EFFECTS OF INSULIN DEFICIENCY ON EXERCISE-INDUCED ACUTE RESPONSES IN THE REGULATION OF FATTY ACID OXIDATION IN MOUSE GASTROCNEMIUS MUSCLES

Tuuli Nissinen

ABSTRACT

Nissinen, Tuuli 2014. Effects of insulin deficiency on exercise-induced acute responses in the regulation of fatty acid oxidation in mouse gastrocnemius muscles. Department of Biology of Physical Activity, University of Jyväskylä, Master's Thesis in Exercise Physiology, 102 p.

Insulin is a hormone that plays an important role in the regulation of the metabolism of all the main nutrients. Its main function is to stimulate glucose uptake and disposal or utilization by the cells and thus to decrease blood glucose concentration. However, it also inhibits breakdown of proteins and lipids and promotes their synthesis. Type 1 diabetes is a disease in which insulin secretion is impaired because of destruction of pancreatic β-cells. It is characterized by hyperglycemia and increased reliance on fat oxidation. This is seen also as altered gene expression patterns. The purpose of this study was to look into the effects of insulin deficiency on exercise-induced acute responses in the expression of genes and the activation of signaling pathways involved in fatty acid oxidation. Male NMRI mice (n = 64) were randomly assigned into three streptozotocin-induced diabetic and three healthy groups. Two healthy and two diabetic groups performed a single one-hour bout of treadmill running (21 m/min, 2.5° incline) and were sacrificed either three or six hours after exercise. Gastrocnemius muscles were dissected and mRNA expression and protein expression and phosphorylation were analyzed with RT-PCR and Western blotting respectively. PGC-1α mRNA expression increased ($p < 0.001$) after exercise in both healthy and diabetic mice, but the response was higher in diabetic mice ($p < 0.05$). PDK4 mRNA expression increased after exercise only in diabetic mice ($p < 0.05$). Diabetic mice showed a more pronounced response in CPT1B mRNA six hours after exercise compared with healthy exercised mice ($p < 0.05$). Contrary to mRNA level results, PGC-1α protein content did not change in response to exercise when compared with sedentary counterparts of the same health status. The analysis of AMPK and p38 MAPK phosphorylation did not suggest activation of these pathways in response to exercise. Even a decrease in AMPK phosphorylation was seen six hours after exercise in healthy mice ($p < 0.05$) while p38 MAPK phosphorylation was decreased in diabetic mice three hours after exercise ($p < 0.05$). There were no significant changes in proteins PDK4, CPT1B, sirtuins 1, 3 and 6, ACC or Cyt c. These results suggest that diabetic mice have more pronounced exercise-induced responses in the expression of genes related to increased fatty acid oxidation. These changes may be mediated by increased PGC-1α activation as no increases were seen in PGC-1α protein expression. The time points were not optimal for protein level analyses and thus, further studies are needed to clarify protein phosphorylation and expression changes and to find out whether the gene expression changes are reflected to the level of substrate metabolism.

Keywords: exercise, diabetes, insulin, fatty acid oxidation

ABBREVIATIONS

ADP	Adenosine diphosphate
ACC	Acetyl-coenzyme A carboxylase
AMP	Adenosine monophosphate
AMPK	5'adenosine 5'AMP-activated protein kinase
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
β-HAD	β-hydroxyacyl-CoA dehydrogenase
cAMP	Cyclic AMP
CHO	Carbohydrate
CoA	Coenzyme A
CPT	Carnitine palmitoyltransferase
CREB	cAMP response element-binding protein
Cs	Citrate synthase
Cyt c	Cytochrome c
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ERK	Extracellular-regulated kinase
ERRα	Estrogen-related receptor α
FABP	Fatty acid binding protein
FAD	Flavin adenine dinucleotide
FAT/CD36	Fatty acid translocase/cluster of differentiation 36
FATP	Fatty acid transport protein
FFA	Free fatty acid
FOXO	Forkhead box protein O
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT4	Glucose transporter type 4
HIF1α	Hypoxia-inducible factor 1α

HK	Hexokinase
HRP	Horseradish peroxidase
HSL	Hormone sensitive lipase
IMTG	Intramuscular triacylglycerols
IRS	Insulin receptor substrate
LCAD	Long chain acyl-CoA dehydrogenase
LCFA	Long chain fatty acid
LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinase
MCAD	Medium chain acyl-CoA dehydrogenase
MCD	malonyl-CoA decarboxylase
NAD	Nicotinamide adenine dinucleotide
NEFA	Non-esterified fatty acid
PCR	Polymerase chain reaction
PCr	Phosphocreatine
PDH	Pyruvate dehydrogenase
PDK4	Pyruvate dehydrogenase kinase isozyme 4
PFK	Phosphofructokinase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PPAR	Peroxisome proliferator-activated receptors
RNA	Ribonucleic acid
SCAD	Short chain acyl-CoA dehydrogenase
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
Sirt	Sirtuin
STZ	Streptozotocin
TAG (TG)	Triacylglycerol (triglyceride)
VLCAD	Very long chain acyl-CoA dehydrogenase
VO_{2max}	Maximal oxygen uptake

CONTENTS

1 INTRODUCTION

Type 1 diabetes mellitus is one of the most prevalent chronic diseases in children, and its incidence has been increasing worldwide. Finland has been the leading country in the incidence of type 1 diabetes in children younger than 15 years, with highest recorded incidence of 64.9 per 100 000 person-years in 2006. (Harjutsalo et al. 2013.) Diabetes mellitus is a disease in which the metabolism of carbohydrates, fats and proteins is impaired. It is caused by either lack of insulin secretion (type 1 or insulin-dependent diabetes) or decreased insulin sensitivity of target tissues (type 2 or non-insulin-dependent diabetes). Both forms are characterized by impaired uptake and utilization of glucose by most of the cells, which results in increased blood glucose concentration, decreased utilization of glucose and consequently increased utilization of fats and proteins by the cells. (Guyton & Hall 2000, 894–895.)

In type 1 diabetes insulin secretion is impaired normally because of autoimmune destruction of pancreatic β-cells. Resulting high blood glucose concentration then causes other dysfunctions including dehydration and abnormal function of blood vessels and nerve cells which then may trigger a number of other disorders and injuries that cause severe harm to multiple tissues. (Guyton & Hall 2000, 894–895.) Insulin deficiency also increases lipolysis and release of fatty acids into the blood stream which eventually causes rise in plasma lipoprotein, cholesterol and phospholipid levels. This can ultimately lead to development of atherosclerosis. In addition, insulin deficiency results in excessive utilization of fat which finally leads to ketosis and acidosis because of accumulation of ketone bodies. (Guyton & Hall 2000, 888–889, 895.)

At least in animal models, the changes in substrate use are seen also in gene expression level: genes related to fatty acid oxidation are up-regulated, whereas those involved in glucose uptake, transport and metabolism are down-regulated. In addition, genes involved in oxidative phosphorylation are down-regulated, which suggests impaired capacity to produce ATP aerobically. (Silvennoinen 2004; Yechoor et al. 2002.) Indeed, both in human and animal

studies, diabetes has been related to lowered aerobic capacity and this is seen at the level of whole organism as impaired VO_{2max} and at the level of skeletal muscle as lowered activity of key oxidative enzymes (el Midaoui et al. 1996; Ianuzzo et al. 1974; Kivelä et al. 2006; Noble & Ianuzzo 1985; Silvennoinen 2004).

Exercise training has been shown to reverse many of the unfavorable effect of diabetes or insulin deficiency on aerobic capacity (Costill et al. 1979; el Midaoui et al. 1996; Ianuzzo et al. 1974; Kivelä et al. 2006; Laaksonen et al. 2000; Noble & Ianuzzo 1985). In addition exercise has many other beneficial effects on health in type 1 diabetics, such as improvements in glycemic control, lipid profile and vascular function. Thus, regular exercise has been recommended to type 1 diabetics. (Costill et al. 1979; Fuchsjager-Mayrl et al. 2002; Laaksonen et al. 2000.)

The purpose of this study was to elucidate the effects of an acute exercise bout on the expression of genes and on the activation intracellular signaling pathways involved in fatty acid oxidation and oxidative metabolism in general in gastrocnemius muscles of healthy and insulin deficient mice. The main research question was, whether insulin deficient mice had different acute responses to an endurance exercise bout compared with healthy mice. The gene expression was studied at the level of both mRNA and protein using quantitative real-time PCR and Western blotting, respectively. In addition, phosphorylation status of certain proteins was studied to evaluate the activation of the key signaling pathways in regulation of oxidative energy metabolism.

2   SKELETAL MUSCLE GLUCOSE METABOLISM

Carbohydrates (CHOs) readily available for energy production are present in blood plasma as glucose and in liver and skeletal muscles as its storage form glycogen, from which glucose can be released when energy demands increase (Nelson & Cox 2013, 543, 612–613, 956). In addition to lipids, carbohydrates are an important source of energy during exercise, especially during exercise at high intensity. Bodily CHOs stores are not even nearly as abundant as those of fat, as they are depleted after one to two hours of intense exercise. However, the advantage of CHO utilization is, that ATP (the form of chemical energy that can be utilized by the cells) can be produced more quickly and both with (aerobically) or without (anaerobically) oxygen. (Horowitz & Klein 2000; Spriet & Watt 2003) The aerobic pathway of glucose catabolism to provide ATP consists of three stages: glycolysis, citric acid cycle and oxidative phosphorylation, the last two of which are common with fatty acid oxidation (Nelson & Cox 2013, 633, 667, 731). This metabolic pathway is described briefly below.

2.1   Energy production from glucose

Glycolysis. The first step of ATP production from glucose is glycolysis. It is a series of 10 enzymatic reactions during which one glucose molecule is degraded to form two molecules of pyruvate. This step is common for both aerobic and anaerobic energy production pathways. Glycolysis can be divided into two phases: The first, preparatory phase consists of five enzymatic reactions during which glucose is phosphorylated and converted to glyceraldehyde 3-phosphate. This first phase requires energy provided by breakdown of two molecules of ATP. During the second, payoff phase glyceraldehyde 3-phosphate is converted to pyruvate. A total of four molecules of ATP are formed during the payoff phase, giving glycolysis a net ATP production of two molecules per one glucose molecule. (Nelson & Cox 2013, 544–546.) Glycogen breakdown can also provide substrates for glycolysis: the consecutive reactions catalyzed by glycogen phosphorylase and phosphoglucomutase detach

glucose 1-phosphate from glycogen and convert it to glucose 6-phosphate which enters glycolysis. This saves one molecule of ATP and thus, the net production of ATP is three molecules instead of two. (Nelson & Cox 2013, 560–561.) Glycolysis liberates also hydrogen ions and electrons that are bound to NAD^+ to form NADH. Two NADH molecules are formed per one glucose molecule. At this point, anaerobic pathway separates from the aerobic. In aerobic conditions, pyruvate is oxidized and the electrons provided by NADH are transferred to oxygen in electron transport chain providing energy for ATP synthesis. In anaerobic glycolysis the next step is formation of lactate in a reaction catalyzed by the enzyme lactate dehydrogenase. In the reaction pyruvate accepts electrons from NADH and is reduced to lactate with concomitant regeneration of NAD^+. This regeneration of NAD^+ allows glycolysis to continue. (Nelson & Cox 2013, 545–546, 555.)

Citric acid cycle. In aerobic pathway the next step is the transport of the formed pyruvate molecules into mitochondria. In mitochondria, pyruvate is oxidized to acetyl coenzyme A (acetyl-CoA) and carbon dioxide by the pyruvate dehydrogenase (PDH) complex. This reaction also liberates electrons that are accepted by NAD^+. Acetyl-CoA then enters the citric acid cycle (also Krebs cycle or tricarboxylic acid (TCA) cycle), where it is oxidized to carbon dioxide. During the first of the eight enzymatic reactions of citric acid cycle acetyl group is attached to four-carbon oxaloacetate. This reaction is catalyzed by citrate synthase and it produces one molecule of 6-carbon citrate and liberates coenzyme A. During the next seven reactions, two molecules of carbon dioxide and one molecule of ATP are liberated and oxaloacetate is reformed enabling the continuation of the cycle. In addition, hydrogen ions and electrons liberated during citric acid cycle are bound to NAD+ and FAD to form NADH and $FADH_2$ respectively. (Nelson & Cox 2013, 633–634, 638–649.)

Electron transport chain and oxidative phosphorylation. The hydrogen ions and electrons liberated during glycolysis and citric acid cycle are directed forward to electron transport chain and oxidative phosphorylation that take place in the inner mitochondrial membrane. In the electron transport chain, electrons derived from glycolysis and citric acid cycle and transported by electron carriers NADH and $FADH_2$ are transferred from complex to another

down the electron transport chain. Finally the electrons are passed by Complex IV to oxygen which is reduced to water. This liberates energy that is harnessed to bump hydrogen ions from mitochondrial matrix to the intermembrane space against their concentration gradient. This produces a proton gradient. Finally, hydrogen ions flow back to the mitochondrial matrix through the enzyme ATP synthase down the electrochemical gradient. This liberates energy that is used by ATP synthase to form ATP from ADP and P_i. The whole oxidative breakdown of glucose yields a total of 30 to 32 molecules of ATP per one molecule of glucose. So, remarkable amount of ATP can be produced aerobically compared with anaerobic energy production, but the cost is a significant reduction in the rate of ATP production. (Nelson & Cox 2013, 732–745, 759–760.)

2.2   Regulation of glucose metabolism

Hormonal regulation. It is crucial to maintain plasma glucose concentration steady and within a narrow range (4–7 mmol/l in normal individuals) as disturbances in glucose balance and its regulation may result in severe complications. The glucose balance is maintained by controlling glucose absorption from the intestine, production by the liver and uptake and metabolism by peripheral tissues. In the center of this regulation is a hormone called insulin. (Saltiel & Kahn 2001.) Insulin is a peptide hormone synthesized and secreted by pancreatic β-cells. It contributes to the regulation of the metabolism of all the main nutrients (glucose, lipids and protein). Insulin promotes uptake of glucose and its storage as glycogen or usage for energy production in most of the cells except brain cells. Thus, insulin tends to decrease blood glucose concentration. This is probably the most visible and widely known effect of insulin. However, insulin has also profound effects on the metabolism of the other two main nutrients: fat and protein. While increasing the utilization of glucose by most of the tissues, insulin decreases utilization of fat and promotes fatty acid synthesis and fat storage. It also increases protein synthesis and inhibits the catabolism of proteins. (Guyton & Hall 2000, 884, 886–889.) Consequently, insulin resistance or deficiency causes profound dysfunctions in the metabolism of all the main nutrients and results in elevated glucose and lipid levels in both fasted and fed states (Saltiel & Kahn 2001). Many hormones

act to increase plasma glucose concentration, in fasted state or during exercise, but insulin is the only hormone promoting the decrease in plasma glucose concentration. Glucagon is considered the main hormone opposing the actions of insulin: It is secreted by pancreatic α-cells in response to lowered blood glucose. It stimulates glycogenolysis and glucose synthesis by gluconeogenesis and inhibits glycolysis in liver. This allows liver to liberate glucose to circulation increasing blood glucose concentration to normal level. Epinephrine exerts similar effects to liver as glucagon, but in addition, it stimulates glycolysis in skeletal muscle. The stress hormone cortisol acts to restore blood glucose levels and to increase glycogen stores by increasing liberation of fatty acids and glycerol (precursor for gluconeogenesis) from adipose tissue and export of amino acids from skeletal muscle to liver and by stimulating gluconeogenesis in liver. (Nelson & Cox 2013, 955–959.)

Insulin signaling. Skeletal muscle is insulin sensitive tissue and it accounts for up to 75% of all insulin-dependent glucose disposal. The mechanism by which insulin increases glucose uptake by skeletal muscle involves translocation of GLUT4 glucose transporter from cytoplasmic storage sites to plasma membrane. The overview of the signaling pathway behind this and the other effects of insulin are shown in figure 1. Briefly, insulin receptor belongs to the family of receptor tyrosine kinases and consists of two α- and two β-subunits. The α-subunit acts as an inhibitory subunit preventing the tyrosine kinase activity of the β-subunit when insulin is not bound to the receptor. Binding of insulin removes this inhibition allowing activation of the receptor by transphosphorylation of the β-subunit. Inside the cell, the receptor then tyrosine phosphorylates insulin receptor substrate (IRS) which starts the intracellular signaling cascades through phosphorylation of target proteins. Probably the most important of these targets is phosphatidylinositol 3-kinase (PI3K) which mediates most metabolic actions of insulin. Ultimately the activation of insulin receptor and its signal transduction pathways increases GLUT4 translocation to the surface of the cell and subsequent glucose uptake, promotes glycogen synthase activity and thus glycogen synthesis and blocks hepatic gluconeogenesis and glycogenolysis thus inhibiting glucose release from the liver. Also, insulin promotes lipid synthesis and inhibits degradation of lipids in lipolysis. The effects of insulin of lipid metabolism are discussed more profoundly later in this re-

view. In addition to substrate metabolism, insulin action promotes cell growth and differentiation and expression of multiple genes via MAPK signaling (briefly reviewed later in this literature review). (Saltiel & Kahn 2001.)
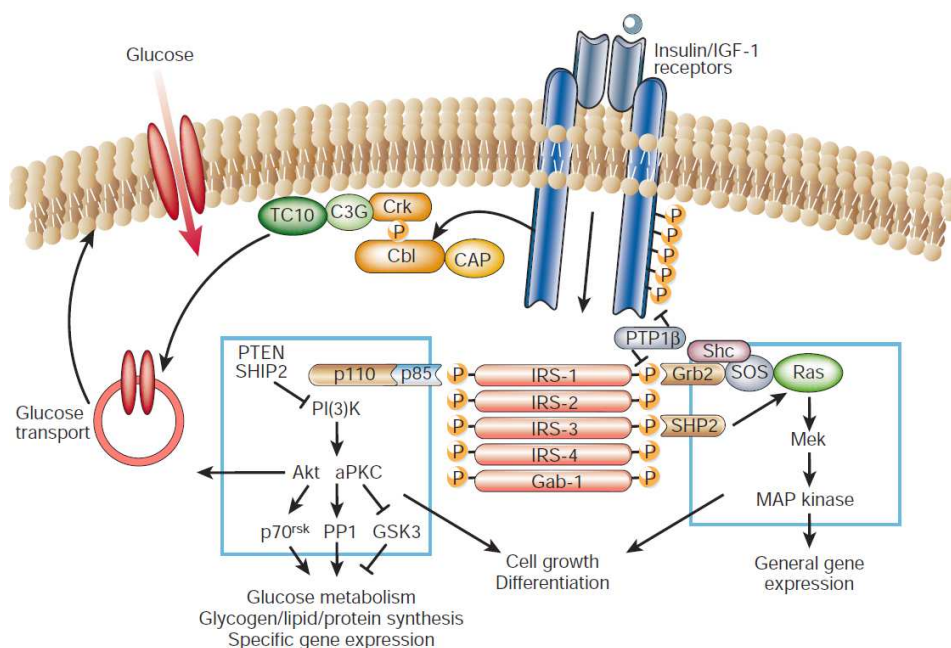
FIGURE 1. Overview of insulin signaling (Saltiel & Kahn 2001).

Intracellular factors in short-term regulation of glucose metabolism. The glycolytic flux is regulated tightly in order to maintain constant ATP levels. Basically, the activity of key enzymes is allosterically regulated by the balance between ATP synthesis and consumption, the ratio between NADH and NAD+ and fluctuations in the concentrations of key metabolites. (Nelson & Cox 2013, 555, 589, 762.) For example, high concentration of ATP indicates that ATP is being produced more than is consumed and this inhibits many of the key enzymes involved in glycolysis and citric acid cycle. On the contrary, when cellular energy consumption increases, accumulation of ADP and AMP activates these enzymes to boost the rate of ATP production. (Nelson & Cox 2013, 604, 654–655.) When oxidative phosphorylation slows down with decreasing energy demands (high ATP and low ADP concentration) NADH starts to accumulate. This inhibits citric acid cycle which then causes accumulation of acetyl-CoA which further inhibits PDH complex. This promotes the switch

from glucose breakdown in glycolysis to gluconeogenesis. (Nelson & Cox 2013, 608.) PDH complex may also be inactivated via covalent protein modification. This modification is performed by pyruvate dehydrogenase kinase (PDK) which phosphorylates and thus inactivates PDH complex. PDK is allosterically activated by high ATP levels, but decline in ATP concentration induces phosphatase activity which reactivates PDH complex. (Nelson & Cox 2013, 654.) The changes in the concentrations of key metabolites reflect the balance between ATP production and consumption. Thus, accumulation of metabolites casts inhibition on the up-stream enzymes to prevent unnecessary progression of glucose catabolism and further accumulation of these products. (Nelson & Cox 2013, 555, 604.) Moreover, certain conditions in addition to increased insulin signaling, such as muscle contraction and subsequent activation of intracellular signaling pathways, promote GLUT4 translocation from intracellular storage sites to plasma membrane and thus regulate cellular glucose metabolism via changes in glucose uptake (Hardie & Sakamoto 2006).

Intracellular factors in long-term regulation of glucose metabolism. In addition to regulation of enzyme activity, some enzymes are regulated through the balance between enzyme synthesis and degradation. Enzyme synthesis is induced via transcription of the gene encoding the enzyme. The regulation of gene expression is induced by a certain signal, such as insulin or muscle contraction, and it is mediated by transcription factors. This regulation is complex, as these transcription factors act in coordination with other transcription factors and they may be activated or inactivated by multiple protein kinases and phosphatases in response to different stimuli. (Nelson & Cox 2013, 608–610.) Some of these factors regulating gene expression are discussed more in detail later in this review.

3 LIPID METABOLISM IN ADIPOSE TISSUE AND SKELE-TAL MUSCLE

3.1 Energy production from fat

Fats, stored as triacylglycerols (TAG) mainly in white adipose tissue but also, to a lesser extent in other tissues, such as skeletal muscle and blood plasma, are the largest energy reserve of the body (Horowitz & Klein 2000; Lass et al. 2011). The amount of energy stored as endogenous TAG represents remarkably larger than the amount of energy stored as the form of glycogen (Horowitz & Klein 2000; Horowitz 2003). TAG consists of one molecule of glycerol to which three fatty acids are bound with ester bonds (Nelson & Cox 2013, 630). Fatty acids can be released from endogenous triacylglycerol stores by lipolysis, that is, hydrolysis of triacylglycerol by the enzyme hormone-sensitive lipase. This is mainly regulated by hormones that either stimulate or inhibit hormone-sensitive lipase. (Horowitz & Klein 2000.) Free fatty acids (FFA) liberated to circulation from adipose tissue are then transported bound to albumin in blood plasma. From plasma, FFAs can be transported to tissues, such as skeletal muscle, to serve as substrates for ATP production. (Lass et al. 2011; Nelson & Cox 2013, 669.)

3.1.1 Lipolysis and mobilization of fatty acids

Lipolysis is a catabolic process where hydrolytic cleavage of TAG results in liberation of three non-esterified fatty acids (NEFA) and one molecule of glycerol. The hydrolysis of primary and secondary ester bonds between long-chain fatty acids (LCFAs) and glycerol is performed by three enzymes (figure 2): First step, generating diacylglycerol (DAG) and NEFA from TAG, is performed by adipose triglyceride lipase (ATGL). This can also be performed by hormone sensitive lipase (HSL) but its role is much more prominent in the second step, which involves a cleavage of another fatty acid yielding monoacylglycerol

(MAG) and another NEFA. The third and last step is to break the last ester bond in a reaction that liberates glycerol and NEFA. This is likely to be performed by the enzyme monoglyceride lipase but other enzymes, such as HSL, might also have significant role in MAG hydrolysis. Also, other enzymes than the previously mentioned might contribute to lipolysis in tissues other than adipose tissue. (Lass et al. 2011.)
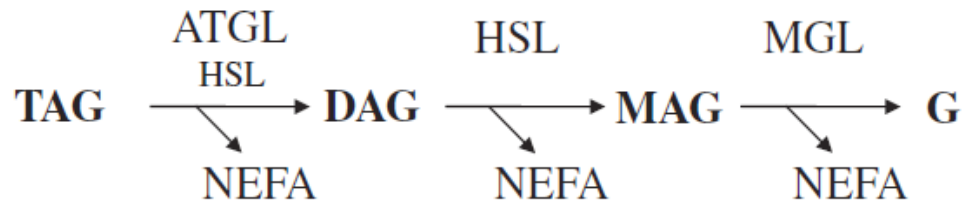
FIGURE 2. Lipolysis is an enzymatic three-step process in which triacylglycerol is degraded into glycerol and three free fatty acids (Lass et al. 2011).

As already stated, adipose tissue isn't, however, the only source of fatty acids for exemple during exercise. It has been found that intramuscular triacylglycerols (IMTG), lipid droplets stored inside muscle cells, may contribute as much as 10–50 % to total amount of fat oxidized during exercise. The hydrolysis of IMTGs provides a source of fatty acids that are more readily available for skeletal muscles to oxidize, and thus they form an attractive source of energy during exercise. In addition, there are lipid droplets also between muscle fibers but their contribution to energy production during exercise is unknown. Circulating plasma TAGs are another potential source of fatty acids for oxidation in active skeletal muscles. Lipoprotein lipase (LPL) is an enzyme located on the capillary endothelium that is able to hydrolyze plasma TAGs. Even though TAGs might be hydrolyzed in skeletal muscle tissue the released FFAs are not necessarily taken up and used locally at the site of LPL activity. (Horowitz 2003.)

