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MOLECULAR REGULATORS OF GLUCOSE AND LIPID METABOLISM IN SKELETAL MUSCLE

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In the middle of difficulty lies opportunity
Albert Einstein, 1879-1955

To my family

ABSTRACT

Skeletal muscle is a primary site of insulin action and insulin-stimulated glucose transport and occupies a center stage in maintaining whole body glucose and lipid homeostasis. Another key feature of a healthy skeletal muscle is its ability to switch between utilization of lipids and glucose as fuel in response to feeding or fasting respectively. This metabolic flexibility is impaired in skeletal muscle from insulin resistant and type 2 diabetic patients. Key molecules such as AMP Kinase [AMPK] and Pyruvate Dehydrogenase Kinase [PDK] play a crucial role in maintaining metabolic flexibility in a healthy skeletal muscle. These molecules have recently emerged as potential drug targets to combat diabetes.

One strategy to activate the AMPK pathway is via altering the expression of AMP-metabolizing enzymes, such as 5'-nucleotidases [NT5C]. The role of cytosolic 5´-nucleotidases was determined in metabolic responses linked to the development of insulin resistance in obesity and type 2 diabetes (Study I). NT5C1A and NT5C2 gene silencing led to increase in the AMP/ATP ratio, increase in phosphorylation of AMPK and ACC, and an increase in palmitate oxidation and glucose transport in mouse and human skeletal muscle.

Another strategy to activate the AMPK system would be to lower the threshold of AMPK activation by rendering AMPK more sensitive to its activators. This strategy has been undertaken in this thesis by pre-treating skeletal muscle with methotrexate [MTX] and targeting 5-Amino-4-imidazolecarboxamide Ribonucleotide Transformylase [ATIC] (Study II). MTX is an inhibitor of ATIC, an enzyme involved in *de-novo* nucleotide biosynthesis. ATIC imposes a metabolic block leading to intracellular ZMP accumulation, lowering the threshold for AMPK activation.

Increased glucocorticoid action leads to reduced whole body insulin action and may predispose to type 2 diabetes. Local conversion of cortisone to active cortisol by the enzyme 11β-hydroxysteroid dehydrogenase [11β-HSD1] in target tissues may regulate tissue specific roles of glucocortioids in pathophysiological states. Chronic high dose exposure to cortisone or cortisol reduced glucose metabolism and enhanced lipid metabolism, via induction of pyruvate dehydrogenase kinase 4 [PDK4] expression in myotubes. siRNA-mediated reduction or pharmacological inhibition of 11β-HSD1 prevented the effects of cortisone but not cortisol, on metabolic responses (Study III).

Type 2 diabetes mellitus is associated with abnormal substrate metabolism, raising the possibility that alterations in the expression of mitochondrial enzymes controlling lipid uptake and metabolism may be altered (Study IV). Evidence that expression of key enzymes regulating mitochondrial function in skeletal muscle is altered in type 2 diabetes mellitus [T2DM] patients is provided.

In summary, activation of AMPK can play a central role in overcoming impairments in skeletal muscle glucose and lipid metabolism and this can be achieved by perturbing the enzymes involved in nucleotide metabolism such as 5'-nucleotidases and ATIC. Alterations in molecular regulators of substrate metabolism such as PDK4, reflect pathogenic condition and could be targeted to restore skeletal muscle energy homeostasis.

LIST OF PUBLICATIONS

I. **Sameer S Kulkarni**, Håkan K.R. Karlsson, Ferenc Szekeres, Alexander V. Chibalin, Anna Krook, Juleen R. Zierath. Suppression of 5'-Nucleotidase enzymes promote AMP activated protein kinase (AMPK) phosphorylation and metabolism in human and mouse skeletal muscle. J Biol Chem. 2011; 286(40):34567-34574.

II. **Sameer S Kulkarni**, Sergej Pirkmajer, Robby Z. Tom, Juleen R. Zierath, Alexander V. Chibalin. Methotrexate enhances AICAR mediated AMPK phosphorylation and lipid oxidation in skeletal muscle. Manuscript

III. Firoozeh Salehzadeh, Lubna Al-Khalili, **Sameer S Kulkarni**, Minghan Wang, Fredrik Lönnqvist, Anna Krook. Glucocorticoid-mediated effects on metabolism are reversed by targeting 11 beta hydroxysteroid dehydrogenase type 1 in human skeletal muscle. Diabetes Metab Res Rev. 2009; 25(3):250-258.

IV. **Sameer S Kulkarni**, Firoozeh Salehzadeh, Toman Fritz, Juleen R. Zierath, Anna Krook, Megan E. Osler. Mitochondrial regulators of fatty acid metabolism reflect metabolic dysfunction in type 2 diabetes mellitus. Metabolism, 2012; 61(2):175-185

CONTENTS

LIST OF ABBREVIATIONS

11β-HSD	11β-hydroxysteroid dehydrogenase
ACC	Acetyl CoA carboxylase
AICAR	5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside
AMPD1	Adenosine monophosphate deaminase 1
AMPK	AMP-activated protein kinase
ATIC	5-Amino-4-imidazolecarboxamide ribonucleotide transformylase
ATP	Adenosine triphosphate
BCA	Bicinchroninic acid
BMI	Body mass index
CPT	Carnitine palmitoyltransferase
DMEM	Dulbecco's minimal essential media
EDL	Extensor digitorum longus
FA	Fatty acid
FBS	Fetal bovine serum
FFA	Free fatty acid
GLUT	Glucose transporter
GMP	Guanine monophosphate
IMP	Ionisine monophosphate
IRS	Insulin receptor substrate
KHB	Krebs-Henseleit buffer
KO	Knock out
LKB	Liver kinase B
MCD	Malonyl coA decarboxylase
MTX	Methotrexate
NADPH	Nicotinamide adenine dinucleotide phosphate
NGT	Normal glucose tolerant
NT5C1A	Cytosolic 5'nucleotidase 1A
NT5C2	Cytosolic 5'nucleotidase 2
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDC	Pyruvate dehydrogenase complex
PDK	Pyruvate dehydrogenase kinase
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1α
RQ	Respiratory quotient
SDS	Sodium dodecyl sulfate
T2D	Type 2 diabetes
TCA	Tri carboxylic acid
XMP	Xanthosine monophosphate
ZMP	AICAR-monophosphate

1 INTRODUCTION AND BACKGROUND

1.1 ENERGY HOMEOSTASIS

Energy homeostasis is critical for the survival of organisms. The control of the metabolic fuel supply and energy homeostasis are central factors in the rising magnitude of obesity, type 2 diabetes and metabolic syndrome. The human body needs to be well adapted to cope with major challenges in the supply and demand of energy. This adaptability requires the body to be capable of utilizing carbohydrate and lipid fuels and to shift from its dependency on these fuel sources. The switch between and lipids and glucose during the fasted and fed state has been described as 'metabolically flexible' (Kelley, 2005).

The primary fuels in humans are carbohydrates (glucose), lipids (fatty acids) and to a lesser extent amino acids, which contribute to whole body energy homeostasis. A western diet consists of approximately 50% of energy contributed by carbohydrates, 33% by fat and 17% by amino acids. However just after an overnight fast, there is a dramatic change in the fuel utilization, such that the carbohydrates account for only 12% of the energy contribution, while 70% of the energy is contributed by fats and 18% by amino acids (Randle, 1995). This ability to switch between utilization of lipids and glucose as oxidative fuel in response to fasting or feeding respectively is a key feature of the skeletal muscle (Figure 1).

The studies described in this thesis are designed to investigate the molecular mechanisms integrating glucose and lipid metabolism and maintaining energy homeostasis and metabolic flexibility in skeletal muscle.

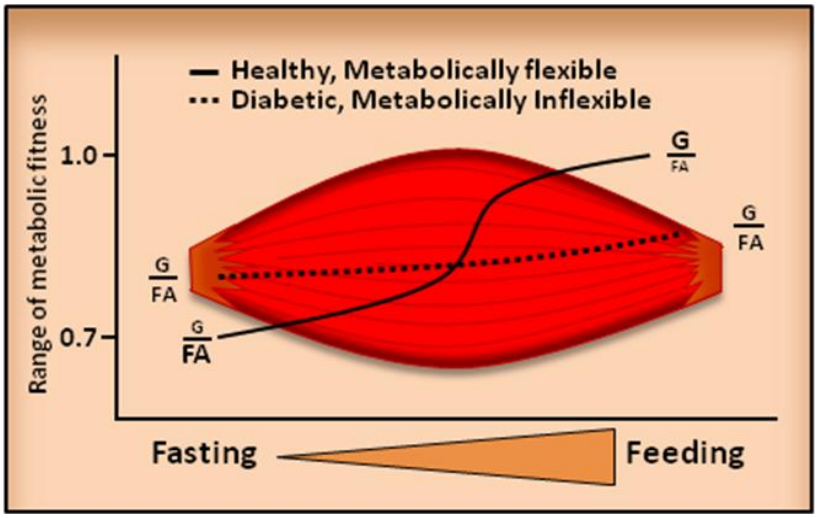

Figure 1: Schematic representation of metabolic flexibility in opposite states of metabolic health: *At rest, a fasted healthy muscle relies primarily on fatty acid oxidation (FA) for energy, which is associated with a lower Respiratory Quotient (RQ) value (~0.7). After a meal, a healthy muscle uses glucose (G) for energy, resulting in an increased RQ value, close to 1. This capability is impaired in a diabetic skeletal muscle rendering it metabolically inflexible. Figure adapted from Osler M.E and Zierath J.R. Endocrinology. 2008 Mar; 149(3):935-41*

1.2 TYPE 2 DIABETES

Type 2 diabetes mellitus is a growing problem across the world. An estimated 246 million people worldwide have type 2 diabetes and approximately 366 million people are estimated to be affected by type 2 diabetes by the year 2030 (Wild et al., 2004). Type 2 diabetes mellitus, also known as adult-onset diabetes, maturity-onset diabetes, or non-insulin-dependent diabetes mellitus is caused due to a combination of defective insulin secretion and insulin resistance, or reduced insulin sensitivity. Insulin resistance exists whenever a normal concentration of insulin produces a less than normal biological response (Kahn, 1978). The state in which insulin resistant individuals are unable to secrete enough insulin to overcome the defect in insulin signaling, leads to development of type 2 diabetes. Several lines of evidence show that genetic susceptibility for type 2 diabetes could induce an early β-cell dysfunction, leading to progression of type 2 diabetes (Gloyn et al., 2009; Lyssenko et al., 2008). Although the underlying cause of diabetes is unknown, insulin resistance clearly plays a major role in the development and progression of the disease (Kadowaki et al., 1984; Taylor et al., 1994).

According to the current recommendation of the World Health Organization, a person is diagnosed as having diabetes mellitus if he or she is diagnosed with a fasting blood glucose ≥7.0 mmol/l or 2 hour plasma glucose ≥ 11.1 mmol/l after an oral glucose tolerance test. In addition to elevated blood glucose levels, type 2 diabetic patients are characterized by a decreased fat oxidative capacity and high levels of circulating free fatty acids (FFAs). The latter is known to correlate with insulin resistance, particularly in skeletal muscle, by reducing insulin-stimulated glucose uptake. Furthermore, type 2 diabetes is associated with impaired "metabolic flexibility" (Figure 1), i.e. an impaired switch from fat to glucose oxidation in response to insulin (Kelley, 2005; Kelley et al., 1999; Kelley et al., 2002). Thus, a reduced ability to oxidize lipids and/or metabolic inflexibility, are important components of skeletal muscle insulin resistance. Additionally, insulin resistance as well as a cluster of risk factors which often coexist, including high plasma triglycerides, low high-density lipoprotein cholesterol and essential hypertension, together increase the risk of cardiovascular disease. This condition has been termed the 'metabolic syndrome', and insulin resistance per se has been postulated as an underlying mechanism (Reaven, 2005a, b). The cause of derangements in skeletal muscle of type 2 diabetic patients remains to be elucidated. Given the current world wide increase in type 2 diabetes prevalence, investigations into the underlying mechanisms of this disease continue to be important.

1.3 SKELETAL MUSCLE AND METABOLISM

Skeletal muscle is one of the major consumers of circulating fuels in the body partly because of its large mass as compared to other tissues, and partly due to higher metabolic demands during strenuous activities. Skeletal muscle is a major site of substrate metabolism and responsible for insulin- and exercise-mediated glucose disposal. Skeletal muscle accounts for ~75% of insulin-mediated glucose uptake (DeFronzo et al., 1985). Glucose is a major fuel for skeletal muscle and an impairment in its transport across the plasma membrane and subsequent utilization is one of the major pathogenic features of type 2 diabetes (DeFronzo et al., 1992).

1.4 INSULIN SIGNALING AND ACTION IN SKELETAL MUSCLE

In the early 1920s, insulin was identified as the major hypoglycemic hormone having the ability to restore normal blood glucose levels in pancreatectomized animals and insulin-deficient humans (Banting et al., 1922). The physiological action of insulin on glucose metabolism primarily takes place in skeletal muscle, liver and adipose tissue. The effects of insulin on skeletal muscle metabolism are mediated by binding of insulin to high affinity, cell surface tyrosine kinase receptors called insulin receptors. Insulin, when bound to these receptors activates the intrinsic tyrosine kinase activity of the receptor's intracellular domain leading to autophosphorylation of the insulin receptor internal kinase domain (Kasuga et al., 1982a; Kasuga et al., 1982b) and phosphorylation of Insulin Receptor Substrates [IRS]. Phosphorylated IRS proteins recruit and activate intracellular effector molecules containing Src Homology 2 domains including regulatory domain of PtdIns (3, 4, 5) [PI] 3-kinase (Backer et al., 1992; Myers et al., 1992). Activation of PI3-kinase leads to activation of several downstream kinases, including Akt/PKB (Alessi et al., 1997), and subsequently the rabGAP proteins TBC1D1/TBC1D4 propagating specific signals that elicit biological effects on glucose uptake in skeletal muscle (Sano et al., 2003; Taniguchi et al., 2006) (Figure 2).

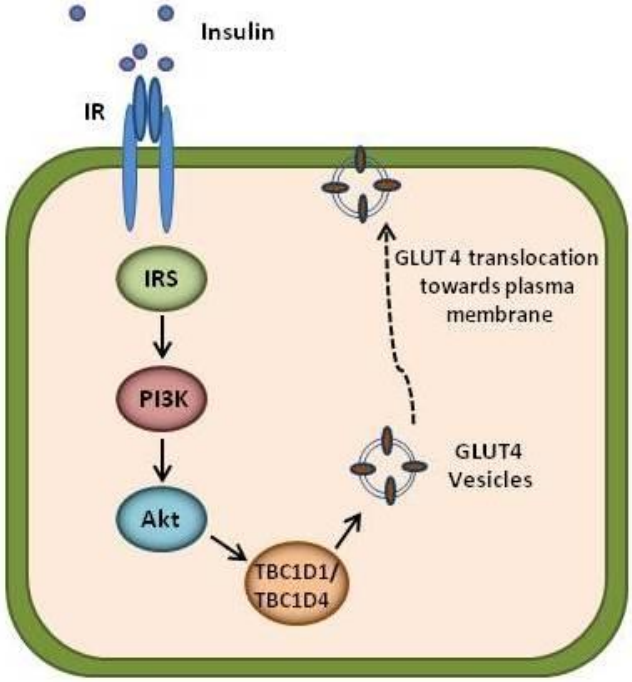

Figure 2: Schematic outline of insulin-mediated GLUT4 translocation to the plasma membrane in skeletal muscle.

