Thesis for doctoral degree (Ph.D.)
2007

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Stockholm 2007

Published by Karolinska Institutet. Printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 978-91-7357-152-4

To my family

ABSTRACT

Skeletal muscle fibers differ considerably in their metabolic and physiological properties. The metabolic properties of skeletal muscle display a high degree of flexibility which adapts to various physiological demands by shifting energy substrate metabolism. Studies were conducted to evaluate the roles of AMP-activated protein kinase (AMPK) and calcineurin in the regulation of skeletal muscle metabolism.

Fasting elicited a coordinated expression of genes involved in lipid utilization and glucose metabolism in white gastrocnemius muscle from wild-type mice. The fasting-induced transcriptional responses were impaired in the AMPKγ3 knockout (*Prkag3*$^{-/-}$) mice. Conversely, in mice transgenic for an activating mutant form of AMPKγ3(R225Q) (*Tg-Prkag3*^{225Q}), an enhanced expression of several fasting-responsive metabolic genes, and a reciprocal down-regulation of glycolytic genes was observed. The results support the role of AMPKγ3 subunit in the coordinated expression of fasting-responsive metabolic genes in skeletal muscle.

Exercise stimulated glucose uptake in EDL (extensor digitorum longus) muscles from wild-type, *Tg-Prkag3*^{225Q} and *Prkag3*$^{-/-}$ mice to the same degree. In *Tg-Prkag3*^{225Q} mice, elevated acetyl-CoA carboxylase phosphorylation, enhanced intramuscular triglyceride utilization and metabolic gene expression was observed after exercise. Conversely, an impaired gene expression was seen in the *Prkag3*$^{-/-}$ mice. Thus, the AMPKγ3 subunit is dispensable for exercise-stimulated glucose transport and the mutant AMPKγ3(R225Q) subunit promotes metabolic and gene expression adaptations in response to exercise.

Enhanced insulin-, but suppressed AICAR-induced glucose uptake was observed in EDL muscles from transgenic mice expressing an activated form of the calcium/calmodulin-dependent protein phosphatase calcineurin (MCK-CnA*). Impaired AMPK-activated glucose uptake was associated with a decrease in the expression of the AMPKγ3 subunit. Contraction-induced glucose uptake however was unaltered in MCK-CnA* mice, despite a decrease in contraction-induced AMPK phosphorylation. Therefore, calcineurin-induced skeletal muscle remodeling altered AMPK-activated glucose uptake.

An enhanced glucose incorporation into glycogen and a reciprocal suppression of glucose oxidation was seen in the EDL muscle from MCK-CnA* mice. These changes were accompanied by an increase in lipid oxidation and lactate release. The alterations in glucose partitioning were supported by a coordinated decrease in glycolytic genes and an elevation in *Pdk4* expression. Consistent with the increase in lipid oxidation, expression of lipid metabolic and mitochondrial genes were activated in EDL muscle from MCK-CnA* mice, concomitant with an induction of several transcription regulators including PPARα, PPARδ and PGC1α. Therefore, calcineurin altered skeletal muscle metabolism via coordinated changes in gene expression.

In conclusion, AMPK regulates skeletal muscle lipid and glucose metabolism, as well as gene regulatory responses to fasting and exercise. Calcineurin-mediated skeletal muscle remodeling alters the expression of AMPK subunits and AMPK-mediated glucose uptake. Furthermore, calcineurin alters lipid and glucose metabolism in skeletal muscle via coordinated changes in gene expression program. Therefore, the flexibility of lipid and glucose utilization in skeletal muscle is not only regulated at the level of covalent and allosteric modifications, but the reciprocity is also conserved at transcriptional level.

Key words: skeletal muscle, metabolism, glucose, lipid, gene expression, AMP-activated protein kinase, calcineurin, exercise

ISBN 978-91-7357-152-4

LIST OF PUBLICATIONS

I. **Long YC**, Barnes BR, Mahlapuu M, Steiler TL, Martinsson S, Leng Y, Wallberg-Henriksson H, Andersson L, Zierath JR. Role of AMP-activated protein kinase in the coordinated expression of genes controlling glucose and lipid metabolism in mouse white skeletal muscle. *Diabetologia* 48: 2354-2364, 2005.

II. Barnes BR*, **Long YC***, Steiler TL, Leng Y, Galuska D, Wojtaszewski JFP, Andersson L, Zierath JR. Changes in exercise-induced gene expression in 5'-AMP-activated protein kinase γ3-null and γ3 R225Q transgenic mice. *Diabetes* 54: 3484-3489, 2005. * Both authors contributed equally to this work.

III. Ryder JW, **Long YC**, Nilsson E, Mahlapuu M, Zierath JR. Effects of calcineurin activation on insulin-, AICAR- and contraction-induced glucose transport in skeletal muscle. *J. Physiol.* 567: 379-386, 2005.

IV. **Long YC**, Glund S, Garcia-Roves PM, Zierath JR. Calcineurin regulates skeletal muscle metabolism via coordinated changes in gene expression. *J. Biol. Chem.* 282: 1607-1614, 2007.

CONTENTS

LIST OF ABBREVIATIONS

ACADVL	Very long chain acyl-CoA dehydrogenase
ACC	Acetyl-CoA carboxylase
AICAR	5-aminoimidazole-4-carboximide-1-β-4-ribofuranoside
AMPK	AMP-activated protein kinase
CaMK	Calmodulin-dependent protein kinase
CPT	Carnitine palmitoyl transferase
CS	Citrate synthase
CYCS	Cytochrome C
DECR1	2,4-dienoyl CoA reductase 1
EDL	Extensor digitorum longus
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT	Glucose transporter
GYS	Glycogen synthase
HAD	3-Hydroxyacyl-CoA dehydrogenase
HK	Hexokinase
IMTG	Intramuscular triglyceride
IR	Insulin receptor
IRS	Insulin receptor substrate
LDH	Lactate dehydrogenase
LPL	Lipoprotein lipase
MCK-CnA*	Transgenic mice expressing activated calcineurin
MEF	Myocyte enhancer factor
MHC	Myosin heavy chain
NEFA	Non-esterified fatty acid
PDK4	Pyruvate dehydrogenase kinase 4
PFK	6-Phosphofrucktokinase 1
PGC1α	Peroxisome proliferator-activated receptor coactivator 1α
PI3-kinase	Phosphatidylinositol 3-kinase
PPAR	Peroxisome proliferator-activated receptor
Prkag3$^{-/-}$	AMPKγ3-knockout mice
Slca25a20	Mitochondrial carnitine/ acylcarnitine translocase
TCA	Tricarboxylic acid
Tg-Prkag3^{225Q}	Transgenic mice expressing mutant R225Q AMPKγ3
UCP3	Uncoupling protein 3

1 INTRODUCTION

Living organisms are challenged with irregular nutrient supply, and the ability to shift substrate utilization is critical for survival. Skeletal muscle accounts for about 75% of glucose disposal under hyperinsulinemic-euglycemic conditions and is a major tissue of lipid utilization. Given its total mass and energy consumption, skeletal muscle plays a critical role in the regulation of glucose homeostasis and lipid utilization. An impaired metabolic response of skeletal muscle is closely associated with metabolic diseases. Indeed, impaired insulin-stimulated skeletal muscle glucose uptake and increased intramuscular triglyceride accumulation are hallmark features of non-insulin dependent (type 2) diabetes. Several studies provided evidence for dysregulated expression of genes regulating skeletal muscle lipid metabolism and mitochondrial function in insulin resistant individuals (Petersen et al. 2003; Petersen et al. 2004). Therefore, an investigation of the signaling pathways and gene regulatory events that govern glucose and lipid metabolism is critical for the understanding of skeletal muscle metabolism and pathophysiology of metabolic diseases.