3.1.2 Beta-oxidation of fatty acids

Fatty acid transport into the mitochondria. After being used for ATP production, fatty acids need to be taken up by the muscles and transported to mitochondria where they are finally oxidized (Horowitz 2003; Nelson & Cox 2013, 670–672). Transport of FFAs from plasma into and inside skeletal muscle cells happens with the help of transport proteins. These include fatty acid translocase (FAT/CD36), fatty acid binding protein (FABP) and fatty acid transport protein (FATP). (Horowitz 2003.) The transport into mitochondria can happen in two manners depending on the length of the fatty acyl chain: fatty acids with 12 or fewer carbons can traverse plasma membrane freely, whereas fatty acids (majority of the fatty acids liberated from adipose tissue) with 14 or more carbons need specific membrane transport proteins. To get into the mitochondria these fatty acids have to go through carnitine shuttle consisting of three enzymatic reactions: 1) formation of fatty acyl-CoA by acyl-CoA synthetase in the outer mitochondrial membrane, 2) carnitine is attached to fatty acid by carnitine acyltransferase I to form fatty acyl-carnitine, which can then move across mitochondrial membranes through pores and acyl-carnitine/carnitine transporter. 3) Finally, in the inner face of the inner membrane, fatty acyl group is detached from carnitine which is replaced by coenzyme A by carnitine acyltransferase II. Then the formed fatty acyl-CoA and carnitine are released into the mitochondrial matrix, and fatty acid oxidation can begin. (Nelson & Cox 2013, 670–672.)

β-oxidation of fatty acids. The first step in fatty acid oxidation is called β-oxidation. It is a series of four enzymatic reactions that take place in the mitochondrial matrix. During these reactions fatty acyl-CoA molecule is modified by four enzymes, acyl-CoA dehydrogenase (VLCAD, MCAD or SCAD depending on the chain length), enoyl-CoA hydratase, β-hydroxyacyl-CoA dehydrogenase (β-HAD) and acyl-CoA acetyltransferase (also thiolase), to detach two carbons from the end of the fatty acyl-CoA chain in the form of acetyl-CoA. In addition, two pairs of electrons and four hydrogen ions are liberated during these reactions. Newly formed acetyl-CoA can then enter citric acid cycle to be oxidized to carbon dioxide and water. Electrons liberated during the reaction of β-oxidation are transferred to

electron carriers of the mitochondrial respiratory chain to be used in oxidative phosphory-
lation. The process of β-oxidation is repeated until the whole fatty acid molecule is degrad-
ed, and thus, the number of repetitions and the amount of ATP produced depends on the
length of the fatty acid. For example, β-oxidation of 16-carbon palmitic acid (palmitoyl-
CoA in the mitochondrial matrix) yields eight acetyl-CoA molecules and 28 ATP mole-
cules. When the formed acetyl-CoA molecules are oxidized in citric acid cycle, additional
80 ATP molecules are produced. Thus, complete oxidation of palmitoyl-CoA yields 108
molecules of ATP compared with 30–32 molecules of ATP produced during the oxidation
of one glucose molecule. (Nelson & Cox 2013, 672–675.)

3.2 Regulation of lipid metabolism

Utilization of adipose tissue -derived TAGs to fuel a bout of endurance exercise, for in-
stance, requires delicate coordination in the regulation of adipose tissue lipolysis, blood
flow, uptake of FFAs by the skeletal muscles and transport into the mitochondria (Horowitz
2003). The mechanisms of this regulation are reviewed briefly below.

Hormonal regulation of adipose tissue lipolysis. Catecholamines, i.e. epinephrine and nore-
pinephrine, and insulin are the main hormones regulating lipolysis in humans. Binding of a
catecholamine to a β-adrenoceptor on the plasma membrane of an adipocyte initiates an
intracellular signaling cascade that ultimately leads to activation of lipolysis. Each β-
adrenoceptor is coupled with a stimulatory G-protein that activates the enzyme adenylate
cyclase which then catalyzes a reaction where ATP is converted to cyclic AMP (cAMP).
cAMP then acts as a second messenger to activate protein kinase A (PKA) which phosphor-
ylates HSL leading to its activation. PKA also phosphorylates perilipins, proteins covering
the lipid droplet, which gives activated HSL access to TAGs within the droplet. Catechola-
mines may also bind to α_2-adrenoceptors located on the surface of adipocytes. Contrary to
β-adrenoceptors, α_2-adrenoceptors are coupled with inhibitory G-proteins casting an inhibi-
tory effect on adenylate cyclase and thus, catecholamine binding to α_2-adrenoceptor inhibits
lipolysis. This is the case at rest when blood catecholamine concentration is low. (Horowitz

2003.) However, during exercise rising catecholamine concentration increases β-adrenergic stimulation, which then causes up-regulation of lipolytic rate (Horowitz & Klein 2000; Horowitz 2003). The effect of insulin on lipolysis contrasts that of β-adrenergic stimulation: even a small increase in plasma insulin concentration may radically reduce lipolysis. Insulin action results in activation of phosphatidylinositol 3-kinase (PI3K) which phosphorylates and activates phosphodiesterase-3. Activated phosphodiesterase-3 then degrades cAMP and thus down-regulates the signaling pathway stimulating the initiation of lipolysis. (Horowitz 2003.) Insulin also promotes lipid synthesis from glucose through increased glucose uptake and activation of enzymes, such as pyruvate dehydrogenase, fatty acid synthase and acetyl-CoA carboxylase, all of which promote lipid synthesis in adipose tissue (Saltiel & Kahn 2001).

Regulation of IMTG lipolysis. Stimulation of β-adrenoceptors has been found to be associated with increased HSL activity and decrease in muscle IMTG content. Thus, exercise-induced increase in plasma epinephrine and resulting β-adrenergic stimulation may contribute also to increase in the lipolysis of IMTGs during exercise. HSL activation might be accomplished through phosphorylation of extracellular-regulated kinase (ERK). This is, however, not the only mechanism of muscle HSL activation, as HSL activation can be induced independently of β-adrenergic stimulation, possibly via Ca^{2+} signaling. (Horowitz 2003.)

Short-term regulation of fatty acid uptake. Fatty acid oxidation is tightly regulated to minimize the amount of fuel consumed (Nelson & Cox 2013, 678). Fatty acid uptake can be regulated at the level of muscle fiber and mitochondria. Short-term regulation of fatty acid uptake into muscle fibers involves translocation of FAT/CD36 from cytosol to plasma membrane (Bonen et al. 2000). Both insulin and muscle contraction have been found to promote this translocation analogous to GLUT4 translocation (Bonen et al. 2000; Luiken et al. 2002). Malonyl-CoA is a compound produced from acetyl-CoA in cytoplasm by acetyl-CoA carboxylase (ACC). It is an intermediate in fatty acid synthesis and inhibitor of CPT1, the rate limiting enzyme of LCFA transport into the mitochondria. (Spriet & Watt 2003.) According to previous studies, malonyl-CoA concentration can be regulated in two main mechanisms:

Firstly, cytosolic citrate is an allosteric activator of ACC, and also substrate for the malo-nyl-CoA precursor, cytosolic acetyl-CoA (Ruderman et al. 1999). It has been found that skeletal muscle malonyl-CoA levels increase during physiological hyperglycemia and hy-perinsulemia, which is accompanied by inhibition of CPT1 activity and LCFA oxidation in humans (Rasmussen et al. 2002). In addition, sustained increase in plasma glucose and/or insulin or inactivity caused by denervation has been found to result in increased muscle malonyl-CoA content due to increased cytosolic citrate concentration in rat skeletal muscle *in vivo* (Saha et al. 1999). Secondly, AMPK phosphorylates and thus inhibits the action of ACC and also its activation by citrate. Inhibition of ACC results in lowered malonyl-CoA concentration, which relieves the inhibition of CPT1. (Nelson & Cox 2013, 679; Rasmussen & Winder 1997; Ruderman et al. 1999; Winder et al. 1997.) AMPK may also activate malo-nyl-CoA decarboxylase (MCD) which catalyzes the opposite reaction than ACC thus favor-ing malonyl-CoA conversion to acetyl-CoA (Saha et al. 2000).

Short-term regulation of β-oxidation. As with glucose oxidation, accumulation of metabo-lites that reflect sufficient cellular energy charge may inhibit some of the enzymes of β-oxidation: high $NADH/NAD^+$ ratio inhibits β-HAD enzyme, whereas accumulation of ace-tyl-CoA inhibits the action of thiolase (Nelson & Cox 2013, 679.) Also, activation of differ-ent intracellular signaling cascades, such as AMPK signaling and sirtuins, may modulate the uptake of fatty acids and activities of the enzymes involved in β-oxidation (Hardie & Sa-kamoto 2006; Houtkooper et al. 2012).

Long-term regulation of fatty acid oxidation. The amount of the enzymes involved in fatty acid oxidation can be changed by transcriptional regulation of the genes encoding these en-zymes. The family of peroxisome proliferator-activated receptors (PPARs) plays a key role in the regulation of the expression of genes involved in fatty acid oxidation. For example PPARα, that acts in skeletal muscle, liver and adipose tissue is capable of inducing the ex-pression of genes related to fatty acid transport and those encoding for enzymes of β-oxidation. (Nelson & Cox 2013, 679, 682.) Also both PPARα and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) have been found to induce the expression of

pyruvate dehydrogenase kinase 4 (PDK4), which inhibits the action of PDH and thus inhibits glucose oxidation and promotes that of fatty acids (Araki & Motojima 2006; Ferré 2004; Wende et al. 2005).

4 REGULATION OF ENERGY METABOLISM DURING EX-ERCISE

4.1 Interplay between fat and carbohydrate utilization during exercise

Carbohydrates and fatty acids are the main sources of energy during exercise, and their relative contribution to fuel utilization is largely dependent on exercise intensity, duration and substrate availability. Carbohydrates are the predominant energy substrate when exercise intensity is high and duration relatively short, while fatty acids provide most of the fuel for more prolonged exercise of lower intensity. In addition, increase in CHO availability increases the utilization of CHOs for energy production and reduces that of FFAs while the opposite is true for increased FFA availability. (Horowitz & Klein 2000; Roepstorff et al. 2005a; Romijn et al. 1993; Spriet & Watt 2003.) The regulatory mechanisms will be discussed later below the heading "4.2 Regulation of fuel selection during exercise".

The effects of exercise intensity and duration have been studied by Romijn et al. in endurance trained men (1993) and women (2000). They found that with increasing exercise intensity, greater reliance on CHOs, especially on muscle glycogen, is apparent, while fat oxidation contributes less to total substrate utilization. The absolute contribution of plasma-derived substrates remains relatively constant over a wide range of exercise intensities, as decreased plasma FFA turnover with increasing exercise intensity is counterbalanced by increased plasma glucose turnover. They found also, that at least in endurance trained subjects, lipolysis in peripheral adipose tissue seems to be already maximally stimulated during low intensity exercise with relatively low catecholamine concentrations, as it is not further stimulated when exercise intensity is increased. However, IMTG lipolysis is stimulated only at higher exercise intensities, suggesting a potentially higher threshold for catecholamine stimulation. (Romijn et al. 1993; Romijn et al. 2000.)

During prolonged exercise at low intensity, i.e. 25% VO_{2max}, availability and oxidation of different substrates don't change much from 30 to 120 minutes of exercise. However, during exercise at moderate intensity, i.e. 65% VO_{2max}, the relative contribution of plasma-derived substrates progressively increases with increasing duration while the contribution of intramuscular substrates decreases. Thus, it seems that at the onset of exercise at moderate intensity, IMTGs are needed to compensate for a slow response in FFA release in plasma. (Romijn et al. 1993.)

4.2   Regulation of fuel selection during exercise

Fatty acids are released from endogenous triacylglycerol stores by lipolysis, that is, hydrolysis of triacylglycerol by the enzyme hormone-sensitive lipase. As previously discussed, this is mainly regulated by hormones that either stimulate or inhibit hormone-sensitive lipase. For example, catecholamines promote and insulin inhibits lipolysis and thus release of fatty acids. Exercise of mild or moderate intensity (25–65 % of VO_{2max}) increases fat oxidation 5–10-fold compared to resting state because of increased energy requirements and fatty acid availability. This results from enhanced adipose tissue lipolysis mediated by β-adrenergic stimulation and increased adipose tissue and skeletal muscle blood flow which facilitates the delivery of fatty acids from adipose tissue to skeletal muscle. The increased fatty acid oxidation during endurance exercise delays glycogen depletion and hypoglycemia and thus permits sustained physical activity. (Horowitz & Klein 2000.)

Exercise intensity has an effect on the rate of fat oxidation and the contribution of different sources of fatty acids to the oxidation. During low-intensity exercise, fatty acids derived mainly from plasma are oxidized. With increasing intensity, the rate of fatty acid oxidation increases along with increasing relative contribution of intramuscular triacylglycerols to fatty acid oxidation. However, during high-intensity exercise (>70 % of VO_{2max}), fatty acid oxidation is suppressed because of reduced release of fatty acids from adipose tissue. This does not result from decreased lipolysis but from reduced adipose tissue blood flow that

impedes the delivery of fatty acids from adipose tissue to skeletal muscle to be oxidized. (Horowitz & Klein 2000.)

There is a bunch of theories concerning the interplay between carbohydrate and fat oxidation during exercise, some of which are more likely to happen during exercise than the others (Spriet & Watt 2003). Some of these theories are briefly described below.

Effect of increased FFA availability. Increased availability of FFAs might decrease CHO oxidation via multiple mechanisms: As early as in the early 1960's Randle and coworkers suggested the existence of so called glucose-fatty acid cycle (also known as "Randle cycle"). The theory suggests that increasing FFA availability increases cellular concentrations of acetyl-CoA, citrate and glucose 6-phosphate (G6-P) (via reduced glycolytic flux). The accumulation of these compounds then leads to inhibition on enzymes pyruvate dehydrogenase (PDH), phosphofructokinase (PFK) and hexokinase (HK), respectively, leading to decreased CHO oxidation and ultimately reduced glucose uptake into the cell. These studies were conducted using contracting cardiac muscle, but as skeletal muscle relies much more on intracellular glycogen stores, the situation in contracting skeletal muscle is obviously different. Indeed, studies examining skeletal muscle tissue during exercise suggest that the inhibition of CHO oxidation happens rather at the level of glycogen phosphorylase and PDH. One hypothesis to explain the mechanisms is that high FFA availability inhibits the decrease in cellular energy charge. First of all, inorganic phosphate acts as a substrate and ADP and AMP as allosteric regulators for phosphorylase and thus, decreased availability of these regulators could explain decreased glycogenolysis by phosphorylase. In addition, high ATP/ADP ratio activates the enzyme PDH kinase (PDK), which phosphorylates and thus inactivates PDH. One possible explanation for increased energy charge with high FFA availability, is increased mitochondrial NADH content. However, this needs more profound investigation to be proven. The results from different studies are somewhat controversial and so, for example during prolonged exercise it is possible that simply the depletion of substrates for phosphorylase (glycogen depletion) and PDH (decreased pyruvate production) account for the decreased activity of these enzymes. Decrease in the CHO availability

and increased reliance on fat oxidation has been shown to induce the expression of PDK4 isoform. As PDK4 inhibits PDH, fatty acid oxidation is favored instead of CHOs. Also, greater IMTG content increases the reliance on fat and decreases that on CHOs to fuel exercise. (Spriet & Watt 2003.)

Effects of increased CHO availability. Increased CHO availability seems to decrease fat oxidation via at least two possible mechanisms: either via inhibition of long-chain fatty acid (LCFA) transport into the mitochondria or secondarily via increased insulin signaling. Carnitine palmitoyltransferase 1 (CPT1) is an enzyme located on the outer mitochondrial membrane that plays an important role in the transport of LCFAs into the mitochondria. This step is believed to be the rate-limiting step in fatty acid oxidation. In addition to CHO ingestion prior to or during exercise, increasing exercise intensity (above 75% VO_{2max}) also attenuates fat oxidation via increased glycolytic flux. At higher intensities, blood glucose concentration increases and cellular CHO metabolism and breakdown are accelerated via glycogenolysis, glycolysis, PDH activation and CHO oxidation. Also, muscle pH tends to drop along with increased flux through glycolysis at high exercise intensity. This slight acidification has been shown to inhibit CPT1 activity which could explain the reduction in fatty acid oxidation. Besides the drop in pH, another mechanism has also been suggested. (Spriet & Watt 2003.)

Malonyl-CoA concentration has also been suggested to rise with increased CHO oxidation and excess of CHO availability. This might cause inhibition of CPT1 and thus decrease in fatty acid oxidation. (Nelson & Cox 2013, 679; Spriet & Watt 2003.) However, this doesn't seem to happen in human skeletal muscle during exercise (Spriet & Watt 2003). For example in the study of Odland et al. (1998) muscle malonyl-CoA content did not increase during high intensity exercise despite significant increase in PDH activation and accumulation of acetyl-CoA and acetylcarnitine all of which result from increased CHO catabolism. Instead, as malonyl-CoA levels have been found to decrease during muscle contractions in rodents and during exercise in humans, it could indicate that malonyl-CoA inhibits CPT1 and thus limits transport of FFAs into the mitochondria at rest and that this inhibition is relieved in

the beginning of exercise to allow greater energy production from fatty acids (Odland et al. 1996; Roepstorff et al. 2005; Spriet & Watt 2003). Indeed, decreased malonyl-CoA levels have been associated with greater fatty acid oxidation during exercise (Roepstorff et al. 2005). However, not all studies have found decreased malonyl-CoA levels after or during exercise at moderate intensity in humans (Dean et al. 2000; Odland et al. 1996; Odland et al. 1998). Also the data concerning different exercise intensities is somewhat controversial (Dean et al. 2000; Odland et al. 1998; Roepstorff et al. 2005). Roepstorff et al. (2005) found a significant decrease in muscle malonyl-CoA concentration after 30 and 60 minutes of exercise at 65% VO_{2max} in subjects with either high or low pre-exercise glycogen content. The malonyl-CoA content tended to decrease more in subjects with low pre-exercise glycogen content. In the study of Dean et al. (2000) subjects exercised for 45 min at 60% VO_{2max}, 10 min at 85% VO_{2max}, and until exhaustion (100% VO_{2max}) with 10 minutes of rest between each exercise bout. Contrary to Roepstorff et al. (2005) they found decreased muscle malonyl-CoA levels only after exercise bouts at high intensities (85 and 100% VO_{2max}). This somewhat contrasts the assumption that decrease in malonyl-CoA content is associated with increased fat oxidation, as CHO utilization increases at higher intensities. Odland et al. (1998) studied changes in muscle malonyl-CoA concentration one and 10 minutes after the onset of exercise at intensities of 35%, 65% and 90% VO_{2max}. The only condition where significant decrease in muscle malonyl-CoA content was found, was 1 minute after the onset of exercise at 35% VO_{2max}. The malonyl-CoA content seemed to have decreased also after one and 10 minutes after the onset of exercise at 90% VO_{2max}, but, even if this result would be consistent with the results of Dean et al. (2000), this decrease wasn't statistically significant. However, in the study of Odland et al. (1998) the subjects had consumed a meal high in CHOs 2–4 hours before the trials which may have had influence on the results as glucose and insulin might have inhibited the decrease in malonyl-CoA (Dean et al. 2000; Odland et al. 1998).

During exercise, cellular AMP levels increase and those of PCr decrease which induces AMPK activation. It has been suggested that this might result in the decrease of malonyl-CoA during exerise. (Rasmussen & Winder 1997; Ruderman et al. 1999; Spriet & Watt

2003.) The fact that increasing AMPK activation with increasing exercise intensity is accompanied by increase in ACC inactivation also supports this theory (Rasmussen & Winder 1997). Roepstorff et al. (2005) studied intracellular mechanisms to regulate fat oxidation in response to altered muscle glycogen content (CHO availability) in human skeletal muscle during submaximal, moderate intensity exercise. They tested two possible mechanism that might play a role in regulating the contributions of fat and carbohydrates to energy production: 1) High glucose levels result in increased malonyl-CoA concentration whereas AMPK activity decreases it, both by regulating acetyl-CoA carboxylase (ACC) activity. Decrease in malonyl-CoA levels relieves the inhibition laid upon CPT1 and fat oxidation is favoured. 2) Carnitine is needed for the transport of fatty acids into the mitochondria. However, carnitine also buffers accumulated acetyl-CoA, e.g. during high intensity exercise, which might result in limited availability of carnitine for fatty acid transport and thus shift energy production towards glucose utilization. They found that during a 60-minute bicycle ergometer test at 65% VO_{2max} both fat and carbohydrate oxidation rates increased in both conditions, high and low pre-exercise glycogen levels, but fat oxidation was significantly higher in subjects with low glycogen levels whereas carbohydrate oxidation was higher in those with high glycogen levels. AMPK phosphorylation and activity increased more when pre-exercise glycogen levels were low, whereas ACC phosphorylation increased similarly in both conditions. Consistent with ACC phosphorylation, malonyl-CoA concentration decreased similarly in both conditions, even if it had a slight tendency to decrease more with low glycogen levels. Acetyl-CoA concentration did not differ at rest between conditions but it decreased during exercise with low glycogen levels and increased with high glycogen levels. After the exercise bout, acetylcarnitine concentration was higher in subjects with high pre-exercise glycogen levels, whereas subjects with low pre-exercise glycogen levels had higher free carnitine concentration. PDH activity was lower with low glycogen levels at rest and after exercise, but the activity increased during exercise irrespective of condition. As ACC activity and malonyl-CoA concentration were not dependent on glycogen availability, this study suggests that greater fatty acid oxidation with low pre-glycogen levels is probably explained by some other mechanism than AMPK mediated ACC inhibition and decrease in malonyl-CoA concentration. Instead, availability of free carnitine had probably

a more pronounced effect on fat oxidation, as the skeletal muscle free carnitine concentration as well as the fat oxidation rate were higher during exercise with low glycogen levels. Thus, increased production of acetyl-CoA by PDH might have resulted in the decreased free carnitine availability with high glycogen availability resulting in impaired capability to fatty acid transport. These results indicate that PDH, acetyl-CoA and acetylcarnitine are potential factors linking the regulation of fat oxidation to carbohydrate availability and glycolytic flux. (Roepstorff et al. 2005.) These results are in line with previous results of Odland et al. (1998), who found that increased reliance on CHOs (based on RER data) at higher exercise intensities was accompanied by increased PDH activity and accumulation of acetyl-CoA and acetylcarnitine without significant alteration in malonyl-CoA concentration.

In the case of glucose ingestion before exercise, fat oxidation might be secondarily inhibited via insulin action and resulting increased availability of CHOs. Decreased transport of LCFAs may lead to accumulation of LCFA-CoA in the cytoplasm and subsequent inhibition of HSL action leading to diminished IMTG hydrolysis. Decreased fatty acid oxidation may also result from decreased availability of FFAs due to insulin's effect on adipose tissue to inhibit lipolysis. (Spriet & Watt 2003.)

4.3   Intracellular regulatory pathways

In addition to hormonal regulation, numerous metabolic, biochemical and mechanical stimuli induced by e.g. exercise or disease have influence on the responses, such as substrate use, of individual cells (Mooren & Völker 2005, 64). Thus, it is also important to look at the intracellular regulators and signaling pathways when trying to clarify molecular mechanisms behind differences in energy metabolism induced by exercise or a disease, such as diabetes.

AMPK signaling. AMPK is a protein kinase that acts as a sensor for cellular energy state: it is allosterically activated by 5'-AMP. Thus high AMP/ATP ratio, resulting from decreased ATP synthesis or increased ATP consumption, stimulates AMPK phosphorylation by up-

stream kinases (e.g. LKB1) and subsequent activation. AMPK has been identified to have a glycogen-binding domain, and it has been proposed that AMPK might be also regulated by muscle glycogen levels. Also, it has been suggested that leptin and adiponectin liberated from adipose tissue activate AMPK in muscle and consequently increase fatty acid oxidation. AMPK activation leads to both phosphorylation of its down-stream targets (acute effects) and changes in gene expression (chronic effects). (Hardie & Sakamoto 2006.) These actions result in expression of genes related to mitochondrial and oxidative metabolism and increased plasma glucose and fatty acid uptake via promotion of glucose and fatty acid transporter translocation and oxidation by the muscle (Canto & Auwerx 2010; Hardie & Sakamoto 2006).

MAPK signaling. The family of mitogen activated protein kinases consists of four members: extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 MAPK, c-Jun NH2-terminal kinases (JNK) and ERK5. MAPKs are stimulated by cytokines, growth factors and cellular stress, such as exercise. MAPK mediate cellular responses by phosphorylating their down-stream targets and thus having influence on substrate metabolism, cell proliferation, differentiation, hypertrophy, apoptosis, inflammation and gene expression via phosphorylation of transcription factors and coactivators. It seems that ERK1/2 participates in regulation of fatty acid uptake and oxidation during exercise, while p38 MAPK potentially regulates gene expression of GLUT4 and PGC-1α in response to exercise to enhance cellular glucose uptake and oxidative capacity. (Kramer & Goodyear 2007.)

AMPK and MAPK signaling during exercise. According to previous studies, exercise increases the activation of AMPK and p38 MAPK via phosphorylation (Akimoto et al. 2005; Gibala et al. 2009; Little et al. 2010; Terada et al. 2002). The activation of these proteins has been shown to up-regulate PGC-1α expression and also its activation via phosphorylation (Akimoto et al. 2005; Arany 2008; Jäger et al. 2007; Pogozelski et al. 2009; Puigserver et al. 2001; Terada et al. 2002; Terada & Tabata 2004). It has also been suggested that exercise-induced AMPK and p38 MAPK activation might promote nuclear translocation of PGC-1α, thus promoting its action as a transcriptional regulator (Little et al. 2010). In addition, there

is evidence that AMPK induced PGC-1α phosphorylation mediates the expression of multiple genes related to oxidative metabolism including the expression of PGC-1α itself (Jäger et al. 2007).