1.4.1 Insulin action on skeletal muscle glucose metabolism

Glucose transport across the plasma membrane is achieved by family of proteins comprising 13 members called "glucose transporter proteins" or "GLUTs" (Joost et al., 2002). Skeletal muscle expresses the glucose transporters GLUT1, GLUT4 and GLUT 12, with GLUT4 playing a role as the predominant isoform (Birnbaum, 1989; James et

al., 1988). GLUT4 is responsible for insulin- and contraction-mediated glucose disposal in skeletal muscle (Douen et al., 1990), while GLUT 12 has been recently implicated in insulin-stimulated glucose transport (Stuart et al., 2009). GLUT1 expression is relatively lower in skeletal muscle compared to GLUT4 and is localized primarily in the plasma membrane and is thus thought to be participating in basal glucose transport (Gulve et al., 1994; Marshall and Mueckler, 1994).

Once glucose is transported through the sarcolemma into the cytosol, it is phosphorylated to glucose-6-phosphate and after which the fate of the glucose molecule inside the cell is diverse. Glucose-6-phosphate is catabolized through glycolysis, the hexose monophosphate shunt and mitochondrial oxidation yielding high energy stores such as ATP and NADPH. Glucose can also be stored in the form of glycogen in skeletal muscle, enter the pentose phosphate pathway or be converted into triglycerides.

Glucose has different metabolic fate depending on whether the conditions are aerobic or anaerobic. Under anaerobic conditions, pyruvate, the end product of glycolysis, is converted into lactate by lactate dehydrogenase enzyme (McLane and Holloszy, 1979). Under aerobic conditions, glucose through glycolysis is converted to pyruvate which is then converted into Acetyl CoA by the pyruvate dehydrogenase complex [PDC] in the mitochondrial matrix, and subsequently undergoes further oxidation through the Tri Carboxylic Acid cycle [TCA] to form carbon dioxide and water as end products.

1.4.2 Insulin action on skeletal muscle lipid metabolism

The concentrations of circulating ketone bodies are low in the fed or insulin-stimulated state and high during the fasted state. Ketone bodies are the end products of fatty acid oxidation in the liver. These observations led to the discovery of role of malonyl CoA in regulating fatty acid oxidation by McGarry and co-workers in 1977. The role of malonyl coA is well-appreciated as being an integrating point between glucose and lipid metabolism. In the fed state, glucose is converted to acetyl CoA by glycolysis and action of the PDC. Mitochondrial acetyl CoA can be exported to the cytosol by converting into citrate and transported by tricarboxylate transporter, then cleaved by ATP citrate lyase, to liberate the acetyl CoA in the cytosol. Cytosolic acetyl CoA is the starting point for *de novo* synthesis of fatty acids. The first step in this process is formation of malonyl CoA by action of acetyl CoA carboxylase enzyme [ACC]. Malonyl CoA was discovered to be potent inhibitor of fatty acid oxidation and ketogenesis (McGarry et al., 1977; Ruderman et al., 1999). Malonyl CoA allosterically inhibits carnitine-palmitoyl transferase-1 [CPT1], responsible for the transport of the fatty acids into the mitochondria for fatty acid oxidation. Thus, in the fed or insulin-stimulated state, malonyl CoA formation inhibits fatty acid oxidation and ketogenesis. In fact, the skeletal muscle isoform of CPT1 is 10-100 times more sensitive to inhibition by malonyl CoA than the liver isoform (Jackson et al., 1999; Zammit, 1999).

1.5 PHARMACOLOGICAL MANIPULATON OF MUSCLE LIPID ACCUMULATION

Obesity is an important risk factor for the development of type 2 diabetes. Weight gain (or loss) is closely correlated with decreasing (or increasing) insulin sensitivity (Friedman et al., 1992). Impairments in fatty acid metabolism are recognized as major components of pathogenesis of obesity and type 2 diabetes. Accumulation of intramyocellular triglycerides is implicated in the development of skeletal muscle insulin resistance (Krssak et al., 1999). The role of intracellular triglycerides in the development of skeletal muscle insulin resistance clearly raises the immediate need for approaches to regulate muscle lipid supply. A critical player in promoting skeletal muscle fatty acid oxidation and thus regulating intramuscular triglycerides is AMP-activated protein kinase [AMPK].

1.6 AMPK AS AN EMERGING ANTI-DIABETIC DRUG TARGET

AMPK has emerged as a major energy sensor and it is recognized as a key player in regulating cellular energy balance as it acts as a master regulator of cellular metabolic homeostasis (Hardie, 2007a; Long and Zierath, 2006; Towler and Hardie, 2007) (Figure 3). AMPK is a phylogenetically conserved enzyme present from unicellular eukaryotes to mammals (Hardie, 2007b). The role of AMPK as a metabolic fuel gauge and master regulator of homeostasis has placed it at the center stage in studies of mechanism contributing to the development of diabetes, obesity and metabolic syndrome.

1.6.1 AMPK- structure and function

AMPK is a heterotrimeric enzyme complex composed of a catalytic α-subunit and regulatory β and γ subunits. Each subunit is encoded by multiple genes. The α-subunit and the β subunit exist as two isoforms (α1, α2 and β1, β2) and the γ subunit exists as three isoforms (γ1, γ2 and γ3), rendering it possible to form 12 different heterotrimeric combinations. The catalytic α-subunit contains a kinase domain and the site T172 in this domain should be phosphorylated to render the kinase active (Hawley et al., 1996). The β-subunit acts as a core of the heterotrimeric structure and serves as a bridge interacting between the α-subunit and the γ-subunit. The γ-subunit contains two pairs of Bateman domains (cystathionine β-synthase), that bind an AMP molecule (Bateman, 1997). The γ-subunit is a functional component of the AMPK heterotrimer rendering it responsive to AMP binding at the Bateman domain. The binding of the AMP molecule at the Bateman domain of the γ-subunit allosterically activates AMPK and also makes it a better substrate for upstream kinases and poorer substrate for phosphatases (Hawley et al., 2005; Hurley et al., 2005; Shaw et al., 2004). The AMPK system is essentially activated through sensing the disturbances in the intracellular abundance of AMP to that of ATP, thus sensing the intracellular nucleotide status. The physiological activation of AMPK in skeletal muscle occurs during conditions of increased binding of AMP and decreased binding of ATP to the γ-subunit. LKB1 has been recognized as an important upstream AMPK kinase regulating AMPK activity (Scott et al., 2002;

Woods et al., 2003). Another upstream kinase, Ca2+/ Calmodulin dependent protein kinase has been identified as an important AMPK Kinase regulating its activity through intracellular calcium sensing (Hawley et al., 2005; Woods et al., 2005). Metabolic stressors, such as deprivation of glucose, hypoxia, and muscle contraction increase the intracellular concentration of AMP relative to ATP and activate the AMPK pathway. Once activated, AMPK switches on the catabolic pathways that generate ATP, while simultaneously switching off the pathways that consume ATP (Figure 3).

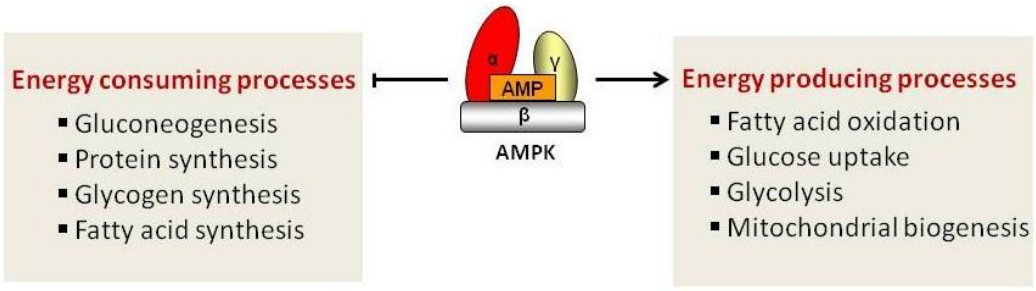

Figure 3: Multiple effects of AMPK activation on skeletal muscle metabolism. *Once activated, AMPK switches on the catabolic pathways that generate ATP, while switches off the pathways that consume ATPs.*

1.6.2 AMPK- Impact on lipid metabolism

AMPK has been proposed as a drug target for the treatment of type 2 diabetes and obesity because of its effect on lipid metabolism (Moller, 2001; Zhang et al., 2009). When activated, AMPK in turn phosphorylates its immediate downstream target ACCβ rendering it inactive (Winder and Hardie, 1996). As ACC catalyses the formation of malonyl CoA, which inhibits CPT1, a decrease in ACC activity decreases the intracellular malonyl CoA concentration, thus releasing the inhibitory loop on CPT1 and allowing the influx of long chain fatty acids into the mitochondria for beta oxidation. Another mechanism through which AMPK is thought to impact lipid metabolism in skeletal muscle is by directly acting on the enzyme malonyl CoA decarboxylase [MCD]. MCD is an enzyme involved in malonyl CoA degradation and its activity is increased following AMPK activation. This increased malonyl CoA clearance in skeletal muscle, releases the inhibition on CPT1 activity, leads to increased fatty acid influx in the mitochondria, and increases mitochondrial beta oxidation (Alam and Saggerson, 1998) (Figure 4). Treatment of rat skeletal muscle with an AMPK activator, AICAR, increases MCD activity and decreases ACC activity (Saha et al., 2000).

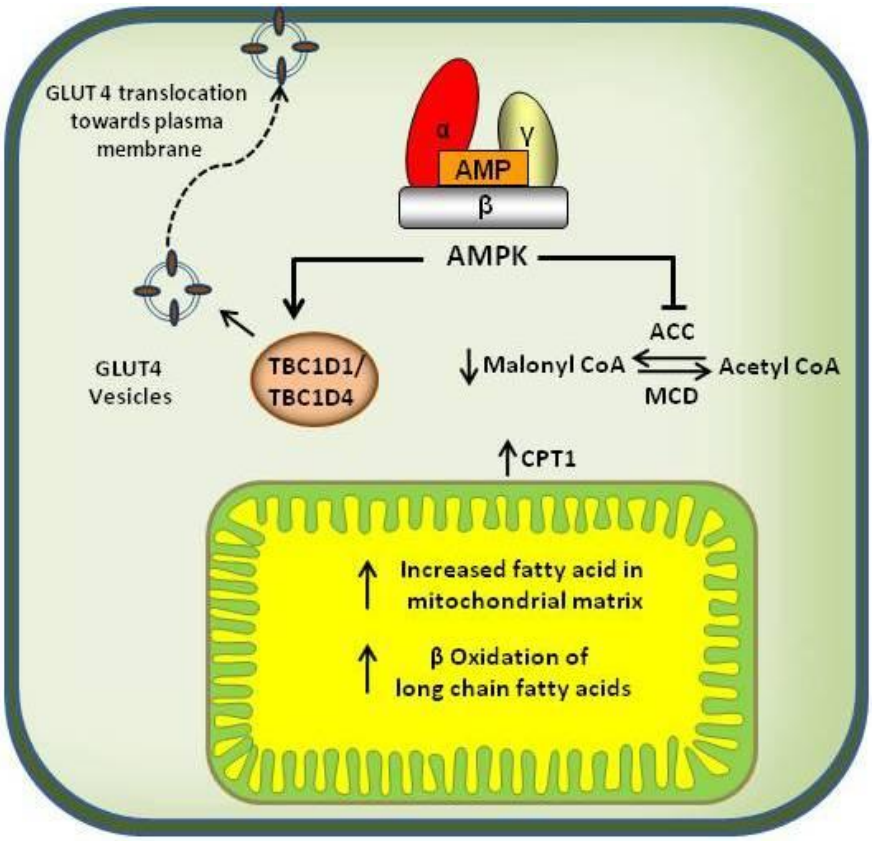

Figure 4: Schematic representation of the impact of AMPK activation on glucose and lipid metabolism.

1.6.3 AMPK- Impact on glucose metabolism

The rate of glucose transport in skeletal muscle can be increased by either insulin stimulation or muscle contraction (i.e. exercise) (Chibalin et al., 2000; Holloszy and Hansen, 1996; Kennedy et al., 1999; Thorell et al., 1999). AMPK plays a critical role in glucose metabolism. Activation of AMPK increases skeletal muscle glucose uptake via increased GLUT4 translocation (Kurth-Kraczek et al., 1999) (Figure 4). This effect of AMPK is independent of pathways regulating insulin-mediated GLUT4 translocation, since the effect is not blocked by PI3 kinase inhibitors, and effects of insulin and AMPK on glucose uptake are additive (Hayashi et al., 1998). In addition to playing a role in GLUT4 translocation to the plasma membrane, AMPK also exerts genomic effects by increasing the GLUT4 protein expression by enhancing the binding of the transcription factor myocyte enhancer factor 2 to the GLUT4 promoter region (Zheng et al., 2001).

1.6.4 Development of AMPK activators

Development of direct and indirect AMPK activators may hold promise as a new strategy to expand the armamentarium against type 2 diabetes (Fogarty and Hardie, 2010; Hardie, 2011; Zhang et al., 2009). While direct activators bind to AMPK and trigger activation (Corton et al., 1995; Fogarty and Hardie, 2010; Guigas et al., 2009), indirect AMPK activators act mainly by increasing AMP/ATP ratio through different

mechanisms (Hawley et al., 2010), including modulation of intracellular nucleotide metabolism (Kulkarni et al., 2011).

1.7 5' NUCLEOTIDASES

The 5'-nucleotidases are a family of enzymes that catalyze the dephosphorylation of nucleoside monophosphates and regulate cellular nucleoside and nucleotide levels (Bianchi and Spychala, 2003). The 5'-nucleotidase was first studied *in vitro* using a semi-purified enzymes extracted from rat and guinea pig skeletal muscle (Cozzani et al., 1969). There are various biochemical pathways that govern purine and pyrimidine nucleotide metabolism. These pathways maintain the levels of purine and pyrimidine nucleotide triphosphates vital to support various cellular processes. 5'-nucleotidases catabolize nucleoside monophosphates and change their abundance. Therefore they are components of cellular energy homeostasis. Besides maintaining balanced ribo and deoxyribonucleotide pools, nucleotidase activities are likely to regulate the activation of nucleoside analogues, a class of anti-cancer and anti-viral agents that rely on the nucleoside kinases for phosphorylation to convert to their phosphorylated active forms (Hunsucker et al., 2005). Early studies on 5'-nucleotidases have revealed a membrane bound ecto-enzyme and soluble form cytosolic enzymes. In humans, seven 5'-nucleotidases have been isolated and characterized that vary in subcellular localization. Out of the seven known 5'-nucleotidases, five are of the soluble form and they are localized to cytosol, one is localized to the mitochondrial matrix (NT5M) and one is bound to the extracellular portion of the plasma membrane (the ecto 5'-nucleotidase or E5'N) (Hunsucker et al., 2005). These enzymes have similar functions in that they hydrolyze 5' nucleoside monophosphates, but they differ in their specificity towards their substrate. Some of these enzymes are ubiquitously distributed and some are tissue-specific. Differences in subcellular localization, specificity towards their substrate and tissue specific distribution allow regulation of nucleotide pools to meet the energy balance and cellular homeostasis. This thesis will focus on two of the soluble 5'-nucleotidases enzymes, namely NT5C1A and NT5C2.