1.1 Contractile and metabolic properties of skeletal muscle fibers

Skeletal muscle fibers display considerable differences with respect to contraction, metabolism and endurance. Muscle fibers can be divided into two major categories based on specialized contractile and metabolic characteristics: fast-glycolytic fibers and slow-oxidative fiber. The classification of muscle fiber contractile properties is based on the myosin heavy chain (MHC) gene that is expressed. In adult rodent skeletal muscle, four major MHC namely type 1, 2A, 2X and 2B have been identified (Schiaffino et al. 1989). Fiber-type varies along a continuum of contraction rate, with type 1 being the slowest and 2B the fastest (Reiser et al. 1985; Bottinelli et al. 1994), however type 1 fibers are more fatigue-resistant when compared to type 2B fibers. The metabolic properties of muscle fibers also regulate the endurance capacity of skeletal muscle. Type 1 fibers express high level of oxidative enzymes and are enriched with mitochondrial proteins, in support of a slow, but long lasting supply of ATP to sustain prolonged recurring activity. Conversely, type 2B fibers have a high level of glycolytic enzymes to generate a rapid source of ATP from glycogen stores independent of oxygen, to support intermittent bursting movement, but compromising the endurance capacity. Although the expression of fiber-type-specific gene programs can be detected during embryonic myoblast development (DiMario et al. 1993; Ontell et al. 1993; Stockdale 1997), it remains malleable in adults by modification in response to contractile load (e.g., muscle contraction and exercise training), hormonal changes, or systemic diseases (Holloszy and Coyle 1984; Ianuzzo et al. 1991; Sabbah et al. 1993) to support shifts in physiological demands.

1.2 Skeletal muscle metabolic flexibility

1.2.1 Metabolic adaptations in response to fasting

The metabolic property of skeletal muscle is highly malleable and adapts to various physiological demands by shifting energy substrate metabolism. Glucose and lipids are the main oxidative fuel substrates in skeletal muscle, and their utilization is coordinated by complex regulatory mechanisms (Jeukendrup 2002; Frayn 2003). Glucose is the main energy substrate of skeletal muscle under fed conditions. However, substrate metabolism shifts from glucose to lipids under fasting conditions (Andres et

al. 1956), for glucose sparing to other organs, particularly the brain. The intimate, but reciprocal relationship between carbohydrate and fatty acid metabolism is described as the Randle cycle (Frayn 2003). Briefly explained, an increase in the glucose concentration induces insulin secretion, which suppresses non-esterified fatty acid release from adipose tissues. This reduces the competition of fatty acids for substrate utilization, and glucose becomes the major fuel. Under fasting conditions, when plasma glucose and insulin concentrations are low, this leads to an increase in the non-esterified fatty acid (NEFA) concentration, so that fatty acids are then the main energy substrate for skeletal muscle. In addition to the control at the level of substrate availability, increased fatty acid oxidation in skeletal muscle reduces glucose utilization, whereas high level of glucose and insulin suppresses fatty acid oxidation. Several fatty acid oxidation-derived metabolites inhibit the activity of glycolytic enzymes (Jeukendrup 2002). Elevation of acetyl-CoA suppresses the activity of pyruvate dehydrogenase (PDH), an increase in the citrate level inhibits phosphofructokinase (PFK), and an accumulation of glucose-6-phosphate (G6P) would in turn inhibit hexokinase 2 (HK2) (Figure 1). Conversely, insulin activates acetyl-CoA carboxylase (ACC) to suppress skeletal muscle fatty acid oxidation (Brownsey et al. 2006). These premises provide a second level of control via allosteric and covalent modifications.

The flexibility of carbon source utilization in skeletal muscle seems to be evolutionarily conserved. In *Saccharomyces cerevisiae*, glucose deprivation induces a shift from anaerobic metabolism of glucose, to aerobic metabolism of alternative carbon fuels (1-3), a critical survival adaptation that is modulated at transcriptional level. In skeletal muscle, the shift towards lipid utilization was supported by a coordinated increase in the expression of genes essential for lipid metabolism, such as lipoprotein lipase 1 (LPL1), carnitine palmitoyl transferase 1 (CPT1) and uncoupling protein 3 (UCP3) (Hildebrandt and Neufer 2000; Pilegaard et al. 2003a; de Lange et al. 2004). Such coordinated transcriptional activation may provide another level of fine-tuning to the balance of the glucose-fatty acid cycle, however the regulatory events remain largely uninvestigated.

1.2.2 Fuel selection in response to exercise

Endurance exercise training induces substantial metabolic and gene expression adaptations in skeletal muscle. The relative contribution of glucose and fatty acid to the energy demand during exercise varies with exercise intensity and duration, as well as the level of endurance training. Generally, oxidation of plasma NEFA is sufficient to meet the energy demand during mild exercise (~ 30% of VO_{2max}). During moderate-intensity exercise (~ 50-70% of $VO_{2max;}$ below lactate threshold), carbohydrate (from intramuscular glycogen and glucose) and fatty acids (from intramuscular triglycerides and plasma NEFA) oxidation contributes almost in equal proportion to the energy expenditure. Glucose (particularly from intramuscular glycogen) becomes the primary fuel during high-intensity exercise (~ 75-85% of $VO_{2max;}$ below lactate threshold) (Holloszy and Kohrt 1996).

During submaximal intense exercise, the contribution of fatty acid oxidation to total energy expenditure is greater in the trained state compared to the untrained state. Based on the principle of glucose-fatty acid cycle, this produces a glucose-sparing effect that results in a slower depletion of intramuscular glycogen stores, and delays the development of exhaustion. The glucose-sparing effect is one of the most critical mechanisms by which training increases the capacity to perform prolonged high-intensity exercise. Consistent with the increased energy input from fatty acid metabolism, endurance training elicits increased capacity for lipid metabolism in

skeletal muscle (Turcotte et al. 1992; Horowitz and Klein 2000; van Loon et al. 2001). This adaptation was associated with increased expression of genes involved in lipid metabolism, including LPL1 (Seip et al. 1995; Kiens et al. 2004), fatty acid transporter CD36 (CD36) (Bonen et al. 1999; Tunstall et al. 2002; Kiens et al. 2004), CPT1 (Tunstall et al. 2002) and activity of β-hydroxyacyl-CoA dehydrogenase (HAD) (Spina et al. 1996). Skeletal muscle glycogen synthesis is enhanced following glycogen-depleting exercise, in order to adapt to an increased energy demand (Bergstrom and Hultman 1966; Ren et al. 1994; Greiwe et al. 1999). In line with this observation, the expression of the glucose transporter 4 (GLUT4) (Kraniou et al. 2000) and hexokinase 2 (HK2) (Koval et al. 1998), two genes that are involved in glucose transport and glycogenesis, are also augmented by exercise training.