Sirtuins. Sirtuins are a family of NAD^+-dependent protein deacetylases that act as important regulators of metabolism and healthspan. As the enzymatic reaction catalyzed by sirtuins is dependent on NAD^+, the sirtuins are responsive to nutritional state of the cell and mediate the responses to energy stress. For example during exercise, NAD^+ levels rise in skeletal muscle, which is accompanied by sirtuin activation. According to current knowledge, the sirtuin family consists of seven members (Sirt1–7), which differ from one another in tissue specificity, subcellular localization, enzymatic activity and targets. Here the focus will be on the sirtuins 1, 3 and 6. Sirt1 is the best described and the most profoundly studied sirtuin. It is localized mainly in the nucleus but it can be exported to cytosol in response to specific signals. This happens for example when insulin signaling is inhibited. Sirt1 is able to activate PGC-1α and FOXO proteins via deacetylation and thus it plays an important role in the regulation of mitochondrial gene expression and substrate metabolism. (Houtkooper et al. 2012.) Sirt1 knockdown has been shown to result in decreased fatty acid oxidation, increased PGC-1α acetylation and down-regulation of mitochondrial and fatty acid oxidation gene expression in cultured cells (Gerhart-Hines et al. 2007). Sirt3 is localized mainly to mitochondria where it deacetylates multiple target enzymes related to aerobic energy metabolism and protection of the cell from oxidative stress. It has been found to promote fatty acid oxidation by deacetylating the enzyme LCAD in response to prolonged fasting. (Houtkooper et al. 2012.) It has also been suggested that, in response to exercise, Sirt3 might induce the expression of PGC-1α via activation of AMPK and/or CREB in skeletal muscle (Palacios et al. 2009). On the other hand, PGC-1α has been shown to potentially control the expression of Sirt3 in an ERRα dependent manner (Giralt et al. 2011). Sirt6 is a nuclear protein deacetylase, showing also ADP-ribosylation activity. Its main functions are related to DNA-stability and repair, but also inhibition of glycolysis via inhibition of HIF1α. It may also stimulate the expression of genes involved in fatty acid oxidation. So, altogether

the current knowledge suggest that these three sirtuins act as boosters of fatty acid oxidation during conditions of energy stress. (Houtkooper et al. 2012.)

PGC-1α. Common to all of these signaling pathways is protein PGC-1α, which has been called a master regulator of mitochondrial biogenesis and aerobic energy metabolism (figure 3). During exercise, cellular energy charge decreases which is demonstrated by increased AMP/ATP ratio. This activates AMPK which in turn phosphorylates and activates PGC-1α. AMPK also activates Sirt1 indirectly via increased NAD$^+$ levels. The action of Sirt1 locks PGC-1α to its active state. Sirt1 also activates PPARα and this together with PGC-1α activation leads to suppression of glycolysis and up-regulation of genes related to fatty acid uptake, β-oxidation and mitochondrial biogenesis. (Canto et al. 2009; Houtkooper et al. 2012; Lomb et al. 2010.) In addition, Sirt1 may also activate AMPK via deacetylation of its upstream activator LKB1 (Lan et al. 2008).
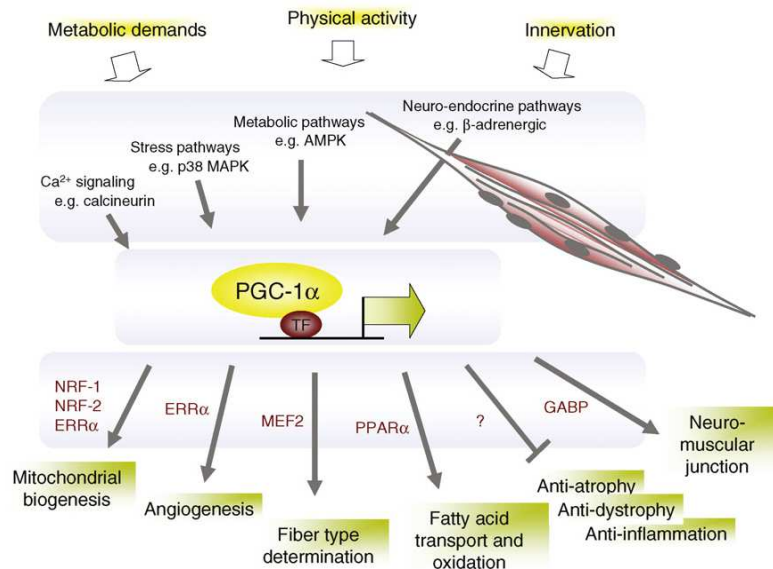

FIGURE 3. Transcriptional regulation of gene expression by PGC-1α (Arany 2008).

Regulation of substrate utilization by PGC-1α. PGC-1α induces transcription of genes favoring the switch from glucose to fatty acid oxidation: It induces the expression of PDK4 which inhibits the rate limiting reaction of glucose oxidation, conversion of pyruvate to ace-

tyl-CoA, by phosphorylating and thus inactivating the enzyme PDH (Araki & Motojima 2006; Wende et al. 2005). Consistently PGC-1α overexpression has been shown to result in reduction in glucose oxidation in cultured myoblasts (Wende et al. 2005). Also, increased Sirt1 activation and PGC-1α deacetylation have been associated to induction of the expression of mitochondrial and fatty acid utilization genes and increased fatty acid oxidation (Gerhart-Hines et al. 2007). PGC-1α transgenic mice are also able to exercise at higher intensity without a shift towards CHO utilization compared with wild type mice, which indicates higher capacity for fatty acid oxidation (Calvo et al. 2008). There is also evidence that PGC-1α plays a role in the expression of genes involved in citric acid cycle, oxidative phosphorylation (e.g. Cyt *c*) and fatty acid uptake (FAT/DC36), transport (FABP3, FATP1, CPT1B) and oxidation (MCAD, LCAD, VLCAD) in skeletal muscle (Calvo et al. 2008; Geng et al. 2010; Leick et al. 2008).
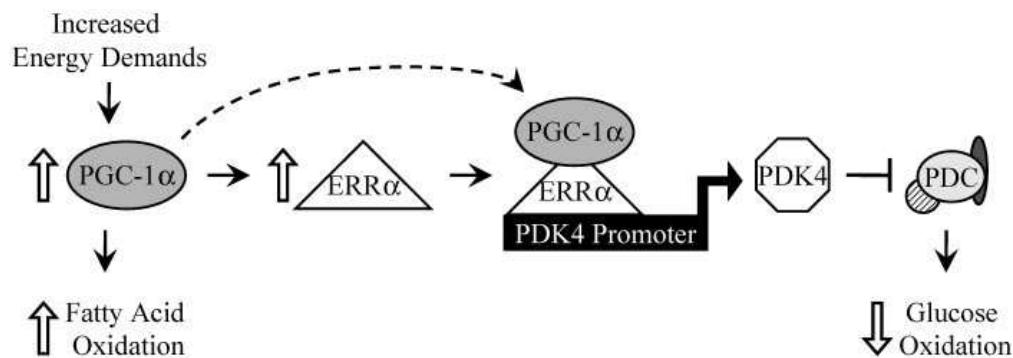
FIGURE 4. Regulation of PDK4 expression by PGC-1α (Wende et al. 2005).

4.4 Long-term adaptations in energy metabolism during exercise

Regular endurance training results in improvement of aerobic capacity which is demonstrated by increased VO_{2max}. This improvement is due to multiple adaptations in cardio-vascular system and skeletal muscle tissue induced by training. (Holloszy & Coyle 1984.) The central cardio-vascular adaptations are out of the scope of this literature review, and thus, adaptations taking place in the peripheral tissues, especially skeletal muscle, are in the focus here.

It has been established that fat oxidation during submaximal exercise increases after endurance training (Carter et al. 2001; Friedlander et al. 1998a; Holloszy & Coyle 1984; Horowitz & Klein 2000; Phillips et al. 1996). This effect is due to many adaptations taking place in skeletal muscle tissue: the delivery of fatty acids to muscle fibers is enhanced by increased capillarization in skeletal muscle tissue, the capacity to transport fatty acids into muscle fibers and mitochondria is increased when the expression of transport proteins increases and finally, the capacity to oxidize fatty acids is improved by increasing mitochondrial density and enzyme activities in muscle fibers. (Holloszy & Coyle 1984; Horowitz & Klein 2000; Kiens et al. 1993; Kiens et al. 1997; Talanian et al. 2007.)

The data obtained from previous research shows, that the increased fat oxidation isn't related to increased lipolysis in adipose tissue. Endurance trained subjects have been found to have similar adipose tissue and whole-body lipolytic responses to epinephrine and to exercise at same absolute intensity, respectively, as untrained subjects. (Horowitz & Klein 2000; Stallknecht et al. 1995.) However, trained subjects seemed to have enhanced epinephrine-stimulated adipose tissue blood flow (Stallknecht et al. 1995). During exercise at same relative intensity, endurance trained subjects have higher whole-body lipolytic rates, which might result from increased delivery of epinephrine to adipose tissue by enhanced adipose tissue blood flow (Horowitz & Klein 2000). In addition, trained individuals may rely more on IMTGs as substrates, as previously untrained subjects have been found to have decreased plasma FFA turnover and oxidation after a 12-week endurance training period despite increased total fat oxidation during prolonged exercise (Horowitz & Klein 2000; Martin et al. 1993).

Contradictory findings exist, however. Friedlander et al. (1999) found that whole-body lipolysis and total fat oxidation were unaffected after 10-week endurance training but FFA rate of appearance (R_a) as well as rate of disappearance (R_d) were increased after training during exercise at both same absolute and same relative intensity in young men. In young women, 12-week but otherwise similar endurance training resulted in increased FFA rate of appearance, rate of disappearance and oxidation measured during exercise at same absolute

and relative intensities, whereas no change was found in whole-body lipolysis. The increased fat oxidation in women after training was mainly due to increased plasma FFA oxidation. (Friedlander et al. 1998a) These results suggest that women may rely more on fat oxidation after endurance training than men (Friedlander et al. 1998a; Friedlander et al. 1999). It has been suggested that women rely more on fat oxidation than men during prolonged moderate intensity exercise also before endurance training (Carter et al. 2001).

Consistent findings have been established for adaptations in CHO metabolism by the same group. Glucose flux (R_a, R_d and metabolic clearance rate, MCR) was decreased after endurance training period in both men and women during exercise at same absolute intensity, but not at same relative intensity. This suggests that glucose flux is related to relative exercise intensity. When it comes to glucose oxidation, it decreased during exercise at both same absolute and relative intensity in men, and only at same absolute intensity in women. In women, also the relative contribution of CHOs to total energy expenditure was decreased after training during exercise at both same absolute and relative intensity, whereas no significant changes were found in men. These values were significantly different between genders. (Friedlander et al. 1997; Friedlander et al. 1998b.) However, in all of these studies by Friedlander et al. (1997; 1998a; 1998b; 1999) the relative improvement in aerobic capacity (VO_{2peak}) was approximately twice as great in women as in men, which might have had something to do with demonstrated gender differences in CHO and lipid metabolism.

In another study, as a consequence of seven weeks of endurance training the proportion of fat oxidized increased during exercise at same absolute intensity but not same relative intensity. This was also seen as lower RER values at same absolute but not at same relative intensity. Consistently, the relative contribution of CHOs to substrate oxidation decreased at same absolute but not at same relative intensity after training. However, glucose flux (R_a, R_d and MCR) was diminished during exercise at same absolute and same relative intensity, but glucose R_a and R_d were higher during exercise at same relative intensity than at same absolute intensity. Glucose uptake was diminished after training at both same absolute and same relative intensity. Glycerol R_a and R_d weren't altered by training period. Plasma norepineph-

rine concentration was lower after training during exercise at same absolute intensity but not at same relative intensity. For men, plasma epinephrine concentration was lower after training during prolonged exercise at both same absolute and same relative intensity, while women showed lowered concentration only at same absolute intensity. Both men and women improved their VO_{2peak} similarly. (Carter et al. 2001.)

Already a short period of endurance training may induce changes in substrate utilization. Phillips et al. (1996) found a 10% increase in fat oxidation due to increased IMTG and decreased glycogen oxidation during exercise at same absolute intensity after only five days of endurance training. After 31 days of training fat oxidation during exercise was increased further with concomitant decrease in CHO oxidation. Total fat oxidation was increased by ~70% mainly due to increased oxidation of IMTGs, while the reduced CHO oxidation was predominantly due to decrease in the rate of glycogen oxidation. Catecholamine response to exercise at same absolute intensity was attenuated in trained state. VO_{2peak} was improved after 30 days of training (10%) but wasn't significantly changed after only eight days of training. (Phillips et al. 1996.)

Contrary to the previously presented results, it has been also suggested that increased fat oxidation during exercise at same absolute intensity after training would result from increased uptake of FFAs into muscle cells due to enhanced transport capacity rather than from increased oxidation of IMTGs. This enhanced capacity for FFA uptake was suggested to be due to either increased capillarization or metabolic changes in trained skeletal muscle that improve the capacity to oxidize FFAs, demonstrated by increased activities of enzymes β-HAD and citrate synthase. It was also concluded that hormones, such as insulin and catecholamines, are not likely to play as important role in the shift of substrate use as the adaptations at the level of trained muscle. (Kiens et al. 1993.)

Also two weeks of high-intensity interval training (HIIT) has been found to increase VO_{2peak} and whole-body fat oxidation and to decrease reliance on muscle glycogen during exercise at 60% of pre-training VO_{2peak} in women. Similarly to more conventional endurance train-

ing, HIIT resulted in increased maximal activities of β-HAD and citrate synthase and in increased protein content of total muscle plasma membrane fatty acid-binding protein (FABP$_{pm}$). No changes were found in fatty acid translocase (FAT)/CD36 or cytosolic HSL content. These results suggest increased capacity to transport and oxidize FFAs during exercise after HIIT. (Talanian et al. 2007.) Increase in skeletal muscle FABP$_{pm}$ after more conventional endurance training has also been reported earlier (Kiens et al. 1997).

Contrary to previously presented results, Tunstall et al. (2002) demonstrated that nine days of endurance training increased the expression of FAT/CD36 both at mRNA and protein levels, without any changes in FABP$_{pm}$ expression. Also CPT1 mRNA expression was increased as a consequence of training suggesting, that the expression of genes related to FFA transport across plasma and mitochondrial membranes could play a role in increased fat oxidation in trained state. However, the mRNA expression of PPARγ was decreased after training, whereas no changes were found in the expression of PPARα, PGC-1α or SREBP-1c, all of which are transcriptional regulators of genes related to fatty acid uptake and oxidation. The expression of none of these factors was changed acutely in response to a single exercise bout. (Tunstall et al. 2002.) These gene expression results are somewhat contradictory to other studies as for example skeletal muscle PPARα protein expression has been found to increase substantially after 12 weeks of endurance training and it has even been suggested to play a regulatory role in increased fat oxidation after training (Horowitz et al. 2000). The interpretation of the results of Tunstall et al. (2002) is limited by the lack of protein expression data, small number of subjects and relatively short training period.

Some discrepancies exist between the results of different studies concerning for example the mechanism and the source of increased fat oxidation, or substrate kinetics during exercise at same absolute and same relative intensity. In some studies increased oxidation of IMTGs has been suggested to be the reason for increased fat oxidation while others suggest that it is due to increased plasma FFA oxidation. Similarly, some results suggest increased FFA transport capacity while others rather increased capacity to metabolize FFAs. The reasons for these differences are not known, but some of them may be at least partially explained by

slightly different exercise intensities used in trials, different exercise training modes or some other differences in the experimental protocols or methods that were used. (Carter et al. 2001; Friedlander et al. 1998a; Horowitz et al. 2000; Kiens et al. 1993; Phillips et al. 1996; Tunstall et al. 2002.)

5   DIABETES MELLITUS AND EXERCISE

5.1   Diabetes mellitus

Diabetes mellitus can be defined as a group of metabolic diseases that are characterized by a rise in blood glucose concentration to abnormal level, i.e. hyperglycemia (figure 4). Diabetes can be caused by defects in either insulin secretion, its action on target tissues, or both. Acute symptoms of the resulting hyperglycemia include polyuria leading to dehydration, polydipsia, weight loss and blurred vision. Uncontrolled diabetes also leads to acute potentially fatal conditions of hyperglycemia with ketoacidosis resulting from excessive utilization of fats, or nonketotic hyperosmolar syndrome. In addition, high blood glucose concentration causes other dysfunctions including abnormal function of blood vessels and nerve cells which then may trigger a number of other disorders and injuries cause severe harm to multiple tissues, highlighting the importance of strict control of glucose balance. Indeed, chronic hyperglycemia is associated with many of the long-term complications of diabetes, including damage, dysfunction and failure of e.g. eyes, nerves, kidneys, heart and blood vessels. Diabetes-induced complications include retinopathy, nephropathy and peripheral and autonomic neuropathies, just to mention a few. Also, patients with diabetes have an increased incidence of atherosclerotic cardiovascular, peripheral arterial and cerebrovascular disease: insulin deficiency increases lipolysis and release of fatty acids into the blood stream which eventually causes rise in plasma lipoprotein, cholesterol and phospholipid levels. This can ultimately lead to development of atherosclerosis. (American Diabetes Association 2013; Guyton & Hall 2000, 888–889, 894–895.)

An individual is considered to have prediabetes, i.e. increased risk for development of diabetes in the future, if their fasting plasma glucose (FPG) ranges from 100 mg/dl (5.6 mmol/l) to 125 mg/dl (6.9 mmol/l) (called impaired fasting glucose (IFG)), 2-hour plasma glucose in the 75-g oral glucose tolerance test (OGTT) falls between 140 mg/dl (7.8 mmol/l) and 199 mg/dl (11.0 mmol/l) (called impaired glucose tolerance (IGT)) or A1C is

5.7–6.4 % (test for glycated hemoglobin that provides information on the average plasma glucose concentrations over the past three months). When the individual meets at least one of the following criteria, they are diagnosed to have diabetes: A1C ≥6.5% or FPG ≥126 mg/dl (7.0 mmol/l) or 2-h plasma glucose ≥200 mg/dl (11.1mmol/l) during an OGTT or (in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis) a random plasma glucose of ≥200 mg/dl (11.1 mmol/l). (American Diabetes Association 2013.)

Stages / Types	Normoglycemia	Hyperglycemia		
	Normal Glucose Regulation	Impaired Glucose Tolerance or Impaired Fasting Glucose (Prediabetes)	Diabetes Mellitus		
			Not insulin requiring	Insulin requiring for control	Insulin requiring for survival
Type 1*	←——————————————————————————→			
Type 2	←————————————————————→			
Other Specific Types**	←————————————————————→			
Gestational Diabetes**	←————————————————————→			

FIGURE 4. Types and stages of glycemic disorders. The degree of hypeglycemia may change over time and is a continuum between normoglycemia and severe hyperglycemia and a state of insulin defiency where insulin is required for survival. (American Diabetes Association 2013.)

The different types of diabetes can be classified according to the etiology (i.e. the cause of the disorder), the pathogenic processes and the symptoms of the disease. Two main etiopathogenic categories can be distinguished: In the first category, named type 1 diabetes (also insulin-dependent diabetes or juvenile-onset diabetes), there is an absolute deficiency of insulin secretion, usually because of cellular-mediated autoimmune destruction of pancreatic (insulin-secreting) β-cells. This type of diabetes accounts for 5–10 % of all the cases of diabetes. On the contrary, diabetes of the other category, type 2 diabetes (also non-insulin-dependent diabetes) results from the combination of resistance to insulin action in

the target tissues and an inadequate compensatory response of insulin secretion (relative insulin deficiency). This type of diabetes accounts for 90–95 % of all the cases of diabetes. In addition to these two categories there are also other types of diabetes that may either fall into one or the other of these categories or be separate of them. Examples of these are gestational diabetes, genetic defects of β-cells or insulin action, diseases of the exocrine pancreas, endocrinopathies, drug- or chemical-induced diabetes and infections, just to mention a few. (American Diabetes Association 2013.)

5.2 Insulin deficiency, diabetes and energy metabolism

Bearing in mind insulin's key role in whole metabolism, it is not surprising that loss of insulin action or insulin deficiency results in multiple changes in cellular function. These changes include alterations in glucose and lipid metabolism and changes in gene expression and protein phosphorylation. Gene expression analysis of the skeletal muscles of insulin deficient and healthy mice revealed that the largest functional group of genes, whose expression was altered in insulin deficient mice, comprised genes related to energy and substrate metabolism. Also groups of genes related to transcriptional regulation and transport were altered in insulin deficiency. As glucose and ketone bodies contribute less to energy metabolism in diabetic skeletal muscle there is an increased need for fatty acid oxidation to meet the energy demands. Consequently the expression of many genes related to fatty acid oxidation were up-regulated in insulin deficient mice. These genes include all the enzymes of β-oxidation of fatty acids, HSL and proteins transporting LCFAs into and inside the cell. Consistently, the genes involved in fatty acid synthesis and ketone body metabolism were down-regulated in diabetic skeletal muscle. Although PPARα has been shown to up-regulate the enzymes of fatty acid oxidation, its expression was down-regulated in diabetic skeletal muscle. This was supposed to result from of a tissue-specific change induced by diabetes or from opposing effects of hyperglycemia and loss of insulin signaling. (Yechoor et al. 2002.) Consistent with these results type 1 diabetic patients have been shown to have higher myocardial fatty acid utilization, myocardial fatty acid oxidation (both absolute and relative) and lower myocardial glucose oxidation despite similar myocardial blood flow and plasma lac-

tate and insulin levels. Plasma glucose (only slightly) and free fatty acid levels as well as myocardial oxygen consumption were higher in type 1 diabetic patients. (Herrero et al. 2006.) In addition, muscle CPT activity has been found to be higher in type 1 diabetic subjects compared with healthy subjects at rest, although *in vitro* ^{14}C-palmityl CoA oxidation rates were similar at rest (Costill et al. 1979).

Glucose disposal is impaired in diabetic skeletal muscle and this was seen also in the changes in the expression of genes involved in glucose transport, uptake and metabolism. For example GLUT4 expression and its translocation to plasma membrane may be impaired in diabetes. In addition, the expression of the rate limiting enzymes of glycolysis and the dehydrogenase component of PDH converting pyruvate to acetyl-CoA were down-regulated in insulin deficiency. (Silvennoinen 2004; Yechoor et al. 2002.) In diabetic skeletal muscle PDH activity would be inhibited even more due to accumulation of acetyl-CoA resulting from fatty acid oxidation and decreased functioning of citric acid cycle causing substantial decrease in glucose oxidation. Furthermore, the expression of α-1,4- to 1,6-glucan-branching enzyme needed to normal glycogen formation was also decreased in insulin deficient mice. (Yechoor et al. 2002.)

As insulin promotes glucose transport into cells and its storage as glycogen by activating glycogen synthase, it can be assumed that skeletal muscle glycogen content would be decreased in diabetic compared to healthy muscle (Guyton & Hall 2000, 887; Saltiel & Kahn 2001). Indeed, impaired glycogen synthase activity and capacity for glycogen synthesis and storage have been found in diabetic individuals (Bak et al. 1989; Shulman et al. 1990).

In addition to previously reviewed changes in gene expression related to lipid and glucose metabolism, also gene expression related to the machinery for oxidative phosphorylation was altered in diabetes: the mRNA expression of the enzymes of electron transport chain were down-regulated in insulin deficient mice. These changes are likely to result in decreased capacity of the mitochondria to produce ATP and increased formation of reactive oxygen species. Some of all the above mentioned changes were responsive to insulin treat-

ment. (Yechoor et al. 2002.) According to the previous findings of our laboratory, some of these adverse effects of diabetes on gene expression, e.g. involved in glucose transport, citric acid cycle and electron transport chain, may be reversed or at least ameliorated by endurance training (Silvennoinen 2004).

5.3 Effects of diabetes and insulin deficiency on aerobic capacity

Type 1 diabetes mellitus has been shown to be associated with impaired aerobic capacity (el Midaoui et al. 1996; Gusso et al. 2008; Ianuzzo et al. 1974; Komatsu et al. 2005). The data from former studies suggests that both central and peripheral alterations may contribute to impairment of aerobic capacity in diabetes.

There are a number of studies suggesting that individuals diagnosed with T1DM have lower aerobic capacity in terms of maximal oxygen consumption and other cardio-respiratory parameters compared with healthy counterparts (Gusso et al. 2008; Komatsu et al. 2005; Komatsu et al. 2010; Niranjan et al. 1997). For example, Komatsu et al. (2005) studied 118 children and adolescents (both male and female) of which 72 had T1DM and the rest were healthy. They found that during an incremental treadmill test for maximal aerobic capacity, the diabetic subjects reached significantly lower values for all the parameters assessed, i.e. peak oxygen consumption (ml/kg/min), peak ventilation (btps), peak heart rate (beats/min) and time to exhaustion, compared to healthy controls. The groups studied did not differ significantly from each other for any of the assessed demographic or clinical parameters (e.g. age, weight, height, BMI and gender distribution), except for HbA1c (%) (glycated hemoglobin), that was obviously higher in diabetic subjects. (Komatsu et al. 2005.) In another study, maximal aerobic capacity and cardio-vascular responses to submaximal exercise were investigated in type 1 (n=12) and type 2 (n=8) diabetic female adolescents and compared with obese (n=10) and non-obese (n=10) controls. Diabetic subjects were found to have lower VO_{2max} (relative to body weight and fat free mass) and maximal heart rate in the maximal cycle ergometer test than non-diabetic controls despite similar reached maximal powers. In addition, during rest and submaximal exercise at fixed heart rate (100 and 120

bpm) type 1 diabetic subjects had lower cardiac output and stroke volume compared to non-diabetic controls. When indexed to fat free mass, the cardiac output and stroke volume responses of both type 1 and type 2 diabetic subjects to submaximal exercise were 30–40% lower than in the non-diabetic groups. (Gusso et al. 2008.) It has also been found that many of the impairments in aerobic capacity are less severe in normoglycemic than hyperglycemic diabetic subjects (Niranjan et al. 1997).