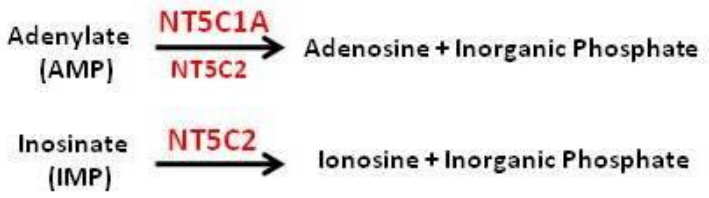

Figure 5: The two enzymes studied in this thesis NT5C1A and NT5C2 have overlapping substrate specificities. *NT5C1A is primarily AMP preferring enzyme while NT5C2 is primarily IMP preferring with overlapping specificity towards AMP. 5'-nucleotidases enzymes act on nucleoside mono-phosphates to produce nucleoside and inorganic phosphate.*

NT5C1A was first described as an AMP preferring enzyme partially purified from pigeon ventricle (Gibson and Drummond, 1972) and was later purified from rabbit, rat, pigeon and dog heart (Darvish and Metting, 1993; Newby, 1988; Truong et al., 1988). In human tissues, NT5C1A mRNA expression is highest in skeletal muscle and is also present in heart, brain, pancreas, kidney, testis and uterus (Hunsucker et al., 2001). NT5C1A is closely related to NT5C1B, but these enzymes have little homology to other intracellular nucleotidases (Sala-Newby and Newby, 2001). While NT5C1A has a broad range of nucleotides and deoxynucleotides as substrates, the enzyme has a preference for AMP and pyrimidine deoxyribonucleotides (Garvey et al., 1998; Hunsucker et al., 2001).

NT5C2 mRNA is ubiquitously expressed in human tissues, with the highest expression in pancreas, skeletal muscle and heart muscle (Allegrini et al., 1997; Rampazzo et al., 1999). Cytosolic NT5C2 was first described as a nucleotidase partially purified from chicken liver that preferentially hydrolyzes 5'-IMP, 5' GMP, 5'-dGMP and XMP (Itoh et al., 1967). NT5C2 not only hydrolyzes IMP, preventing conversion to GMP or AMP, but also hydrolyzes GMP to directly regulate guanine nucleotide pools. AMP is catabolized to IMP by AMP deaminase; subsequently, NT5C2 converts IMP to inosine. Thus, NT5C2 and AMP deaminase are important regulators of purine nucleotide pools.

Another approach to activate the AMPK system would be to use a lower dose of an AMPK activator to amplify its ability to activate AMPK. This may decrease unwanted side effects caused by the unavailability of specific AMPK activators. For example, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside [AICAR], a commonly used activator of AMPK, is known to activate several other kinases (Lefebvre et al., 2001; Moopanar et al., 2006).

1.8 ATIC

The 5-aminoimidazole-4-carboxamide ribonucleotide formytransferase / inosine monophosphate [IMP] cyclohydrolase [ATIC], a bifunctional enzyme, which catalyzes the conversion of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside 5'-monophosphate [ZMP, AICAR-monophosphate] to IMP in the final two steps of the *de novo* purine biosynthetic pathway (Allegra et al., 1985; Allegra et al., 1987; Baggott and Morgan, 2007). MTX and its analogue pemetrexed [PMX] primarily exert antineoplastic effects by disrupting DNA and RNA synthesis, but recent evidence associates these proteins with AMPK activation in cancer cells (Beckers et al., 2006; Racanelli et al., 2009; Rothbart et al., 2010). The molecular basis of MTX-mediated AMPK activation involves inhibition of ATIC. ZMP is also the biologically active form of the widely used cell-permeable AMPK activator AICAR (Corton et al., 1995; Guigas et al., 2009), which is phosphorylateded to ZMP by the intracellular adenosine kinase (Sabina et al., 1985; Samari and Seglen, 1998; Vincent et al., 1996). By preventing ZMP entry into the *de novo* biosynthetic pathway, MTX markedly potentiates AMPK activation in AICAR-treated cancer cells (Beckers et al., 2006). MTX can substantially increase the ZMP concentration and/or reduce adenylate energy charge in some, but not all, cell types (Allegra et al., 1987; Beckers et al., 2006;

Bokkerink et al., 1986a; Bokkerink et al., 1986b; Kaminskas, 1982; Kaminskas and Nussey, 1978; Racanelli et al., 2009), indicating it may also act as an indirect AMPK activator under certain conditions (Figure 10).

1.9 ROLE OF GLUCOCORTICOIDS IN PATHOPHYSOLOGY OF METABOLIC SYNDROME

Endogenous glucocorticoids are steroid hormones secreted from the adrenal cortex under the influence of the hypothalamic–pituitary–adrenal axis, which is an integral part of the stress response modulating a large number of physiological processes. Glucocorticoids bind to the glucocorticoid receptors, which are nuclear receptors and exert metabolic action by modulating the expression of target genes. Excess of circulating glucocorticoids have been linked to clinical manifestations overlapping with that of metabolic syndrome. These clinical characteristics include central obesity, hypertension, hyperlipidemia, insulin resistance and glucose intolerance (Figure 11). These symptoms are exacerbated in patients with Cushing's syndrome, which is a result of glucocorticoid excess due to a pituitary adenoma. Patients administered with glucocorticoids to treat inflammatory diseases suffer adverse metabolic effects such as weight gain, hypertension and insulin resistance (Davis, 1986; Gallant and Kenny, 1986). All these observations form the basis of a hypothesis to regulate pre-receptor glucocorticoid metabolism as a potential therapeutic strategy in treatment of metabolic syndrome.

1.9.1 Glucocorticoid metabolism and action on skeletal muscle

The intracellular glucocorticoid concentration is regulated by the 11β-hydroxysteroid dehydrogenase [11β-HSD1] enzyme, which converts inactive cortisone to active cortisol and thus modulates glucocorticoid action locally (Morton, 2010; Paterson et al., 2004). 11β-HSD1 is expressed in variety of tissues such as liver, adipose, brain and kidney, while 11β-HSD2, an isozyme, responsible to convert cortisol to cortisone, is primarily expressed in kidneys and salivary glands (Walker and Stewart, 2003). Pathophysiological effects of glucocorticoids occur due to their actions on variety of tissues. Glucocorticoids enhance hepatic glucose production by inducing the enzyme phosphoenolpyruvate carboxykinase and thus positively affecting gluconeogenesis. Insulin secretion from pancreatic beta cells is inhibited by glucocorticoids. In skeletal muscle, excess glucocorticoids directly interfere with insulin signaling and decrease insulin-stimulated glucose uptake by preventing translocation of the glucose transporters from intracellular compartments to the plasma membrane (Morgan et al., 2009; Nechushtan et al., 1987). In adipose tissue and liver, glucocorticoids act in conjugation with insulin to drive lipogenic enzymes and lipid uptake, causing hepatic lipid accumulation (Wang et al., 2004). These findings provide evidence to suggest that metabolic actions of glucocorticoids are exerted in multiple tissues and excess glucocorticoids contribute to the progression of metabolic syndrome.

The enhanced rate of peripheral glucocorticoid clearance would help to curtail the metabolic complications mediated by excess local or circulating glucocorticoids. This

is supported by the finding that insulin sensitivity is increased and gluconeogenesis is decreased as a consequence of reduced intracellular glucocorticoids in mice with a targeted deletion of 11β-HSD (Kotelevtsev et al., 1999). Transgenic mice overexpressing 11β-HSD1 selectively in adipose tissue have increased visceral adiposity, insulin resistance, hyperlipidemia and hyperphagia (Masuzaki et al., 2001). Taken together these findings suggest that downregulation of 11β-HSD1 activity locally could act to limit the pathophysiological consequences of high glucocorticoid metabolism and curtailing associated conditions such as obesity and type 2 diabetes.

1.10 MITOCHONDRIAL PROTEINS AS NOVEL CANDIDATES FOR MAINTAINING METABOLIC FLEXIBILITY?

Skeletal muscle substrate metabolism plays a fundamental role in whole body nutrient utilization and homeostasis. A number of key mitochondrial enzymes determine the balance between glucose and fatty acid metabolism. Mitochondrial metabolism results from the concerted action of several biochemical reactions that are coordinately regulated. Some of the key enzymes include pyruvate dehydrogenase kinases [PDKs], CPT1b and MCD. Genotypic and physiological factors contribute to adversely affect mitochondrial metabolism, which perhaps also affects lipid metabolism governed by these mitochondrial genes.

The pyruvate dehydrogenase complex [PDC] occupies a central position in mitochondrial metabolism. Since PDC represents a flux point for the entry of the carbohydrate derived fuel into the mitochondria for oxidation, it regulates overall substrate selection in skeletal muscle. The activity of the pyruvate dehydrogenase complex is negatively regulated by PDKs. There are four PDK isoforms (PDK1, 2, 3, 4). In skeletal muscle the predominant form is PDK4 and to a lesser extent PDK2 (Bowker-Kinley et al., 1998). Pyruvate dehydrogenase kinase phosphorylates PDC, leading to inhibition of the complex and reduced carbohydrate oxidation. Thus, PDC inhibition is recognized as a flux point at which fuel selection in skeletal muscle can be shifted towards fat oxidation (Sugden and Holness, 2006). Pyruvate dehydrogenase activity is decreased in response to fasting or high fat diet in human skeletal muscle (Putman et al., 1993). Exercise and contraction increase pyruvate dehydrogenase activity in human and rat skeletal muscle (Dohm et al., 1986a; Dohm et al., 1986b; Dyck et al., 1993).

PDK4 is crucial for the regulation of pyruvate dehydrogenase activity and might be regulated at the level of transcription in response to exercise. PDK4 transcription and PDK4 mRNA are markedly increased in human skeletal muscle during exercise of acute high-intensity or prolonged low-intensity (Pilegaard and Neufer, 2004). Increased pyruvate dehydrogenase kinase activity in skeletal muscle suppresses glucose oxidation, and thus may cause or exacerbate hyperglycemia. Mice with a targeted deletion of PDK4 have lower blood glucose levels and slightly improved glucose tolerance compared to wild-type mice after an 18 week high fat diet (Jeoung and Harris, 2008), highlighting a role of PDK4 in the development of hyperglycemia. During a euglycemic hyperinsulinemic clamp, insulin exposure reduces PDK4 mRNA

expression in normal glucose tolerant, but not in insulin resistant people, indicating elevations in PDK4 expression and activity may further increase plasma glucose level (Kim et al., 2006; Lee et al., 2004).

Mitochondrial activity and function in skeletal muscle is a highly controlled process, under the influence of a variety of nuclear and mitochondrial factors that act as metabolic sensors, which adapt to perturbations in cellular nutrient and energy status.
MCD and CPT1 play a pivotal role in fuel selection and drive increases in fatty acid oxidation. MCD and CPT1b (muscle isoform) coordinate fuel balance during exercise and states of metabolic disease. Exercise reduces skeletal muscle malonyl CoA (Roepstorff et al., 2005) and increases expression of MCD1 (Kuhl et al., 2006), which may contribute to the increase in lipid oxidation at the onset of exercise (Roepstorff et al., 2005). Increased levels of malonyl CoA have been reported in insulin resistant animal models (Assifi et al., 2005). However, MCD knockdown protects against the development of dietary-induced whole body insulin resistance in mice (Koves et al., 2008) and enhances insulin-stimulated glucose uptake in human muscle cells (Bouzakri et al., 2008). CPT1b expression and function has been linked to the regulation of insulin sensitivity. Chemical inhibition of CPT1b with the pharmacological agent Etomoxir increases lipid deposition and exacerbates insulin resistance in rats fed a high-fat diet (Dobbins et al., 2001), while overexpression of CPT1b in rat hindlimb muscle by electrotransfer prevents the dietary-induced insulin-resistance on glucose uptake (Bruce et al., 2009). Whether MCD or CPT1b levels are altered or related to the metabolic phenotype in type 2 diabetes is unknown.

This thesis aims to investigate new strategies to combat impairments in skeletal muscle glucose and lipid metabolism and restore the overall skeletal muscle energy homeostasis.

2 AIMS AND OBJECTIVES

One of the key features of healthy skeletal muscle is the ability to switch between utilization of lipids and glucose as fuel in response to feeding or fasting respectively. This metabolic flexibility is impaired in skeletal muscle from insulin resistant and type 2 diabetic patients. Molecules such as AMPK and PDK play crucial roles in maintaining metabolic flexibility in healthy skeletal muscle. The overall goal of this thesis work is to identify and validate novel molecules which are involved in maintaining the metabolic flexibility of the skeletal muscle.

2.1 SPECIFIC GOALS

2.1.1 Specific aims of the present investigation are as follows:

- To determine whether cytosolic 5'-nucleotidases play a role in maintaining skeletal muscle energy metabolism. Could targeting 5´-nucleotidases be a potential strategy to activate AMPK pathway?

- To determine whether modulation of the enzymes of nucleotide metabolism reduce the threshold for AMPK activation and potentiate AICAR-stimulated AMPK activity.

- To determine whether reduction in 11β-HSD1 prevents cortisone- or cortisol-mediated metabolism in skeletal muscle. What is the molecular link for the glucocorticoid-mediated shift in metabolism in skeletal muscle?

- To determine whether alterations in skeletal muscle expression and/or regulation of mitochondrial proteins involved in fatty acid metabolism reflect metabolic dysfunction. Does increase in physical activity restore metabolic dysfunction and reflect normal mitochondrial fatty acid metabolism?

3 EXPERIMENTAL PROCEDURES

3.1 MATERIALS AND METHODS

3.1.1 Recruitment of subjects for lifestyle intervention

This investigation included 112 individuals without severe physical or cardiovascular impairments aged 61 (57–64) years, with BMI of 29 (27.5–32.0) kg/m^2. Subjects were stratified based on normal glucose tolerance (n = 79) or type 2 diabetes mellitus (n = 33). Of the total number of volunteers, a subgroup of male participants in each category (NGT n=23, T2DM n=17) underwent an exercise intervention. Subjects were directed to engage in Nordic walking with walking poles, 5 hours per week for 4 months. Self-reported physical activity was assessed at the time of inclusion and after 4 months. Remaining volunteers maintained their habitual lifestyle. Written informed consent was obtained from all participants. The study was approved by the Ethics Committee of Karolinska Institutet, Stockholm.

3.1.2 Human *Vastus Lateralis* muscle biopsies

Skeletal muscle biopsies were obtained (Study IV) from subjects in the morning, following an overnight fast. Local anesthesia (lidocaine hydrochloride, 5 mg/ml) was administered, and an incision (5 mm long/10 mm deep) was made in the skin and skeletal muscle fascia. A biopsy (20-100 mg) was obtained from *vastus lateralis* portion of thigh muscle using a conchotome tong. Biopsies were immediately frozen in liquid nitrogen until analysis.