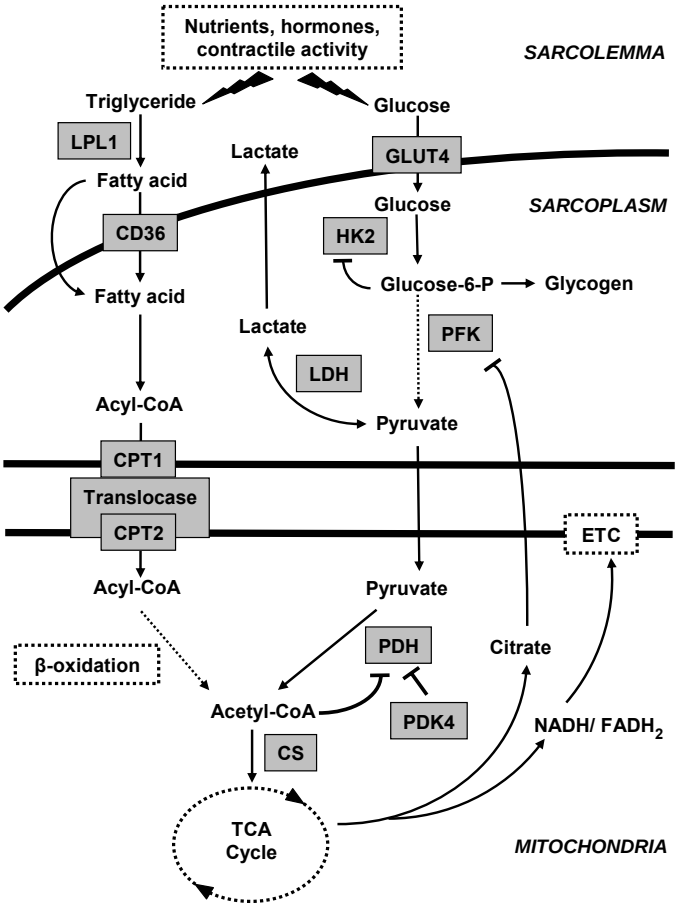

Figure 1: Regulation of glucose and fatty acid metabolism in skeletal muscle. Substrate-utilization is controlled at several levels. The availability and utilization of glucose and fatty acid (or triglyceride), and their selective transport across the sarcolemma is regulated by food intake, hormones and skeletal muscle contractile activity (such as exercise). Increased fatty acid oxidation generates metabolites such as acetyl-CoA and citrate, which inhibits PDH and PFK, respectively. Decreased PFK activity leads to an accumulation of G-6-P, which in turn inhibits HK2. Exercise and fasting induce a coordinated change in the expression of lipid metabolic genes, thus providing another level of fine-tuning for the influx of fatty acid into the TCA cycle. Such regulation ensures efficient energy production from the utilization of substrates, which itself is malleable to the whole-body physiological condition or demand.

1.2.3 Neural regulation of skeletal muscle metabolic properties

Signals from the motor neurons have a profound influence on the metabolic properties of skeletal muscle (Hughes 1998; Pette and Vrbova 1999). The impact of motor neuron activity on skeletal muscle has been emphasized by experiments that involved cross-innervation and chronic delivery of tonic low-frequency electrical impulses (that mimics the firing pattern of slow muscle fiber motor neurons) through implanted electrodes. Denervated fast-glycolytic skeletal muscle, when reinnervated with motor neurons of slow-oxidative muscle, displayed a fast-to-slow contractile property reprogramming (Schiaffino and Reggiani 1996). When skeletal muscle was stimulated by chronic low-frequency electrical impulses, the expression of genes essential for lipid utilization including *Lpl* (Hamilton et al. 1998), *Cd36* (Bonen et al. 1999), *Had* (Theriault et al. 1994) and citrate synthase (*Cs*) (Theriault et al. 1994; Nuhr et al. 2003) were activated. Moreover, genes that are critical for uptake and storage of glucose, such as glucose *Glut4* and *Hk2* are increased in skeletal muscle by electrical stimulation (Kong et al. 1994). Chronic low-frequency stimulation also induced a marked shift from MHC type 2B to type 1 fibers (Mayne et al. 1993; Windisch et al. 1998). Generally, the extent of adaptive changes induced by increased neuromuscular activity in response to physical training in humans or animals have led to less dramatic changes when compared to that of electrical stimulation. The major adaptations typically involved include increases in enzyme activities of oxidative metabolism, with undetectable or subtle changes (from type 2B to 2A) in MHC conversion. Although electrical stimulation represents an artificial model of neuromuscular activity (Pette and Vrbova 1999), this model provided compelling evidence that neural signals regulate metabolic gene expression in skeletal muscle.

1.2.4 Insulin-regulated skeletal muscle metabolism

Insulin plays a primary role in the control of skeletal muscle glucose and lipid metabolism. Apart from the regulation of NEFA availability, the hormone exerts direct effects on the metabolic signaling cascade in skeletal muscle. When insulin binds to α subunits of the insulin receptor, it stimulates autophosphorylation of tyrosine residues in the β subunits, which in turn phosphorylate intracellular proteins including the insulin receptor substrates (IRS) 1 through IRS4 (White 2003; Taniguchi et al. 2006). In cultured myotubes, IRS1 and 2 play specialized roles in the regulation of metabolic and mitogenic responses (Bouzakri et al. 2006), while IRS3 and 4 are not expressed in skeletal muscle. Phosphorylation of IRS on several critical tyrosine residues increases the binding of proteins that contains the *src* homology (SH) 2 domains, such as the phosphatidylinositol (PI) 3-kinase to IRS (White 2003; Taniguchi et al. 2006). Binding of PI3-kinase to IRS recruits the kinase close to the plasma membrane, and thereby increases its lipid substrate availability for the generation of second messengers including PIP3. The binding of PIP3 to the PH domain of 3-phosphoinositide-dependent protein kinase (PDK) activates the kinase, which in turn phosphorylates and activates AKT (also known as protein kinase B). Multiple effects of insulin-induced skeletal muscle substrate metabolism are mediated by AKT: (1) AKT phosphorylates the AKT substrate 160 kDa (AS160), which is critical for the translocation of GLUT4 to the plasma membrane to mediate glucose transport (Sano et al. 2003). (2) AKT inhibits glycogen synthase kinase 3 via direct phosphorylation (Cross et al. 1995) and decreases its inhibitory action towards glycogen synthase, which leads to an increase in the rate of glycogen synthesis. (3) AKT phosphorylates PFK and activates glycolysis (Deprez et al. 1997). (4) AKT phosphorylates and inhibits TSC2 (tuberous sclerosis complex-2), which leads to activation of the mTOR (mammalian target of rapamycin)

pathway for protein synthesis, including p70 S6 kinase and eukaryotic translation initiation factor 4E binding protein-1 (Um et al. 2006).

1.3 Exercise-induced skeletal muscle glucose transport

When glycogen is depleted after exercise, the subsequent accumulation of glycogen in response to a high carbohydrate-diet can far exceed levels found in well-fed sedentary subjects. This phenomenon is known as "glycogen supercompensation". Exercise or muscle contraction potently stimulates glucose transport in skeletal muscle, to meet the elevated demand of glucose for glycogen resynthesis (Holloszy and Kohrt 1996). The stimulatory effect of muscle contraction on glucose transport is substantial and parallels the magnitude of the insulin response to promote glucose uptake. Although muscle contraction and insulin exposure stimulate GLUT4 translocation to the plasma membrane to accelerate glucose transport, the two stimuli elicit glucose transport through separate signaling pathways.

In isolated skeletal muscle, contraction-induced glucose uptake is additive to the maximal effect of insulin on glucose uptake (Nesher et al. 1985; Zorzano et al. 1986; Wallberg-Henriksson et al. 1988). Furthermore, exercise/muscle contraction did not appear to activate the IR (Goodyear et al. 1995; Koval et al. 1999), IRS1(Goodyear et al. 1995; Koval et al. 1999), PI3-kinase (Goodyear et al. 1995; Koval et al. 1999), or AKT (Brozinick and Birnbaum 1998; Widegren et al. 1998). Furthermore, an inhibition of PI3-kinase by wortmannin abolished insulin-, but not contraction-induced skeletal muscle GLUT4 translocation (Lund et al. 1995) and glucose transport (Lee et al. 1995; Lund et al. 1995; Yeh et al. 1995). Insulin and muscle contraction have also been proposed to recruit different intracellular pools of GLUT4-containing vesicles (Douen et al. 1990; Coderre et al. 1995). Recent studies however have suggested that the two signaling pathways may converge at the point of AS160. Contraction and insulin induced phosphorylation of AS160 (Kramer et al. 2006; Treebak et al. 2006), and their effects were additive(Kramer et al. 2006), in line with the additive effects of both stimuli on glucose transport. In contrast to our better understanding of signaling pathways governing insulin-stimulated glucose uptake, little is known about the effectors by which contraction stimulates this response.