However, there are some studies that haven't found any difference in aerobic capacity between diabetic and healthy subjects. For example, in a study by Fintini et al. (2012) cardiovascular capacity was similar between type 1 diabetic and healthy children even if the diabetic children were less active than their healthy counterparts. However, HbA1c negatively correlated with VO_2 in multivariate analysis which might indicate a connection between glycemic control and cardiovascular performance. (Fintini et al. 2012.) Similarly, Veves et al. (1997) reported similar maximal oxygen uptake values for type 1 diabetic and healthy exercising individuals and for type 1 diabetic and healthy sedentary individuals. However, the exercising type 1 diabetic individuals with cardiac autonomic neuropathy had lower maximal oxygen uptake than the other exercised groups but similar to the sedentary groups. They concluded that diabetes in itself doesn't affect the aerobic capacity in physically active individuals, and that the reduction in aerobic capacity could be due to neuropathy. As levels of physical activity or the presence of autonomic neuropathy weren't reported in the studies reviewed above, the causes behind the impaired aerobic capacity can only be speculated. But, as was discussed by Gusso et al. (2008), the lower peak heart rate of the diabetic subjects might be due to impaired autonomic response to exercise, or cardiac autonomic neuropathy that lowers the normal exercise-induced heart rate response (Veves et al. 1997). This is also supported by the study of Costill et al. (1979) in which diabetic, not showing any symptoms of neuropathy, and non-diabetic subjects had similar pre-training values for VO_{2max} and maximal heart rate in a maximal treadmill test. However, the possibility that the diabetic subjects just had lower physical activity levels prior to the studies cannot be fully excluded.

Diabetic skeletal muscle has been reported in numerous studies to have lowered oxidative capacity. For example, diabetes has been shown to result in impaired capacity to oxidize palmitate-1-[^{14}C] and pyruvate, decreased activity of oxidative marker enzymes (e.g. citrate synthase, succinate dehydrogenase, and 3-hydroxyacyl-CoA dehydrogenase), lower myoglobin content of skeletal muscle and slower production of ATP by mitochondria relative to the mitochondrial protein content. (el Midaoui et al. 1996; Ianuzzo et al. 1974; Kivelä et al. 2006; Noble & Ianuzzo 1985.) Also, greater reliance on anaerobic glycolysis has been reported in type 1 diabetic individuals at rest and during an isometric muscle contraction exercise. In that study, the oxidative capacity of the muscles was evaluated by measuring the recovery rate of phosphocreatine (PCr) after a 30-s isometric contraction of ankle dorsiflexors. Diabetic subjects had significantly lower rates of PCr replenishment indicating lower oxidative capacity of the muscles. (Crowther et al. 2003.) However, insulin deficiency has been shown to result in decreased expression of creatine kinase homologs, which could also account for impaired PCr replenishment (Silvennoinen 2004; Yechoor et al. 2002).

A study comparing healthy and diabetic athletes and healthy and diabetic non-athlete controls found that diabetic athletes had similar VO_{2max} as healthy athletes but lower anaerobic thresholds. The authors speculated that this could be due to both metabolic issues, such as hyperglycemia and low lactic acid clearance during and after exercise, and respiratory problems. (Komatsu et al. 2010.) This is somewhat in accordance with the results of Crowther et al. (2003) who found greater reliance in anaerobic glycolysis, although the exercise mode was very different from the study of Komatsu et al. (2010).

5.4 Effects of diabetes and insulin deficiency on responses to exercise

Crowhter et al. (2003) reported greater reliance on anaerobic glycolysis during both rest and exercise in men with well-controlled type 1 diabetes compared with healthy counterparts. This was manifested by lower muscle pH at rest and during 120-s sustained isometric contraction of ankle dorsiflexors at 70–75 % of maximal voluntary contraction (MVC). In addition, during exercise, the pH dropped more quickly and the onset of glycolytic flux, meas-

ured as the accumulation of glycolytic hydrogen ions, happened earlier in the diabetic subjects. The peak glycolytic flux rate was also found to be higher in diabetic subjects. (Crowther et al. 2003.) Type 1 diabetic athletes have been reported to have lower anaerobic thresholds than non-diabetic athletes despite similar VO_{2max} values (Komatsu et al. 2010). This suggests that diabetic individuals might rely more on anaerobic glycolysis also during aerobic exercise of relatively high intensity.

As already stated, diabetic individuals have been suggested to have impaired glycogen synthase activity and capacity for glycogen synthesis and storage (Bak et al. 1989; Shulman et al. 1990). In addition, low glycogen levels have been shown to result in increased fat oxidation during exercise (Roepstorff et al. 2005). So, it could be assumed, that insulin deficiency would result in increased reliance on fat oxidation during exercise. This is supported by the fact, that capacity to use fatty acids seems to be higher in type 1 diabetes, measured at the levels of both gene expression and enzymatic activity (Costill et al. 1979; Yechoor et al. 2002).

According to the knowledge of the author, there are no studies available at present comparing the acute responses to exercise at the level of intracellular signaling between healthy and type 1 diabetic individuals or animals.

5.5 Training adaptations and beneficial effects of exercise

Exercise has been shown to reverse many of the impairments in aerobic capacity, such as lowered VO_{2max} and capacity of skeletal muscle to produce ATP aerobically, related to diabetes or insulin deficiency (Costill et al. 1979; el Midaoui et al. 1996; Ianuzzo et al. 1974; Kivelä et al. 2006a; Laaksonen et al. 2000; Noble & Ianuzzo 1985). In addition, exercise has been shown to have many other positive, health-related effects, such as improved glycemic control, lipid profile and vascular function, in patients with type 1 diabetes. Thus, regular exercise has been recommended to individuals with diabetes. (Costill et al. 1979;

Fuchsjager-Mayrl et al. 2002; Laaksonen et al. 2000.) Training adaptations and some health benefits of exercise training for the diabetic patients will be discussed briefly below.

Training adaptations. Endurance training has been proved to improve the aerobic capacity of type 1 diabetic individuals (Costill et al. 1979; Laaksonen et al. 2000). Costill et al. (1979) found that diabetic subjects not exhibiting any symptoms of neuropathy showed similar adaptations to 10 weeks of endurance training (30 min on 5 days per week) as the healthy controls: Firstly, the VO_{2max} of the diabetic subjects increased 11.0% while the increase for the healthy controls was 12.9%. Secondly, the activities of enzymes lipoprotein lipase (LPL, hydrolysis of plasma TAGs), carnitine palmityltransferase (CPT, fatty acid transport to mitochondria), succinate dehydrogenase (SDH, citric acid cycle)) and hexokinase (glycolysis) in skeletal muscle increased markedly during the training period and this increase was similar to both diabetics and healthy controls. Malate dehydrogenase (MDH, citric acid cycle) activity increased also with training but this increase was significant only for the non-diabetic subjects. Before training, both groups had similar capacities for ^{14}C-palmityl-CoA oxidation in skeletal muscle (measured *in vitro*) and the rate of oxidation increased significantly with training for both groups indicating an increase in the capacity of muscles to oxidize lipids. However, after the training period the maximal rate of ^{14}C-palmityl CoA oxidation averaged 41% higher in diabetic subjects compared with healthy controls. (Costill et al. 1979.)

Glycemic control. VO_{2max} has been found to correlate inversely with HbAl and required insulin dose (Austin et al. 1993). In the study of Costill et al. (1979) the diabetic subjects were able to reduce their insulin dose 23% on average during the first two to three weeks of endurance training. Despite this reduction, the serum glucose concentration was significantly lower after the 10 weeks of training. Also, serum concentration of triglycerides was significantly reduced after the training period. Exercise training has also been found to restore the reported impaired glycogen synthase activity in diabetic individuals (Bak et al. 1989). When athletes and sedentary individuals with type 1 diabetes mellitus were compared, it was found that diabetic athletes had higher basal glycogen synthase activity compared with

sedentary diabetics. Glycogen synthase activity also correlated with VO_{2max}. However, there were no differences in muscle glycogen content between athletes and sedentary subjects and the difference in glycogen synthase activity disappeared during insulin infusion. Athletes had smaller insulin doses, potentially to avoid hypoglycemia, and it was suggested that the relative insulin deficiency increased the use of FFAs as a fuel, worsened hyperglycemia and possibly prevented improvement in insulin sensitivity. (Ebeling et al. 1995.)

Lipid profile. Physical fitness and daily energy expenditure on aerobic activity have been found to be associated with better plasma lipid profile in type 1 diabetic individuals (Austin et al. 1993; Laaksonen et al. 2000). In a study investigating the relationship between physical fitness and lipid profile in type 1 diabetic adolescents, VO_{2max} correlated inversely with blood cholesterol, LDL, triglycerides and lipoprotein(a) (Austin et al. 1993). In another study, daily energy expenditure on aerobic activity was found to correlate positively with serum apo A-I/apo B ratio and negatively with triglyceride levels. It also tended to correlate with HDL/total cholesterol ratio. In the same study, endurance training of 12–16 weeks resulted in decrease in serum levels of total and LDL cholesterol and apolipoprotein B (apo B). This was accompanied by a concomitant increase in HDL and apo A-1 levels as well as in HDL/LDL, HDL/apo A-I and apo A-I/apo B ratios. When relative changes in different lipid parameters were compared, favorable changes in HDL/LDL and apo A-I/apo B ratios and apo B and triglyceride levels were significantly greater in the endurance trained group compared with control group. In addition, it seemed that the training-induced relative changes in the HDL/LDL and apo A-I/apo B ratios were greater in the individuals with low HDL/LDL levels at baseline. Also, apo B levels decreased more in individuals that were less physically active before the training period. (Laaksonen et al. 2000.) Even though, Laaksonen et al. (2000) did not find significant decrease in serum triglyceride levels after endurance training in type 1 diabetic subjects, this kind of result has been reported elsewhere (Costill et al. 1979). These results suggest a positive effect of exercise on blood lipid profile, which might be especially beneficial for type 1 diabetic individuals already at higher risk for cardiovascular complications.

6 RESEARH QUESTIONS AND HYPOTHESES

6.1 Research questions

1. Are the key genes related to regulation of aerobic energy metabolism differently expressed (mRNA and protein level) or signaling pathways differently activated in gastrocnemius muscles of insulin deficient mice compared to healthy mice?

2. What kind of acute effects does a single exercise bout have on the expression of the key genes (mRNA and protein level) and on the activation of signaling pathways related to regulation of aerobic energy metabolism in healthy and insulin deficient mice? Is the response different in insulin deficient mice compared to healthy mice?

6.2 Hypotheses

1. The expression of genes (PGC-1α, PDK4, CPT1 and sirtuins) and the activation of signaling pathways (AMPK) favoring fatty acid oxidation, are up-regulated or at similar level in the gastrocnemius muscles of insulin deficient mice compared to healthy mice.

2. A single exercise bout acutely increases the expression of the genes favoring fatty acid oxidation in the gastrocnemius muscles of both healthy and insulin deficient mice but the response is more pronounced in insulin deficient mice. Consistently, the activation of the signaling pathways favoring fatty acid oxidation is more visible in the gastrocnemius muscles of the insulin deficient mice in comparison to healthy mice.

- AMPK and ACC phosphorylation increase in response to exercise and insulin deficiency.

- p38 MAPK phosphorylation increases in response to exercise, but decreases in response to insulin deficiency alone.

- mRNA and protein expression of PGC-1α, PDK4, CPT1B increase in response to exercise.

- Sirtuin protein expression increases (Sirt1) or stays the same (Sirt3) in response to exercise.

- The genes related to aerobic capacity (Cs and Cyt *c*) are down-regulated in insulin deficiency but up-regulated in response to exercise.

7 MATERIALS AND METHODS

7.1 Animals and experimental design

All the experimental procedures were approved by the Animal Care and Use Committee of the University of Jyväskylä. Male NMRI mice (n = 64, Harlan, The Netherlands) aged 10–15 weeks were used in this study. The mice were housed in standard conditions (temperature 22°C, humidity 60 ± 10 %, light from 8.00 am to 8.00 pm) and they were fed *ad libitum*. The mice were randomly assigned into healthy or diabetic groups (figure 5). Experimental type 1 diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, Sigma-Aldrich, France, 180 mg/kg), a compound that destroys pancreatic β-cells, dissolved in sodium citrate buffer solution (0.1 mol/l, pH 4.5). An injection of an equal volume, containing only citrate buffer, was administered to the healthy mice. The development of diabetes was confirmed with blood glucose test (HemoCue B-Glucose analyzer, Ängleholm, Sweden) three days after injection. The mice were considered diabetic when their blood glucose concentration exceeded 15 mmol/l. The mice were not treated with insulin during the experiment.
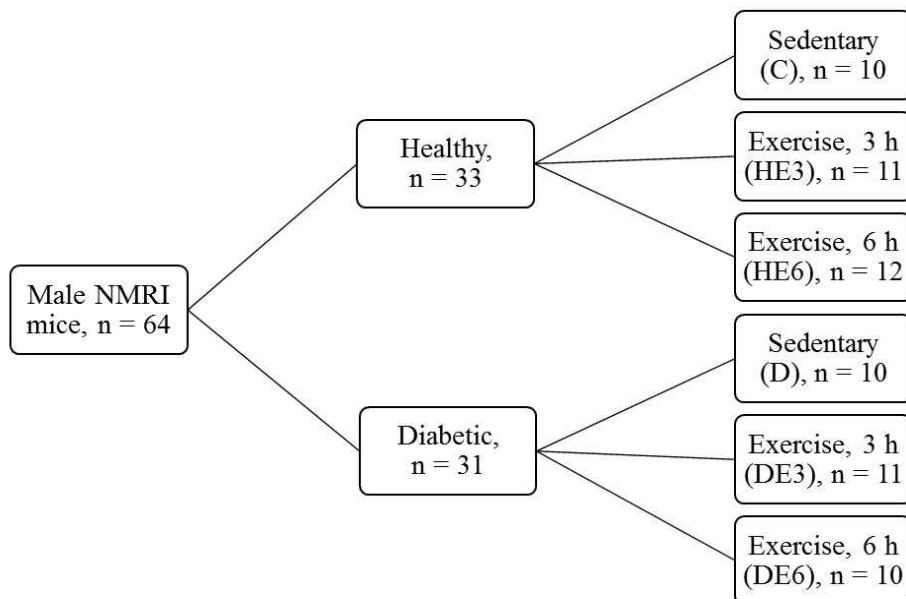

FIGURE 5. Grouping of the mice for the experiment.

Ten days after injection both healthy and diabetic mice were further assigned into three groups. One healthy and one diabetic group (H and D) served as sedentary controls while the other groups performed 1 hour running exercise on a treadmill (speed 21 m/min with an incline of 2.5°). Of the exercise groups, one healthy and one diabetic group were sacrificed 3 h (HE3 and DE3), and the other two groups 6 h post exercise (HE6 and DE6). The mice were familiarized with treadmill running prior to the experiment and only the mice capable of running on a treadmill were included in this study. Grouping was performed after the elimination of the mice not capable of running on a treadmill.

7.2   Data collection and tissue preparation

The mice were weighed and their plasma glucose concentration was measured (HemoCue B-Glucose analyzer, Ängleholm, Sweden) prior to and after the experiment. After the experiment, the mice were killed by cervical dislocation. Gastrocnemius muscles were collected, snap frozen in liquid nitrogen and then stored at $-80^{o}C$ before further RT-PCR and Western blotting analyses. For RNA extraction, whole cross sections were taken from the distal part of the muscle in order to take the heterogeneity of the muscle sample into account. Whole gastrocnemius muscle was used for Western blot analyses.

7.3   RNA extraction and reverse-transcription to cDNA

Total RNA was extracted from the gastrocnemius muscles with TRIzol reagent (Invitrogen-Life Technologies). Muscle samples were homogenized in TRIzol, which stabilizes RNA molecules and lyses cells and cell components. FastPrep® instrument (MP Biomedicals, California, USA) and compatible tubes were utilized for homogenization. Chloroform was added to the homogenate to separate it into two phases, organic and water phases, during centrifugation. Water phase containing the RNA molecules was collected and RNA was precipitated with isopropanol. RNA pellet was then dissolved in small amount of RNase free water (DEPC-water). The total RNA concentration as well as the purity of the samples were determined spectrophotometrically by measuring the absorbance of the samples at

wavelengths of 260 and 280 nm with NanoDrop spectrophotometer (Thermo Scientific, USA). Concentration was calculated with the formula A_{260nm} * 40 µg/ml and the purity was determined from the ratio A_{260nm}/A_{280nm}. The purity was considered adequate when the ratio was 1.8–2.0. The integrity of RNA was confirmed with agarose-formaldehyde (1.2%) gel electrophoresis. The gel was imaged in UV light to visualize RNA stained with ethidium bromide. RNA was considered intact when two bands were visible on the gel: the upper band for 28S and the lower for 18S ribosomal RNA. Samples were stored at –80 ºC.

After RNA extraction, RNA was reverse-transcribed to complementary DNA (cDNA) with a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, USA). This reaction requires an enzyme, reverse transcriptase, which produces single stranded DNA using RNA molecules as templates. In addition to the enzyme, the kit contains also random primers, nucleotides (A, T, C, G) and RT buffer to yield optimal chemical conditions for the reaction.

7.4 Quantitative real-time PCR

TaqMan[®] Gene Expression Assay (Applied Biosystems) -based quantitative real-time PCR was used to analyze expression of the genes of interest using the ABI Prism Sequence Detection System 7300 (Applied Biosystems). TaqMan[®] primer and probe sets for Cs (Mm00466043_m1), CPT1B (Mm00487200_m1), PGC-1α (Mm00447183_m1), PDK4 (Mm00443325_m1) and GAPDH (Mm99999915_g1) were designed and synthesized by Applied Biosystems. Primer pairs were designed so that they overlapped an exon–exon boundary to avoid interference from possible genomic DNA contamination. Each reaction contained 4 µl (containing 8 ng RNA equivalents, 2 ng RNAeq/µl) of sample, 1 µl of TaqMan[®] Gene Expression Assay (containing primers and probes), 10 µl of TaqMan[®] Mastermix (containing the enzyme, nucleotides and buffers, Applied Biosystems) and 5 µl of RNase free water, the total reaction volume being thus 20 µl. The following thermal profile was applied to carry out the PCR reactions: +50 ^{o}C for 2 min for activation of the reaction, +95 ^{o}C for 10 min for denaturation, and then 40 cycles at +95 ^{o}C for 15 s, and +60 ^{o}C for 1 min, for annealing and extension respectively. The expressions of target genes were quanti-

fied according to the corresponding gene-specific standard curve. All samples were analyzed in triplicate.

GAPDH was used as an endogenous control gene and the raw data of each gene was normalized to the raw data of GAPDH to correct for potential variation in total RNA loading. GAPDH was chosen because its expression has been shown in microarray analysis to be stable in a study with similar experimental diabetes and exercise protocol (Kivelä et al. 2006). In addition, the mRNA expression of GAPDH was in line with the picogreen results for total RNA content. GAPDH raw data was normalized to picogreen data and statistical analyses were carried out to evaluate the stability of GAPDH mRNA expression among the groups. Kruskall-Wallis P-value was 0.987 and the mean values and standard deviations were similar between the groups. So it can be concluded that the mRNA expression of GAPDH wasn't affected by the experimental protocols.

7.5 Muscle sample homogenization, protein extraction and total protein analysis

For protein extraction, the gastrocnemius muscles were pulverized in liquid nitrogen. Approximately 50 mg of muscle powder was then weighed and 15-fold volume of Hepes homogenization buffer (20mM Hepes pH 7.4, 1mM EDTA, 5mM EGTA pH 7.4, 0,2% sodium deoxy cholate, 10mM $MgCl_2$, 2mM DTT, 1% NP-40, 3% protease-phosphatase inhibitor (Halt Protease and Phosphatase Inhibitor Single-Use Cocktail #78443, Pierce Protein Biology Products, Thermo Scientific), 1mM Na_3VO_4, 100mM β-glycerophosphate, ddH_2O) was added for homogenization and protein extraction. The samples were rotated for 30 min at $+4^oC$ and then centrifuged at $+4^oC$ for 10 min at 10 000 g in order to separate the insoluble material to the bottom of the tube. Supernatants were collected and stored at -80^oC for further analysis. Total protein concentrations of the samples were determined with protein BCA kit (Pierce™ BCA Protein Assay Kit, #23227) using automated Konelab 20 XTi device (Thermo Scientific, Vantaa, Finland).

7.6 Western blotting protein analysis

SDS-PAGE. Changes in expression and activities of key proteins in regulation of fatty acid oxidation were analyzed with western blotting. The samples were diluted so that 15 µl contained 40 µg of total protein. Then 3 µl of 6x sample buffer (1.5 ml glycerol, 750 µl β-mercaptoethanol, 1.0 g SDS, 940 µl 1 M Tris (pH 6.8), 0.6 mg bromophenol blue, ddH$_2$O to attain end volume of 5 ml) or 15 µl of 2x Laemmli sample buffer (Bio-Rad #161-0737) with 5% β-mercaptoethanol was added and the samples were centrifuged briefly and then heated for 10 min at 95°C. 6x sample buffer was used for the analysis of proteins p-ACC, ACC, p-AMPK, AMPK, Cyt *c* and Sirt1, 3 and 6, and 2x Laemmli sample buffer for the analysis of PGC-1α, PDK4, CPT1B, p-p38 MAPK and p38 MAPK. After heating, the samples were centrifuged again briefly and put back on ice for 5 minutes. Samples were loaded to the gel (4–20% Criterion^{TM} TGX^{TM} Precast Gels, Bio-Rad #567-1094), so that each well contained approximately 40 µg total protein. First well of each gel was also loaded with molecular weight marker (Odyssey^{TM} Protein Molecular Weight Marker (10–250 kDa) P/N 928-40000 or Precision Plus Protein^{TM} Dual Color Standards, Bio-Rad #161-0374). Electrophoresis chamber was filled with EF running buffer (2.5 mM Tris Base, 19.2 mM glycine, 0.01% SDS, ddH$_2$O) an electrophoresis was run with voltage of 250 V, for approximately 45 minutes, at +4°C on ice. SDS-PAGE is based on the negative charge of SDS and the migration of proteins in the gel when voltage gradient is applied: SDS binds to proteins and in consequence, proteins get a negative charge that is proportional to their size and molecular weight. In gel electrophoresis, proteins are exposed to voltage gradient in which proteins start to migrate towards the anode. Smaller proteins migrate more quickly through the gel and consequently, proteins become separated according to their size/molecular weight.

Blotting. After SDS-PAGE proteins were transferred from gel to an absorbent membrane in a process called blotting. Nitrocellulose membrane (Hybond ECL, GE Healthcare Life Sciences, RPN303D) was used for proteins Sirt1, 3 and 6, AMPK (total and phospho), ACC (total and phospho) and Cyt *c* and PVDF (Hybond-P, GE Healthcare Life Sciences, RPN303F) for p38 MAPK (total and phospho), PGC-1α, PDK4 and CPT1B. Gel was bal-

anced in transfer buffer (2.5 mM Tris Base, 19.0 mM glycine, (pH adjusted to 8.3 with HCl), 10% methanol, ddH$_2$O) for 15–30 minutes. Membrane sheets were activated either with distilled water for 10 minutes (nitrocellulose) or methanol for 10 seconds (PVDF). After activation, membrane sheets were also balanced in transfer buffer for ~15 minutes. Then the blotting sandwich was built: a scotch-brite pad and a sheet of blotting paper were immersed in transfer buffer. The gel was then placed onto the blotting paper and membrane sheet onto the gel. All the air bubbles were carefully removed. Then another sheet of pre-soaked blotting paper and a pad were placed onto the membrane and blotting cassette was closed. Cassettes were immersed in transfer buffer in the blotting chamber so that the side of the membrane was against the anode of the chamber. An ice brick was placed into the chamber to avoid excessive heating during blotting. Blotting was performed with electric current of 300 mA, for approximately 2.5 hours, at +4$^\mathrm{o}$C on ice. Magnetic mixing was used to stir the transfer buffer during blotting.

Blocking, antibodies and detection. After blotting, membranes were stained with Ponceau S to confirm successful transfer and to enable later quantitation of relative total protein content of each lane. Membranes were imaged with Molecular Imager ChemiDoc XRS System (Bio-Rad) and Quantity One 4.6.3 -software (Bio-Rad). Then membranes were cut into strips at appropriate sites so that each strips contained only one, or in certain cases two, proteins of interest. The strips were blocked for 1–2 hours at room temperature in gentle rocking with either Odyssey™ Blocking Buffer (Li-Cor, P/N 927-40000) (p-AMPK, AMPK, Cyt *c*, Sirt3), TBS containing 5% non-fat dry milk (p-ACC, ACC, Sirt1, Sirt6) or TBS-Tween (TBS + 0.1% Tween20) containing 5% non-fat dry milk (CPT1B, p-p38 MAPK, p38 MAPK, PDK4, PGC1α). After blocking, strips were incubated with primary antibodies overnight at +4$^\mathrm{o}$C in gentle rocking. See appendix 1. for more detailed information on the antibodies.