3.1.3 Human *Rectus Abdominis* muscle biopsies

For Study I, III, IV muscle biopsies were obtained from subjects scheduled for abdominal surgery at Karolinska University Hospital, Stockholm, Sweden. Skeletal muscle biopsies (*rectus abdominis*) were obtained during scheduled abdominal surgery with informed consent from the donors. The subjects were 61±5 years (BMI 26 kg/m^2), with no known metabolic disorder. The study was approved by the Ethics Committee of Karolinska Institutet, Stockholm

3.1.4 Isolation of human skeletal muscle satellite cells

Satellite cells were isolated from the *rectus abdominis* biopsies by trypsin and collagenase digestion and grown to myoblasts and differentiated to myotubes (Al-Khalili et al., 2003; Al-Khalili et al., 2004). The biopsy material was collected in cold phosphate buffered saline (PBS) containing 1% penstrep (100 units/ml penicillin/ 100 µg/ml streptomycin) and 1% fungizone Gibco BRL (Invitrogen, Stockholm, Sweden). The connective tissue was teased out by dissection while the muscle was finely diced and bundles of muscle fibers were isolated and then transferred to the trypsin-EDTA digestion solution Gibco, BRL (Invitrogen, Stockholm, Sweden). The muscle fibers were incubated in trypsin digestion solution at 37ºC for 15-20 minutes with gentle agitation every 5 minutes. The tube was subjected to centrifugation 350 g for 10

minutes to pellet down the undigested tissue. The supernatant containing satellite cells was collected in a separate tube and mixed with the growth medium (Hams F10 with 20% FBS, 1% penstrep, 1% fungizone) in 1:1 ratio. The remaining undigested muscle was then transferred to a fresh trypsin digestion solution and incubated at 37ºC for another 15 minutes, centrifuged and resultant supernatant was pooled with the previously collected supernatant. The pooled supernatant was then subjected to centrifugation at 2000 g for 10 minutes. The supernatant was discarded and the pelleted cells were resuspended in 5 ml of the growth media and incubated in a bacteriological non-coated culture dish to promote the adherence of non-myogenic cells for 30-40 minutes. After incubation, the media containing the isolated satellite cells was transferred to a new 25 cm^2 culture flask. The culture flask media was changed every 2 days with fresh medium.

Dulbecco's modified Eagle's medium (DMEM), Ham's F-10 medium, fetal bovine serum, penicillin, streptomycin and fungizone were obtained from Gibco BRL (Invitrogen, Stockholm, Sweden). Unless specified, all reagents were purchased from Sigma. Radioactive reagents were purchased from Amersham.

3.1.5 Human skeletal muscle cell culture

Cell were seeded at a density of 2-3 X 10^4 cells /cm^2 and cultivated in growth media (Ham's F-10 media with 20% FBS, 1% penstrep, 1% fungizone) in tissue culture dishes. When cells were almost 80% confluent, they were subjected to the start of the differentiation program by supplementing them with a differentiation medium (DMEM 1 g/L glucose media with 2% FBS, 1% penstrep and 1% fungizone). Upon initiating the differentiation program, the myoblasts fuse to form multinucleated myotubes that are visible by the third day of the differentiation. All the experiments were performed on fully differentiated myotubes around 5-6^{th} day of differentiation.

3.1.6 Rat L6 muscle cell culture

Rat L6 skeletal muscle cells were cultivated in alpha-MEM growth media containing 10% FBS (Study II). Once the seeded cells reached 80% confluence, similar to human skeletal muscle cells, a differentiation program was initiated by supplementing the cells with alpha-MEM differentiation medium (2% FBS) instead of the alpha MEM growth medium. All the experiments were performed on the fully differentiated myotubes around 6^{th} day of differentiation.

3.1.7 siRNA transfections

3.1.7.1 Human skeletal muscle cells

Myotubes cultured in 6-well plates were transfected using Lipofectamine 2000 reagent (Invitrogen) following manufacturer's directions. Differentiation medium was changed to antibiotic-free growth medium on day 0 and 2 of the myotube differentiation protocol. On days 0 and 2, myotubes were transfected with individual siRNAs (1 μg/ml) using Lipofectamine 2000 in serum-free DMEM. In Study I, pools of four siRNAs directed against human NT5C2 or a scrambled sequence were used. In Study

III, 11β-HSD1 or PDK4-directed siRNAs were transfected into cells following four days of exposure to glucocorticoid hormones. siRNA oligonucleotides for NT5C2 (Study I) and 11β-HSD1 and PDK4 (Study II) were purchased from Dharmacon (Chicago, IL). Myotubes were washed with PBS, and 2 ml of DMEM containing 2% fetal bovine serum was then added to each well.

3.1.7.2 Rat L6 muscle cells

Myotubes were cultured in 6-well plates and transfected with 100 nM pools of siRNAs (Dharmacon, Chicago, IL) against rat ATIC mRNA (Study II) using calcium phosphate (CellPhect Transfection kit; GE Healthcare) on days 2 and 4 of differentiation. siRNA-calcium phosphate precipitates were removed 16–18 h after the addition of siRNA by washing with PBS and adding 2 ml αMEM supplemented with 2% FBS. Experiments were performed 48–72 h after the last transfection. A scrambled siRNA was used as a control in all experiments. On day 6 of the differentiation protocol, the myotubes were serum starved for 4 h before the start of the experiment.

3.1.8 Gene expression measurement

Gene expression (Study I, III, and IV) was measured by real-time polymerase chain reaction (RT-PCR) using TaqMan technology. In the TaqMan chemistry, a fluorogenic probe is used to add specificity to the reaction. The probe has a reporter fluorescent dye at the 5' end and a quencher dye at the 3' end, and anneals downstream of the primer on the target sequence. When the probe is intact, the quencher dye greatly reduces the reporter dye. During the PCR reaction the Taq polymerase cleaves the probe and the reporter dye no longer is inhibited by the quencher dye, giving a signal responding to the amplification of the target sequence.

3.1.9 Metabolic assays

3.1.9.1 Glucose uptake

Radio labeled 2-deoxy-D-glucose was utilized to determine the glucose uptake in the cultured human myotubes (Study I, III). 2-deoxy-D-glucose is a glucose analogue that is taken up by the cell and phosphorylated by the enzyme hexokinase to 2-deoxy-D-glucose-6-phosphate which cannot be further metabolized. Hence the phosphorylated form of 2-deoxy-D-glucose is trapped inside the cell and can be measured by scintillation liquid counting and an estimate of glucose accumulated inside the cell can be determined. Myotubes were grown and differentiated in six-well plates and serum starved overnight. Cells were pre-incubated in the absence or presence of insulin (120 nM) or AICAR (1 mM) for 1 hour in glucose- and serum-free DMEM. Thereafter, myotubes were incubated in glucose-free DMEM containing [$^{1,2\text{-}3}$H]-2-deoxy-D-glucose glucose (0.33 μCi/mL) and 10 μM unlabeled 2-deoxy-D-glucose and glucose uptake (Al-Khalili et al., 2003; Al-Khalili et al., 2004). Experiments were performed in triplicate and results were normalized by protein concentration determined by the BCA method (BCA Protein Assay Kit, Thermo Scientific, Rockford, IL). The radioactivity in 20 μl of incubation media with known concentration was measured; results are

expressed in terms of the amount of glucose taken up by the cells per minute per milligram protein or by representing as fold over basal.

3.1.9.2 Glucose oxidation

Myotubes were incubated for 4 hours in 1 ml serum-free DMEM supplemented with 0.1% fatty acid free BSA, D-[U-^{14}C] glucose (1 µCi/ml; final specific activity, 0.18 µCi/µmol, Amersham), 120 nM insulin, 1 mM AICAR to the respective wells. Thereafter, 150 µl solvable (aqueous based tissue solubilizer, Perkin Elmer Life Science and Analytical Science, Boston, MA) was dispensed into a center-well positioned in each dish and 150 µl of 35% perchloric acid [PCA] was added to the medium. Myotubes were incubated for an additional hour, after which the center well was removed subjected to scintillation analysis (Study I).

3.1.9.3 Glucose incorporation into glycogen

Glycogen synthesis was determined by assessing the conversion of labeled glucose into glycogen (Study I, III) (Al-Khalili et al., 2003; Al-Khalili et al., 2004). Skeletal muscle myotubes were grown on a six-well plates and serum starved for 12-16 hours following five days of differentiation. Myotubes were incubated in DMEM containing 5 mM glucose and D-[U-^{14}C] glucose (1 µCi/ml; final specific activity, 0.18 µCi/µmol). Myotubes were incubated in the absence or presence of insulin (120 nM) for 90 minutes at 37^{o}C. Experiments were performed in triplicate. The reaction was terminated by placing the six-well plates on ice; myotubes were washed three times with ice-cold PBS and lysed in 1 ml of 0.03% sodium dodecyl sulfate [SDS]. After an overnight freeze thaw cycle, 0.85ml of lysate was transferred to 10 ml tubes and 100 µl of carrier glycogen (20 mg/ml) was added and samples were heated to 100ºC for 1-2 hours. Three ml of 98% ethanol was added to the samples and incubated at -20^{o}C overnight to precipitate the glycogen. The samples were subjected to centrifugation at 1700 g for 35 minutes. The glycogen pellet was washed once with 70% ethanol and resuspended in 400 µl of distilled water, which was used for liquid scintillation counting to measure the radioactivity (WinSpectral 1414 Liquid Scintillation Counter; Wallac/PerkinElmer, Waltham, MA, USA). The remaining lysate from the experiment was used to determine protein concentration by the BCA method which was used for normalization.

3.1.9.4 Palmitate oxidation method I

Lipid oxidation was assessed by exposing myotubes to [^{3}H] palmitic acid and measuring the tritiated water produced (Study I) (Rune et al., 2009). Myotubes were cultured in six well plates and serum starved overnight. Cells were washed once with PBS and exposed to 1 ml of DMEM (1 g glucose/L), supplemented with 0.2% fatty acid free BSA and 0.5 µCi palmitic acid [9-10(n)-^{3}H], and incubated in the absence or presence of 1 mM AICAR for 4 hours. To adsorb non-metabolized palmitate, 0.2 ml of cell supernatant was mixed with 0.8 ml charcoal slurry (0.1 g charcoal powder in 1 ml 0.02 M tris-HCl buffer, pH 7.5) and shaken (30 minutes). Samples were subjected to centrifugation for 15 minutes at 13,000 rpm, after which 0.2 ml of supernatant with

tritium bound water was withdrawn and radioactivity was measured by liquid scintillation counting (WinSpectral 1414 Liquid Scintillation Counter; Wallac, Turku, Finland). Each experiment was performed in triplicate.

3.1.9.5 Palmitate oxidation method II

Myoblasts were grown in 25 cm^2 cell culture flasks and differentiated to myotubes at >80% confluence (Study III). Before starting the experiment, a 2 mm hole was made in the lid of each flask and two sheets of 24 mm Whatman filter (Cat No 108340-24, VWR International AB, Sweden) were covered with a gauze bandage circle, prepared by cutting gauze into the same shape as the Whatman filter. The filter and gauze sandwich was then pressed into the inside of the culture flask lid, and the lid was sealed with Parafilm. Overnight serum starved myotubes (eight days after differentiation) were treated for 180 min with 0.4 μCi of [1-^{14}C] palmitate in 2 ml serum-free DMEM at 37ºC, in 5% CO_2–95% O_2 with the filterlid tightly closed and sealed with Parafilm (Nordic EM Supplies, Espoo, Finland). Thereafter, 200 μl of Solvable reagent (benzethonium hydroxide, Packard, PerkinElmer Sweden) was added drop-wise through the hole of the lid to soak the filter, followed by 250 μl of 35% perchloric acid. The lids were then sealed with Parafilm to prevent escape of gas from the flasks through the hole. Flasks were gently agitated for 1 h at room temperature. The filter compass was removed to a scintillation tube and 10 mL of scintillation liquid and 200 μl of icecold methanol were added. The level of trapped $^{14}CO_2$ in the filter was determined in a liquid scintillation counter.

3.1.9.6 Media lactate concentration

Myotubes were incubated overnight in the presence or absence of AICAR (1 mM) in serum-free DMEM. Media (100 μl) was collected in duplicate and lactate concentration was determined (Bouzakri et al., 2008) using a commercially available kit (Study I) (A-108; Biochemical Research Service Center, University of Buffalo, Buffalo, NY).

3.1.10 Extraction of nucleotides

Differentiated skeletal muscle myotubes from 10 cm dishes were washed three times with ice-cold PBS and scraped in 300 μl of ice cold 5% perchloric acid. *Tibialis anterior* muscle (~50 mg) was powdered in liquid nitrogen and then homogenized in 0.2 ml of 5% (by volume) perchloric acid. Samples were subjected to centrifugation (14,000 rpm for 3 minutes at 4ºC) to remove insoluble material. A solution containing an equal volume of 1, 1 tri-n-octylamine and 1, 1, 2,-trichlorotrifluoro ethane (total volume 220 μl) was added to the supernatant and the sample was rigorously vortexed. After centrifugation, the upper aqueous layer was removed and extracted a second time with a further addition of 220 μl of the same organic solvent mixture as identified above (Study I). An aliquot (20 μl) of the final aqueous phase was analyzed by capillary electrophoresis with on-column isotachophoretic pre-concentration, using a leading buffer consisting of 50 mM sodium phosphate and 50 mM NaCl (pH 5.2), and a tailing buffer containing 100 mM MES/Tris pH 5.2. To each buffer, 0.2% hydroxyethyl-cellulose was added to decrease the electro-osmotic flow. Nucleotide

peaks were detected by UV absorbance at 260 nM (ref. 400 nM), and integrated using System Gold Software (Beckman). Nucleotide ratios were calculated from peak areas after correction for retention times (Sakamoto et al., 2005).

3.1.11 Determination of protein concentration

3.1.11.1 Protein concentration in cell culture

To determine the total protein concentration, myotubes were grown and differentiated in six welled plates or 100 mm dishes and cells were lysed in 50 µl/well or 300 µl/ dish homogenization buffer. The homogenates were solubilized by vortexing and subjected to centrifugation at 4ºC at 12000 g for 10 minutes. The supernatant was collected and total protein was determined by BCA method using a commercially available kit (Pierce, Rockford, IL). The supernatant was stored at -80ºC for preparation of samples for immunoblot analysis.

3.1.11.2 Protein concentration in skeletal muscle biopsy

Frozen muscle biopsies were homogenized in ice cold buffer (10% glycerol, 5 mM sodium pyrosulfate, 13.7 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 20 mM Tris (pH 7.8), 1% Triton X100, 10 mM NaF, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 0.5 mM sodium vandanate, 1 mM benzamidine, 1 µM microcystin) for 20 seconds using a motor-driven pestle. Homogenates were rotated end-over-end for 1 hour at 4^{o}C and then subjected to centrifugation (12,000 g for 10 minutes at 4^{o}C). The supernatant was collected and total protein was determined by BCA method using a commercially available kit (Pierce, Rockford, IL). The supernatant was stored at -80ºC for preparation of samples for immunoblot analysis.

3.1.12 Immuno blot analysis (Western blot)

Protein expression or phosphorylation in either cultured myotubes or muscle biopsies was accessed by western blot analysis. Equal concentration of the homogenized lysate was mixed with 4X Laemmli buffer containing β-mercaptoethanol and heated at 56ºC for 30 minutes. Proteins were subjected to SDS-PAGE and separated on a 4-12% precast gradient agarose gel (Criterion, Bio-Rad). The separated proteins were then transferred to polyvinylidenediflouride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA, USA). After the protein transfer, the membranes were incubated in 7.5% low fat milk in Tris-buffered Saline and Tween 20 [TBST] for 1-2 hours at room temperature. The blocked membranes were then washed in TBST to remove the milk and then incubated in respective primary antibody diluted in TBST and 0.01% sodium azide overnight at 4ºC (16 hours). The incubated membranes were then washed in TBST for 4 times 10-15 minutes each and incubated in appropriate horse radish peroxidase conjugated secondary antibody at room temperature for 1 hour. After washing the membrane to remove the secondary antibody for 3 times 10-15 minutes each, the proteins were visualized by enhanced chemiluminescence using ECL reagent (GE Healthcare, UK) and quantified by densitometry. Results were expressed as arbitrary units normalized to loading controls.