1.4 Exercise enhances insulin-stimulated glucose uptake

Despite the divergence in the signaling intermediates of pathways regulating exercise- and insulin-induced glucose uptake, exercise training has long been recognized to enhance insulin-stimulated glucose uptake in skeletal muscle. One of the proposed mechanism for such an improvement is the increased in GLUT4 protein after endurance exercise (Ren et al. 1994; Greiwe et al. 1999), such that more GLUT4 is available for translocation to the plasma membrane (Ren et al. 1994; Kawanaka et al. 1999; Kawanaka et al. 2000). Several studies showed that maximal insulin- and contraction-induced glucose uptake was increased in proportion to the elevation in GLUT4 protein (Ren et al. 1994; Host et al. 1998; Kawanaka et al. 1999; Kawanaka et al. 2000). However, protein synthesis inhibition did not prevent increase in insulin-induced glucose uptake observed 2-4 h after exercise, suggesting that GLUT4 is not essential for the acute effect of exercise to enhance insulin sensitivity (Fisher et al. 2002).

The enhancement in insulin-stimulated glucose uptake after exercise training is not limited to the increase in GLUT4, and may also be related to improvements in insulin signal transduction of skeletal muscle. Regular exercise led to marked increase

in IRS1 tyrosine phosphorylation and IRS-1-associated PI3-kinase activity, despite a reduction in IRS-1 protein expression (Chibalin et al. 2000). A similar enhancement in the improvement of IRS-1-associated PI3-kinase activity was also observed in human skeletal muscle after regular exercise (Kirwan et al. 2000). Therefore, the improvement in insulin signaling could be mediated by a more efficient signal transduction via IRS-1. Insulin-stimulated IRS-2 associated PI3-kinase activity was also increased after exercise (Chibalin et al. 2000; Howlett et al. 2002) and this effect was impaired in IRS-2-knockout mice (Howlett et al. 2002). However, insulin-stimulated skeletal muscle glucose uptake after exercise was not different between IRS-2-knockout and wild-type mice (Howlett et al. 2002). These results suggest that IRS-1 and 2 may have specialized role in the transduction of insulin signal in response to exercise. Regular exercise training also enhanced insulin-stimulated PI3-kinase activity (Houmard et al. 1999; Chibalin et al. 2000; Kirwan et al. 2000; Tanner et al. 2002) and Akt phosphorylation (Chibalin et al. 2000), suggesting an enhancement at multiple steps of the insulin signaling pathway can contribute to the overall exercise-induced improvements in metabolism.

1.5 Fuel selection and insulin resistance in skeletal muscle

Increased intramuscular triglyceride accumulation is closely associated with insulin resistance. In lean and obese individuals, IMTG content was inversely related to insulin sensitivity (Goodpaster et al. 1997; Pan et al. 1997; Greco et al. 2002), and the association was maintained even after corrected for visceral adiposity (Goodpaster et al. 1997; Pan et al. 1997). Whether increased triglyceride content is a cause or effect of insulin resistance remains unclear. However, when the plasma free fatty acid level was acutely altered via experimental perturbation, there were corresponding changes in the IMTG concentration with the development of insulin resistance (Boden et al. 2001), indicating IMTG as a causal factor for insulin resistance.

Skeletal muscle of healthy lean subjects switches fuel utilization from lipid oxidation under fasting conditions (Andres et al. 1956) to glucose utilization with a concomitant suppression of lipid oxidation under insulin-stimulated conditions (Kelley et al. 1990). In obese individuals and individuals with type 2 diabetes, the metabolic flexibility of fuel selection was severely impaired (Kelley and Mandarino 1990; Kelley et al. 1999). Under fasting conditions, skeletal muscle fatty acid oxidation failed increase in these individuals; instead, a high rate of glucose oxidation was observed compared to the insulin-sensitive subjects. Furthermore, while there was a sharp increase in glucose oxidation under insulin-stimulated condition in the lean control subjects, such an increase was blunted in the obese and diabetic subjects, with a lower rate of glucose oxidation observed in diabetic individuals. The failure to turn on fatty acid oxidation in obese and diabetic subjects may be a key mechanism leading to IMTG accumulation (Kelley and Mandarino 2000).

1.6 AMP-activated protein kinase as an energy sensor

The ability of skeletal muscle to adapt in response to metabolic demands requires a signal integrator that is capable of sensing the energy state and to activate downstream adaptive events. AMP-activated protein kinase (AMPK) has been implicated in the transduction of such metabolic signals. An important feature of AMPK as a putative signal transducer for skeletal muscle metabolic adaptations is its capacity to monitor and response to the cellular energy status. AMPK is a heterotrimeric complex composed of a catalytic α, and regulatory β and γ subunits (Kemp et al. 2003; Carling 2004; Hardie 2004) (Figure 1). Each α and β subunits are

encoded by distinctive genes (α1, α2 and β1, β2), whereas the γ subunit is encoded by three genes (γ1, γ2 and γ3). AMPK is activated by an increase in the ratio of AMP:ATP within the cell, and therefore it functions as an efficient metabolic sensor. Binding of AMP to the γ-subunit activates AMPK allosterically, and promotes the phosphorylation of threonine residue (Thr-172) within the activation domain of α subunit by an upstream kinase, the tumor suppressor LKB1 (Carling 2004; Hardie 2004; Kahn et al. 2005). This phosphorylation is further sustained by an inhibitory effect of AMP on dephosphorylation at Thr-172 by protein phosphatases (Davies et al. 1995). The marked sensitivity of AMPK to the AMP:ATP ratio is also conferred by an antagonistic effect of high ATP on the AMP-mediated activation of AMPK. Recent studies have identified the calmodulin-dependent protein kinase kinase (CaMKK) as an additional upstream kinase of AMPK (Hawley et al. 2005; Hurley et al. 2005; Woods et al. 2005). Activation of AMPK by CaMKK is stimulated by an increase in intracellular calcium ions, which appears to be independent of changes in AMP:ATP ratio (Hawley et al. 2005). Although CaMKK is highly expressed in the central nervous system, lower levels are detected in other tissues such as skeletal muscle and liver, suggesting that the regulation of the AMPK pathway is likely to be tissue-specific (Birnbaum 2005).

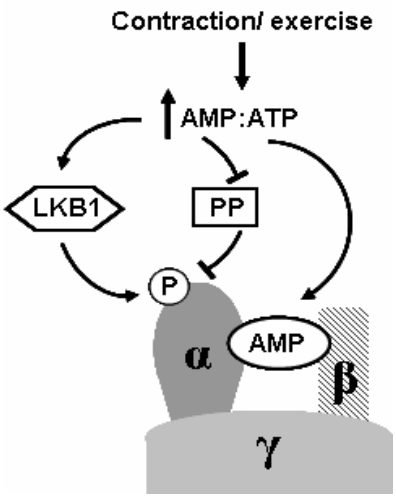

Figure 2: Putative mechanism for the activation of AMPK in skeletal muscle. Contraction or exercise increases energy utilization in skeletal muscle, leading to increase in AMP: ATP ratio. Binding of AMP to the γ-subunit of AMPK activates the kinase allosterically, and enhances the phosphorylation of the α subunit at Thr-172 by upstream kinase LKB1, which is also activated by AMP. Phosphorylation at Thr-172 is necessary for the activation of AMPK. Binding of AMP to AMPK also decreases the susceptibility of dephosphorylation at Thr-172 by phosphatases, thereby amplifying the sensitivity of AMPK to energy state.