The next day, strips were rinsed and washed 4 x 5 minutes with TBS-Tween and then incubated with secondary antibodies for 1 hour at room temperature in gentle rocking. Either fluorescently labelled IRDye$^{®}$ secondary antibodies or HRP-conjugated secondary antibod-

ies (see appendix 1) were used. When fluorescently labelled IRDye-antibodies were used, strips were protected from light from this point on. After secondary antibody incubation, strips were again rinsed and washed 5 x 5 minutes with TBS-Tween. When fluorescently labelled IRDye-antibodies were used, strips were then scanned with Odyssey® CLx Imager. When HRP-conjugated secondary antibodies were used, strips were incubated for 5 minutes with detection kit (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce Protein Biology Products, Thermo Scientific #34096). The strips were then imaged with Molecular Imager ChemiDoc XRS System (Bio-Rad) and Quantity One 4.6.3 -software (Bio-Rad) with varying exposure times/lengths of exposure.

Stripping. In certain cases blots needed to be stripped to remove the bound antibodies and to enable the detection of another protein from the same strip. Two different protocols were used: In the first protocol, right after scanning the blots were washed for 10 minutes with TBS. Then, the blots were stripped with Restore™ Western Blot Stripping Buffer (Thermo Scientific, #21059) for 10 minutes at room temperature in moderate rocking. After stripping, the blots were washed for another 10 minutes. This protocol was used to strip p-AMPK and to detect total AMPK. In the other protocol, blots were incubated with Restore™ Western Blot Stripping Buffer (Thermo Scientific, #21059) for ~20 minutes at room temperature in moderate rocking. Then, blots were rinsed five times with distilled water and subsequently washed 3 x 5 minutes with TBS-Tween. This protocol was applied to strip p-p38 MAPK and to detect total p38 MAPK. After these stripping procedures, all the steps starting from blocking were repeated with new antibodies.

Quantitation and data analysis. To determine the relative quantities of each protein in each sample, protein bands were quantified with Quantity One 4.6.3 -software (Bio-Rad) for proteins detected with ECL and with Image Studio Software (Li-Cor) for proteins detected with infrared fluorescence (Odyssey®). Quantitation is based on the relationship between the intensity and area of the light signal and the amount of protein. The larger the quantity of protein, the more antibody will bind which results in bigger and the more intensive the band. The bands were defined as accurately as possible, and the software then determined a value

for each band based on the intensity and area of the band. In case there were some non-specific background bands, the correct band for quantitation was chosen according to the molecular weight marker and the knowledge on the size of the protein based on literature (appendix 1). In addition, actin band (42 kDa) was quantified from Ponceau S stained membranes for total protein normalization purposes (figure 6).
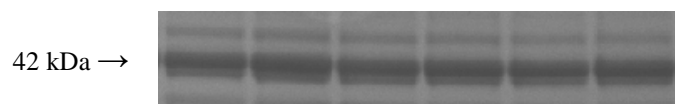
42 kDa →

FIGURE 6. Example of Ponceau S staining.

The raw data for each protein was normalized with Ponceau S staining of the same membrane to exclude the effect of potential differences in total protein loading. Ponceau S staining was chosen for normalization because it represents the total protein loading and, in addition, the mean values did not differ between groups. Then the values obtained for each sample were divided by the mean value of the control samples of the same gel to minimize the effect of gel to gel variation.

7.7 Statistical analysis

For repeated measurements (plasma glucose and body weight) both absolute and relative changes were calculated. For mRNA and protein expression measurements, relative differences between sedentary and exercised groups of same health status were calculated to evaluate differences in responses to exercise between healthy and insulin deficient mice. Statistical analyses were carried out using IBM SPSS Statistics 20.0 for Windows (SPSS, Chigago, IL, USA). The Shapiro-Wilk test was used to test whether the variables were normally distributed and the Levene's test was used to analyze the homogeneity of variances. Due to small group size and random violations in the normal distribution assumption, non-parametric tests were chosen. Kruskall-Wallis test with Mann-Whitney U-test was used to analyze differences between the groups. For repeated measurements, the significances of the

PRE–POST changes were analyzed with Wilcoxon Matched-Pairs Signed-Ranks test. Significance level was set to p < 0.05. All the results are expressed as mean ± standard deviation (SD) unless otherwise stated.

8   RESULTS

8.1   Body weight and plasma glucose concentration

In the beginning of the experiment there were no significant differences in the body weight between the groups. The body weight of the insulin deficient mice decreased significantly ($p < 0.05$, $p < 0.01$ and $p < 0.01$ in groups D, DE3 and DE6 respectively) during the experiment regardless of the group. The reductions in body weight were $9.5 \pm 9.8\%$, $7.7 \pm 5.9\%$ and $11.2 \pm 6.0\%$ for the groups D, DE3 and DE6 respectively. In healthy mice the body weight either did not change (groups C and HE3) or increased slightly ($2.8 \pm 4.7\%$) but significantly (group HE6, $p < 0.05$) during the experiment. After the experiment the insulin deficient mice were significantly ($p < 0.01$ when compared with healthy controls and $p < 0.05$ when compared to healthy exercised counterparts of the same time point) lighter than the healthy counterparts regardless of the exercise status. The body weights of the mice with same health status weren't significantly different after the experiment.

In the beginning of the experiment plasma glucose concentrations were similar among the healthy groups. The same was true for the insulin deficient groups. However, the insulin deficient mice had significantly ($p < 0.001$) higher plasma glucose concentrations than the healthy mice both in the beginning and at the end of the experiment. The plasma glucose concentration of the control group and one of the healthy exercised groups (HE3) increased significantly ($p < 0.01$) during the experiment. In the group HE6, plasma glucose concentration tended to increase during the experiment but the change was not significant ($p < 0.1$). The post-experiment values for plasma glucose weren't significantly different between the healthy groups. Contrary to the healthy mice, the plasma glucose concentration of the insulin deficient mice decreased significantly ($p < 0.01$) during the experiment in all groups (~28% on average). The post-experiment values for plasma glucose were similar among the insulin deficient groups. Plasma glucose concentrations are presented in the figure 7.
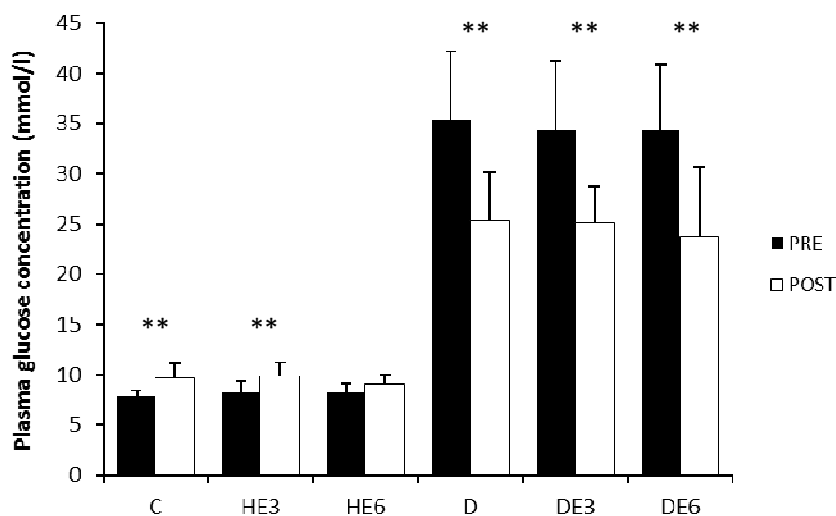
FIGURE 7. The changes in the plasma glucose concentration during the experiment (n = 64). ** indicates significant difference between PRE- and POST-values, p < 0.01.

8.2 Gene expression at mRNA level

PGC-1α. Three hours after acute exercise PGC-1α mRNA expression was significantly (p < 0.001) higher, almost three-fold, in healthy exercised group compared to control mice (figure 8). Six hours after exercise the expression was still significantly (p < 0.001) higher compared to control mice but the expression was significantly lower than three hours after exercise (p < 0.05) being only two-fold compared to control mice. The expression of PGC-1α wasn't different between healthy and insulin deficient sedentary mice. In insulin deficient mice the expression of PGC-1α was also significantly (p < 0.001) higher three hours after acute exercise compared to both healthy and insulin deficient sedentary counterparts, but contrary to healthy mice, the elevated expression was sustained until six hours after exercise (p < 0.001) being almost four-fold compared to healthy and insulin deficient sedentary controls (p < 0.001 for both time points). The expression of PGC-1α was significantly higher both three (p < 0.05) and six hours (p < 0.01) after exercise in insulin deficient mice compared to healthy exercised counterparts. Also, when relative differences between sedentary and exercised groups of same health status were compared, insulin

deficient mice had significantly more pronounced responses to exercise at both time points observed (p < 0.05 three hours after exercise and p < 0.01 six hours after exercise).
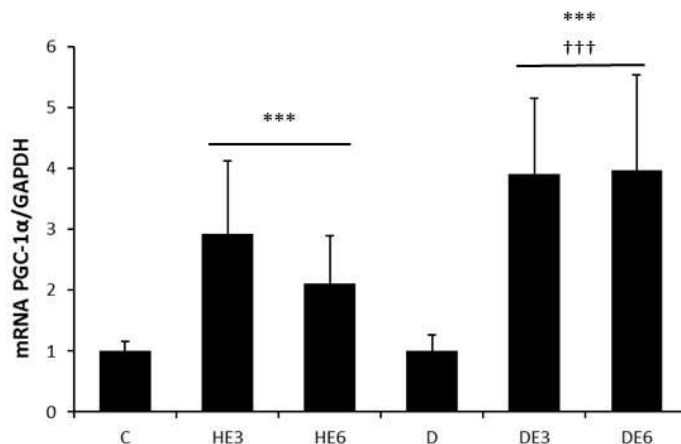

FIGURE 8. PGC-1α mRNA expression in healthy and insulin deficient mice three and six hours after acute exercise compared with sedentary controls (n = 64). *** = significantly different from control group (p < 0.001). ††† = significantly different from sedentary insulin deficient group (p < 0.001).

PDK4. There were no significant differences in PDK4 mRNA expression between healthy and insulin decifient sedentary mice (figure 9). In healthy mice, exercise did not cause any changes to PDK4 mRNA expression at the time points observed. In insulin deficient mice, however, the expression of PDK4 was significantly higher both three (p < 0.01 compared with control mice and p < 0.05 compared with diabetic controls) and six hours (p < 0.001 compared with control mice and p < 0.01 compared with diabetic controls) after exercise compared with healthy and insulin deficient sedentary counterparts. There was no significant difference between the time points. In addition, when relative differences between sedentary and exercised groups of same health status were compared, insulin deficient mice tended (p < 0.1) to have greater response to exercise in PDK4 expression compared to healthy mice both three and six hours after exercise (p = 0.056 and p = 0.059 respectively).
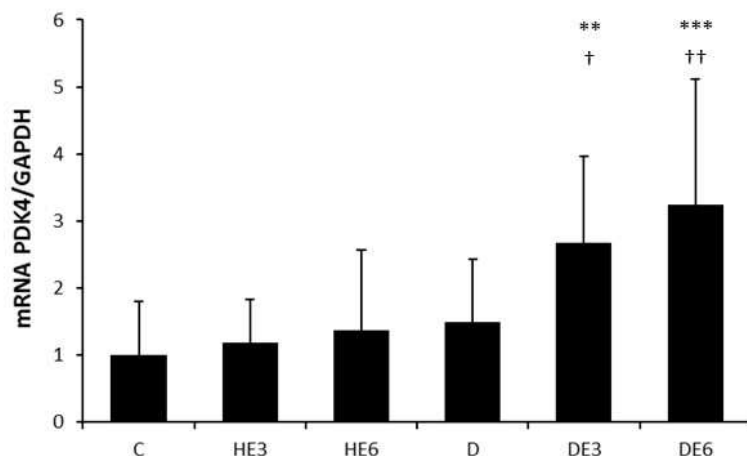
FIGURE 9. PDK4 mRNA expression in healthy and insulin deficient mice three and six hours after acute exercise compared with sedentary controls (n = 64). ** and *** = significantly different from control group (p < 0.01 and p < 0.001). † and †† = significantly different from sedentary insulin deficient group (p < 0.05 and p < 0.01).

CPT1B. There were no significant differences between the groups in CPT1B mRNA expression at the time points observed (figure 10). However, the insulin deficient mice had signifiantly (p < 0.05) more pronounced response to exercise in CPT1B expression than the healthy mice. This was visible six hours after the cessation of exercise.
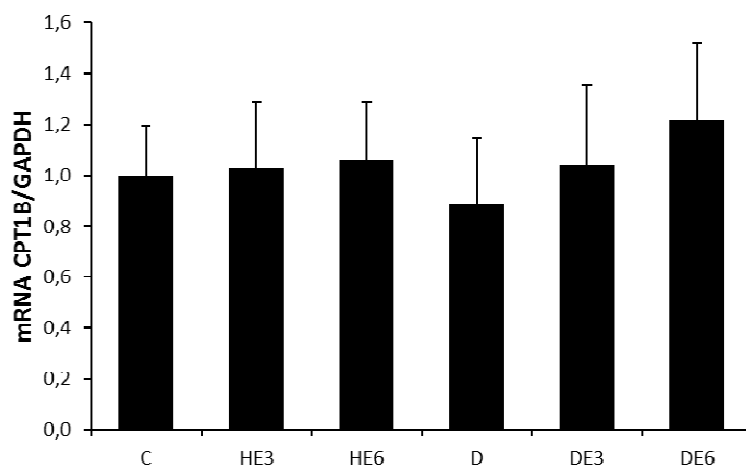

FIGURE 10. CPT1B mRNA expression in healthy and insulin deficient mice three and six hours after acute exercise compared with sedentary controls (n = 64).

Citrate synthase. The mRNA expression of citrate synthase was significantly lower ($p <$ 0.05) in insulin deficient sedentary mice compared to healthy controls (figure 11). Three hours after exercise, insulin deficient mice still tended to have lower expression of Cs compared with healthy controls but this difference did not reach statistical significance ($p < 0.1$). The exercised groups did not differ significantly from sedentary counterparts regardless of the health status in terms of citrate synthase mRNA expression at the time points observed. The same was true, when only exercised groups were compared to one another. However, six hours after exercise bout, insulin deficient mice tended to have lower Cs expression compared to healthy exercised mice ($p < 0.1$).
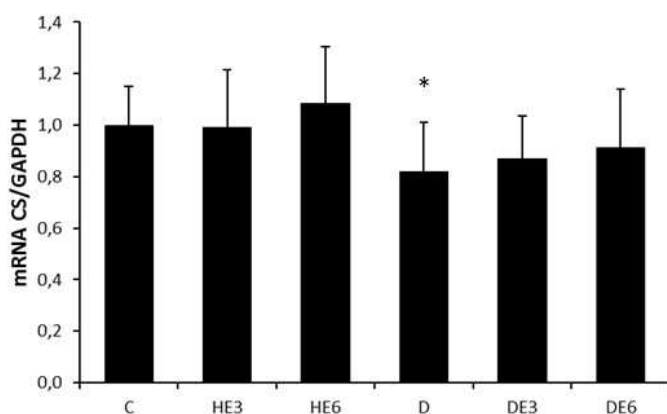

FIGURE 11. Citrate synthase mRNA expression in healthy and insulin deficient mice three and six hours after acute exercise compared with sedentary controls (n = 64). * = significantly different from control group ($p < 0.05$).

8.3 Protein expression and phosphorylation

In healthy mice, AMPK phosphorylation level was significantly decreased ($p < 0.05$) six hours after exercise compared to healthy controls. Insulin deficient sedentary mice did not differ significantly from healthy controls. However, the decrease in AMPK phosphorylation after exercise wasn't seen in insulin deficient mice. The ratio of phosphorylated AMPK and total AMPK followed the same pattern but without any statistical significances. Neither

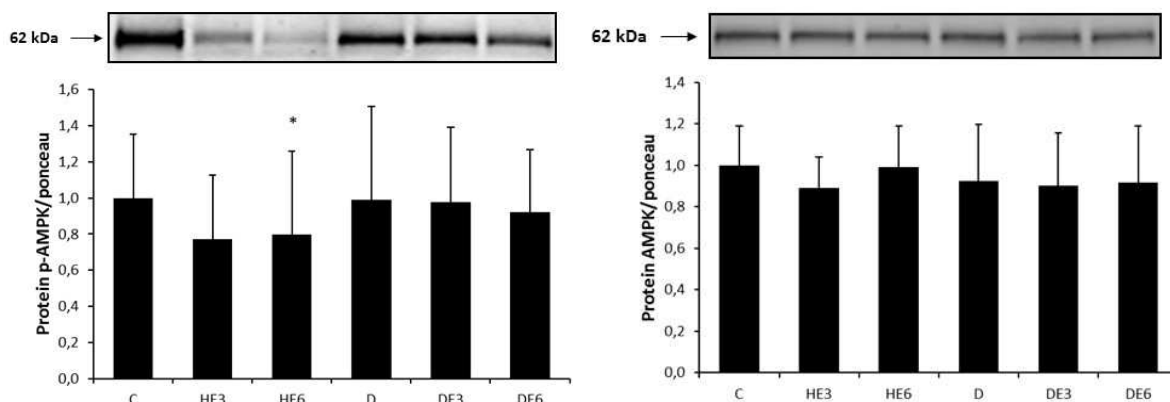insulin deficiency nor acute exercise seemed to induce any significant changes in AMPK protein expression.

FIGURE 12. AMPK protein phosphorylation (left) and expression (right) in healthy and insulin deficient mice three and six hours after acute exercise compared with sedentary controls (n = 60). * = significantly different from control group (p < 0.05).
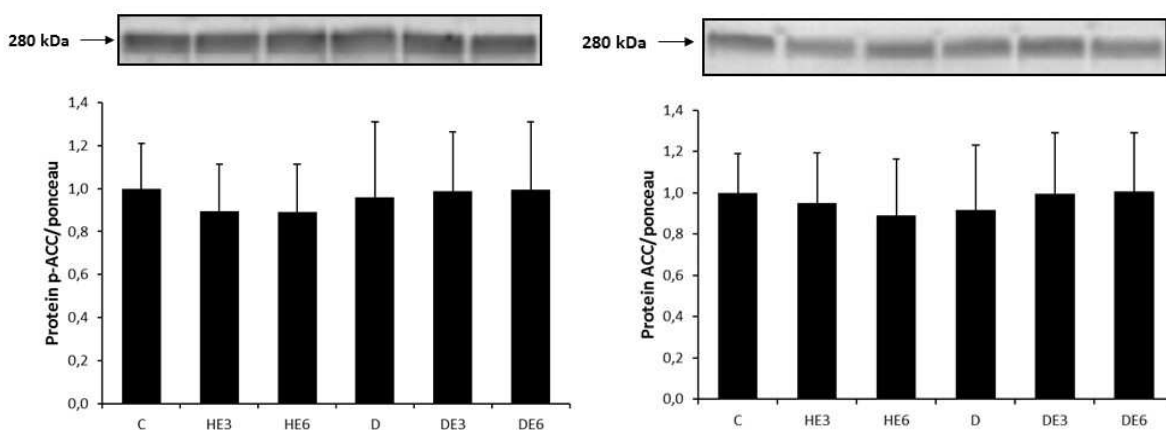

FIGURE 13. ACC protein phosphorylation (left) and expression (right) in healthy and insulin deficient mice three and six hours after acute exercise compared with sedentary controls (n = 59).

Despite the significant decrease in AMPK phosphorylation there weren't any significant changes in ACC phosphorylation in response to exercise (figure 13). Neither did insulin deficiency have any significant effect on ACC phosphorylation. However, insulin deficient mice tended to maintain ACC phosphorylation level higher after exercise than healthy counterparts, but exercise did not change ACC phosphorylation in insulin deficient mice

either. Neither acute exercise nor insulin deficiency elicited any changes in ACC protein level.

In healthy mice, acute exercise bout did not induce any significant changes to p38 phosphorylation or expression (figure 14). In addition, sedentary insulin deficient mice did not differ from the healthy controls in terms of p38 phosphorylation or expression. However, in insulin deficient mice p38 phosphorylation level was significantly lower in exercised mice three hours after exercise compared with both healthy and insulin deficient controls. The phosphorylation status tended to be lower also when compared with healthy exercised mice at the same time point ($p < 0.1$). The same tendency to lower phosphorylation status in insulin deficient exercised mice compared with healthy controls extended to the time point six hours after exercise but the difference did not reach statistical significance ($p < 0.1$). The expression of p38 seemed to follow the same pattern as the phosphorylation status but without significant differences. Total p38 tended to be lower in insulin deficient mice six hours after exercise compared with healthy sedentary controls ($p < 0.1$).
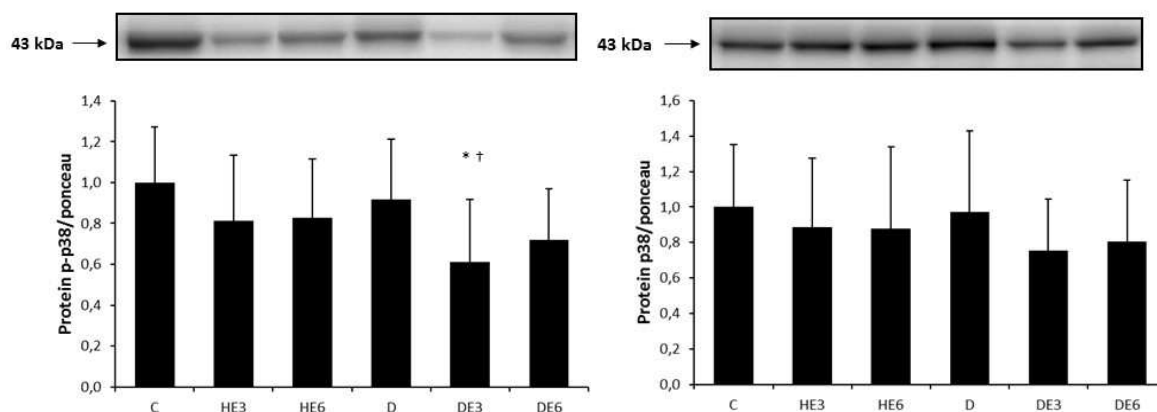

FIGURE 14. p38 MAPK protein phosphorylation (left) and expression (right) in healthy and insulin deficient mice three and six hours after acute exercise compared with sedentary controls (n = 60). * = significantly different from control group ($p < 0.05$). † = significantly different from sedentary insulin deficient group ($p < 0.05$).

The protein expression of PGC-1α stayed the same across the healthy groups in spite of acute exercise bout (figure 15). Neither did insulin deficiency in itself induce any change in PGC-1α expression. However in exercised insulin deficient mice the expression on PGC-1α was significantly lower ($p < 0.05$) three hours after exercises compared with healthy exercised counterparts of the same time point and tended to be lower compared with healhy controls ($p < 0.1$). Similarly, the expression of PGC-1α was significantly lower in insulin deficient mice six hours after exercise compared to healthy control group but it only tended to be lower compared with healthy exercised counterparts of the same time point ($p < 0.1$). However, the expression of PGC-1α did not differ significantly between sedentary and exercised insulin deficient mice.

FIGURE 15. PGC-1α protein expression in healthy and insulin deficient mice three and six hours after acute exercise compared with sedentary controls (n = 60). * = significantly different from control group, $p < 0.05$; ‡ = significantly different from healthy exercised group of the same time point, $p < 0.05$.

The protein expressions of PDK4 (figure 16), CPT1B (figure 17) or Cyt *c* (figure 18) weren't significantly affected by neither exercise protocol nor insulin deficiency in this experimental design.

FIGURE 16. PDK4 protein expression in healthy and insulin deficient mice three and six hours after acute exercise compared with sedentary controls (n = 60).
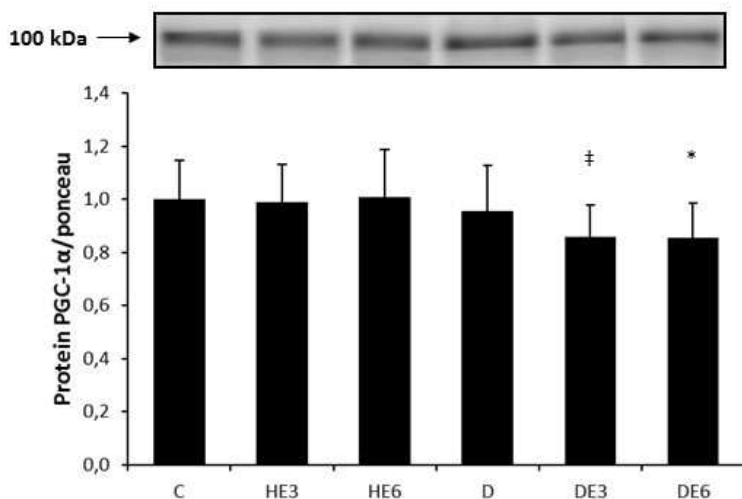

FIGURE 16. CPT1B protein expression in healthy and insulin deficient mice three and six hours after acute exercise compared with sedentary controls (n = 60).

FIGURE 18. Cyt *c* protein expression in healthy and insulin deficient mice three and six hours after acute exercise compared with sedentary controls (n = 60).