3.1.12.1 Membrane stripping

When required and specified, PVDF membranes were stripped in buffer (62.5 mM Tris-HCl pH 6.8, 10 mM β-mercaptoethanol and 2% (w/v) SDS) for 40 minutes at 56ºC to remove the primary and secondary antibodies followed by washing in TBST and further incubation in 7.5% low fat milk dissolved in TBST for 2 hours. After a brief wash to remove the milk particles, the membrane was incubated in the primary antibody of interest overnight at 4ºC.

3.1.13 Animal models

The C57BL/6 mice were used in Study I and AMPKγ3 KO mice and their wild-type littermates were investigated in Study II. Animals were maintained on a 12 hour light-dark cycle and were allowed to have a free access to standard rodent chow. Animals were fasted for 4 hours before the experiments. All the experiments were approved by the Regional Animal Ethical Committee Stockholm, Sweden. The C57BL/6 mice were purchased from Charles River Laboratories (Germany) and the AMPKγ3 KO and their respective wild-type littermates were bred in-house. The generation of the AMPKγ3 KO mice has been previously described (Barnes et al., 2004).

3.1.14 Isolated skeletal muscle incubations

Mice were anesthetized via an intraperitoneal injection 2.5% Avertin (2, 2, 2-tribromo ethanol 99% and Tertiary amyl alcohol) (0.02 ml/g of body weight) and *extensor digitorum longus* (EDL) and *Soleus* muscle were carefully dissected and removed further analysis. Krebs-Henseleit buffer [KHB] supplemented with 5 mM glucose, 15 mM mannitol, 5 mM HEPES and 0.1% BSA (RIA grade) was pre-gassed (95% oxygen, 5% CO_2) and used as a basal incubation buffer. Immediately after dissection, the muscles were allowed to recover for 20 minutes in basal incubation buffer. The isolated muscles were incubated at 30ºC in a shaking water bath under a constant gas phase of 95% oxygen, 5% CO_2.

3.1.14.1 Palmitate oxidation in isolated skeletal muscle

On the day of experiment, mice were fasted for 4 hours and anesthetized with intraperitoneal injection of Avertin (0.02 ml/g of body weight). EDL and *soleus* muscles were quickly removed and then incubated in KHB, containing 5 mM glucose and 15 mM mannitol for a recovery period of 20 minutes at 30°C, oxygenated with 95% O_2/5% CO_2 gas mixture. Muscles were pre-incubated in KHB in the presence or absence of MTX (10 µM) for 2 hours, which was followed by analysis of palmitate oxidation. For palmitate oxidation assay (Study II) EDL and *soleus* were incubated in KHB containing [^{3}H]-palmitate (0.4 µCi/ml) complexed with 20% fatty acid free BSA and 0.3 mM cold palmitate for a period of 2 hours with or without AICAR (0.2 mM). At the end of the experiment, muscles were taken out of the medium, washed in ice-cold KHB, briefly blotted on Whatman filter paper, freeze-clamped with aluminium tongs snap-frozen in liquid nitrogen and stored at -80°C until further analysis. The medium was collected in 1.5 ml eppendorf tubes and stored at -20°C until analysis. For the determination of palmitate oxidation, an aliquot (200 µl) of the medium was

thoroughly mixed with 800 µl of 10% charcoal slurry (0.02 M Tris-HCl, pH 7.5). The mixture was then subjected to centrifugation at 16,000 g for 15 minutes and the supernatant (200 µl) was counted for tritium labelled water. The final results are normalized per mg of muscle weight.

3.1.14.2 Gene transfer by electroporation into intact mouse muscle

Adult C57BL/6 mice (20-25 g) were anesthetized by isoflurane. The hindlimbs of the mice were shaved using a regular shaving apparatus to visualize the *tibialis anterior* muscle. Hyaluronidase (30 µl of 1 U/µl) was then injected in the *tibialis anterior* muscle through the skin and the mouse was returned to a separate cage for 2 hours. Mice were anesthetized by isoflurane using a gas anesthesia machine (Harvard Scientific Instruments) and a pool of plasmids (30 µg) encoding either a scrambled sequence or shRNA against NT5C1A (SABiosciences, Frederick, MD) was injected in the *tibialis anterior* muscle of each leg through the skin (Study I). Electroporation was performed by delivering 220 V/cm of 8 pulses of 20 milliseconds using an ECM 830 electroporator (BTX, Harvard Apparatus, Holliston, MA).

3.1.14.3 Glucose uptake in intact tibialis anterior muscle

Seven days after the electroporation procedure, mice were fasted for 4 hours (Study I). Thereafter, a bolus of glucose (3 g/kg) was administered by gavage, and [^{3}H] glucose (4.5 µl of 2-[^{3}H]deoxy-D-glucose/ 100 µl of saline/animal, 1 mCi/ml) was administered intraperitoneally. Mice were anesthetized 120 minutes after the glucose gavage, and the *tibialis anterior* muscle was dissected and directly frozen in liquid nitrogen for subsequent determination of [^{3}H]glucose accumulation. Frozen muscle samples were homogenized in ice-cold buffer (10% glycerol, 5 mM sodium pyrosulfate, 13.7 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 20 mM Tris (pH 7.8), 1% Triton X-100, 10 mM NaF, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 0.5 mM sodium vanadate, 1 mM benzamidine, and 1 µM microcystin) for 20 seconds using a motor-driven pestle. Homogenates were rotated end-over-end for 1 hour at 4 °C and then subjected to centrifugation at 12,000 g for 10 minutes at 4 °C. The supernatant (30 µl) was analyzed by liquid scintillation counting. A portion of the remaining supernatant was stored at -80 °C for immunoblot analysis.

3.1.15 Nucleotidase activity assay

5'-nucleotidase activity was assayed (Study I) as the release of [^{3}H]adenosine from 2-[^{3}H] AMP or [^{3}H]inosine from 8-[^{3}H] IMP. An aliquot (20 µl) of homogenate was incubated for 15 min at 30°C in a total volume of 50 µl containing 100 mM-Tris-HCl, 2 mM $MgCl_2$ and 10 mM β-glycero P-Na, with either 2-[^{3}H] AMP or 8-[^{3}H] IMP (200 µM of AMP or IMP and 5 µCi of AMP or 1 mCi of IMP, respectively). The incubation was terminated by addition of 10 µl of 150 mM $ZnSO_4$, followed by addition of 10 µl saturated $Ba(OH)_2$ to precipitate unhydrolyzed AMP or IMP. Samples were placed on ice for 10 minutes and subjected to centrifugation (13000 rpm for 15 minutes at 4°C). The supernatant was collected for determination of the radioactivity by scintillation counting (Belsham et al., 1980).

3.1.16 Statistical analysis

Data are presented as mean ± SEM. Statistical evaluation between groups was performed by one-way ANOVA, two-way ANOVA or Students *t*-test as appropriate and as specified in each study. Comparisons were considered statistically significant at $P < 0.05$. Natural log transformation was applied when data were not normally distributed. Analyses were performed using either MS Excel version 2010 or SPSS version 17.0 (SPSS, Chicago, IL).

4 RESULTS AND DISCUSSION

4.1 MODULATION OF NUCLEOTIDE METABOLISM AS A STRATEGY TO MAINTAIN SKELETAL MUSCLE ENERGY HOMEOSTASIS

4.1.1 Role of 5'-nucleotidases in skeletal muscle metabolism

Obesity and type 2 diabetes are characterized by an increase in whole-body lipid accumulation and decreased insulin-stimulated glucose disposal (DeFronzo and Tripathy, 2009). Skeletal muscle is one of the largest organs to utilize the body fuels and is a natural target to be affected by the disturbance in substrate metabolism and fuel selection in obesity, insulin resistance and type 2 diabetes. Efforts are underway to validate new fuel sensing molecules in skeletal muscle to regulate the skeletal muscle fuel homeostasis and energy metabolism. One of the molecules which has emerged as a critical player in diabetes research and metabolic flexibility is AMPK. In Study I, the role of 5'-nucleotidases in skeletal muscle energy metabolism has been dissected by linking it to the regulation of AMPK. An alternative strategy to activate the AMPK system and mediate its beneficial metabolic effects is proposed (Figure 6).

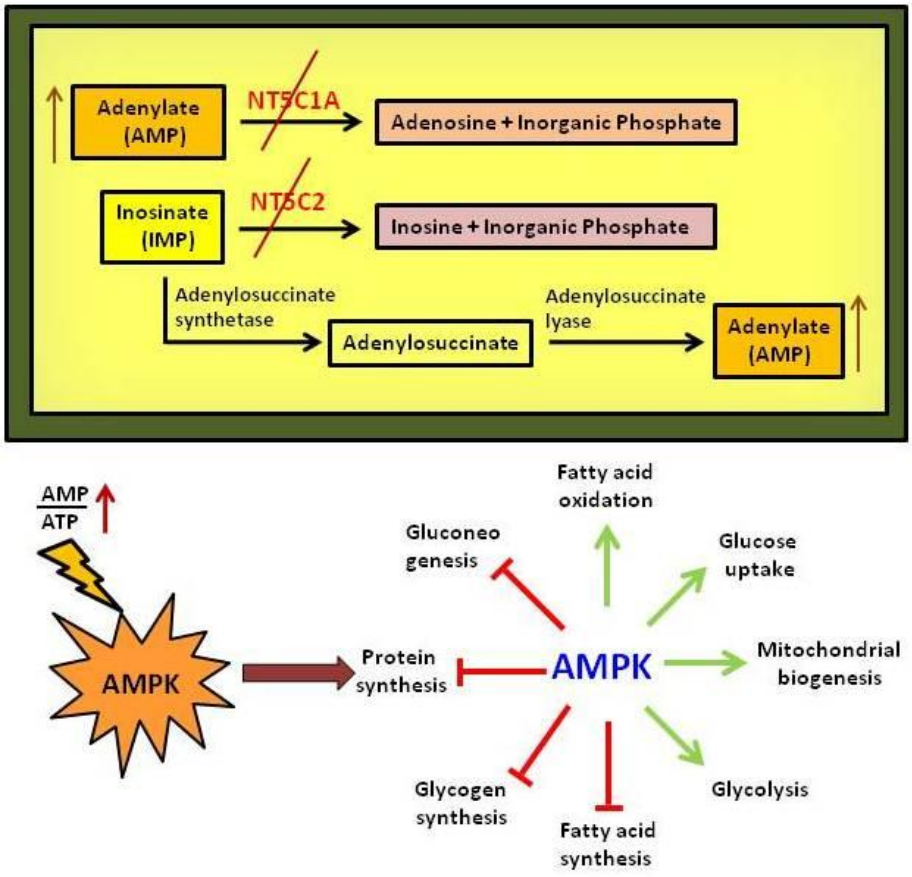

Figure 6: Working hypothesis to illustrate the role of 5'-nucleotidases (NT5C1A and NT5C2) and effect of silencing on AMPK and metabolism in skeletal muscle.

4.1.1.1 Effect of 5'-nucleotidases silencing in human and mouse skeletal muscle

Enzymes involved in cytosolic AMP catabolism, including cytosolic 5'-nucleotidases have been implicated in balance of intracellular nucleotide pools (Hunsucker et al., 2005). These enzymes are important for maintaining the appropriate levels of AMP and ATP and thus may influence AMPK activity. We hypothesized gene silencing of 5'-nucleotidases enzymes would increase the intracellular availability of AMP relative to ATP and may trigger the activation of the AMPK system (Figure 6). Relative mRNA expression of NT5C1A and NT5C2 was determined in primary human myotubes. NT5C1A mRNA was either low or undetectable, whereas NT5C2 mRNA was readily detectable in primary human myotubes. Given that NT5C2 was the only detectable enzyme among the two targets studied in human myotubes, gene silencing was directed against this enzyme. Although intracellular IMP has not been reported to contribute to AMPK activation, IMP is metabolized and converted into adenylosuccinate by adenylosuccinate synthetase and is then converted into AMP by adenylosuccinate lyase, which may subsequently increase intracellular AMP. Gene silencing of NT5C2 reduced mRNA expression (Study I, Figure 1A) and protein content (Figure 8) 75% and 70%, respectively, followed by a 2-fold increase in the AMP/ATP ratio (Study I, Figure 1E). Preliminary data indicate that NT5C1A mRNA expression is increased 1.5-fold in *vastus lateralis* muscle from type 2 diabetic patients, compared to normal glucose tolerant people (Kulkarni et. al. Unpublished data, Figure 7). The effect of NT5C1A silencing was determined in adult mouse skeletal muscle using the electroporation technique. Contralateral muscles were transfected with either a scrambled sequence or shRNA against NT5C1A. Gene silencing resulted in 60% decrease of NT5C1A protein content (Figure 8) versus the sham-treated contralateral muscle, followed by a 17% increase in the AMP/ATP ratio (Study I, Figure 6C).

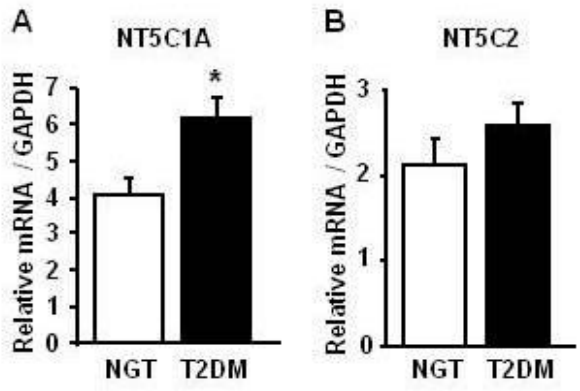

Figure 7: NT5C1A and NT5C2 mRNA in human skeletal muscle. *NT5C1A and NT5C2 mRNA expression was determined in skeletal muscle from normal glucose tolerant (NGT) and type 2 diabetic (T2DM) subjects. NT5C1A mRNA expression was increased 1.5-fold in T2DM, compared to NGT subjects. Conversely, mRNA expression of NT5C2 was similar between T2DM and NGT subjects (B). *P < 0.05*

4.1.1.2 Effect of 5'-nucleotidases silencing on AMPK and ACC phosphorylation

We hypothesized that siRNA based gene silencing of NT5C2 may increase intracellular availability of AMP and IMP, which may alter AMP/ATP ratio and increase AMPK activity. NT5C2 silencing in the human cultured myotubes, increased basal AMPK phosphorylation 2.0-fold (Figure 8). AMPK phosphorylation was further enhanced in NT5C2-silenced myotubes upon AICAR exposure. NT5C2 silencing also increased basal ACC phosphorylation 3.6-fold, which was further enhanced in the presence of AICAR (Figure 8). In case of mouse skeletal muscle, gene silencing of NT5C1A was associated with a 60% increase in AMPK phosphorylation (Figure 8) and a 50% increase in ACC phosphorylation (Figure 8), whereas AMPK and ACC protein content remained unaltered.

