Figure 6). It was therefore concluded that the cDNA isolated previously was appropriate to use in Studies II and III. The relationship between γ1, γ2 and γ3 cannot be compared since the amount of cDNA was calculated from dilution curves without an exact standard.
5.2.2 AMPK expression

In Study II it was shown that skeletal muscle mRNA expression of AMPK $\alpha_1$ and $\alpha_2$ differed significantly ($P<0.05$) between spinal cord injured and able-bodied subjects. $\alpha_1$ and $\alpha_2$ mRNA was reduced in the spinal cord injured to a level of 46% and 19% respectively, compared to able-bodied subjects ($P<0.05$). Furthermore, mRNA expression of $\beta_1$ and $\beta_2$ isoforms were also significantly reduced ($P<0.05$) in spinal cord injured, 27 and 34%, respectively. In contrast, there was a significant increased mRNA expression of the $\gamma_1$ and $\gamma_3$ isoforms ($P<0.05$) observed in the spinal cord injured subjects, whereas $\gamma_2$ expression was unchanged.

5.2.3 AMPK expression post exercise

Four of the spinal cord injured subjects (A-D) participated in an eight week exercise training program consisting of ESLC 7 sessions per week for 8 weeks. The time since injury differed between the four subjects. Subject A was injured 23 years before the study, the corresponding numbers for the other subjects are: B 11, C 7 and D 6. The ESLC resulted in changed mRNA expression for all the subjects. The two subjects with the shortest time since injury, C and D, showed an increase of expression of all AMPK isoforms (Table 5). Subject A and B had a different pattern compared to C and D. They both decreased the expression of $\alpha_1$, $\alpha_2$, $\gamma_1$ and $\gamma_3$. $\gamma_2$ was increased whereas the $\beta_1$ increased in subject A and decreased in subject B. The opposite was noted with the $\beta_2$ expression that decreased in subject A and increased in B.

<table>
<thead>
<tr>
<th>Subject</th>
<th>A1</th>
<th>A2</th>
<th>B1</th>
<th>B2</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

Table 5. Changes in AMPK gene expression post training.

It has previously been reported that the protein expression of $\alpha_1$, $\beta_2$, and $\gamma_1$ is increased after endurance exercise, while $\gamma_3$ has been shown to decrease (Langfort et al., 2003; Nielsen et al., 2003; Frosig et al., 2004). The same protein expression pattern has also been observed after strength training (Wojtaszewski et al., 2005). The mRNA levels of the $\alpha$ and $\beta$ AMPK isoforms were unchanged between one exercised leg and one untrained leg after 6 weeks of strength training 3 times a week, $\gamma_1$ and $\gamma_2$ were also unchanged whereas $\gamma_3$ decreased (Wojtaszewski et al., 2005).

The two individuals that were injured 6 and 7 years before the biopsies were taken, C and D, responded differently to the exercise in means of gene expression than A and B that were injured 23 and 11 years prior to the ESLC. Whether this is due to some morphological changes that occur in innervated muscle between 7 and 11 years of injury, or other factors, needs to be further studied. It is worth emphasising that spinal cord injured subjects are a heterogeneous group, depending on for example the degree of the injury, which makes the results more difficult to interpret.
5.2.4 Relative expression of AMPK isoforms

The majority of AMPK complexes in human muscle contain $\alpha_2$ and $\beta_2$. Of these complexes about 20% has been shown to be associated with $\gamma_3$ and the remaining most probably associates with $\gamma_1$ (Wojtaszewski et al., 2005). This is in agreement with data from mouse skeletal muscle in which $\gamma_3$ was also found to be associated with $\alpha_2$ and $\beta_2$, but not with $\alpha_1$ and $\beta_1$ (Mahlapuu et al., 2004). Based on mRNA measurements, the $\gamma_3$ isoform is the predominant $\gamma$ isoform expressed in mouse muscle representing glycolytic fibres (Mahlapuu et al., 2004), indicating that the $\alpha_2/\beta_2/\gamma_3$ complexes are the major AMPK complex in this fibre type of mouse skeletal muscle.

The high content of $\gamma_3$ mRNA in the spinal cord injured subjects may be explained by the decreased number of type I oxidative fibres and the increase of glycolytic fibres. When comparing the mean values after the ESLC (Figure 7), a changed relationship between the $\gamma$-subunits was observed, indicating that $\gamma_3$ is decreased and hence more similar to the expression profile in the control group. It should be noted again that the values after training only is based on four subjects. The major proportion of AMPK complexes in skeletal muscle consists of $\alpha_2/\beta_2/\gamma_1$ isoforms (Wojtaszewski et al., 2005). Thus, the reported mRNA expression differs from the protein expression in skeletal muscle in humans. This may be explained by the mixed fibre type composition in the human vastus lateralis muscle, or also by post translational modifications.