1.7 AMPK and skeletal muscle glucose and lipid metabolism

1.7.1 AMPK and skeletal muscle glucose uptake

During exercise and contraction, energy stores in skeletal muscle are markedly depleted and there is a concomitant increase in glucose uptake and fatty acid oxidation to meet the rise in energy demand. Nonetheless, the intracellular events that trigger such acute metabolic response remain largely uncharacterized. The AMPK system is a putative signaling pathway for contraction-induced skeletal muscle glucose uptake and lipid oxidation because it is activated in response to an energy deficit, such as an elevation in AMP and a reduction in creatine phosphate energy stores (Hutber et al. 1997). Treatment of skeletal muscle with 5-aminoimidazole-4-carboxamide riboside (AICAR, a pharmacological activator of AMPK) *in vitro* increased glucose uptake (Merrill et al. 1997), concomitant with GLUT4 fusion with plasma membrane (Kurth-Kraczek et al. 1999). AICAR-stimulated glucose uptake was unaffected by inhibition of the insulin-dependent PI3-kinase pathway, and was additive to insulin-induced glucose

uptake. These characteristics mirror that of contraction-induced glucose uptake. Nonetheless, in rat slow-oxidative soleus muscle, AICAR had no stimulatory effect on glucose uptake, despite an increase in phosphorylation of AMPK (Wright et al. 2005). Furthermore, in rat soleus muscle, contraction induced glucose uptake without any apparent increase in AMPK activity when glycogen content was elevated (Derave et al. 2000). Given that AICAR induces glucose uptake concomitant with AMPK phosphorylation in fast-glycolytic rat epitrochlearis muscle, AMPK-mediated skeletal muscle glucose uptake is likely to be fiber-type dependent. Whether the differential effects of AMPK activation on fast- and slow-twitch skeletal muscle glucose uptake is due to distinctive expression pattern of the various AMPK subunit isoforms is unknown.

Part of the challenge to directly link AMPK with contraction-induced glucose transport has been due to a lack of specific inhibitor of this kinase. Moreover, earlier studies have been correlative in nature. Various genetically modified mouse models of AMPK have improved our understanding of the AMPK system in contraction-induced glucose uptake. Overexpression of a dominant negative $\alpha 2$ subunit in skeletal muscle blunted AICAR-, but only modestly impaired contraction-induced glucose transport (Mu et al. 2001). Moreover, genetic knockout of AMPK $\alpha 2$ and $\gamma 3$ subunit did not impair contraction-stimulated glucose uptake, although the AICAR effect was abolished (Barnes et al. 2004; Jorgensen et al. 2004; Fujii et al. 2005). In the skeletal muscle-specific LKB1 knockout mice however, both AICAR- and contraction-induced glucose uptake was impaired (Sakamoto et al. 2005). Thus, AMPK mediates the effects of AICAR on glucose uptake; however, its role in contraction-induced glucose uptake remains uncertain (Figure 3).

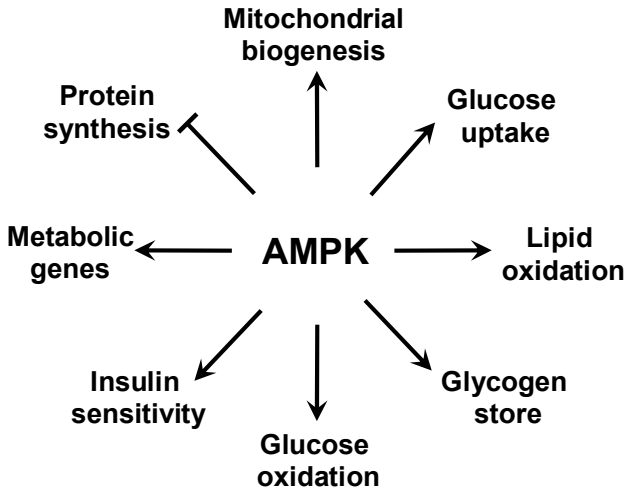

Figure 3: The effects of AMPK activation in skeletal muscle. Activation of AMPK induces lipid oxidation, glucose uptake and glucose oxidation in skeletal muscle. Conversely, AMPK inhibits skeletal muscle protein synthesis. These acute metabolic responses aim to restore energy balance rapidly by inducing pathways that produces energy and block biochemical processes that consume energy. Chronic activation of AMPK leads to mitochondrial biogenesis, increased insulin sensitivity, enhanced metabolic gene expression, as well as elevated glycogen content in skeletal muscle. These long-term modifications allow skeletal muscle to adapt and respond efficiently to future metabolic challenges.

1.7.2 AMPK signaling in skeletal muscle fatty acid oxidation

An increase in lipid oxidation during exercise is a critical adaptation for skeletal muscle to generate ATP to meet the energy demand of the working muscle, and AMPK has been proposed to mediate this effect. During exercise (Winder and Hardie 1996) and muscle contraction (Hutber et al. 1997), AMPK phosphorylates and inhibits acetyl-CoA carboxylase (ACC) (Winder and Hardie 1996; Winder et al. 1997), an enzyme that controls the synthesis of malonyl CoA (Trumble et al. 1995), an allosteric inhibitor of CPT1 (Ruderman et al. 1999). Inhibition of ACC reduces synthesis of malonyl-CoA, thereby relieving the inhibition of CPT1, such that the transfer of long-chain acyl-CoA into the mitochondria for β-oxidation is increased (Merrill et al. 1997; Merrill et al. 1998). In mice that are deficient in ACC2 (the predominant isoform in skeletal muscle and heart) (Brownsey et al. 2006)), there was an elevation in fatty acid oxidation that was insensitive to the inhibitory effect of insulin (Abu-Elheiga et al. 2001). These results underscore the central role of the AMPK-ACC system in the regulation skeletal muscle fatty acid oxidation. Given the close association between insulin sensitivity and IMTG deposition, activation of fatty acid oxidation by the AMPK-ACC system to lower intramuscular triglyceride deposition is considered an important feature for the insulin-sensitizing effect of AMPK activation.

1.7.3 AMPK and skeletal muscle gene expression

Besides acute metabolic responses such as glucose uptake and lipid oxidation, endurance exercise training induces chronic adaptations including enhanced skeletal muscle insulin sensitivity (Zierath 2002; Holloszy 2005), mitochondrial biogenesis, and the oxidative capacity (Holloszy 1975). Although the chronic adaptations to exercise have long been recognized, the signaling pathways that mediate such responses remain uncharacterized. Given that AMPK is activated during exercise, and activation of AMPK produces several aspects of exercise-induced adaptations, several lines of evidence supports its role in the regulation of skeletal muscle gene expression (Zierath 2002; Holloszy 2005).

Long-term *in vivo* AICAR treatment increased skeletal muscle GLUT4 and HK2 protein content (Holmes et al. 1999; Song et al. 2002) and enhanced insulin-stimulated glucose transport (Buhl et al. 2001; Iglesias et al. 2002) as well as GLUT4 translocation (Buhl et al. 2001). When skeletal muscles were studied *in vitro*, activation of AMPK by hypoxia or AICAR similarly enhanced insulin sensitivity for glucose transport (Fisher et al. 2002), suggesting the effect could be achieved in a cell autonomous manner, and excluded the influence of systemic factors. These changes could be explained in part by alterations in gene expression. Activation of AMPK induced the expression of GLUT4 via increased expression of transcription factors of the GLUT4 gene promoter (Ojuka et al. 2002), myocyte enhancer factor 2 (MEF2). Furthermore, activation of AMPK via diet-induced chronic energy deprivation (via increase in AMP:ATP ratio) in rodents induced skeletal muscle mitochondrial biogenesis (Bergeron et al. 2001; Zong et al. 2002), concomitant with increased NRF-1 activity (Bergeron et al. 2001) and PGC1 content (Zong et al. 2002), both which are critical transcriptional regulators for mitochondrial gene expression (Puigserver and Spiegelman 2003). The direct role of AMPK in these adaptations was supported by the findings that overexpression of a dominant negative form of AMPK α2 subunit abolished these effects (Zong et al. 2002).