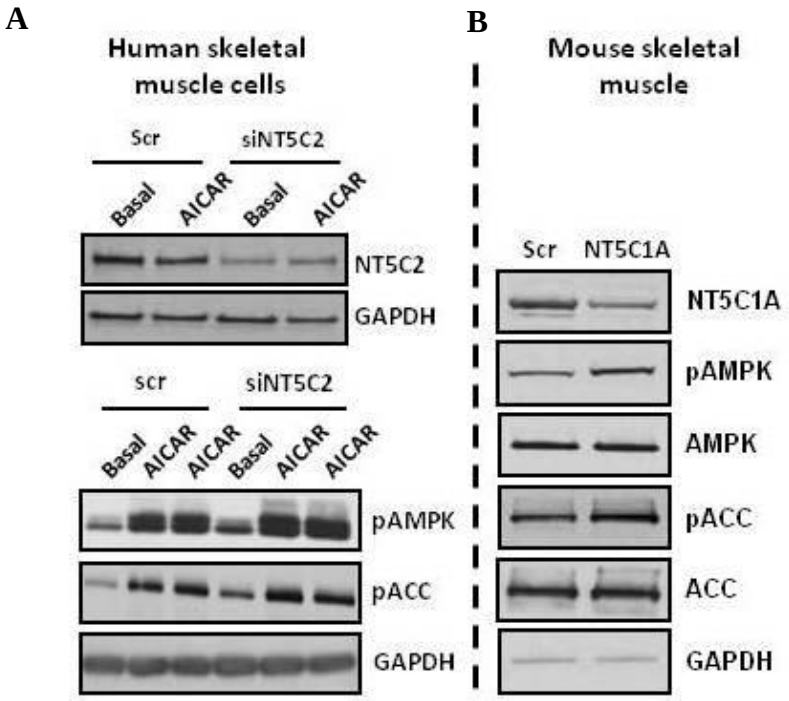

Figure 8: Effect of siRNA-mediated silencing of NT5C2 (A) and NT5C1A (B) on protein phosphorylation of AMPK and ACC in primary human myotubes or mouse tibialis anterior muscle.

4.1.1.3 Effect of 5'-nucleotidases silencing on lipid oxidation and glucose transport

In cultured human myotubes, NT5C2 silencing increased basal palmitate oxidation 1.8-fold (Figure 9). NT5C2 silencing did not modify palmitate oxidation under insulin-stimulated conditions; however, the response to AICAR was enhanced 1.5-fold (Figure 9). Activated AMPK phosphorylates and inhibits ACC at Ser^{79}, leading to a decrease in malonyl CoA, which releases the inhibitory loop on CPT1 and simulates β-oxidation of long chain acyl-CoAs in the mitochondrial matrix (Hardie, 1989; Merrill et al., 1997; Winder and Hardie, 1996). NT5C2 silencing in the cultured human myotubes increased glucose uptake under basal and insulin-stimulated conditions. Conversely, AICAR-mediated glucose uptake was unaltered by NT5C2 silencing (Figure 9). *In vivo* electrotransfer to overexpress DNA from endogenous (Bruce et al., 2009; Mauvais-Jarvis et al., 2002) or mutant proteins (Treebak et al., 2010; Witczak et al., 2010) in skeletal muscle has been used to address the role of specific genes in signal transduction and metabolism. Taking advantage of this technique, shRNA against NT5C1A was used to silence the gene expression in the mouse contralateral *tibialis anterior* muscle. Parallel to the observation in human cultured myotubes, the electroporation resulted in ~60% decrease in NT5C1A protein expression (Figure 8), increased AMPK and ACC phosphorylation and a ~20% increase in glucose uptake in intact *tibialis anterior* muscle *in vivo* (Figure 9).

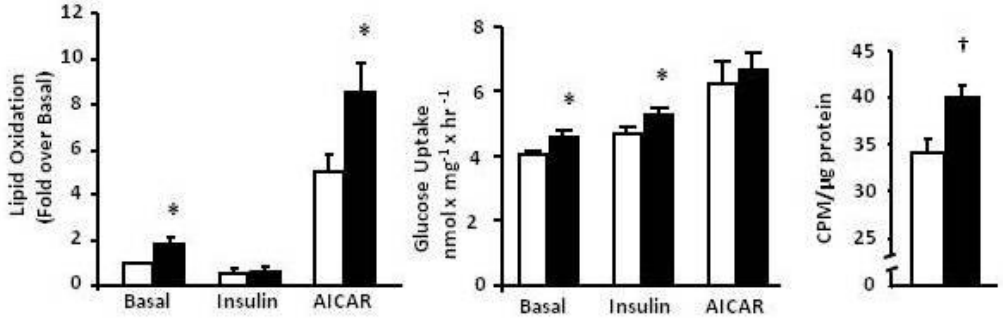

Figure 9: Effect of NT5C2 silencing on muscle metabolism: *Primary human myotubes were transfected with siRNA against a scrambled sequence (white bars) or NT5C2 (black bars) and incubated in the absence (Basal) or presence of 120 nM insulin or 1 mM AICAR for measurement of glucose uptake. NT5C2 silencing increased basal palmitate oxidation 1.8-fold and the response to AICAR was enhanced 1.5-fold. Glucose uptake was increased under basal and insulin stimulated condition by 20% and 15% respectively. While silencing of NT5C1A in mouse tibialis anterior muscle led to a 20% increase in glucose uptake. Results are mean ±SEM, *P < 0.05.*

Another critical enzyme involved in the intracellular availability of AMP is AMP deaminase 1 [AMPD1]. AMPD1 plays a major role in regulating cellular AMP levels by converting AMP to IMP (Sabina and Mahnke-Zizelman, 2000). Defects in the AMPD1 gene increase AMP accumulation in skeletal muscle. The common C34T polymorphism in the AMPD1 gene is associated with lower prevalence of type 2 diabetes, reduced frequency of obesity, and lower systolic blood pressure in people with coronary artery disease without heart failure (Safranow et al., 2009), possibly

through increased AMPK activity. Variations in the AMPD1 gene are associated with alterations in the metabolic clearance rate of insulin (Goodarzi et al., 2005). Conversely, adenylate kinase 1-deficient mice have reduced levels of AMP and exhibit decreased contraction- induced AMPK phosphorylation (Hancock et al., 2006) and glucose transport (Janssen et al., 2000) in skeletal muscle.

NT5C1A is also expressed in cardiac muscle, where it has a physiological function in the generation of adenosine during ischemic conditions and protects the myocardial and cerebrovascular systems against ischemia-induced damage (Sala-Newby et al., 1999). The family of soluble 5'-nucleotiodases seems to have an increasing clinical potential, since 5'-nucleotidases activity is linked with efficacy of certain nucleoside analogues that are anti-cancer and anti-viral drugs (Hunsucker et al., 2005). Since these drugs rely on their phosphorylation by nucleoside kinases, increased 5'-nucleotidases activity may lead to drug resistance (Hunsucker et al., 2005).

In this study a novel role for 5'-nucleotidases in maintaining the intracellular energy status via AMPK is highlighted. Alterations in the adenine nucleotide levels may have beneficial effects on glucose and energy homeostasis.

4.1.2 Methotrexate enhances AICAR mediated AMPK phosphorylation and lipid oxidation in skeletal muscle

Methotrexate (MTX) is a folic acid analog, which has been used for many years to treat cancer and rheumatic disease. Methotrexate is a potent inhibitor of 5-aminoimidazole-4-carboxamide ribonucleotide formytransferase / inosine monophosphate cyclohydrolase (ATIC), which converts intracellular ZMP to IMP. MTX treatment in humans (Baggott et al., 1999; Luhby and Cooperman, 1962; Lulenski et al., 1970; Morgan et al., 2004) and animals (Baggott and Morgan, 2007) increases excretion of ZMP/AICAR metabolite 5-aminoimidazole-4-carboxamide (AICA, Z-base). This implies that MTX effectively inhibits ATIC and thereby imposes a metabolic block and build-up of ZMP (Cronstein, 2010; Cronstein et al., 1993).

To test the hypothesis that intracellular ZMP accumulation would alter AMPK activity, ATIC enzyme activity was inhibited or suppressed by either pretreating the skeletal muscle with MTX or by siRNA silencing ATIC in cultured skeletal muscle, respectively. We reasoned this strategy would ultimately lead to enhanced sensitivity of AMPK towards its activator AICAR, and lead to further AMPK-mediated changes in skeletal muscle metabolism (Figure 10).

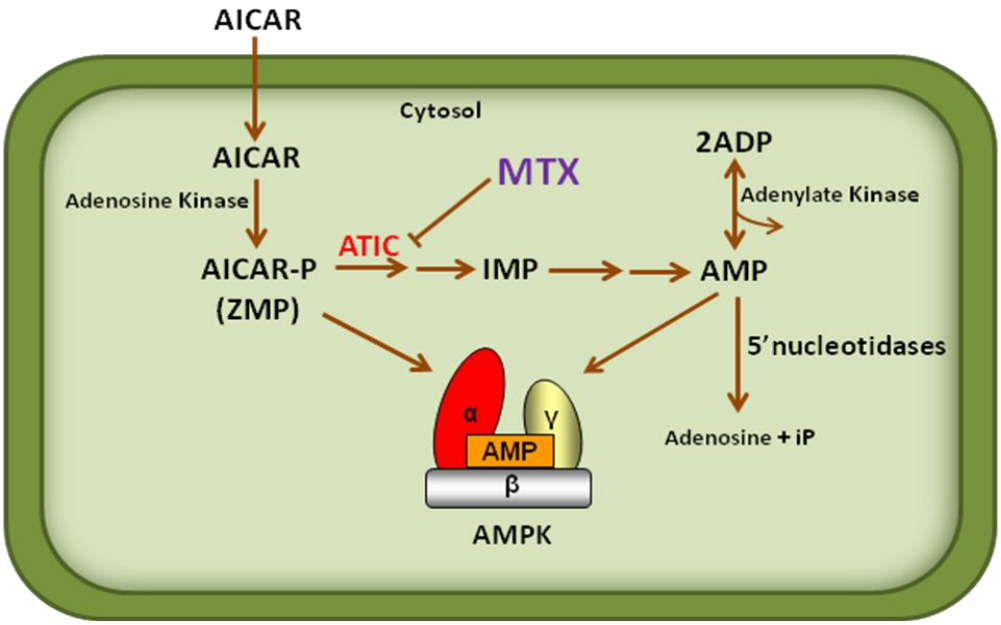

Figure 10: Working hypothesis to illustrate the activation of AMPK by AICAR-P and effect of MTX treatment on ATIC mediated intracellular nucleotide metabolism. *Inhibition of ATIC by MTX or siRNA silencing ATIC potentiated AICAR-mediated AMPK phosphorylation in skeletal muscle.*

4.1.2.1 Effect of Methotrexate pretreatment on AICAR-mediated AMPK activation in cultured rat and human skeletal muscle.

MTX treatment in humans and animals increases endogenous production of ZMP (Cronstein, 2010; Cronstein et al., 1993) and its metabolites like AICA (Baggott and Morgan, 2007; Baggott et al., 1999; Luhby and Cooperman, 1962; Lulenski et al., 1970). This suggests MTX could activate AMPK and/or reduce the threshold for its activation by AICAR. Pretreatment of the L6 myotubes and differentiated human skeletal myotubes with MTX (5 µM) for 16 hours, followed by a 5-hour treatment with 0.2 mM AICAR, robustly increased AMPK and ACC phosphorylation (Study II, Figure 1A, B, 2 A, B). Palmitate oxidation remained unaltered in L6 myotubes treated with 0.2 mM AICAR or MTX alone, but MTX-pretreated L6 myotubes when stimulated with 0.2 mM AICAR enhanced palmitate oxidation by 15% compared to treatment with 0.2 mM AICAR alone (Study II Figure 1C). The MTX-mediated reduction in the threshold for AMPK activation could not be considered as an idiosyncratic reaction of L6 cell line, since primary human myotubes (Study II, Figure 2) and murine wild-type EDL and soleus (Study II, Figure 4) displayed essentially the same response.

In this study the effects of MTX on signal transduction and metabolism was assessed in skeletal muscle. MTX enhanced AMPK signaling in AICAR-treated myotubes and isolated murine EDL and *soleus*, which translated into substantially increased palmitate oxidation. These effects were at least partially dependent on the muscle-specific AMPK

γ3 isoform, since MTX was without effect on AMPK signaling and palmitate oxidation in AICAR-treated EDL from AMPK $\gamma3^{-/-}$ mice.

Patients treated with MTX and other disease modifying anti-rheumatic drugs have reduced cardiovascular mortality and an improved metabolic profile, including reduction in insulin resistance (Dessein and Joffe, 2006). Data regarding the clinical metabolic effects of MTX remains equivocal and it is far from clear whether the observed clinical benefit is simply due to immunosupressive and anti-inflammatory action of MTX or whether its modulation of AMPK function, observed in cell culture of cancer cell, may also contribute. MTX is an inhibitor of ATIC, which is an enzyme involved in *de novo* nucleotide biosynthesis, that imposes a metabolic block, leading to intracellular ZMP accumulation, lowering the threshold for AMPK activation.

4.1.3 Targeting 11β-HSD1 for reversal of glucocorticoid action on human skeletal muscle cells

Impairments in the glucocorticoid metabolism have been linked to obesity, insulin resistance and metabolic syndrome (Chapman and Seckl, 2008; Qi and Rodrigues, 2007; Walker, 2007; Wang, 2005). The action of glucocorticoids is dependent on an enzyme 11β-HSD1, which converts cortisone in an inactive form to cortisol in its active form. Local conversion of cortisone to cortisol in target tissues may determine tissue-specific roles of glucocorticoids in pathophysiological conditions. In this study the intracellular role of 11β-HSD1 in mediating the local glucocorticoid effects on skeletal muscle and its impact on glucose and lipid metabolism was determined.

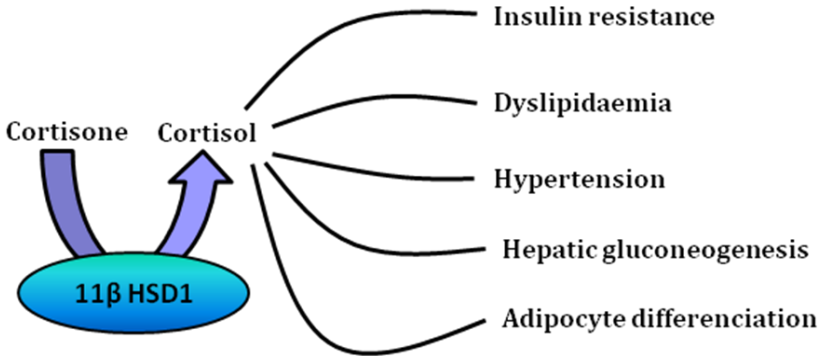

Figure 11: Targeting pre-receptor metabolism of cortisol - Potential treatment for type 2 diabetes? *Detrimental effects of high circulating cortisol on metabolism.*

4.1.3.1 *Effect of glucocorticoids on skeletal muscle substrate metabolism: role in shift of metabolism*

The effects of chronic exposure of glucocorticoids i.e, cortisone (inactive) and cortisol (active) were determined on skeletal muscle metabolism by assessing glucose and lipid metabolism. Glucose metabolism was assessed by measuring glucose uptake (Figure 12) and glycogen synthesis (Study III, Figure 1C), while the lipid metabolism was assessed by measuring palmitate oxidation (Study III, Figure 1D). Chronic exposure of skeletal muscle to active or inactive glucocorticoids had a negative impact on glucose metabolism, indicating a shift in metabolism from glucose to lipid oxidation. This also indicated the presence of a functioning machinery to convert inactive cortisone to active cortisol, since the treatment of cells with inactive cortisone paralleled the effect produced by active cortisol exposure (Study III, Figure 1).