![Figure 7. AMPK isoform expression in relation to $\alpha_1$.](image)

5.3 STUDY III: GENE EXPRESSION AND FIBRE TYPES

In the same material from spinal cord injured subjects as in Study II, proteins important for fibres type regulation, i.e. calcineurin $\alpha$ and $\beta$, PPAR$\alpha$ and $\delta$, and PGC-1$\alpha$ and $\beta$ were also assed in Study III. Expression of these factors was compared to skeletal muscle from elite endurance trained athletes. Skeletal muscle from spinal cord injured subjects have almost no type I fibres, while muscle from endurance trained individual contain a high degree of these oxidative fibres. Gene expression of the previously mentioned factors shown to affect the expression of genes important for oxidative fibre types and mitochondrial biogenesis was determined.
5.3.1 Subject characteristics

Athletes and sedentary controls were matched for age and BMI (Table 4). The athletes had a significantly increased VO$_2$ max (P<0.05), as compared to sedentary controls. The spinal cord injured subjects were older than the able-bodied subjects, but had similar BMI. The athletes had a significant increased proportion of type I fibres (P<0.05), while the sedentary controls had an increased proportion of type IIB fibres. Conversely, spinal cord injured subjects had a decreased proportion of type I fibres and an increased proportion of type IIB fibres, as compared to the able-bodied subjects and athletes.

5.3.2 Gene expression

Expression of calcineurin $\alpha$ showed a tendency to be increased in spinal cord injured, compared to controls and athletes, but this did not reach statistical significance. Expression of the transcriptional co-activator PGC-1$\alpha$ and the related PGC-1$\beta$ were higher in athletes, as compared to control subjects (p<0.05). In spinal cord injured subjects, mRNA expression of PGC-1$\alpha$ and $\beta$ was reduced, as compared to control subjects (p<0.05). The expression profile of the nuclear receptors PPAR$\alpha$ and PPAR$\delta$ was similar to that of PGC-1$\alpha$ and $\beta$. Thus mRNA expression of PPAR$\alpha$ and PPAR$\delta$ was higher in athletes, as compared to control subjects. In spinal cord injured subjects, mRNA expression of PPAR$\alpha$ and PPAR$\delta$ was reduced, as compared to control subjects (p<0.05).

5.3.3 Correlations to muscle fibre type

In order to further test the hypothesis that the target genes were associated with the oxidative slow-twitch muscle fibre phenotype, individual gene expression data was correlated with individual fibred type composition of all subjects (Table 6).

<table>
<thead>
<tr>
<th>fibre type</th>
<th>%</th>
<th>PPAR$\alpha$</th>
<th>PPAR$\delta$</th>
<th>PGC1$\alpha$</th>
<th>PGC-1$\beta$</th>
<th>Calcineurin $\alpha$</th>
<th>Calcineurin $\beta$</th>
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<td>+</td>
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<td>-</td>
<td>Ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>IIB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ns</td>
<td>Ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

Table 6. Correlation between type I fibres and gene expression.

Percentage of type I muscle fibres was positively correlated with mRNA expression of PPAR$\alpha$ (r=0.59; p=0.003), PPAR$\delta$ (r=0.64; p=0.002) and PGC-1$\alpha$ (r=0.83; p<0.001). mATPase Type IIB muscle fibre type content was negatively correlated with mRNA expression of PPAR$\alpha$ (r=-0.55; p=0.007), PPAR$\delta$ expression (r=-0.65; p=0.002) and PGC-1$\alpha$ (r=-0.77; p<0.001). Type IIA muscle fibre type content was negatively correlated with mRNA expression of PGC1$\alpha$ (r=-0.56; p=0.015). Other correlations were insignificant.

PPAR$\alpha$, PPAR$\delta$, PGC1$\alpha$, and calcineurin $\alpha$ have been shown in animal studies to transform fast glycolytic fibres to a slow-twitch oxidative phenotype (Naya et al., 2000; Chin et al., 1998; Wu et al., 2000; Lin et al., 2002; Wang et al., 2004). These factors are reported to be activated in response to exercise, and it is well documented that elite endurance athletes possess a higher percentage of type I slow twitch fibres.
in the muscles they use in their sport, compared to untrained individuals (Andersen et al., 2000). It is unknown whether the percentage of type I slow twitch fibres in elite endurance athletes is due to genetic endowment or years of rigorous training. To further study the genetic or adaptive contribution to fibre type distribution it would probably be necessary to analyse muscle biopsies of elite athletes before and after they started exercise training and excelling in their respective sport. Spinal cord injured individuals increase their amount of type I fibres after an intense training program in eight week (Hjeltnes et al., 1997). In Study III a significant correlation between the gene expression of PPARα, PPARδ and PGC-1α and a high amount of type I fibres, and also a low amount of type IIB fibres was observed. This is in line with what the animal experiments mentioned above showed. In contrast to previous animal studies, the expression of calcineurin was not significantly changed between the three groups of athletes, spinal cord injured individuals and controls. However, because of scarcity of material we could not confirm our mRNA data with neither protein expression nor activity of the proteins, which could explain the discrepancy. It might also be so that since the athletes already have a higher proportion of type I fibres with high oxidative capacity and mitochondrial biogenesis, they may also have a higher expression of PCG-1 and PPAR. In supportive of data presented in Study III, calcineurin has been shown to be dispensable for the exercise-induced increase in mitochondrial biogenesis in skeletal muscle (Garcia-Roves et al., 2006).
6 SUMMARY

• Insulin-stimulated glucose uptake with the maximal insulin concentration (120nM) was reduced by 38% (p<0.05) despite unchanged insulin-stimulated Akt and AS160 phosphorylation and total GLUT4 expression. This might indicate that the impaired glucose transport is an early metabolic defect in the pathogenesis of type 2 diabetes. Further gene expression of the key transcriptional factors and co-regulators of mitochondrial biogenesis PGC-1α, PGC-1β, PPAR-δ, NRF-1, and UCP-3 under basal conditions were unchanged.