Nevertheless, activation of AMPK by chronic AICAR administration only selectively increased activity of a few mitochondrial enzymes in rat skeletal muscle, an effect that is less extensive compared to exercise (Winder et al. 2000). Furthermore, exercise-induced gene expression is unaltered in the α1- and α2-knockout mice

(Jorgensen et al. 2005). However, in skeletal muscle of the α1- and α2-knockout mice, elevated expression of the other α subunit was observed, and it is unclear whether such expression has any compensatory effects that would have obscured the results. In contrast to the activating effects of exercise on skeletal muscle mitochondrial biogenesis and lipid metabolic gene expression, chronic AICAR treatment was without effect on the expression of genes for lipid utilization. Thus, the role of AMPK in the regulation of skeletal muscle gene expression is clearly unresolved.

1.8 Role of AMPK γ3 subunit in skeletal muscle metabolism

Although AMPK is ubiquitously expressed in multiple tissues, the regulatory γ3 subunit is the predominant isoform expressed in skeletal muscle, and specifically fast-glycolytic muscle (Mahlapuu et al. 2004). In contrast, the expression of the γ1 and 2 subunits display wide tissue distribution. Based on immunoprecipitation experiments, the γ3 subunit appears to associate predominantly with the α2 and β2 subunits (Mahlapuu et al. 2004). The selective association of these subunits is of particular interest because genetic knockout of either α2 or γ3 subunits both abolished AICAR-induced glucose uptake, suggesting the central role of the α2β2γ3 heterotrimer in mediating the AICAR effect. Furthermore, in skeletal muscle from pigs carrying a naturally occurring mutation of γ3 (R225Q), there is an elevation in glycogen content (Estrade et al. 1993; Milan et al. 2000), as well as activity of CS and HAD (Estrade et al. 1993; Lebret et al. 1999). In skeletal muscle of rats treated chronically with AICAR, the enhancement in glucose uptake and GLUT4 expression was limited to fast-glycolytic muscles including epitrochlearis, EDL and white gastrocnemius, and was undetected in slow-oxidative muscles, such as soleus and red gastrocnemius (Buhl et al. 2001). The lack of an AICAR response on glucose uptake was also observed in rat soleus muscle (Wright et al. 2005). Thus, the AMPK γ3 subunit is likely to play a specific role in fast-glycolytic muscle metabolic responses. Thus, the expression of γ3 subunit may be altered by fast-to-slow twitch muscle reprogramming, which in turn could alter AMPK-mediated metabolic responses. Given the distinctive contractile and metabolic characteristics of fast-glycolytic myofibers, the role of γ3 subunit in this muscle type deserves special consideration.

1.9 Calcineurin and skeletal muscle contractile and metabolic properties

1.9.1 Calcineurin as a sensor of neuronal activity

Fast-glycolytic and slow-oxidative myofibers express distinctive sets of genes that regulate contraction and substrate metabolism in skeletal muscle (Booth and Thomason 1991; Pette and Staron 2001). A central role of motor neuron activity in the regulation of fiber-type-specific programs of gene expression was established by earlier studies involving cross-innervation and electrical stimulation (Vrbova 1963; Williams et al. 1986; Dirk Pette 1999). Slow fibers have been suggested to maintain a higher concentration of cytosolic calcium due to more frequent neuronal stimulation (Hennig and Lomo 1985; Williams et al. 1986; Sreter et al. 1987), but the mechanisms by which calcium may transduce the neuronal signal to changes in skeletal muscle gene expression remains unclear.

Calcineurin is a heterodimeric protein phosphatase (PP2B) composed of a calmodulin-binding catalytic A subunit and a calcium-binding regulatory B subunit that has been proposed to act as a calcium sensor to couple neuronal signals to the

activation of slow-oxidative fiber-specific gene program (Chin et al. 1998; Bassel-Duby and Olson 2006). The binding of calcium to calmodulin activates calcineurin via the regulatory subunit (Dolmetsch et al. 1997), allowing it to dephosphorylate the nuclear factor of activated T-cells (NFAT), a major downstream substrate of calcineurin. Upon dephosphorylation, NFAT translocates from the cytoplasm into the nucleus where it binds to consensus DNA sequences of gene promoter region, as well as other transcription factors including MEF2 and GATA-2 for activation of target genes (Chin et al. 1998; Olson and Williams 2000; Wu et al. 2000). Calcineurin is expressed in fast- and slow-twitch myofibers, and the ability of calcineurin to discriminate the calcium signal evoked by different motor neuron activity patterns is central for the selective activation of the slow fiber gene program. Motor neurons that innervate slow-twitch myofibers induce low (100 – 300 nM) but sustained levels of intracellular calcium, a pattern proposed to activate calcineurin (Chin et al. 1998; Bassel-Duby and Olson 2006). Conversely, motor neuron activity of fast-twitch myofibers stimulate transient high calcium levels (~ 1μM) that are predicted to be insufficient to activate calcineurin-mediated signaling (Timmerman et al. 1996; Dolmetsch et al. 1997).

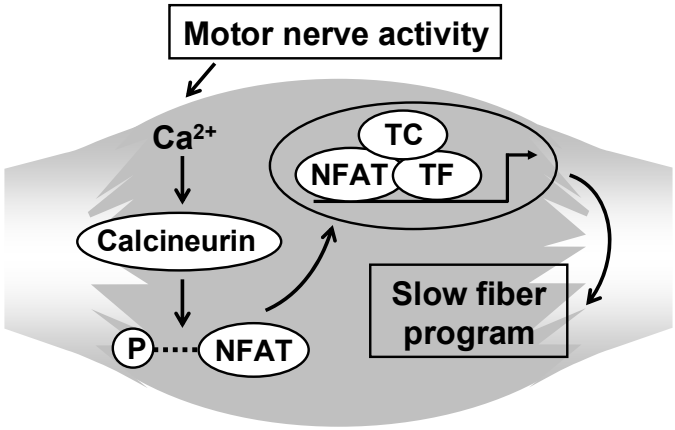

Figure 4: Schematic representation of calcineurin pathway in skeletal muscle. Motor neuron activity induces an elevation in intracellular calcium ions, which activates calcineurin. Activated calcineurin dephosphorylates NFAT, thereby inducing its translocation from the cytoplasm into the nucleus. In the nucleus, NFAT binds to the promoter regions of slow-fiber specific genes in cooperation with other transcription factors (TF) or coactivators (TC), resulting in transcriptional activation of the slow-fiber program.

1.9.2 Activation of the slow fiber program by calcineurin

Beside the ability to discriminate different neuronal firing pattern, the specificity of the calcineurin-induced slow fiber program is also conferred by the selectivity of calcineurin-dependent transcriptional activation. Transfection of a constitutively active form of calcineurin in cultured myotubes activated the promoters of myoglobin and troponin I (Chin et al. 1998), two genes that are preferentially expressed in slow-oxidative myofibres. The activation of the slow-fiber-specific promoters by calcineurin requires NFAT binding-consensus sequence motifs, suggesting a role for NFAT in mediating the transcriptional activation (Chin et al. 1998). In contrast, activation of calcineurin was without effect on the promoter of

muscle creatine kinase (Chin et al. 1998), a gene that is selectively expressed in fast-glycolytic fibers. Transgenic expression of an activated form of calcineurin in fast-glycolytic skeletal muscle induces fast-to-slow skeletal muscle reprogramming *in vivo*, with increased slow myosin ATPase activity (Naya et al. 2000). In addition, these mice display increased expression of slow-fiber genes such as myoglobin, troponin I and sarcomeric mitochondrial creatine kinase, and a reciprocal suppression of fast-fiber genes including parvalbumin and muscle creatine kinase (Naya et al. 2000). Conversely, in mice lacking both isoforms of calcineurin A (α and β), there is a marked reduction in the slow-oxidative fiber program in both slow and fast-twitch muscle (Parsons et al. 2003). A similar reduction in slow-oxidative fibers is also observed in skeletal muscle-specific calcineurin β1 knockout mice (Parsons et al. 2004).