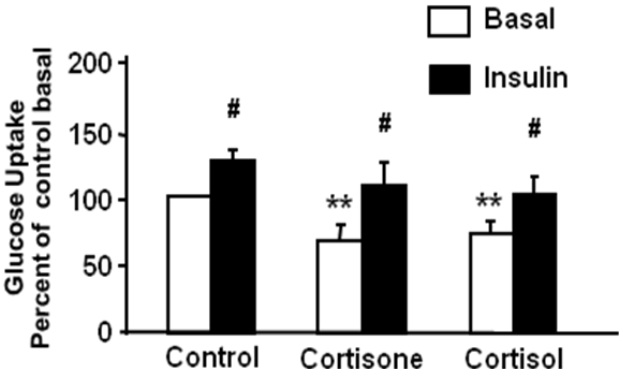

Figure 12: Effect of glucocorticoid exposure on skeletal muscle glucose uptake. *Primary cultures of human skeletal muscle were exposed to 0.5 μM cortisol or 0.5 μM cortisone for eight days. Insulin-stimulated glucose uptake was determined. Results are mean ± SEM. Basal (open box), insulin (closed box),*P < 0.05, **P < 0.01 vs. control basal, # P < 0.05 vs. basal for each condition.*

4.1.3.2 *Role of 11βHSD1 in glucocorticoid-mediated effects on skeletal muscle metabolism*

The pre-receptor metabolism of the glucocorticoids determine the tissue specific sensitivity, which is also partly regulated by enzyme 11β-HSD1 that converts inactive cortisone to active cortisol (Salehzadeh et al., 2009). Increased adipose tissue 11β-HSD expression has been observed in obesity, which may lead to increased local glucocorticoid signaling (Stimson et al., 2009). Transgenic mice overexpressing HSD1 in either liver or adipose tissue have features resembling that noted in the metabolic syndrome (Paterson et al., 2004) while transgenic deletion of 11β-HSD1 prevents adverse metabolic complications of obesity (Kotelevtsev et al., 1997).

The role of 11β-HSD1 in the skeletal muscle glucocorticoid-mediated changes in the metabolism was determined. Chronic exposure of cultured myotubes to either cortisone or cortisol resulted in a 2-fold increase in mRNA (Study III, Figure 2A) expression and 4-fold increase in protein (Figure 13) expression of 11β-HSD1. This is in agreement with earlier observations exhibiting positive effects of glucocorticoids on

HSD1 expression in cultured human skeletal muscle (Whorwood et al., 2002) and other cell types (Hammami and Siiteri, 1991; Sun and Myatt, 2003). These previous observations indicate increased skeletal muscle glucocorticoid sensitivity. siRNA against 11β-HSD1 was used to reverse the glucocorticoid-mediated effects on metabolism. siRNA against 11β-HSD1 prevented the cortisone, but not cortisol-mediated reduction in glucose incorporation into glycogen (Study III, Figure 3A). The siRNA-mediated reduction of 11β-HSD1 decreased cortisone-, but not cortisol-mediated increases in lipid oxidation (Study III, Figure 3B); rather an unexpected increase in cortisol-mediated palmitate oxidation was observed. Using a pharmacological inhibitor against 11β-HSD1, similar results on metabolism was observed (Study III, Figure 5).

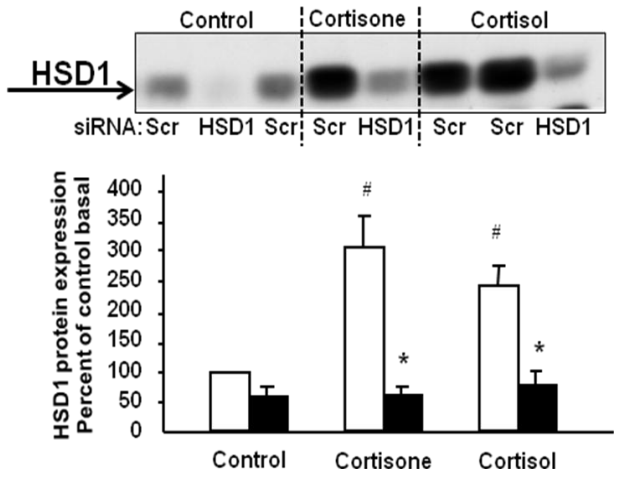

Figure 13: Effect of HSD1 siRNA on protein expression. *Myotubes were exposed to glucocorticoids as described in Figure 12. Myotubes were transfected with siRNA against a scrambled sequence (open box) or 11β-HSD1 (closed box) and 11β-HSD1 protein content was determined. Upper panel shows representation of immunoblot of 11β-HSD1 protein content. Scr: Scrambled siRNA, 11β-HSD1: siRNA targeted against 11β-HSD1. Results are mean ± SEM. *P < 0.05 transfected myotubes vs. scramble-control for each condition, #P < 0.05 treated-scramble vs. scramble-control.*

4.1.3.3 Glucocorticoid-mediated changes in gene expression in human skeletal muscle

This study focused on the direct effects of glucocorticoids on primary human skeletal muscle cells. Expression of key regulatory genes involved in glucose and lipid metabolism remained unaltered after chronic exposure of the cultured myotubes to either 0.5μM cortisone or cortisol (Study III, Table 2). However, expression of PDK4 mRNA and protein was increased after an 8 day treatment of human cultured myotubes with either cortisone or cortisol (Study III, Figure 4A). A key mechanism in regulation of tissue fuel selection is at the level of mitochondrial pyruvate dehydrogenase complex, an enzyme that catalyzes the conversion of pyruvate to acetyl CoA, which is also regulated negatively by PDK enzymes. We determined the role of PDK4 in glucocorticoid-mediated effects on skeletal muscle. Gene silencing of PDK4 by siRNA prevented the cortisol-mediated reduction in glycogen synthesis (Study III, Figure 4C), suggesting PDK4 may play a key role in the cortical-mediated metabolic shift in substrate utilization in human skeletal muscle. Our findings are in agreement

with previous findings reporting an enhanced glucose oxidation in isolated skeletal muscle of mice with targeted deletion of PDK (Jeoung and Harris, 2008).

Collectively, these data indicate that PDK4 plays a key role in mediating the effects of cortisol to attenuate skeletal muscle glucose utilization, and subsequent increase in lipid utilization. 11β-HSD1 could be a target molecule to overcome the deleterious effects of cortisone-mediated shift in the metabolism. Pre-receptor metabolism of glucocorticoids thus, could be a novel strategy in treatment of diabetes and obesity.

4.1.4 Mitochondrial regulators of fatty acid metabolism as targets for muscle fuel homeostasis

The mitochondria is the aerobic powerhouse of the cell, since it converts and conserves energy obtained from various metabolic processes such as oxidation of the macronutrients and thus contributing to the overall energy balance. Skeletal muscle mitochondrial activity is a controlled process, which is governed by a variety of internal stimuli. The ability of the mitochondria to sense the metabolic perturbations and accordingly adapt to maintain cellular energy homeostasis, places it at a center stage in treatment of diabetes, obesity and metabolic syndrome. Moreover, mitochondrial proteins such as PDK4, CPT1 are located at the metabolic flux points playing key roles in fuel selection and metabolic flexibility (Figure 14). Impairments in mitochondrial metabolism and diminished oxidative capacity is directly associated with impairments in skeletal muscle insulin signaling, accumulation of intramyocellular triglycerides and progression of type 2 diabetes and obesity (Kelley et al., 1999). The diminished mitochondrial capacity in type 2 diabetics could be due to changes in the expression profile of the key genes such as PDK2, PDK4, CPT1 and MCD located at critical metabolic flux points governing mitochondrial fatty acid metabolism. The aim of this study was to profile the expression pattern of PDK4, PDK2, CPT1b, MCD in skeletal muscles obtained from type 2 diabetic patients and matched normal glucose tolerant volunteers.

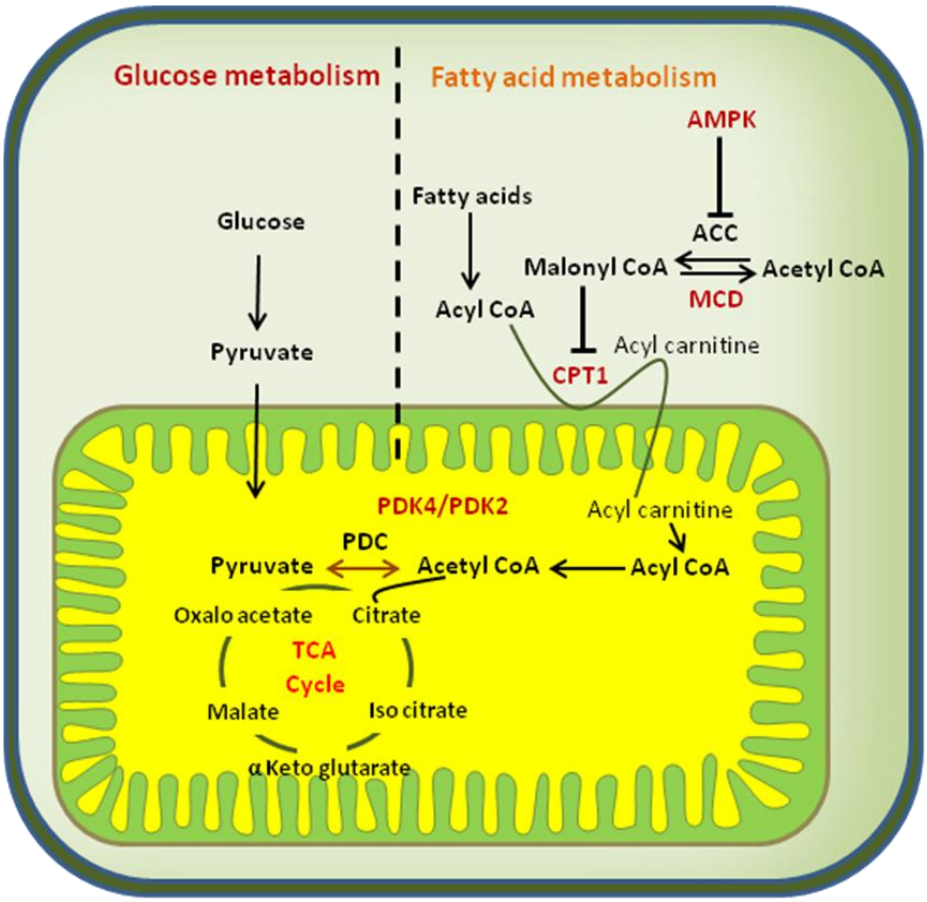

Figure 14: Regulation of glucose and fatty acid metabolism in skeletal muscle. *Substrate utilization is controlled at several steps. The gate keeper proteins such as PDK4, PDK2, CPT1, MCD and AMPK occupy central roles in regulation of the substrate switching and muscle metabolism.*

4.1.4.1 Skeletal muscle gene expression profile of key mitochondrial regulators of substrate switching

Type 2 diabetes mellitus is associated with abnormal substrate metabolism, raising the possibility that alterations in the expression of mitochondrial enzymes controlling lipid uptake and metabolism may by altered. Skeletal muscle expression of key mitochondrial genes that orchestrate the switch of substrate utilization between glucose and lipid sources was determined. mRNA expression of PDK4, PDK2, CPT1 and MCD was assessed in skeletal muscle from normal glucose tolerant and type 2 diabetic patients matched for age and body mass index [BMI]. PDK4 mRNA expression was increased 70% in skeletal muscle from type 2 diabetic patients compared to normal glucose tolerant subjects (Study IV, Figure 1A). Skeletal muscle mRNA expression of PDK2 and MCD was increased 50% in type 2 diabetic patients (Study IV, Figure 1B, and D).

Elevated PDK4 gene expression has been associated with various clinical manifestations, such as high circulating lipids (Sugden and Holness, 2006). In rodents,

a 4-week high fat diet increases skeletal muscle PDK4 expression (Holness et al., 2000). However in normal glucose tolerant subjects and type 2 diabetic patients plasma triglyceride levels are unaltered. Thus in this cohort, PDK4 mRNA expression is unrelated to circulating triglycerides. This discrepancy to previous results may be due to the fact that a subset of the type 2 diabetic cohort was treated with cholesterol lowering drugs that might have masked the previously observed relationships. However, a significant correlation was observed between PDK4 mRNA expression and BMI (Figure 15), highlighting the close relationship between expression of mitochondrial genes related to lipid metabolism and body mass index. A close positive relationship was also observed between PDK4, PDK2, MCD and CPT1, further strengthening the observation that this cluster of genes were similarly coordinated and regulated in type 2 diabetic and healthy individuals (Study IV, Figure 2A).

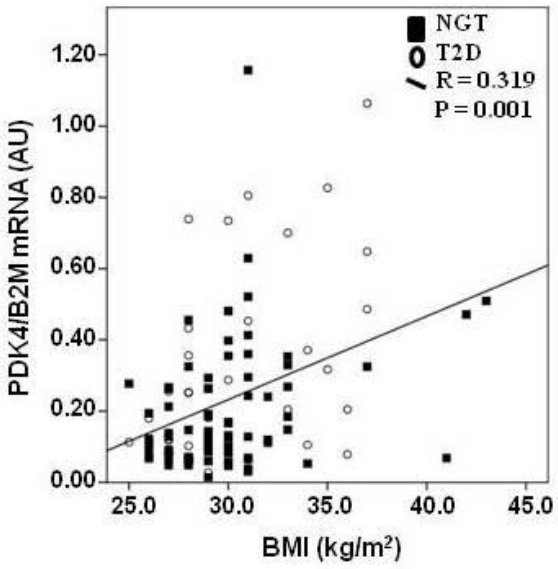

Figure 15: A significant correlation was observed between PDK4 mRNA expression and body mass index.

4.1.4.2 Evidence of epigenetic regulation of PDK4 promoter and gene expression

The methylation status of cytosines in the PDK4 promoter region was determined in genomic DNA extracted from skeletal muscle biopsies obtained from type 2 diabetic and normal glucose tolerant participants. DNA methylation is a major epigenetic modification that regulates the gene expression by altering the accession of the transcription factors to the promoter regions in the DNA (Cedar and Bergman, 2009). The PDK4 promoter in skeletal muscle obtained from type 2 diabetic patients was found to be hypo-methylated compared to skeletal muscle of normal glucose tolerant volunteers, with the mRNA expression being inversely regulated (Study IV, Figure 3A, B). Hypomethylation of the PDK4 promoter in skeletal muscle of type 2 diabetic patients is coincident with an impaired response of PDK4 mRNA after exercise. Evidence is emerging that epigenetic modifications through DNA methylation may

contribute to the increased risk and development of metabolic disease by modifying the expression of genes controlling whole body energy and glucose homeostasis (Barres et al., 2009; Klose and Bird, 2006). Increased promoter methylation of peroxisome proliferator-activated receptor 1α (PGC1α), another key regulator of mitochondrial activity and metabolism, has been reported from skeletal muscle of individuals with impaired glucose tolerance or type 2 diabetes mellitus (Barres et al., 2009). Evidence for the effect of nutrition and metabolic status of an individual on the epigenetic regulation in type 2 diabetes (Pembrey et al., 2006) raises the possibility that epigenetic modifications of genomic DNA may contribute to the development of metabolic diseases.