• The relationship between the seven AMPK isoforms in human skeletal muscle from able-bodied controls and spinal cord injured subjects before and after training is reported. Training led to a different pattern of changes in AMPK mRNA expression depending on the time since injury. Exercise tended to normalise expression levels similar to that observed for able-bodied controls.

• The mRNA expression of PGC-1α, PGC-1β, PPARα and PPARδ is related to physiological and pathological variations in skeletal muscle fibre type in humans, while mRNA expression of calcineurin Aα and Aβ is unrelated.
7 CONCLUSION

In this thesis, the expression of genes involved in mitochondrial regulation in human skeletal muscle in different conditions and metabolic statuses has been assessed. The insulin signalling pathway has also been explored in healthy first degree relatives of subjects with type 2 diabetes.

In Study I, skeletal muscle biopsies from normal glucose tolerant first-degree relatives to subjects with type 2 diabetes were obtained, and mRNA levels of PGC-1α, PGC-1β, PPARδ, NRF-1 and UCP-3 measured. All these genes showed similar expression to well-matched controls, when corrected to either β-actin or GAPDH, indicating that disturbances in mitochondrial function are unlikely to be the initial cause of insulin resistance and type 2 diabetes. Mitochondrial function is known to be disturbed in type 2 diabetic patients. Type 2 diabetic patients also feature defects in the insulin signalling pathway and decreased glucose uptake. We did not provide any evidence that the increased probability of developing type 2 diabetes is preceded by changes in mitochondrial function or decreased insulin signalling in skeletal muscle of first-degree relatives to subjects with type 2 diabetes before the onset of the disease. However, decreased glucose uptake was observed in the relatives at a supraphysiological insulin stimulation, which could be interpreted as an early defect in skeletal muscle.

In Study III, mRNA expression of PPARα, PPARδ, PGC-1α, PGC-1β, as well as calcineurin Aα and calcineurin Aβ was determined. All these factors have been suggested to influence fibre type distribution and mitochondrial biogenesis in rodent skeletal muscle. In contrast to results in Study I in first degree relative subjects, in groups of subjects selected to represent different extremes of fibre types and exercise habits, mRNA expression of PPARα, PPARδ, PGC-1α, PGC-1β correlated with fibre types in human skeletal muscle, whereas calcineurin Aα and Aβ expression was unrelated. The mRNA levels were measured in skeletal muscle biopsies from elite athletes with a higher portion of type I fibres compared to sedentary controls. It would have been very interesting to measure the expression of these genes in the spinal cord injured subjects after training. The expected result from such an analysis would be increased expression of genes related to mitochondrial biogenesis, since these subjects had an increased proportion of oxidative type I fibres. It would perhaps also give more information about how time since injury influences the properties of the skeletal muscle.

This thesis work also provides data on the relative expression pattern of AMPK α1, α2, β1, β2, γ1, γ2 and γ3 isoforms in skeletal muscle from able-bodied subjects and spinal cord injured individuals. The relationship between all isoforms has not been previously determined. To be able to use activators of AMPK as pharmaceutical agents, it is of great importance to study how the trimeric AMPK complexes are composed in different tissues. We were able to demonstrate that α2, β2 and γ3 show the highest mRNA expression in skeletal muscle in all the groups studied, possibly indicating that this is the preferred heterotrimeric AMPK composition in human vastus lateralis. Since studied mRNA levels were determined, it remains important to verify
results at the protein level. The effects on mRNA expression of an eight week training study, where spinal cord injured subjects have been cycling seven days a week, was also determined. Interestingly, the expression of AMPK seemed to differ depending on time since injury.

The members of the signalling pathways controlling fibre types and mitochondrial biogenesis presented in this thesis work, and how they interact, are shown in Figure 8. It is a complex network that needs to be studied further in order to fully understand the physiological and pathophysiological changes that occur in skeletal muscle.

In summary, this thesis work has provided evidence that exercise leads to a more oxidative skeletal muscle phenotype, with increased glucose uptake. An opposing pattern is seen in skeletal muscle that has been unused for several years. The positive effects of exercise delays and may even fully prevent the onset of type 2 diabetes, despite the fact that people with a genetic predisposition to the disease seems to have intact insulin receptor signalling and mitochondrial function, before the onset of the disease.

Figure 8. Schematic presentation of some factors important for gene expression of slow contractile genes, fatty acid oxidation and mitochondrial biogenesis.


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9 REFERENCES