Neither exercise nor insulin deficiency seemed to have any significant effect on the expression of the studied sirtuin proteins (figures 19 and 20).
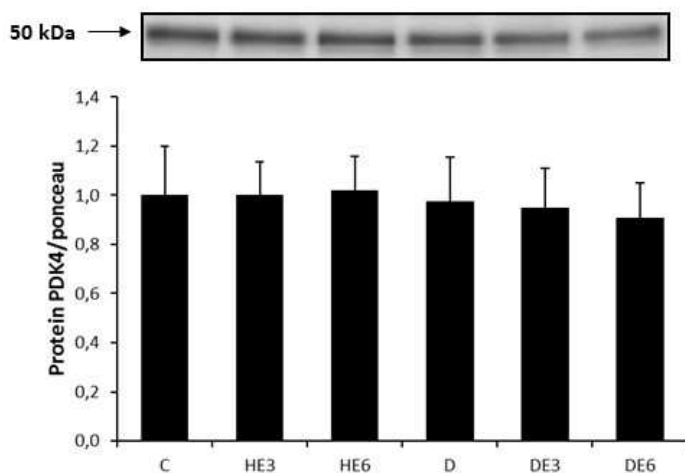

FIGURE 19. Sirt1 protein expression in healthy and insulin deficient mice three and six hours after acute exercise compared with sedentary controls (n = 60).
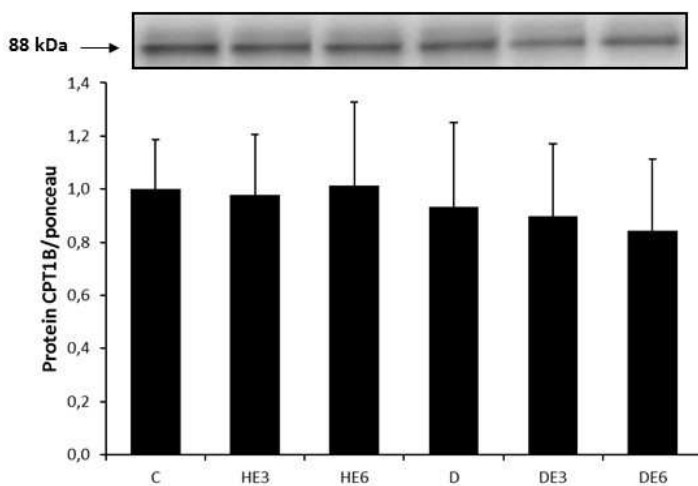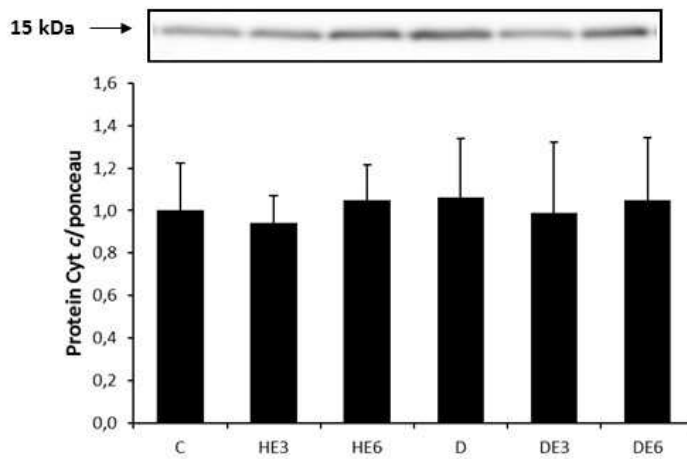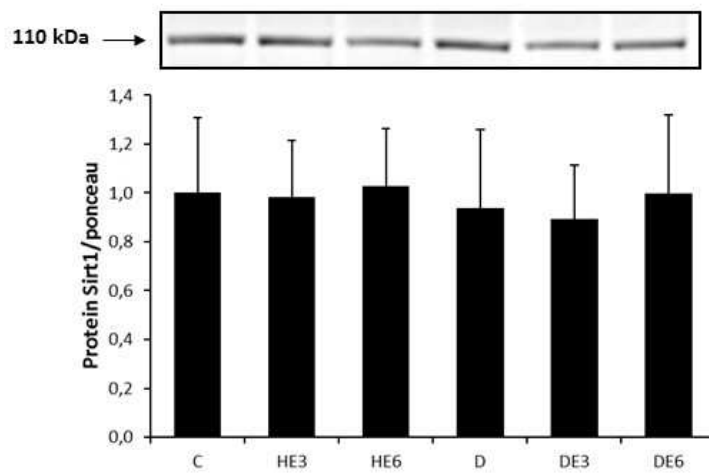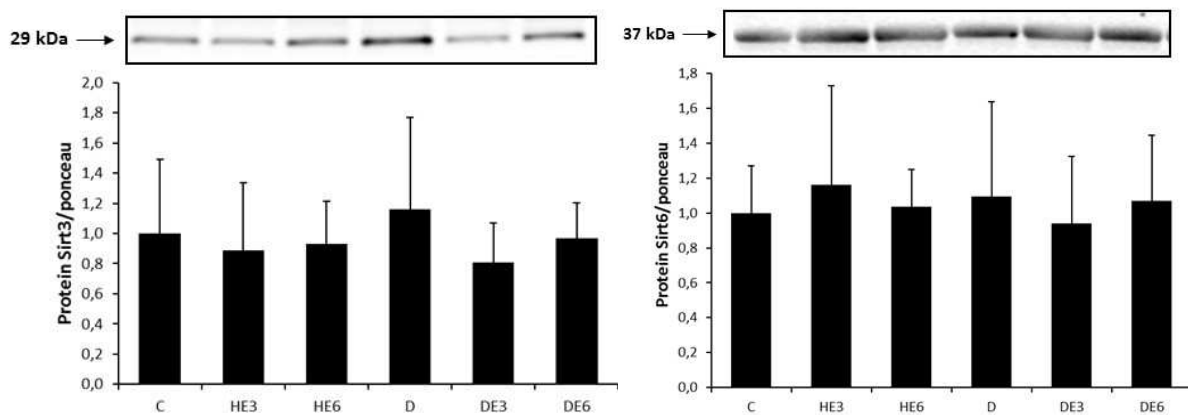
FIGURE 20. Protein expression of Sirt3 and Sirt6 in healthy and insulin deficient mice three and six hours after acute exercise compared with sedentary controls (n = 60).

9   DISCUSSION

The purpose on the present study was to examine the acute effects of a single exercise bout on the expression of genes and on the activation intracellular signaling pathways involved in fatty acid oxidation in gastrocnemius muscles of healthy and insulin deficient mice. The main finding of the study was that insulin deficient mice have more pronounced exercise-induced responses in mRNA expression of genes related to increased fatty acid oxidation (PGC-1α, PDK4 and CPT1B). However, the changes in mRNA expression did not reflect to protein level.

Gene expression is a process that is strictly regulated at multiple steps. First of all, gene transcription in nucleus is in itself under tight regulation, e.g. by chromatin structure and transcription factors. After transcription, formed transcript needs to be processed and then transported to cytoplasm for translation. So, transcription and translation are separated from one another in space and time. Even after having been transported to cytosol, immediate initiation of translation is not guaranteed: translation is regulated by many proteins that may either promote or repress the initiation of translation. In addition, small RNAs called microRNAs may interact with mRNAs and either degrade them or prevent their translation. After translation, the newly formed polypeptide needs still to be folded and processed before it takes its functional form. Moreover, some proteins require also some post-translational modifications, such as phosphorylation, to be fully activated. (Nelson & Cox 2013, 1136, 1155, 1175–1180, 1184–1185.) This explains why it is possible that not all stimuli that induce changes in mRNA expression result in immediate changes in protein expression and why even changes in protein expression don't always induce physiological or metabolic responses.

The whole signaling network studied in this thesis is overviewed in figure 21. The following parts of discussion will focus on each branch of it one at the time.
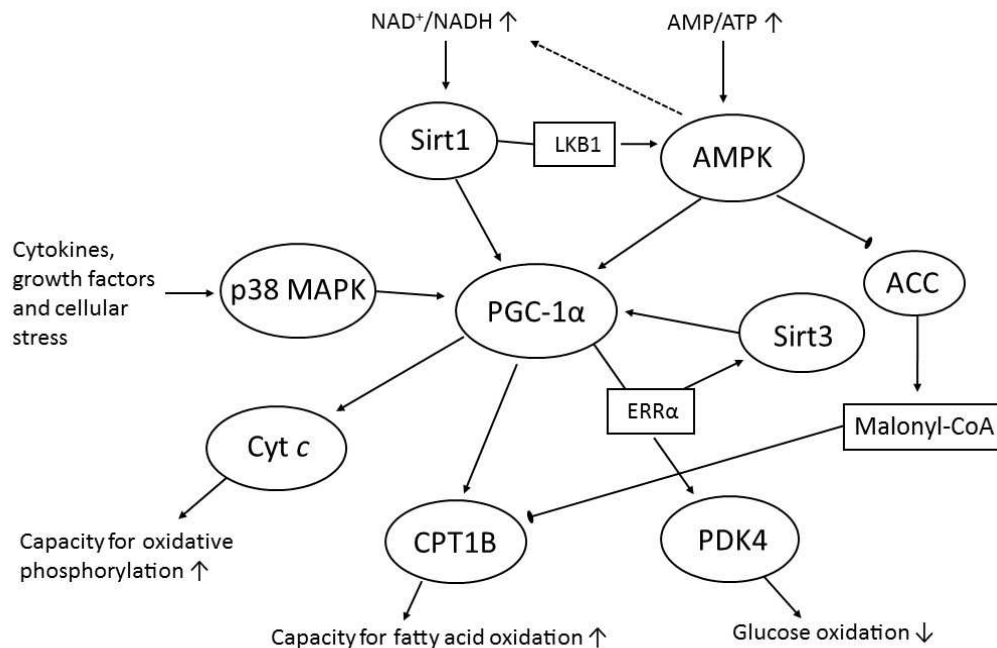
FIGURE 21. Overview of the studied signaling network.

9.1 Effects of insulin deficiency on signaling and oxidative capacity

Insulin deficient mice lost weight significantly during the experiment and were significantly lighter after the experiment compared with healthy mice. This is in line with previous studies using the same experimental model of type 1 diabetes (Hulmi et al. 2012; Kivelä et al. 2006). Insulin deficient mice had also dramatically higher plasma glucose concentrations than healthy mice both in the beginning and at the end of the experiment. This result was expected as the insulin deficient mice with too low glucose concentration were excluded from the study. However, it is interesting that blood glucose concentration of the insulin deficient mice decreased significantly during the experiment while that of healthy mice even increased. The mechanisms underlying this response can be only speculated. It is possible that the glucose concentration increased to high values acutely after destruction of pancreatic β-cells and the consequent cessation of insulin secretion, but some compensatory mechanisms were activated after the acute phase of insulin deficiency. The decrease was of similar magnitude in all the insulin deficient groups so it is not probable that the decrease was due

to the exercise bout. In humans this kind of effect is known as "honeymoon remission", i.e. partial remission phase, which is characterized by improved glycemic control and reduced insulin requirement, due to a boost in the residual β-cell function in order to compensate for the already destroyed β-cells (Abdul-Rasoul et al. 2006). In the case of untreated experimental type 1 diabetes in mice this may be also due to some other, yet unknown compensatory mechanisms that are launched after the destruction of β-cells, but none of these possible explanations can be confirmed.

Consistently with the previous microarray results of our laboratory, insulin deficient mice had lower basal citrate synthase expression compared with healthy mice (Silvennoinen 2004). This could indicate impaired functioning of citric acid cycle and thus impaired capacity to produce ATP aerobically in insulin deficient mice, as also decreased citrate synthase activity has been reported in insulin deficient mice with the same experimental model of type 1 diabetes. In these studies, endurance training was shown to restore citrate synthase mRNA expression and activity. (Kivelä et al. 2006b; Silvennoinen 2004.) In this study, however, a single bout of exercise did not elicit any significant increase in citrate synthase mRNA expression in insulin deficient mice. However, after exercise, citrate synthase mRNA expression did not differ significantly from healthy controls either, suggesting potentially a partial restoration in citrate synthase mRNA expression. It is thus suggested, that a single bout of endurance exercise isn't probably enough to elicit substantial increases in citrate synthase expression in insulin deficient mice, at least in the six-hour time frame used in this study, and repeated exercise bouts are potentially required to induce significant increase in citrate synthase mRNA expression. The responses to exercise were of similar magnitude and also non-significant in healthy mice.

Partially contrary to the hypotheses, there weren't any significant differences in the basal protein levels or protein phosphorylation between healthy and insulin deficient mice. The hypothesis was that insulin deficient mice would have increased AMPK phosphorylation because of cellular energy stress, and decreased p38 MAPK phosphorylation as this kind of an effect has been shown in another experiment using the same experimental model of type

1 diabetes. In that other study, however, the mice had higher blood glucose concentra-
tions despite similar duration of insulin deficiency. (Hulmi et al. 2012.) If diabetes was
more severe in the other study, it might also explain the differences in the activation of sig-
naling pathways.

Gene expression analyses have revealed that genes related to electron transport chain and
oxidative phosphorylation, such as Cyt *c*, are down-regulated in streptozotocin-induced ex-
perimental type 1 diabetes, indicating impaired capacity for aerobic ATP production
(Yechoor et al. 2002). Contrary to these results, the present study did not find any decrease
in Cyt *c* protein expression in insulin deficient mice. This is contradictory to the assumption
that insulin deficiency results in impaired capacity to oxidative phosphorylation. However,
PGC-1α and AMPK seem to play a role in Cyt *c* expression (Bergeron et al. 2001; Calvo et
al. 2008; Geng et al. 2010; Leick et al. 2008). In this study, insulin deficiency did not cause
any baseline changes to PGC-1α expression or AMPK expression or phosphorylation,
which is in line with Cyt *c* expression. This may indicate that the capacity to electron
transport and oxidative phosphorylation wasn't impaired in these mice. However, this
doesn't exclude the possibility that the expression of some other proteins involved in these
processes, but not analyzed in this study, might have changed.

9.2 Effects of exercise and insulin deficiency on mRNA and protein ex-
pression

PGC-1α plays a crucial role in regulation of aerobic metabolism in skeletal muscle. Exer-
cise, both endurance and high-intensity interval exercise, has been shown to increase PGC-
1α expression at mRNA and protein level (Akimoto et al. 2005; De Filippis et al. 2008;
Gibala et al. 2009; Terada et al. 2002; Terada & Tabata 2004; Terada et al. 2005). Con-
sistent with previous studies, this study showed a substantial increase in PGC-1α mRNA
expression elicited by exercise in both healthy and insulin deficient mice. The effect was
even more pronounced and sustained in insulin deficient mice. However, contrary to our
hypotheses and existing research evidence, exercise did not have any significant effect on

PGC-1α protein expression in healthy mice and it almost decreased in insulin deficient mice. Previously, prolonged running exercise has been shown to increase PGC-1α protein content in rat soleus, plantaris and red gastrocnemius muscles but not in white gastrocnemius. This effect was observed six hours after exercise. (Terada & Tabata 2004.) In another study, aerobic interval exercise resulted in increased PGC-1α mRNA and protein expression already 30 minutes after exercise with a more pronounced increase observed five hours after exercise. This effect was delayed and blunted in insulin resistant, non-diabetic subjects for mRNA expression and increase in protein content was totally prevented. (De Filippis et al. 2008.) As the time frames used in these experiments are similar to the present study, the differences in the results may be explained by the differences in the exercise protocol and studied muscles. In the present study, the mice ran on a treadmill for one hour at relatively high speed (21 m/min with an incline of 2.5°) while in the study of Terada and Tabata (2004) the exercise protocol consisted of two subsequent bouts of three hours of running at low intensity (13 m/min, no incline) separated by 45 minutes of rest (a total of six hours of running). It could be speculated that either more prolonged and lower intensity exercise is more optimal to induce PGC-1α protein expression and the present protocol was insufficient to induce translation from mRNA to protein, or that there was just more time for translation as PGC-1α mRNA expression increases already during prolonged exercise (Akimoto et al. 2005). However, this explanation is not in line with the results of De Filippis et al. (2008), who had rather intensive 48-minute exercise protocol. In the present study, only gastrocnemius muscle (including both red and white parts) was analyzed for mRNA and protein expression. Hence, another possibility is that there actually was an increase in PGC-1α protein content in the red part of gastrocnemius but the effect was diluted by "non-responsive" white part of gastrocnemius. Again, this assumption somewhat contrasts the results of De Filippis et al. (2008) who took biopsies from vastus lateralis that can be assumed to be also a muscle consisting of different types of muscle fibers. In addition PGC-1α mRNA has been found to increase also in white gastrocnemius, but the response being somewhat blunted compared with soleus (Leick et al. 2008). All in all, it is possible that the exercise intensity in the study by Terada and Tabata (2004) was so low, that white gastrocnemius wasn't sufficiently recruited to elicit any responses in gene expression, and thus,

fiber type composition is probably not the explanation for not seeing exercise-induced increase in PGC-1α.

To support further the previous assumption, this latter speculation of muscle fiber types doesn't explain the discrepancy between mRNA and protein level results. Besides, this is not the only study not reporting changes in skeletal muscle PGC-1α protein content despite increase in mRNA expression: for example Gibala et al. (2009) did not find any increase in PGC-1α protein despite substantial increase in PGC-1α mRNA in human vastus lateralis muscle three hours after high-intensity interval exercise on a cycle ergometer. Another study reported similar results after 180 minutes of moderate intensity cycling exercise. However, in this latter study, muscle biopsies were taken immediately after exercise and PGC-1α protein content tended to increase although the change wasn't statistically significant. (Watt et al. 2004.)

One possible explanation to discrepancy between PGC-1α mRNA and protein level results may lie behind the different regulation of different PGC-1α isoforms, i.e. splice variants. The antibody used to detect PGC-1α protein recognized the PGC-1α1 splice variant while the primer probes used to detect PGC-1α mRNA recognized splice variants PGC-1α1 and PGC-1α4. PGC-1α1 originates from the proximal promoter of PGC-1α whereas PGC-1α4 originates from alternative promoter both of which are activated during exercise. The proximal promoter is more dependent on AMPK activation while the alternative promoter is also influenced by β-adrenergic stimulation. (Norrbom et al. 2011; Ruas et al. 2012; Ydfors et al. 2013.) Catecholamine levels were not analyzed in this experiment, but it could be speculated that as there were no differences in PGC-1α protein content, the induction of PGC-1α4 from the alternative promoter might account for the increase in PGC-1α mRNA level. Similarly to the other suggested explanations, this assumption can be discussed but not confirmed without additional studies. Type 1 diabetics have been found to have increased plasma catecholamine responses to exercise compared with healthy controls (Tamborlane et al. 1979). So if the insulin deficient mice had higher catecholamine response to exercise in this

study, it could explain the higher response in PGC-1α mRNA and support the assumption that this response was due to induction of the alternative promoter.

PDK4 is an enzyme that phosphorylates and thus inhibits the rate-limiting enzyme, PDH, in glucose oxidation and thus switches substrate use from CHO to fat oxidation (Nelson & Cox 2013, 654). It has been established that PGC-1α directly induces PDK4 gene expression in an ERRα dependent manner and that this effect is independent of PPARα (Araki & Motojima 2006; Wende et al. 2005). Two bouts of exercise, performed on two consecutive days, have been shown to induce both PGC-1α and PDK4 mRNA expression 12 hours after the last bout in mouse gastrocnemius muscles. A single bout of acute exercise has been found to increase the mRNA expression of PGC-1α already one hour after exercise followed by increased PDK4 mRNA three hours after exercise. PGC-1α mRNA expression was still elevated three hours after exercise compared with sedentary state, but it had reduced from over 12-fold two three-fold elevation. (Wende et al. 2005.) In humans, PDK4 mRNA expression has been found to increase after acute bouts of both endurance and resistance exercise (Yang et al. 2005). In this study, exercise-induced increase in PDK4 mRNA was seen only in insulin deficient mice.

The acute increase in PGC-1α mRNA expression observed in this study is in line with the study by Wende et al. (2005): The magnitude of the increase in PGC-1α mRNA is similar three hours after exercise in healthy mice and higher in insulin deficient mice. However, the results of Wende et al. (2005) suggest that in the present study, we might have missed the peak PGC-1α mRNA expression as substantially higher expression was observed already one hour after exercise. In this study, the expression also dropped slightly in healthy mice from three to six hours after exercise, although the expression remained significantly higher compared with sedentary controls. Even though we did not observe any significant changes in PDK4 mRNA expression in healthy mice in response to exercise, insulin deficient mice showed an increase in PDK4 mRNA three hours after exercise and the increment was even more substantial six hours after exercise. Despite the changes at mRNA level, there were no changes in PDK4 protein expression. These results suggest, that in response to acute exer-

cise, at least the PGC-1α–PDK4 axis of intracellular signaling favoring the switch from glucose to fatty acid oxidation is up-regulated in insulin deficient mice compared with healthy counterparts. As PGC-1α protein expression did not increase in insulin deficient mice after exercise, it is possible that the increase in PDK4 expression was due to increased activation and/or nuclear translocation of already existing PGC-1α protein. Nuclear PGC-1α content has been shown to be increased in rat skeletal muscle immediately after two hours of swimming (Wright et al. 2007).

In the present study, healthy mice had significantly lower AMPK phosphorylation six hours after exercise compared with control group. This seemed to be the case already three hours after exercise, but without statistical significance. This observed decrease in AMPK phosphorylation after exercise below the value of the control group may be due to excessive down-regulation of the phosphorylation after the acute exercise-induced increase in phosphorylation. This explanation is only speculation and cannot be fully confirmed because of the lack of samples from the time period immediately after exercise. This assumption is at least partially supported by the results of some previous studies: It has been reported that phosphorylation and activation of AMPK increase immediately after exercise but that this effect starts to fade or totally disappears during the first two to four hours after exercise (Dreyer et al. 2006; Gibala et al. 2009; Sriwijitkamol et al. 2007; Terada et al. 2002). However, some differences in the results exist between different exercise intensities, AMPK isoforms and populations studied (Sriwijitkamol et al. 2007). In insulin deficient mice, AMPK phosphorylation level stayed the same despite exercise. This might reflect more pronounced and sustained energy stress in insulin deficient mice after exercise which then could have prevented the fall in AMPK phosphorylation.

As AMPK has been suggested to regulate the expression of PGC-1α, the increased PGC-1α mRNA expression could potentially reflect AMPK activation during and/or immediately after exercise (Jäger et al. 2007; Terada et al. 2002; Terada & Tabata 2004). In addition, insulin deficient mice had higher and more sustained PGC-1α mRNA response and concom-

itantly, no decrease in AMPK phosphorylation after exercise compared with sedentary counterparts.

Although the results obtained from AMPK phosphorylation were unexpected, they were in line with observed ACC phosphorylation, which behaved the same way as AMPK. This was expected since ACC is a down-stream phosphorylation target of AMPK. However, the hypothesis was that both of these would be phosphorylated after exercise especially in insulin deficient mice. As no changes were observed in ACC phosphorylation and malonyl-CoA and/or acetyl-CoA levels were not measured, nothing can be said about whether insulin deficient mice had lower level of CPT1B inhibition compared with healthy mice. However, protein phosphorylation changes in response to acute exercise are transient, and it is thus possible that there were changes in AMPK and ACC phosphorylation, which had already faded before the first time point. Leick et al. (2008) found significantly increased AMPK and ACC phosphorylation immediately after exercise but this effect was lost already two hours after exercise. In addition, insulin deficient mice tended to maintain ACC phosphorylation higher after exercise, which might indicate higher phosphorylation level during and/or immediately after exercise. Unfortunately, this speculation cannot be confirmed with existing data.

Phosphorylation of p38 MAPK did not change in response to exercise in healthy mice in this study. This was contrary to what was expected, but as with AMPK, this can be explained by the time points of observation: previously, exercise-induced phosphorylation of p38 MAPK has been observed immediately after exercise but not anymore three hours after exercise (Gibala et al. 2009). All in all, these results suggest that the optimal window to observe exercise-induced acute changes in protein phosphorylation was already closed before the first time point of this study. In insulin deficient mice, p38 went through dephosphorylation after exercise as the p38 phosphorylation level was significantly lower in insulin deficient mice three hours after exercise compared with healthy and insulin deficient sedentary mice. It cannot be confirmed whether this effect was due to excessive down-

regulation of p38 phosphorylation after exercise-induced increase in phosphorylation, as speculated with AMPK, or an actual response to exercise bout.

Sirtuins have mainly been studied in conditions of fasting and caloric restriction, but there is only a limited number of studies investigating the effects of exercise on sirtuin expression. Consequently, it is difficult to put up hypothesis concerning the behavior of sirtuin expression in response to acute exercise. As cellular NAD^+/NADH increases during exercise, it can be assumed that sirtuin activity increases during exercise (Houtkooper et al. 2012). At least exercise training has been found to increase Sirt1 activation in rat skeletal muscle. This resulted from the exercise-induced activation of nicotinamide phosphoribosyltransferase (NAMPT) and consecutive production of NAD^+. (Koltai et al. 2010.) However, in this study the activities of sirtuins of interest weren't measured, and so, it is possible to only assess their activity based on the expression and/or activity of their down-stream targets, such as PGC-1α.