4.1.4.3 Effect of life style modification on anthropometry and skeletal muscle mRNA

The effect of a 4-month lifestyle intervention on anthropometry and mRNA expression was determined (Figure 16). The intervention involved 4 hours of Nordic walking per week. Muscle biopsies were obtained from study participants before and after the 4 month intervention. The lifestyle intervention was accompanied by weight loss, decrease in waist circumference and BMI in both normal glucose tolerant volunteers and type 2 diabetic patients (Table 1) while decrease in a 2-hour plasma glucose levels was observed only in type 2 diabetic patients (Table 1). Skeletal muscle PDK4 mRNA expression was increased only in the normal glucose tolerant subjects in response to the lifestyle intervention, but not in type 2 diabetic patients (Study IV, Figure 4A). This differential response between normal glucose tolerant humans and type 2 diabetic patients could be due to a higher compliance with the exercise program in normal glucose tolerant subjects or due to an inherent difference in exercise-dependent gene regulation in type 2 diabetic patients. Marked reduction of skeletal muscle PDK4 expression is observed in morbidly obese patients who underwent gastric bypass surgery (Rosa et al., 2003) and a decrease in PDK4 expression has been associated with increased insulin sensitivity (Rosa et al., 2003). Weight loss after the exercise intervention in the present study was modest, yet PDK4 mRNA expression was elevated in normal glucose tolerant subjects after intervention. In contrast, skeletal muscle mRNA expression of PDK2, CPT1 and MCD remained unaltered after the exercise intervention (Study IV, Figure 4B-D).

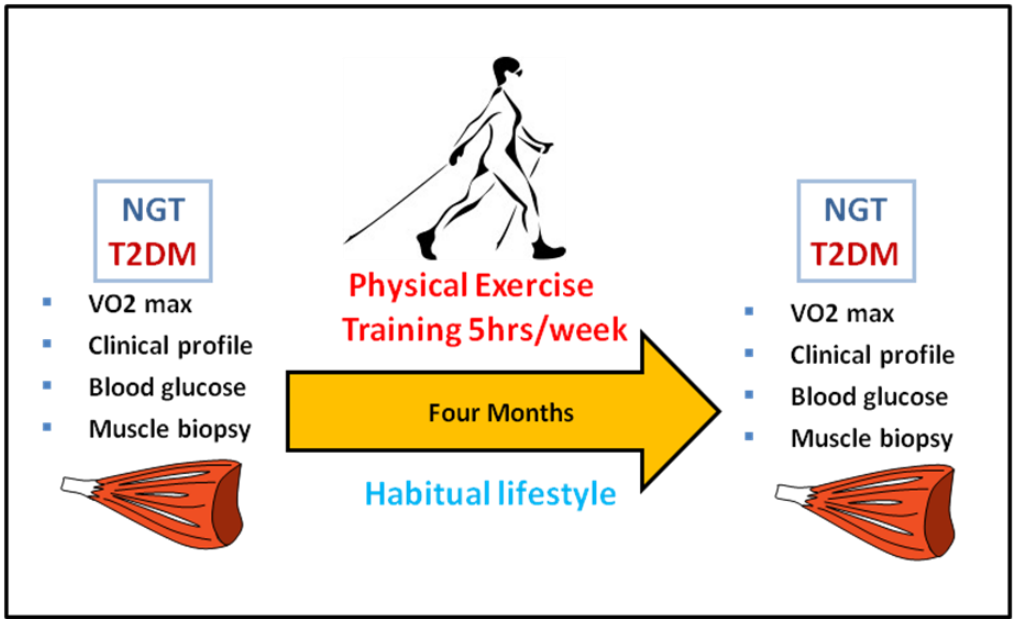

Figure 16: Effect of lifestyle modification on overall health and fitness. *In this exercise intervention Study, both normal glucose tolerant and type 2 diabetic male and female subjects were recruited and divided in two groups. One group was instructed to increase their physical activity by 5 hours/ week for four months by performing Nordic walking exercise while the other to maintain their habitual lifestyle. Blood glucose, VO_2 max, anthropometric measurements and a muscle biopsy was collected both before and after the intervention.*

	NGT			T2D		
	Pre-exercise	*Post-exercise*	*P value*	*Pre-exercise*	*Post-exercise*	*P value*
Weight (kg)	89.9 ± 2.0	88.5 ± 2.1	0.003*	94.1 ± 2.7	92.5 ± 2.7	0.018*
BMI (kg/m2)	28.7 ± 0.4	28.1 ± 0.4	0.001*	29.9 ± 0.8	29.5 ± 0.7	0.042*
Waist circumference (cm)	101.5 ± 1.6	98.0 ± 1.6	0.0001*	105.1 ± 1.6	103.0 ± 2.0	0.005*
FBG (mmol/l)	5.5 ± 0.1	5.4 ± 0.1	0.434	8.3 ± 0.5	8.01 ± 0.5	0.207
2-h BG (mmol/l)	7.0 ± 0.2	6.8 ± 0.2	0.392	15.1 ± 0.8	13.3 ± 1.0	0.006*

Table 1: Anthropometric measurements and metabolic parameters in normal glucose tolerant (NGT) and type 2 diabetic subjects (T2D). *Data are presented as means ± SEM. BMI indicates body mass index; BG, blood glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure. *Significant at P < 0.01 level*

4.1.4.4 Effect of exercise mimetics on cultured human skeletal muscle cells

In an attempt to dissect the influence of factors that are altered in response to exercise, mRNA expression of PDK4, PDK2, CPT1b and MCD was determined in cultured human myotubes after exposure to different agents including caffeine, AICAR and palmitate. These factors were selected to mimic some of the in vivo changes noted following exercising intervention. PDK4 mRNA expression was robustly increased after incubation with caffeine or palmitate (Study IV, Figure 5A). PDK2 mRNA expression was increased only after incubation with caffeine. CPT1b mRNA expression was markedly increased by palmitate exposure (Study IV, Figure 5B), while both caffeine or palmitate increased the MCD mRNA expression (Study IV, Figure 5C, D). However, mRNA expression of all four genes studied was unaltered after exposure to AICAR.

5 SUMMARY

The overall goal of this study was to identify and validate novel molecules involved in maintaining the metabolic flexibility of skeletal muscle.

- In **Study I**, the role of 5'-nucleotidases in maintaining overall skeletal muscle metabolism and energy homeostasis is highlighted. Targeting skeletal muscle 5'-nucleotidases (NT5C1A and NT5C2) could be one potential strategy to activate AMPK and thus mediate the beneficial effects of AMPK in promoting skeletal muscle metabolic flexibility.

- In **Study II**, evidence that MTX treatment results in inhibition of enzymes involved in nucleotide metabolism leading to a reduction in the threshold for AMPK activation; thus potentiating AICAR-stimulated AMPK activity is provided.

- In **Study III**, 11β-HSD1 is validated as a potential skeletal muscle anti-diabetic target. siRNA-mediated reduction of 11-βHSD1 prevents the effects of cortisone but not cortisol on metabolism via a PDK4-dependent mechanism in skeletal muscle.

- In **Study IV**, evidence that skeletal muscle expression of mitochondrial regulators of fatty acid metabolism reflects metabolic dysfunction in type 2 diabetes is provided. Skeletal muscle expression of PDK4 and related genes regulating mitochondrial function reflects alterations in substrate utilization and clinical features associated with type 2 diabetes mellitus. Furthermore, hypomethylation of the PDK4 promoter in skeletal muscle of type 2 diabetic patients was coincident with an impaired response of PDK4 mRNA after exercise.

6 CONCLUSIONS

The results presented in this thesis highlight novel molecular strategies to bypass impairments in skeletal muscle glucose and lipid metabolism and restore skeletal muscle insulin sensitivity (Figure 17).

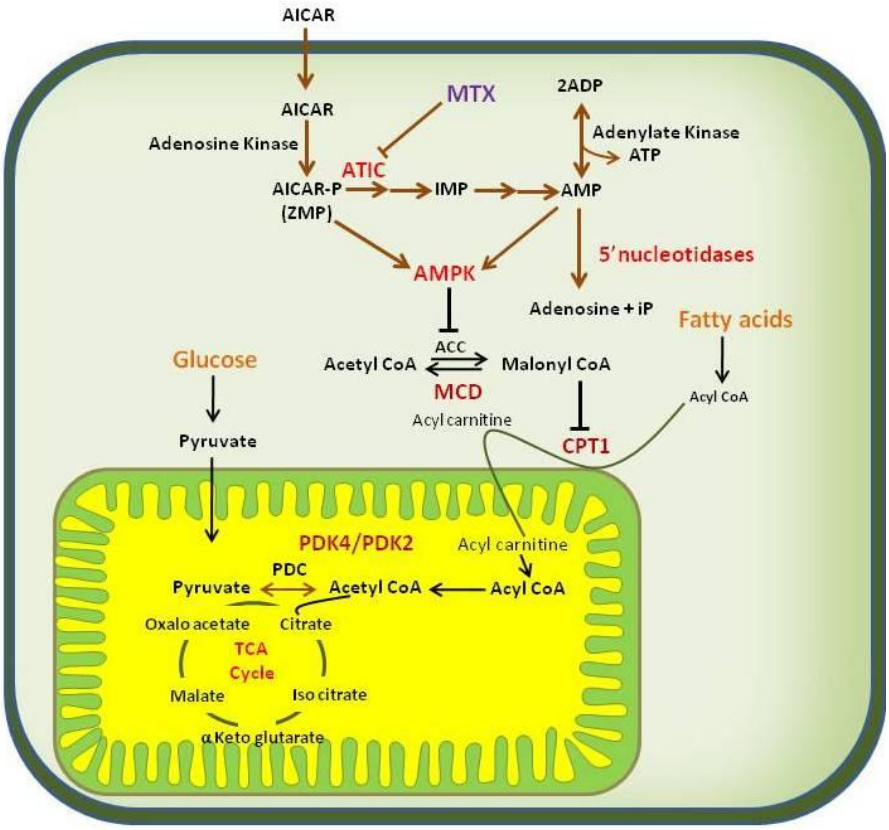

Figure 17: Summary of molecular mechanisms which maintain cellular glucose and lipid metabolism investigated in this thesis.

In skeletal muscle, AMPK can be activated by metabolic stress such as hypoxia, exercise and glucose deprivation. This thesis presents novel strategies to activate AMPK by altering select enzymes of nucleotide metabolism including 5'-nucleotidases and ATIC. Silencing of 5'-nucleotidases enzymes, NT5C1A and NT5C2 by RNAi technology activated the AMPK system in skeletal muscle by altering the cellular AMP: ATP ratio. Pre-treating the skeletal muscle with MTX rendered AMPK more sensitive to endogenous and/or exogenous activator [AICAR] via inhibition of ATIC. Our results indicate that targeting enzymes controlling specific steps in intracellular nucleotide metabolism could be a novel approach to activate the AMPK system and mediate beneficial metabolic effects.

Skeletal muscle mRNA expression of key enzymes involved in substrate metabolism, including PDK4, PDK2 and MCD are altered in skeletal muscle of type 2 diabetes mellitus patients. Increased expression of PDK4 mRNA was coincident with decreased PDK4 promoter methylation, indicative of altered epigenetic regulation. How altered

PDK4 promoter methylation affects muscle PDK4 expression requires further investigation. Low intensity exercise resulted in increased PDK4 mRNA expression only in healthy subjects, but not in type 2 diabetic patients reflecting inflexibility to adapt to exercise responses. Thus it is tempting to speculate that epigenetic alterations underlie the impaired exercise-induced response.

Evidence that PDK4 plays a pivotal role in mediating deleterious effects of glucocorticoid excess on skeletal muscle glucose metabolism is provided. Thus PDK4 plays a central role in directing muscle nutrient metabolism. Direct manipulation of PDK4 expression in human skeletal muscle is a challenging proposition. However, we have validated that inhibition of the enzyme 11β-HSD1, markedly reducing local glucocorticoid signaling, inhibits glucocorticoid-mediated induction of PDK4. Further studies are warranted to explore different approaches to regulating skeletal muscle PDK4 expression.

Taken together, results presented in this thesis provide evidence for novel mechanisms which act as entry points for therapeutic interventions for the treatment of insulin resistance and type 2 diabetes.

7 FUTURE PERSPECTIVES

The primary aim of this thesis was to investigate key molecular regulators involved in skeletal muscle glucose and lipid metabolism. Therapeutic strategies to enhance whole-body lipid or glucose metabolism may improve insulin sensitivity and energy homeostasis in type 2 diabetic patients.

The energy sensing enzyme AMPK has been the focus of many investigation and is considered an attractive anti-diabetic target. A major aim of this thesis was to delineate the mechanisms governing the AMPK activity in skeletal muscle. One challenge is to develop a drug that is specific to AMPK. The question remains whether a drug that specifically activates AMPK would yield therapeutic effects without having deleterious side effects. AICAR, the most widely used AMPK activator has positive effects on metabolism. However, this drug is a long way from clinical treatment of insulin resistance and type 2 diabetes, since it is not specific to AMPK and it activates several other kinases. One way to activate the AMPK system as highlighted in this thesis would be to alter the expression of AMP-metabolizing enzymes, such as 5-nucleotidases and ATIC. Silencing of 5'-nucleotidases, NT5C1A and NT5C2 in rodent muscle and human skeletal muscle cell culture respectively increased AMPK and ACC phosphorylation and enhanced glucose and lipid metabolism, indicating their role in restoring skeletal muscle energy homeostasis. These results provide proof-of-principle that skeletal muscle specific inhibitors of 5'-nucleotidases enzymes may be beneficial to improve metabolism in type 2 diabetes. Since this approach was skeletal muscle specific, deleterious cardiac effects could be avoided. This thesis investigated the transient effects of inhibition of 5´-nucleotidases. Future studies to assess the consequence of long-term inhibition of 5'-nucleotidases enzymes is warranted.

Studies in this thesis were designed to test hypothesis that the AMPK system can be activated by lowering the threshold of AMPK activation to render AMPK more sensitive to endogenous and/or exogenous activators. In this way, one could expect to have a maximum AMPK activation with administration of relatively lower dose of the activator compound and curtail unwanted side effects. This strategy has been partly demonstrated in this thesis by pre-treating skeletal muscle with MTX and inhibiting ATIC (Study II). MTX is used as an anti-rheumatic drug, and patients with MTX treatment have reduced cardiovascular mortality, improved metabolic profile and improved insulin sensitivity. Co-treatment with multiple chemical compounds that have complementary mechanisms of action or with compounds that elicit effects through different pathways, often provide insight towards more effective intervention. The development of more selective inhibitors of ATIC may improve efficacy of therapeutic intervention for treatment of obesity and diabetes.

A second focus of this thesis was to identify and validate metabolic "gate keeper" molecules; i.e. key molecules regulating the shift in substrate metabolism. In obesity, expression of 11β-HSD1 is increased and this can increase local glucocorticoid signaling. A central role of PDK4 in the cortisol-mediated shift in cellular metabolism was highlighted. The effects of glucocorticoids to increase lipid oxidation and reduce glucose metabolism appears to be dependent on induction of PDK4 expression.

Increased PDK4 expression would inhibit glucose oxidation and this may be an important adaptive mechanism to conserve glucose in the fasting state, when glucose is scarce. Insulin acts to suppress PDK4 in the fed states when glucose is abundant. Increases in muscle PDK4 expression with type 2 diabetes may be largely due to reduced insulin action (i.e. insulin resistance or relative insulin deficiency), rather than to increases in circulating FFAs (Kim et al., 2006). Whether an increase in expression is a cause or a consequence of insulin resistance remains to be determined. This key question remains to be addressed to further design therapeutic interventions against type 2 diabetes mellitus.

Physical activity and exercise have beneficial effects on substrate metabolism and play crucial role in prevention and management of type 2 diabetes. Nevertheless, exercise intervention may not be feasible for all subjects with type 2 diabetes, and in some cases, a combination of exercise and pharmacological intervention could prove to be efficacious. Thus further studies on skeletal muscle insulin sensitivity are necessary to validate novel targets for the development of drugs in attempt to combat the world wide epidemic of type 2 diabetes mellitus.

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