The utilization of cyclosporine A, an antagonist of calcineurin and immunosuppressant currently in clinical use, has provided substantial evidence for a role of calcineurin in the maintenance of the skeletal muscle slow-fiber program. In rats treated with cyclosporin A, there was a suppressed slow myosin expression and a reciprocal increase in fast myosin expression, showing a remodeling of the slow-to-fast contractile phenotype (Chin et al. 1998; Bigard et al. 2000). In skeletal muscle of mice lacking calsarcin-1 (a protein inhibitor of calcineurin), there is an increase in type 1 myosin heavy-chain expression, concomitant with an elevation in calcineurin activity (Frey et al. 2004).

1.9.3 Calcineurin signaling in skeletal muscle metabolism

Although several lines of evidence support calcineurin as an activator of the slow-fiber program, much work has focused on contractile property conversion, with little emphasis on metabolic properties as a marker for skeletal muscle remodeling. If remodeling of skeletal muscle to express slower and more energy-efficient contractile proteins is to cope with the physiological demand, it is likely that a compatible metabolic reprogramming would be elicited to meet the energy cost. Mice that overexpress an activated form of calcineurin in fast-twitch muscle (EDL) displayed increased protein content of IR, Akt and GLUT4, resulted in elevated skeletal muscle glucose uptake and insulin sensitivity, characteristic of a slow-twitch skeletal muscle (Ryder et al. 2003). Inhibition of calcineurin by treatment of cyclosporine A in rats led to decreased proportion of slow myosin heavy chain, and an increase in activities of metabolic enzymes including cytosolic creatine kinase and lactate dehydrogenase (LDH) that are predominantly expressed in fast-twitch fibers (Bigard et al. 2000). However, inhibition of calcineurin activity by overexpression of a protein inhibitor of calcineurin, RCAN1 (also known as MCIP-1), results in loss of type I fiber without alteration in oxidative capacity and mitochondrial content in skeletal muscle (Oh et al. 2005). The role of calcineurin in regulation of skeletal muscle metabolism remains largely unknown.

2 AIMS

Skeletal muscle plays an important role in the maintenance of whole-body energy homeostasis. Glucose and lipid are two major energy substrates for skeletal muscle, which utilization is governed by multiple factors such as food intake, hormones and exercise. Impaired glucose and lipid metabolism in skeletal muscle is associated with metabolic diseases such as type 2 diabetes, and investigation of skeletal muscle metabolism would enhance our knowledge of skeletal muscle physiology, disease pathophysiology, and conceivably lead to novel therapeutic and preventive strategies. Thus, the overall objective of this thesis is to identify and validate signaling pathways that regulate skeletal muscle metabolism. Studies were conducted to explore the roles of AMPK and calcineurin in the regulation of metabolic events in skeletal muscle.

The specific questions that were raised are:

- Does the AMPK γ3 subunit play a role in the coordinated expression of genes essential for glucose and lipid metabolism in white skeletal muscle?
- Is the AMPK γ3 subunit involved in exercise-induced metabolic and gene regulatory responses?
- Does calcineurin activation alter insulin-, AICAR- and contraction-stimulated glucose uptake in skeletal muscle?
- Is skeletal muscle glucose and lipid metabolism regulated by a coordinated alteration in gene expression in response to activated calcineurin?

3 EXPERIMENTAL PROCEDURE

3.1 Mouse models

All animals used in the studies were maintained on a 12-h light-dark cycle and allowed free access to water and standard rodent chow. Mice were anesthetized via intraperitoneal injection of 2.5% avertin (0.02ml/g of body weight), and EDL, soleus and gastrocnemius muscles were removed for experiments or analysis. Mice were studied in the fed or fasted (16 h) conditions, and were humanely killed by cervical dislocation immediately after muscle dissection. The generation of mutant transgenic AMPK γ3 R225Q (*Tg-Prkag3*^{225Q}) and AMPK γ3 knockout (*Prkag3*$^{-/-}$) mice has been described previously (Barnes, 2004). The mutant AMPK γ3 R225Q transgene is driven by the promoter for skeletal muscle myosin light chain (MLC), with SV40 poly A signal and MLC1 enhancer inserted at 3' UTR. Traditional gene targeting techniques were utilized for the generation of *Prkag3*$^{-/-}$, with knockout targeting construct that caused major frameshifts and premature stop codon. A colony of transgenic mice expressing an activated form of calcineurin driven by skeletal muscle creatine kinase promoter/enhancer was established using MCK-CnA* mice (a kind gift from Dr. Eric N. Olson, University of Texas Southwestern Medical Center, Dallas). Male *Tg-Prkag3*^{225Q} and *Prkag3*$^{-/-}$ mice were used in Paper I and II, while female MCK-CnA* mice were used in Paper III and IV. The Ethics Committee on Animal Research in Northern Stockholm approved all experimental procedures.

3.2 RNA purification and cDNA synthesis

Skeletal muscle was homogenized in Trizol reagent (Sigma) and RNA was purified according to recommendations of the manufacturer. Purified RNA was subjected to DNAse treatment, using DNA-free kit (Ambion, Huntingdon, Cambridgeshire, UK) according to the manufacturer's protocol. DNAse-treated RNA was used for cDNA synthesis by using SuperScript First Strand Synthesis System (Invitrogen, Carlsbad, CA) with oligo (dT) primers. A reaction without reverse transcriptase was included for each sample as reverse transcriptase-minus control.

3.3 Quantitative real-time PCR

The quantity of cDNA for each gene transcript was determined by using real-time PCR with the ABI PRISM 7000 Sequence Detector System and fluorescence-based SYBR-green technology (Applied Biosystems, Warrington, UK). PCR was carried out in a final volume of 25 µl consisting of diluted cDNA sample, 1x SYBR-green PCR Master Mix (Applied Biosystems), primers optimized for each target transcript and water. All samples were analyzed in duplicate. Relative abundance of target transcripts were calculated from duplicate samples after normalization of the data against a housekeeping gene (acidic ribosomal phosphoprotein PO) using the standard curve method. Primers were designed by using Primer Express computer software (Applied Biosystems). Transcript sequences obtained from ENSEMBL database were *Cpt1* (ENSMUST00000023287), *Cd36* (ENSMUST00000003024), *Cs* (ENSMUST00000005826), GLUT4 (*Slc2a4*, ENSMUST00000018710), glycogen synthase (ENSMUST00000003964), short chain HAD (*Hadsc*) (ENSMUST00000029610), *Lpl* (ENSMUST00000015712), muscle 6-

phosphofructokinase (*Pfkm*, ENSMUST00000043950), pyruvate dehydrogenase kinase 4 (*Pdk4*, ENSMUST00000019721), *Ucp3* (ENSMUST00000032958), and NCBI Genbank database were acidic ribosomal phosphoprotein PO (BC003833), aldolase A (*Aldoa*, NM007438), *Cpt2* (NM009949), cytochrome C (NM007808), 2,4-dienoyl CoA reductase 1 (*Decr1*, NM026172), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, BC095932), hexokinase II (Y11666), lactate dehydrogenase (*Ldh*, NM008492), mitochondrial carnitine/ acylcarnitine translocase (*Slc25a20*, NM020520), *PPARα* (NM011144), *PPARδ* (NM011145) and *PPARγ* (NM011146), and very long chain acyl-CoA dehydrogenase (*Acadvl*, NM017366).