Sirt1 has been found to be indispensable for the induction of mitochondrial fatty acid oxidation in response to nutrient deprivation. It has been suggested that this effect is mediated by PGC-1α with following arguments: Sirt1 was able to deacetylate PGC-1α. Also, the expression of PGC-1α target genes was inhibited in skeletal muscle cells with Sirt1 knock-down. In addition, decrease in glucose concentration increased fatty acid oxidation and concentration of Sirt1 activator NAD^+ in C2C12 myotubes. It also decreased PGC-1α acetylation and induced the expression PGC-1α target genes involved in mitochondrial and fatty acid utilization genes. However, Sirt1 knock-out and knock-down prevented these effects. So, it was concluded that low glucose availability increases cellular NAD^+, which activates Sirt1. Sirt1 then deactylates PGC-1α which ultimately results in expression of mitochondrial and fatty acid oxidation genes and increased fatty acid oxidation to maintain the bioenergetic state of the cell and to spare glucose for neuronal and red blood cells in the case of food deprivation. (Gerhart-Hines et al. 2007.) Given these results, it would be tempting to hypothesize that as insulin deficiency presumably results in low intracellular glucose availability, this same mechanism would apply to insulin deficient skeletal muscle. In the case of this study, the

contribution of Sirt1 to probable PGC-1α activation and resulting induction of the expression of PGC-1α responsive genes (PDK4, Cyt *c*, CPT1B) can be only speculated as Sirt1 activity is not known and there are a number of other factors capable of activating PGC-1α.

In line with the results of this study, Hokari et al. (2010) did not find any significant change in Sirt1 protein expression eight hours after acute exercise bout, nor after four weeks of training. However, Sirt1 mRNA expression was induced in rat soleus muscle by acute exercise. However, in another study, Sirt1 protein expression was found to be increased two hours after acute exercise in rat soleus. Sirt1 protein level was also increased after two weeks of low-intensity and high-intensity training in rat soleus and after high-intensity training in plantaris muscle. (Suwa et al. 2008.) Interestingly, Sirt1 protein expression was increased two hours after acute brief high intensity sprint exercise (30-s Wingate test) in human vastus lateralis muscle (Guerra et al. 2010).

In skeletal muscle Sirt3 expression has been found to be induced by exercise training, caloric restriction and fasting (Hokari et al. 2010; Jing et al. 2011; Palacios et al. 2009). Contrary to Palacios et al. (2009), Jing et al. (2011) found that Sirt3 mRNA and protein were downregulated during 24-hour fasting but that this effect was reversed by refeeding. In addition, high-fat feeding and streptozotocin-induced experimental type 1 diabetes have been reported to result in decreased expression of Sirt3 mRNA and protein in skeletal muscle (Jing et al. 2011; Palacios et al. 2009). However, this study did not find any differences in Sirt3 expression between healthy and insulin deficient mice. Exercise training has been shown to increase Sirt3 protein, but not mRNA, expression in skeletal muscle, this effect being more profound in female than male rats (Hokari et al. 2010; Palacios et al. 2009). However, the increase in Sirt3 expression has not been seen after acute exercise bout, which is in line with the results of this study (Hokari et al. 2010). Also, while Sirt3 is more highly expressed in slow oxidative type muscles, there seem to be differential responses in different muscles to different modes of training (Hokari et al. 2010; Palacios et al. 2009).

Sirt3 knock-out (KO) has been shown *ex vivo* to reduce fatty acid oxidation in various oxidizing tissues, e.g. liver, cardiac muscle, mixed skeletal muscle and brown adipose tissue, with high lipid availability. It has been suggested that, at least in liver, Sirt3 increases fatty acid oxidation by deacetylating and thus activating the enzyme long-chain acyl coenzyme A dehydrogenase (LCAD), which is involved in LCFA oxidation. (Hirschey et al. 2010.) Whether this happens in skeletal muscle, is still unknown. In skeletal muscle, exercise training has been shown to increase not only Sirt3 but also phosphorylation of CREB (activator of PGC-1α transcription) and expression of PGC-1α. In the same study, caloric restriction was found to induce AMPK phosphorylation in addition to Sirt3 protein expression. Additionally, Sirt3 knock-out resulted in decreased AMPK and CREB phosphorylation, i.e. activation, and decreased PGC-1α expression. Thus it was suggested that the induction of Sirt3 may cause increase in PGC-1α via phosphorylation and subsequent activation of AMPK and/or CREB. (Palacios et al. 2009.) According to present knowledge, this would lead to switch from glucose to FFA oxidation (Lomb et al. 2010; Wende et al. 2005).

However, opposite function has also been suggested to Sirt3: It has recently been shown that Sirt3 knock-out and knock-down result in impaired insulin signaling and insulin resistance possibly via increased JNK phosphorylation and subsequent serine phosphorylation of IRS-1 by JNK (Jing et al. 2011). Also, Sirt3 deletion has been found to result in impaired glucose oxidation and inhibition of insulin-mediated suppression of fatty acid oxidation with concomitant accumulation of pyruvate and lactate metabolites in skeletal muscle. This effect was suggested to be due to hyperacetylation of E1α subunit of PDH, resulting from absence of Sirt3 action, which leads to alteration in PDH phosphorylation. This results then in suppressed PDH activity and consequently in decreased CHO oxidation and increased lactate production and fatty acid oxidation even in fed state. (Jing et al. 2013.) These results are in line with the finding of Jing et al. (2011) that Sirt3 content is reduced after fasting (increased fatty acid oxidation and sparing of glucose) and that refeeding increases Sirt3 (in fed state fatty acid synthesis and glucose utilization are favored). In other words, these results suggest, that in fasted state, Sirt3 protein content decreases leading to hyperacetylation and subsequent inactivation of PDH resulting in conservation of pyruvate, lactate and ala-

nine for gluconeogenesis and greater reliance on fatty acids in ATP production, whereas in fed state increased Sirt3 deacetylates PDH enabling its activation and thus production of acetyl-CoA from glucose derived pyruvate for citric acid cycle and subsequent ATP production and fatty acid synthesis (Jing et al. 2011; Jing et al. 2013; Kwon & Harris 2004).

In short, Sirt3 has been suggested to have an important role in regulation of oxidative metabolism, substrate use and metabolic homeostasis in various tissues and conditions. However, these functions may be different, even opposite, in different tissues and in response to different metabolic, physiologic or pathologic stimuli, e.g. fasting, diabetes, insulin and exercise (Jing et al. 2011). Consequently, more studies are needed to elucidate the exact functions and mechanisms of Sirt3 that take action in skeletal muscle in response to exercise in diabetes.

Contrary to the results presented above, this study found no association between Sirt3 expression and AMPK phosphorylation or PGC-1α mRNA expression in response to exercise. However, the pattern of p38 phosphorylation was similar to PGC-1α and Sirt3 protein expression (decrease after exercise in insulin deficient mice and no change in healthy mice), despite the fact that the Sirt3 content did not differ significantly between the groups. PGC-1α expression and/or activity have been shown to be regulated at least partially by p38 (Akimoto et al. 2005; Arany 2008; Pogozelski et al. 2009). As for Sirt3, besides having been suggested to be an up-stream activator of PGC-1α expression, it has also been shown to be a down-stream target of PGC-1α (Kong et al. 2010). So, these results might suggest that the regulatory pathway consisting of these proteins is down-regulated in the skeletal muscle of insulin deficient mice after exercise. Given the previously discussed discrepancy and possible dual activity of Sirt3 in regulation of substrate use, the meaning of this possible down-regulation is unclear. As Sirt3 increases fatty acid oxidation at least in liver, via enzyme deacetylation, this response would indicate down-regulation of fatty acid oxidation in insulin deficient mice in response to an acute exercise bout (Hirschey et al. 2010). However, as Sirt3 has been also shown to deacetylate and activate the enzyme PDH, this down-

regulation might also mean decreased activity of PDH and thus switch towards fatty acid oxidation (Jing et al. 2013).

To add still a little complexity to the interpretation of these results, increased rather than decreased, p38 phosphorylation has been found in Sirt3 knock-down myoblasts (Jing et al. 2011). However, the changes in Sirt3 in response to exercise were not significant and these results don't provide any information on p38 phosphorylation immediately after exercise. Moreover, PGC-1α activity wasn't analyzed and no explanation was found to the question, why PGC-1α mRNA and protein responded differently in insulin deficient mice. Consequently, no solid conclusions of what is happening in diabetic skeletal muscle in response to exercise can be drawn from these results.

Acute exercise has been shown to increase the expression of Cyt *c* mRNA in rodent skeletal muscles, without changes in Cyt *c* protein expression two or six hours after exercise (Leick et al. 2008; Wright et al. 2007). This is in line with the results of the present study, as no changes were observed in Cyt *c* protein expression three or six hours after exercise. However, four to five weeks of exercise training has been shown to increase both Cyt *c* mRNA and protein levels in mouse skeletal muscle (Geng et al. 2010; Leick et al. 2008).

Transgenic overexpression of PGC-1α and chronic AMPK activation have been shown to result in increased Cyt *c* mRNA and protein expression, respectively (Bergeron et al. 2001; Calvo et al. 2008). PGC-1α knock out mice seem to have lower skeletal muscle Cyt *c* mRNA and protein levels. In addition the exercise-induced changes in Cyt *c* mRNA expression are inhibited in PGC-1α knock-out mice. However, exercise training seems to increase the expression of Cyt *c* mRNA and protein in both wild type and PGC-1α knock out mice despite lower expression level in knock out mice in both trained and sedentary state. These results show, that PGC-1α is an important regulator of Cyt *c* expression, but not mandatory for the training-induced adaptations. (Leick et al. 2008.) On the contrary, in another study PGC-1α knock out resulted in significantly attenuated training-induced response in Cyt *c*

protein expression supporting the importance of PGC-1α in the regulation of Cyt *c* expression (Geng et al. 2010).

In many of the previous human and animal studies, acute exercise or exercise training have had little or no effect at all on the expression of CPT1 mRNA in skeletal muscle (Huang et al. 2006; Narkar et al. 2008; Yang et al. 2005; Zhou et al. 2000). However, in one study six weeks of endurance training resulted in increased CPT1B mRNA in mouse skeletal muscle, while no change was seen four hours after acute exercise (Burch et al. 2010). In addition, inactivity has been shown to significantly decrease the mRNA expression of CPT1B in human skeletal muscle and there is a linear relationship between CPT1B and PGC-1α expression in this condition (Timmons et al. 2006). In liver, however, exercise training and dietary restriction have been found to induce increase in CPT1 mRNA in a model of type 2 diabetes (Huang et al. 2006). In this study, there were no significant differences between the groups in CPT1B mRNA or protein expression. This is in line with previous studies. However, insulin deficient mice had significantly more pronounced increase in CPT1B mRNA expression in response to exercise compared with healthy mice. This is consistent with the hypothesis apart from the fact that there were no differences in CPT1B protein expression.

Fasting and transgenic expression of AMPK γ3 subunit have been found to increase CPT1 mRNA expression in mouse skeletal muscle, while knock-out of AMPK γ3 subunit expression attenuates the effect of fasting (Long et al. 2005). CPT1B expression has been found to increase in response to transgenic PGC-1α expression and pharmacological PPARδ activation in mouse skeletal muscle (Calvo et al. 2008; Narkar et al. 2008). The regulation of muscle CPT1 by PPARβ/δ has also been shown to be dependent on PGC-1α (Dressel et al. 2003). Thus, it is possible, that the higher response in CPT1B mRNA expression in insulin deficient mice results from higher AMPK and PGC-1α activities during exercise. This speculation cannot be confirmed, because PGC-1α activity or subcellular localization wasn't analyzed and the time points weren't optimal to observe acute changes in protein phosphorylation.

The lack of hypothesized changes at protein level can be explained at least partially by the time points of sample collection: The experiment was designed to study changes in gene expression at mRNA level and the time points three and six hours after exercise are perhaps not optimal to study protein expression. For some proteins that time frame is not long enough to reveal the changes in translation and thus protein expression: For example in one study, increased PGC-1α protein expression wasn't observed until 18 hours after an acute bout of exercise (Suwa et al. 2008). In another study, PGC-1α protein expression was elevated already three and 12 hours after exercise but the increase was more pronounced 18 hours after exercise (Wright et al. 2007). In addition, protein phosphorylation in response to an acute stimulus is immediate and transient. Thus, to detect acute changes in protein phosphorylation status, the samples should have been collected immediately after the exercise. For example, increase in phosphorylation of p38, AMPK and ACC has been observed immediately after exercise, but not anymore three hours after exercise (Gibala et al. 2009). So, in the present case, the phosphorylation responses were probably already returned to baseline levels three hours after the cessation of the exercise. More specific time-course studies would be needed to confirm these speculations.

9.3   Limitations of the study

One of the limitations of the study was that the time points chosen for sample collection were not optimal for all the parameters examined. The addition of time points immediately after the exercise and for example 12 and 18 hours after the cessation of exercise could have provided more information on the changes in protein phosphorylation and expression. In addition, the analysis of respiratory gases during exercise, activities of some oxidative (citrate synthase, β-HAD) and glycolytic (PFK) enzymes and potentially the blood and muscle concentrations of energy substrates (FFAs, triacylglycerols, glycogen), could have provided more profound information and supported the conclusions drawn from this present data.

Also, it has to be kept in mind that streptozotocin-induced type 1 diabetes is only an experimental model of diabetes and thus, the results cannot be directly applied to type 1 diabetic

patients. It cannot be ruled out that some of the results may not result from the actual insulin deficiency or resulting hyperglycemia, but some other yet unknown effects of streptozotocin. Another problem in comparing the diabetes studies conducted using human subjects and animal models lies in the fact that in humans, diabetes has to be controlled with regular insulin injections for survival, whereas in animal experiments diabetes is usually uncontrolled. This complicates thus not only the comparison of these results to other studies, but also the application of these results to practice.

10 CONCLUSION AND FUTURE STUDY PROPOSALS

In this study the acute responses to a single endurance exercise bout were studied at the level of mRNA and protein expression as well as at the level of activation of certain intracellular signaling pathways in insulin deficient and healthy mice. In conclusion, these results suggest that insulin deficiency may lead to more pronounced exercise-induced responses in mRNA expression of certain genes that favor the switch from glucose to fatty acid oxidation. These changes in gene expression may be mediated by increased PGC-1α activation and/or nuclear localization as no increases were found in PGC-1α protein expression. The basal protein levels were not affected by insulin deficiency and it is possible that also the exercise-induced changes in phosphorylation were missed because the time points were not optimal. The same might be true for exercise-induced changes in protein expression. Thus, the mechanisms underlying the changes in mRNA expression can be only speculated.

The research questions of this study remained partially unanswered for the part of activation of intracellular signaling pathways, because the experimental design wasn't optimal to study acute protein phosphorylation or expression responses. Consequently, further studies, with an experimental design aiming to protein level analysis, should be conducted to provide more reliable information on the effects of insulin deficiency on the acute protein level responses to a single bout of exercise in skeletal muscle. In addition, measurements of the levels of intramuscular lipid and glycogen stores, the activities of key enzymes involved in aerobic energy metabolism as well as analysis of substrate use during exercise would provide more profound information on the metabolism of diabetic skeletal muscle and allow more solid conclusions to be drawn.

11 REFERENCES

Abdul-Rasoul, M., Habib, H. & Al-Khouly, M. 2006. 'The honeymoon phase' in children with type 1 diabetes mellitus: frequency, duration, and influential factors. Pediatric diabetes 7; 2, 101-107.

Akimoto, T., Pohnert, S. C., Li, P., Zhang, M., Gumbs, C., Rosenberg, P. B., Williams, R. S. & Yan, Z. 2005. Exercise stimulates Pgc-1alpha transcription in skeletal muscle through activation of the p38 MAPK pathway. The Journal of biological chemistry 280; 20, 19587-19593.

American Diabetes Association 2013. Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 36, S67-S74.

Araki, M. & Motojima, K. 2006. Identification of ERRalpha as a specific partner of PGC-1alpha for the activation of PDK4 gene expression in muscle. The FEBS journal 273; 8, 1669-1680.

Arany, Z. 2008. PGC-1 coactivators and skeletal muscle adaptations in health and disease. Current opinion in genetics & development 18; 5, 426-434.

Austin, A., Warty, V., Janosky, J. & Arslanian, S. 1993. The relationship of physical fitness to lipid and lipoprotein(a) levels in adolescents with IDDM. Diabetes care 16; 2, 421-425.

Bak, J. F., Jacobsen, U. K., Jorgensen, F. S. & Pedersen, O. 1989. Insulin receptor function and glycogen synthase activity in skeletal muscle biopsies from patients with insulin-dependent diabetes mellitus: effects of physical training. The Journal of clinical endocrinology and metabolism 69; 1, 158-164.

Bergeron, R., Ren, J. M., Cadman, K. S., Moore, I. K., Perret, P., Pypaert, M., Young, L. H., Semenkovich, C. F. & Shulman, G. I. 2001. Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. American journal of physiology.Endocrinology and metabolism 281; 6, E1340-6.

Bonen, A., Luiken, J. J., Arumugam, Y., Glatz, J. F. & Tandon, N. N. 2000. Acute regulation of fatty acid uptake involves the cellular redistribution of fatty acid translocase. The Journal of biological chemistry 275; 19, 14501-14508.

Burch, N., Arnold, A. S., Item, F., Summermatter, S., Brochmann Santana Santos, G., Christe, M., Boutellier, U., Toigo, M. & Handschin, C. 2010. Electric pulse stimulation of cultured murine muscle cells reproduces gene expression changes of trained mouse muscle. PloS one 5; 6, e10970.

Calvo, J. A., Daniels, T. G., Wang, X., Paul, A., Lin, J., Spiegelman, B. M., Stevenson, S. C. & Rangwala, S. M. 2008. Muscle-specific expression of PPARgamma coactivator-1alpha improves exercise performance and increases peak oxygen uptake. Journal of applied physiology (Bethesda, Md.: 1985) 104; 5, 1304-1312.

Canto, C. & Auwerx, J. 2010. AMP-activated protein kinase and its downstream transcriptional pathways. Cellular and molecular life sciences: CMLS 67; 20, 3407-3423.

Canto, C., Gerhart-Hines, Z., Feige, J. N., Lagouge, M., Noriega, L., Milne, J. C., Elliott, P. J., Puigserver, P. & Auwerx, J. 2009. AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. Nature 458; 7241, 1056-1060.

Carter, S. L., Rennie, C. & Tarnopolsky, M. A. 2001. Substrate utilization during endurance exercise in men and women after endurance training. American journal of physiology.Endocrinology and metabolism 280; 6, E898-907.

Costill, D. L., Cleary, P., Fink, W. J., Foster, C., Ivy, J. L. & Witzmann, F. 1979. Training adaptations in skeletal muscle of juvenile diabetics. Diabetes 28; 9, 818-822.

Crowther, G. J., Milstein, J. M., Jubrias, S. A., Kushmerick, M. J., Gronka, R. K. & Conley, K. E. 2003. Altered energetic properties in skeletal muscle of men with well-controlled insulin-dependent (type 1) diabetes. American journal of physiology.Endocrinology and metabolism 284; 4, E655-62.

De Filippis, E., Alvarez, G., Berria, R., Cusi, K., Everman, S., Meyer, C. & Mandarino, L. J. 2008. Insulin-resistant muscle is exercise resistant: evidence for reduced response of nuclear-encoded mitochondrial genes to exercise. American journal of physiology.Endocrinology and metabolism 294; 3, E607-14.

Dean, D., Daugaard, J. R., Young, M. E., Saha, A., Vavvas, D., Asp, S., Kiens, B., Kim, K. H., Witters, L., Richter, E. A. & Ruderman, N. 2000. Exercise diminishes the activity of acetyl-CoA carboxylase in human muscle. Diabetes 49; 8, 1295-1300.

Dressel, U., Allen, T. L., Pippal, J. B., Rohde, P. R., Lau, P. & Muscat, G. E. 2003. The peroxisome proliferator-activated receptor beta/delta agonist, GW501516, regulates the expression of genes involved in lipid catabolism and energy uncoupling in skeletal muscle cells. Molecular endocrinology (Baltimore, Md.) 17; 12, 2477-2493.

Dreyer, H. C., Fujita, S., Cadenas, J. G., Chinkes, D. L., Volpi, E. & Rasmussen, B. B. 2006. Resistance exercise increases AMPK activity and reduces 4E-BP1 phosphorylation and protein synthesis in human skeletal muscle. The Journal of physiology 576; Pt 2, 613-624.

Ebeling, P., Tuominen, J. A., Bourey, R., Koranyi, L. & Koivisto, V. A. 1995. Athletes with IDDM exhibit impaired metabolic control and increased lipid utilization with no increase in insulin sensitivity. Diabetes 44; 4, 471-477.

el Midaoui, A., Tancrede, G. & Nadeau, A. 1996. Effect of physical training on mitochondrial function in skeletal muscle of normal and diabetic rats. Metabolism: clinical and experimental 45; 7, 810-816.

Ferré, P. 2004. The biology of peroxisome proliferator-activated receptors: relationship with lipid metabolism and insulin sensitivity. Diabetes 53 Suppl 1, S43-50.

Fintini, D., Di Giacinto, B., Brufani, C., Cafiero, G., Patera, P. I., Turchetta, A., Giordano, U., Nobili, V., Pelliccia, A., Calzolari, A. & Cappa, M. 2012. Impaired energy expenditure despite normal cardiovascular capacity in children with type 1 diabetes. Hormone research in paediatrics 78; 1, 1-7.

Friedlander, A. L., Casazza, G. A., Horning, M. A., Buddinger, T. F. & Brooks, G. A. 1998a. Effects of exercise intensity and training on lipid metabolism in young women. The American Journal of Physiology 275; 5 Pt 1, E853-63.

Friedlander, A. L., Casazza, G. A., Horning, M. A., Huie, M. J. & Brooks, G. A. 1997. Training-induced alterations of glucose flux in men. Journal of applied physiology (Bethesda, Md.: 1985) 82; 4, 1360-1369.

Friedlander, A. L., Casazza, G. A., Horning, M. A., Huie, M. J., Piacentini, M. F., Trimmer, J. K. & Brooks, G. A. 1998b. Training-induced alterations of carbohydrate metabolism in women: women respond differently from men. Journal of applied physiology (Bethesda, Md.: 1985) 85; 3, 1175-1186.

Friedlander, A. L., Casazza, G. A., Horning, M. A., Usaj, A. & Brooks, G. A. 1999. Endurance training increases fatty acid turnover, but not fat oxidation, in young men. Journal of applied physiology (Bethesda, Md.: 1985) 86; 6, 2097-2105.

Fuchsjager-Mayrl, G., Pleiner, J., Wiesinger, G. F., Sieder, A. E., Quittan, M., Nuhr, M. J., Francesconi, C., Seit, H. P., Francesconi, M., Schmetterer, L. & Wolzt, M. 2002. Exercise training improves vascular endothelial function in patients with type 1 diabetes. Diabetes care 25; 10, 1795-1801.

Geng, T., Li, P., Okutsu, M., Yin, X., Kwek, J., Zhang, M. & Yan, Z. 2010. PGC-1alpha plays a functional role in exercise-induced mitochondrial biogenesis and angiogenesis but not fiber-type transformation in mouse skeletal muscle. American journal of physiology.Cell physiology 298; 3, C572-9.

Gerhart-Hines, Z., Rodgers, J. T., Bare, O., Lerin, C., Kim, S. H., Mostoslavsky, R., Alt, F. W., Wu, Z. & Puigserver, P. 2007. Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha. The EMBO journal 26; 7, 1913-1923.

Gibala, M. J., McGee, S. L., Garnham, A. P., Howlett, K. F., Snow, R. J. & Hargreaves, M. 2009. Brief intense interval exercise activates AMPK and p38 MAPK signaling and increases the expression of PGC-1alpha in human skeletal muscle. Journal of applied physiology (Bethesda, Md.: 1985) 106; 3, 929-934.

Giralt, A., Hondares, E., Villena, J. A., Ribas, F., Diaz-Delfin, J., Giralt, M., Iglesias, R. & Villarroya, F. 2011. Peroxisome proliferator-activated receptor-gamma coactivator-1alpha controls transcription of the Sirt3 gene, an essential component of the thermogenic brown adipocyte phenotype. The Journal of biological chemistry 286; 19, 16958-16966.

Guerra, B., Guadalupe-Grau, A., Fuentes, T., Ponce-Gonzalez, J. G., Morales-Alamo, D., Olmedillas, H., Guillen-Salgado, J., Santana, A. & Calbet, J. A. 2010. SIRT1, AMP-

activated protein kinase phosphorylation and downstream kinases in response to a single bout of sprint exercise: influence of glucose ingestion. European journal of applied physiology 109; 4, 731-743.

Gusso, S., Hofman, P., Lalande, S., Cutfield, W., Robinson, E. & Baldi, J. C. 2008. Impaired stroke volume and aerobic capacity in female adolescents with type 1 and type 2 diabetes mellitus. Diabetologia 51; 7, 1317-1320.

Guyton, A. C. & Hall, J. E. 2000. Textbook of Medical Physiology. (10. edition) W.B. Saunders Company, USA.

Hardie, D. G. & Sakamoto, K. 2006. AMPK: a key sensor of fuel and energy status in skeletal muscle. Physiology (Bethesda, Md.) 21, 48-60.

Harjutsalo, V., Sund, R., Knip, M. & Groop, P. H. 2013. Incidence of type 1 diabetes in Finland. JAMA : the journal of the American Medical Association 310; 4, 427-428.

Herrero, P., Peterson, L. R., McGill, J. B., Matthew, S., Lesniak, D., Dence, C. & Gropler, R. J. 2006. Increased myocardial fatty acid metabolism in patients with type 1 diabetes mellitus. Journal of the American College of Cardiology 47; 3, 598-604.

Hirschey, M. D., Shimazu, T., Goetzman, E., Jing, E., Schwer, B., Lombard, D. B., Grueter, C. A., Harris, C., Biddinger, S., Ilkayeva, O. R., Stevens, R. D., Li, Y., Saha, A. K., Ruderman, N. B., Bain, J. R., Newgard, C. B., Farese, R. V.,Jr, Alt, F. W., Kahn, C. R. & Verdin, E. 2010. SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. Nature 464; 7285, 121-125.

Hokari, F., Kawasaki, E., Sakai, A., Koshinaka, K., Sakuma, K. & Kawanaka, K. 2010. Muscle contractile activity regulates Sirt3 protein expression in rat skeletal muscles. Journal of applied physiology (Bethesda, Md.: 1985) 109; 2, 332-340.

Holloszy, J. O. & Coyle, E. F. 1984. Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. Journal of applied physiology: respiratory, environmental and exercise physiology 56; 4, 831-838.

Horowitz, J. F. 2003. Fatty acid mobilization from adipose tissue during exercise. Trends in endocrinology and metabolism: TEM 14; 8, 386-392.

Horowitz, J. F. & Klein, S. 2000. Lipid metabolism during endurance exercise. The American Journal of Clinical Nutrition 72; 2 Suppl, 558S-63S.

Horowitz, J. F., Leone, T. C., Feng, W., Kelly, D. P. & Klein, S. 2000. Effect of endurance training on lipid metabolism in women: a potential role for PPARalpha in the metabolic response to training. American journal of physiology.Endocrinology and metabolism 279; 2, E348-55.

Houtkooper, R. H., Pirinen, E. & Auwerx, J. 2012. Sirtuins as regulators of metabolism and healthspan. Nature reviews.Molecular cell biology 13; 4, 225-238.

Huang, H., Iida, K. T., Sone, H., Yokoo, T., Yamada, N. & Ajisaka, R. 2006. The effect of exercise training on adiponectin receptor expression in KKAy obese/diabetic mice. The Journal of endocrinology 189; 3, 643-653.

Hulmi, J. J., Silvennoinen, M., Lehti, M., Kivela, R. & Kainulainen, H. 2012. Altered REDD1, myostatin, and Akt/mTOR/FoxO/MAPK signaling in streptozotocin-induced diabetic muscle atrophy. American journal of physiology.Endocrinology and metabolism 302; 3, E307-15.

Ianuzzo, C. D., Lesser, M. & Battista, F. 1974. Metabolic adaptations in skeletal muscle of streptozotocin-diabetic rats following exercise training. Biochemical and biophysical research communications 58; 1, 107-111.

Jäger, S., Handschin, C., St-Pierre, J. & Spiegelman, B. M. 2007. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. Proceedings of the National Academy of Sciences of the United States of America 104; 29, 12017-12022.

Jing, E., Emanuelli, B., Hirschey, M. D., Boucher, J., Lee, K. Y., Lombard, D., Verdin, E. M. & Kahn, C. R. 2011. Sirtuin-3 (Sirt3) regulates skeletal muscle metabolism and insulin signaling via altered mitochondrial oxidation and reactive oxygen species production. Proceedings of the National Academy of Sciences of the United States of America 108; 35, 14608-14613.

Jing, E., O'Neill, B. T., Rardin, M. J., Kleinridders, A., Ilkeyeva, O. R., Ussar, S., Bain, J. R., Lee, K. Y., Verdin, E. M., Newgard, C. B., Gibson, B. W. & Kahn, C. R. 2013. Sirt3 regulates metabolic flexibility of skeletal muscle through reversible enzymatic deacetylation. Diabetes 62; 10, 3404-3417.

Kiens, B., Essen-Gustavsson, B., Christensen, N. J. & Saltin, B. 1993. Skeletal muscle substrate utilization during submaximal exercise in man: effect of endurance training. The Journal of physiology 469, 459-478.

Kiens, B., Kristiansen, S., Jensen, P., Richter, E. A. & Turcotte, L. P. 1997. Membrane associated fatty acid binding protein (FABPpm) in human skeletal muscle is increased by endurance training. Biochemical and biophysical research communications 231; 2, 463-465.

Kivelä, R., Silvennoinen, M., Touvra, A. M., Lehti, T. M., Kainulainen, H. & Vihko, V. 2006. Effects of experimental type 1 diabetes and exercise training on angiogenic gene expression and capillarization in skeletal muscle. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 20; 9, 1570-1572.

Koltai, E., Szabo, Z., Atalay, M., Boldogh, I., Naito, H., Goto, S., Nyakas, C. & Radak, Z. 2010. Exercise alters SIRT1, SIRT6, NAD and NAMPT levels in skeletal muscle of aged rats. Mechanisms of ageing and development 131; 1, 21-28.

Komatsu, W. R., Barros Neto, T. L., Chacra, A. R. & Dib, S. A. 2010. Aerobic exercise capacity and pulmonary function in athletes with and without type 1 diabetes. Diabetes care 33; 12, 2555-2557.

Komatsu, W. R., Gabbay, M. A., Castro, M. L., Saraiva, G. L., Chacra, A. R., de Barros Neto, T. L. & Dib, S. A. 2005. Aerobic exercise capacity in normal adolescents and those with type 1 diabetes mellitus. Pediatric diabetes 6; 3, 145-149.

Kong, X., Wang, R., Xue, Y., Liu, X., Zhang, H., Chen, Y., Fang, F. & Chang, Y. 2010. Sirtuin 3, a new target of PGC-1alpha, plays an important role in the suppression of ROS and mitochondrial biogenesis. PloS one 5; 7, e11707.

Kramer, H. F. & Goodyear, L. J. 2007. Exercise, MAPK, and NF-kappaB signaling in skeletal muscle. Journal of applied physiology (Bethesda, Md.: 1985) 103; 1, 388-395.

Kwon, H. S. & Harris, R. A. 2004. Mechanisms responsible for regulation of pyruvate dehydrogenase kinase 4 gene expression. Advances in Enzyme Regulation 44, 109-121.

Laaksonen, D. E., Atalay, M., Niskanen, L. K., Mustonen, J., Sen, C. K., Lakka, T. A. & Uusitupa, M. I. 2000. Aerobic exercise and the lipid profile in type 1 diabetic men: a

randomized controlled trial. Medicine and science in sports and exercise 32; 9, 1541-1548.

Lan, F., Cacicedo, J. M., Ruderman, N. & Ido, Y. 2008. SIRT1 modulation of the acetylation status, cytosolic localization, and activity of LKB1. Possible role in AMP-activated protein kinase activation. The Journal of biological chemistry 283; 41, 27628-27635.

Lass, A., Zimmermann, R., Oberer, M. & Zechner, R. 2011. Lipolysis - a highly regulated multi-enzyme complex mediates the catabolism of cellular fat stores. Progress in lipid research 50; 1, 14-27.

Leick, L., Wojtaszewski, J. F., Johansen, S. T., Kiilerich, K., Comes, G., Hellsten, Y., Hidalgo, J. & Pilegaard, H. 2008. PGC-1alpha is not mandatory for exercise- and training-induced adaptive gene responses in mouse skeletal muscle. American journal of physiology.Endocrinology and metabolism 294; 2, E463-74.

Little, J. P., Safdar, A., Cermak, N., Tarnopolsky, M. A. & Gibala, M. J. 2010. Acute endurance exercise increases the nuclear abundance of PGC-1alpha in trained human skeletal muscle. American journal of physiology.Regulatory, integrative and comparative physiology 298; 4, R912-7.

Lomb, D. J., Laurent, G. & Haigis, M. C. 2010. Sirtuins regulate key aspects of lipid metabolism. Biochimica et biophysica acta 1804; 8, 1652-1657.

Long, Y. C., Barnes, B. R., Mahlapuu, M., Steiler, T. L., Martinsson, S., Leng, Y., Wallberg-Henriksson, H., Andersson, L. & Zierath, J. R. 2005. Role of AMP-activated protein kinase in the coordinated expression of genes controlling glucose and lipid metabolism in mouse white skeletal muscle. Diabetologia 48; 11, 2354-2364.

Luiken, J. J., Dyck, D. J., Han, X. X., Tandon, N. N., Arumugam, Y., Glatz, J. F. & Bonen, A. 2002. Insulin induces the translocation of the fatty acid transporter FAT/CD36 to the plasma membrane. American journal of physiology.Endocrinology and metabolism 282; 2, E491-5.

Martin, W. H.,3rd, Dalsky, G. P., Hurley, B. F., Matthews, D. E., Bier, D. M., Hagberg, J. M., Rogers, M. A., King, D. S. & Holloszy, J. O. 1993. Effect of endurance training on plasma free fatty acid turnover and oxidation during exercise. The American Journal of Physiology 265; 5 Pt 1, E708-14.

Mooren, F. & Völker, K. 2005. Molecular and Cellular Exercise Physiology. Human Kinetics, Champaign, IL. USA.

Narkar, V. A., Downes, M., Yu, R. T., Embler, E., Wang, Y. X., Banayo, E., Mihaylova, M. M., Nelson, M. C., Zou, Y., Juguilon, H., Kang, H., Shaw, R. J. & Evans, R. M. 2008. AMPK and PPARdelta agonists are exercise mimetics. Cell 134; 3, 405-415.

Nelson, D. L. & Cox, M. M. 2013. Lehninger Principles of Biochemistry. (6. edition) W. H. Freeman and Company, New York, USA.

Niranjan, V., McBrayer, D. G., Ramirez, L. C., Raskin, P. & Hsia, C. C. 1997. Glycemic control and cardiopulmonary function in patients with insulin-dependent diabetes mellitus. The American Journal of Medicine 103; 6, 504-513.

Noble, E. G. & Ianuzzo, C. D. 1985. Influence of training on skeletal muscle enzymatic adaptations in normal and diabetic rats. The American Journal of Physiology 249; 4 Pt 1, E360-5.

Norrbom, J., Sallstedt, E. K., Fischer, H., Sundberg, C. J., Rundqvist, H. & Gustafsson, T. 2011. Alternative splice variant PGC-1alpha-b is strongly induced by exercise in human skeletal muscle. American journal of physiology.Endocrinology and metabolism 301; 6, E1092-8.

Odland, L. M., Heigenhauser, G. J., Lopaschuk, G. D. & Spriet, L. L. 1996. Human skeletal muscle malonyl-CoA at rest and during prolonged submaximal exercise. The American Journal of Physiology 270; 3 Pt 1, E541-4.

Odland, L. M., Howlett, R. A., Heigenhauser, G. J., Hultman, E. & Spriet, L. L. 1998. Skeletal muscle malonyl-CoA content at the onset of exercise at varying power outputs in humans. The American Journal of Physiology 274; 6 Pt 1, E1080-5.

Palacios, O. M., Carmona, J. J., Michan, S., Chen, K. Y., Manabe, Y., Ward, J. L.,3rd, Goodyear, L. J. & Tong, Q. 2009. Diet and exercise signals regulate SIRT3 and activate AMPK and PGC-1alpha in skeletal muscle. Aging 1; 9, 771-783.

Phillips, S. M., Green, H. J., Tarnopolsky, M. A., Heigenhauser, G. F., Hill, R. E. & Grant, S. M. 1996. Effects of training duration on substrate turnover and oxidation during exercise. Journal of applied physiology (Bethesda, Md.: 1985) 81; 5, 2182-2191.

Pogozelski, A. R., Geng, T., Li, P., Yin, X., Lira, V. A., Zhang, M., Chi, J. T. & Yan, Z. 2009. P38gamma Mitogen-Activated Protein Kinase is a Key Regulator in Skeletal Muscle Metabolic Adaptation in Mice. PloS one 4; 11, e7934.

Puigserver, P., Rhee, J., Lin, J., Wu, Z., Yoon, J. C., Zhang, C. Y., Krauss, S., Mootha, V. K., Lowell, B. B. & Spiegelman, B. M. 2001. Cytokine stimulation of energy expenditure through p38 MAP kinase activation of PPARgamma coactivator-1. Molecular cell 8; 5, 971-982.

Rasmussen, B. B., Holmback, U. C., Volpi, E., Morio-Liondore, B., Paddon-Jones, D. & Wolfe, R. R. 2002. Malonyl coenzyme A and the regulation of functional carnitine palmitoyltransferase-1 activity and fat oxidation in human skeletal muscle. The Journal of clinical investigation 110; 11, 1687-1693.

Rasmussen, B. B. & Winder, W. W. 1997. Effect of exercise intensity on skeletal muscle malonyl-CoA and acetyl-CoA carboxylase. Journal of applied physiology (Bethesda, Md.: 1985) 83; 4, 1104-1109.

Roepstorff, C., Halberg, N., Hillig, T., Saha, A. K., Ruderman, N. B., Wojtaszewski, J. F., Richter, E. A. & Kiens, B. 2005. Malonyl-CoA and carnitine in regulation of fat oxidation in human skeletal muscle during exercise. American journal of physiology. Endocrinology and metabolism 288; 1, E133-42.

Romijn, J. A., Coyle, E. F., Sidossis, L. S., Gastaldelli, A., Horowitz, J. F., Endert, E. & Wolfe, R. R. 1993. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. The American Journal of Physiology 265; 3 Pt 1, E380-91.

Romijn, J. A., Coyle, E. F., Sidossis, L. S., Rosenblatt, J. & Wolfe, R. R. 2000. Substrate metabolism during different exercise intensities in endurance-trained women. Journal of applied physiology (Bethesda, Md.: 1985) 88; 5, 1707-1714.

Ruas, J. L., White, J. P., Rao, R. R., Kleiner, S., Brannan, K. T., Harrison, B. C., Greene, N. P., Wu, J., Estall, J. L., Irving, B. A., Lanza, I. R., Rasbach, K. A., Okutsu, M., Nair, K. S., Yan, Z., Leinwand, L. A. & Spiegelman, B. M. 2012. A PGC-1alpha isoform induced by resistance training regulates skeletal muscle hypertrophy. Cell 151; 6, 1319-1331.

Ruderman, N. B., Saha, A. K., Vavvas, D. & Witters, L. A. 1999. Malonyl-CoA, fuel sensing, and insulin resistance. The American Journal of Physiology 276; 1 Pt 1, E1-E18.

Saha, A. K., Laybutt, D. R., Dean, D., Vavvas, D., Sebokova, E., Ellis, B., Klimes, I., Kraegen, E. W., Shafrir, E. & Ruderman, N. B. 1999. Cytosolic citrate and malonyl-CoA regulation in rat muscle in vivo. The American Journal of Physiology 276; 6 Pt 1, E1030-7.

Saha, A. K., Schwarsin, A. J., Roduit, R., Masse, F., Kaushik, V., Tornheim, K., Prentki, M. & Ruderman, N. B. 2000. Activation of malonyl-CoA decarboxylase in rat skeletal muscle by contraction and the AMP-activated protein kinase activator 5-aminoimidazole-4-carboxamide-1-beta -D-ribofuranoside. The Journal of biological chemistry 275; 32, 24279-24283.

Saltiel, A. R. & Kahn, C. R. 2001. Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414; 6865, 799-806.

Shulman, G. I., Rothman, D. L., Jue, T., Stein, P., DeFronzo, R. A. & Shulman, R. G. 1990. Quantitation of muscle glycogen synthesis in normal subjects and subjects with non-insulin-dependent diabetes by 13C nuclear magnetic resonance spectroscopy. The New England journal of medicine 322; 4, 223-228.

Silvennoinen, M. 2004. Tyypin 1 diabeteksen ja kestävyysharjoittelun vaikutukset energiametaboliaan liittyvien geenien ilmenemiseen hiirten raajalihaksissa. Master's thesis. University of Jyväskylä, Jyväskylä, Finland.

Spriet, L. L. & Watt, M. J. 2003. Regulatory mechanisms in the interaction between carbohydrate and lipid oxidation during exercise. Acta Physiologica Scandinavica 178; 4, 443-452.

Sriwijitkamol, A., Coletta, D. K., Wajcberg, E., Balbontin, G. B., Reyna, S. M., Barrientes, J., Eagan, P. A., Jenkinson, C. P., Cersosimo, E., DeFronzo, R. A., Sakamoto, K. & Musi, N. 2007. Effect of acute exercise on AMPK signaling in skeletal muscle of subjects with type 2 diabetes: a time-course and dose-response study. Diabetes 56; 3, 836-848.

Stallknecht, B., Simonsen, L., Bulow, J., Vinten, J. & Galbo, H. 1995. Effect of training on epinephrine-stimulated lipolysis determined by microdialysis in human adipose tissue. The American Journal of Physiology 269; 6 Pt 1, E1059-66.

Suwa, M., Nakano, H., Radak, Z. & Kumagai, S. 2008. Endurance exercise increases the SIRT1 and peroxisome proliferator-activated receptor gamma coactivator-1alpha protein expressions in rat skeletal muscle. Metabolism: clinical and experimental 57; 7, 986-998.

Talanian, J. L., Galloway, S. D., Heigenhauser, G. J., Bonen, A. & Spriet, L. L. 2007. Two weeks of high-intensity aerobic interval training increases the capacity for fat oxidation during exercise in women. Journal of applied physiology (Bethesda, Md.: 1985) 102; 4, 1439-1447.

Tamborlane, W. V., Sherwin, R. S., Koivisto, V., Hendler, R., Genel, M. & Felig, P. 1979. Normalization of the growth hormone and catecholamine response to exercise in juvenile-onset diabetic subjects treated with a portable insulin infusion pump. Diabetes 28; 8, 785-788.

Terada, S., Goto, M., Kato, M., Kawanaka, K., Shimokawa, T. & Tabata, I. 2002. Effects of low-intensity prolonged exercise on PGC-1 mRNA expression in rat epitrochlearis muscle. Biochemical and biophysical research communications 296; 2, 350-354.

Terada, S., Kawanaka, K., Goto, M., Shimokawa, T. & Tabata, I. 2005. Effects of high-intensity intermittent swimming on PGC-1alpha protein expression in rat skeletal muscle. Acta Physiologica Scandinavica 184; 1, 59-65.

Terada, S. & Tabata, I. 2004. Effects of acute bouts of running and swimming exercise on PGC-1alpha protein expression in rat epitrochlearis and soleus muscle. American journal of physiology.Endocrinology and metabolism 286; 2, E208-16.

Timmons, J. A., Norrbom, J., Scheele, C., Thonberg, H., Wahlestedt, C. & Tesch, P. 2006. Expression profiling following local muscle inactivity in humans provides new perspective on diabetes-related genes. Genomics 87; 1, 165-172.

Tunstall, R. J., Mehan, K. A., Wadley, G. D., Collier, G. R., Bonen, A., Hargreaves, M. & Cameron-Smith, D. 2002. Exercise training increases lipid metabolism gene expression

in human skeletal muscle. American journal of physiology.Endocrinology and metabolism 283; 1, E66-72.

Veves, A., Saouaf, R., Donaghue, V. M., Mullooly, C. A., Kistler, J. A., Giurini, J. M., Horton, E. S. & Fielding, R. A. 1997. Aerobic exercise capacity remains normal despite impaired endothelial function in the micro- and macrocirculation of physically active IDDM patients. Diabetes 46; 11, 1846-1852.

Watt, M. J., Southgate, R. J., Holmes, A. G. & Febbraio, M. A. 2004. Suppression of plasma free fatty acids upregulates peroxisome proliferator-activated receptor (PPAR) alpha and delta and PPAR coactivator 1alpha in human skeletal muscle, but not lipid regulatory genes. Journal of Molecular Endocrinology 33; 2, 533-544.

Wende, A. R., Huss, J. M., Schaeffer, P. J., Giguere, V. & Kelly, D. P. 2005. PGC-1alpha coactivates PDK4 gene expression via the orphan nuclear receptor ERRalpha: a mechanism for transcriptional control of muscle glucose metabolism. Molecular and cellular biology 25; 24, 10684-10694.

Winder, W. W., Wilson, H. A., Hardie, D. G., Rasmussen, B. B., Hutber, C. A., Call, G. B., Clayton, R. D., Conley, L. M., Yoon, S. & Zhou, B. 1997. Phosphorylation of rat muscle acetyl-CoA carboxylase by AMP-activated protein kinase and protein kinase A. Journal of applied physiology (Bethesda, Md.: 1985) 82; 1, 219-225.

Wright, D. C., Han, D. H., Garcia-Roves, P. M., Geiger, P. C., Jones, T. E. & Holloszy, J. O. 2007. Exercise-induced mitochondrial biogenesis begins before the increase in muscle PGC-1alpha expression. The Journal of biological chemistry 282; 1, 194-199.

Yang, Y., Creer, A., Jemiolo, B. & Trappe, S. 2005. Time course of myogenic and metabolic gene expression in response to acute exercise in human skeletal muscle. Journal of applied physiology (Bethesda, Md.: 1985) 98; 5, 1745-1752.

Ydfors, M., Fischer, H., Mascher, H., Blomstrand, E., Norrbom, J. & Gustafsson, T. 2013. The truncated splice variants, NT-PGC-1alpha and PGC-1alpha4, increase with both endurance and resistance exercise in human skeletal muscle. Physiological reports 1; 6, e00140.

Yechoor, V. K., Patti, M. E., Saccone, R. & Kahn, C. R. 2002. Coordinated patterns of gene expression for substrate and energy metabolism in skeletal muscle of diabetic mice.

Proceedings of the National Academy of Sciences of the United States of America 99; 16, 10587-10592.

Zhou, M., Lin, B. Z., Coughlin, S., Vallega, G. & Pilch, P. F. 2000. UCP-3 expression in skeletal muscle: effects of exercise, hypoxia, and AMP-activated protein kinase. American journal of physiology.Endocrinology and metabolism 279; 3, E622-9.

APPENDIX 1. Information on the antibodies used in Western blotting analysis.

Protein	Host, isotype	Clonality	Product No.	Manufacturer	Blocking	Dilution	Buffer	Secondary Antibody	Detection	kDa
Phospho-ACC (Ser79)	Rabbit	polyclonal	#3661	Cell Signaling Technology	5% non-fat dry milk diluted in TBS	1:1000	5% non-fat dry milk diluted in TBS	HRP-conjugated Goat anti-Rabbit IgG Sigma A9169 (1:40000)	ECL	280
ACC	Rabbit, IgG	monoclonal	#3676	Cell Signaling Technology	5% non-fat dry milk diluted in TBS	1:1000	5% non-fat dry milk diluted in TBS	HRP-conjugated Goat anti-Rabbit IgG Sigma A9169 (1:40000)	ECL	280
Phospho-AMPKα (Thr172) (D79.5E)	Rabbit, IgG	monoclonal	#4188	Cell Signaling Technology	Odyssey Blocking Buffer	1:1000	Odyssey Blocking Buffer diluted 1:1 in TBS	926-68023 IRDye® 680LT Donkey anti-Rabbit IgG (1:20000)	Infrared fluorescence	62
AMPKα (23A3)	Rabbit, IgG	monoclonal	#2603	Cell Signaling Technology	Odyssey Blocking Buffer	1:1000	Odyssey Blocking Buffer diluted 1:1 in TBS	926-68023 IRDye® 680LT Donkey anti-Rabbit IgG (1:20000)	Infrared fluorescence	62
CPT1B	Rabbit	polyclonal	NBP1-59576	Novus Biologicals	5% non-fat dry milk diluted in TBS-Tween	1:3000	2.5% non-fat dry milk diluted in TBS-T	HRP-conjugated anti-rabbit Jackson ImmunoResearch (1:10000)	ECL	88
Cytochrome C (C-20)	Goat	polyclonal	sc-8385	Santa Cruz Biotechnology	Odyssey Blocking Buffer	1:1000	Odyssey Blocking Buffer diluted 1:1 in TBS	926-68024 IRDye® 680LT Donkey anti-Goat IgG (1:20000)	Infrared fluorescence	15
Phospho-p38MAPK (Thr180/Tyr182)	Rabbit	polyclonal	#9211	Cell Signaling Technology	5% non-fat dry milk diluted in TBS-Tween	1:1500	2.5% non-fat dry milk diluted in TBS-T	HRP-conjugated anti-rabbit Jackson ImmunoResearch (1:10000)	ECL	43
p38MAPK	Rabbit	polyclonal	#9212	Cell Signaling Technology	5% non-fat dry milk diluted in TBS-Tween	1:1500	2.5% non-fat dry milk diluted in TBS-T	HRP-conjugated anti-rabbit Jackson ImmunoResearch (1:10000)	ECL	43
PDK4	Rabbit, IgG	polyclonal	ab38242	Abcam	5% non-fat dry milk diluted in TBS-Tween	1:2500	2.5% non-fat dry milk diluted in TBS-T	HRP-conjugated anti-rabbit Jackson ImmunoResearch (1:10000)	ECL	50
PGC-1α, C-terminal (777-797)	Rabbit, IgG	polyclonal	516557	Calbiochem	5% non-fat dry milk diluted in TBS-Tween	1:3000	2.5% non-fat dry milk diluted in TBS-T	HRP-conjugated anti-rabbit Jackson ImmunoResearch (1:10000)	ECL	~100
Sirt1	Rabbit, IgG	polyclonal	ab28170	Abcam	5% non-fat dry milk diluted in TBS	1:1000	5% non-fat dry milk diluted in TBS	926-68023 IRDye® 680LT Donkey anti-Rabbit IgG (1:20000)	Infrared fluorescence	110
Sirt3	Goat, IgG	polyclonal	ab118334	Abcam	Odyssey Blocking Buffer	1:800	Odyssey Blocking Buffer diluted 1:1 in TBS	926-68024 IRDye® 680LT Donkey anti-Goat IgG (1:20000)	Infrared fluorescence	29
Sirt6	Rabbit, IgG	polyclonal	ab62739	Abcam	5% non-fat dry milk diluted in TBS	1:1000	5% non-fat dry milk diluted in TBS	926-32213 IRDye® 800CW Donkey anti-Rabbit IgG (1:20000)	Infrared fluorescence	37