3.4 Muscle incubations

Incubation media were prepared from pre-gassed (95% O2, 5% CO2) Krebs-Henseleit buffer (KHB) supplemented with 5 mM HEPES and 0.1% bovine serum albumin (radioimmunoassay grade). EDL and/ or soleus muscles were excised and incubated at 30°C in a shaking water bath under a constant gas phase of 95% O2 and 5% CO2, unless stated otherwise.

3.5 In vitro muscle contraction

Muscles were positioned between two platinum electrodes with the distal tendon fixed to the bottom of an incubation chamber containing 4 ml of KHB maintained at 30°C. The proximal tendon was fixed to an isometric force transducer and resting tension was adjusted to 0.5 g. Muscles were forced to contract via electrical stimulation (pulse duration, 0.2 ms; amplitude 10 V, frequency 100 Hz) at a rate of one 0.2-s contraction every 2 s for 10 min. Basal muscles were treated identically without the application of electrical stimulation. Thereafter, muscles were frozen immediately between aluminum tongs cooled to the temperature of liquid nitrogen or further incubated for the assessment of glucose transport.

3.6 Glucose transport

Following pre-incubation, muscles were transferred into KHB buffer containing 1 mM 2-deoxy[^{3}H]glucose (2.5 mCi/ml) and 17 mM (when 2 mM AICAR was added) or 19 mM [^{14}C]mannitol (0.7 mCi/ml). Insulin or AICAR was added at concentrations identical to pre-incubation conditions. Transport of 2-deoxyglucose into the muscle was assessed for 20 min at 30°C. The total time of AICAR or insulin exposure (inclusive of pre-incubation and glucose transport assay) was 60 min. After incubation, muscles were homogenized in 0.5 m NaOH. Sample aliquots were used for protein determination using a commercially available kit (Coomassie Plus, Pierce, Inc., Rockford, IL, USA). Extracellular space and intracellular 2-deoxyglucose concentration were determined by liquid scintillation counting. Glucose transport activity is expressed as μmol 2-deoxyglucose per ml intracellular water per hour (in Paper II) or nmol 2-deoxyglucose per mg protein per 20 min (in Paper III).

3.7 Glucose oxidation, glycogen synthesis and lactate release

Muscles were incubated (30°C for 60 min) in 1 ml of KHB containing 8 mM U-^{14}C-glucose (0.3 μCi/ml) in the presence or absence of insulin (12 nM). Vials were sealed with a rubber stopper, through which a center-well was fitted. Muscles were oxygenated for the first 60 min of the incubation period with a needle inserted through the stopper. Thereafter, muscles were removed, trimmed of excess tendon, and weighed. Protosol (0.2 ml) (PerkinElmer Life Sciences) was injected via a rubber stopper inserted into the center-well, and the medium was acidified by injection of 0.5

ml of 15% perchloric acid. Liberated CO_2 was collected for 60 min, and center-wells were transferred into vials for liquid scintillation counting. Rate of glucose incorporation into glycogen was determined by accumulation of ^{14}C into glycogen. The muscles were homogenized in 0.5 ml of 1 M NaOH and subsequently neutralized with 0.5 ml of 20 % trichloroacetic acid. The homogenates were subjected to centrifugation for 15 min at 3500 g and the supernatant was collected for analysis. Glycogen in the supernatant was precipitated by adding 200 µl of 110 mM glycogen and 2 ml of 95 % ethanol. The glycogen precipitate was then collected after centrifugation at 2000 g for 15 min, and dissolved in water for liquid scintillation counting. For measurement of lactate release, non-radiolabeled glucose was used. After 60 min of incubation with or without insulin, muscles were weighed and medium was collected. Concentration of lactate in the medium was measured by using a colorimetric Lactate Assay Kit (Biomedical Research Service Center, University at Buffalo) according to the manufacturer's instruction.

3.8 Oleate oxidation

Muscles were trimmed of excess tendon and weighed before pre-incubated for 40 min in KHB supplemented with 5 mM HEPES, 3.5% fatty acid-free bovine serum albumin, 5 mM glucose in the presence or absence of 12 nM insulin. Thereafter, muscles were transferred into vials containing 1 ml of identical media with the addition of 0.3 mM [1-^{14}C]oleate (0.2 µCi/ml) and incubated for 60 min. The vials were sealed with a rubber stopper, which was fitted with a center-well. Muscles were oxygenated for the first 15 min of the incubation time via a needle inserted through the stopper. Thereafter, the needle was removed to seal the system. After 60 min, 0.2 ml of Solvable® (PerkinElmer Life Sciences) was injected into the center-well through the rubber stopper, and 0.5 ml of 15% perchloric acid was injected into the medium. Liberated CO_2 was collected for 60 min, and center-well was transferred into a vial for liquid scintillation counting after addition of 47 µl of 5 M HCl.

3.9 Glycogen and triglyceride analyses

For glycogen analysis, skeletal muscle was homogenized in 0.5 ml 1M HCl and then incubated at 100^{o}C for 1 hr to induce hydrolysis of glycogen. Glycogen content was determined fluorometrically based on liberated glucose via an enzymatic assay (Barnes et al. 2005). For triglycerides analysis, skeletal muscle (15-20 mg) was homogenized in 0.3 ml heptane-isopropanol-Tween mixture (3:2:0.01 by volume) and subjected to centrifugation (1500g for 15 min at 4^{o}C). The upper phase that contained extracted triglycerides was collected and dried using vacuum centrifuge. Triglyceride content was measured in duplicate samples using a triglycerides/glycerol blanked kit (Roche) and Seronorm lipid (SERO) standards according to the manufacturer's recommendation.

3.10 Sample preparation for immuno-blotting

Muscles were pulverized in microcentrifuge tubes over liquid nitrogen and homogenized in 0.3 ml of ice-cold lysis buffer (20 mM Tris (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 10 mM NaF, 1 mM MgCl, 1 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, 10% glycerol, 1% Triton X 100, 1 µg/ml aprotinin and 1 µg/ml leupeptin) using a motor-driven pestle. Homogenates were solubilized by end-over-end mixing at 4°C for 60 min and subjected to centrifugation for 10 min at 12000 g and 4°C. Protein concentration was determined using a commercially available kit (Pierce, Rockford, IL). For extraction of cytoplasmic and total membrane fractions,

pulverized muscle was homogenized using an electrical homogenizer in 0.4 ml of ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA and 20 mM Tris-HCl, pH 7.5). The homogenate was subjected to centrifugation (800 g for 15 min at 4°C) and supernatant was collected. The pellet was homogenized and supernatant was collected after centrifugation (800 g for 15 min at 4°C). The supernatants were combined and centrifuged (190 000 g for 1 hour at 4°C). The supernatant (cytoplasmic fraction) was collected, and the pellet of crude membranes was dissolved in lysis buffer. Total protein of cytoplasmic and membrane fractions was determined by using a commercial kit based on the Bradford method (Bio-Rad, Hercules, CA).

3.11 Western blot analysis

Proteins (50 μg) solubilized in Laemmli sample buffer were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Western blot analysis was performed using the following antibodies against aldolase (Biodesign, Saco, ME), CD36 (Cayman Chemical, Ann Arbor, U.S.A.), GLUT 4 (Biogenesis, Poole, U.K.), PDK4 (Abgent, San Diego, CA), PGC1α (Chemicon, Temecula, CA), GS and HK2 (kind gifts from O. Pedersen, Steno Memorial Hospital, Gentofte, Denmark), GAPDH, PPARα, PPARδ and UCP3 (Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies against NADH-ubiquinol oxidoreductase (NUO), succinate-ubiquinol oxidoreductase (SUO), ubiquinol-cytochrome c oxidoreductase subunit II (Core II), cytochrome c oxidase subunit I (COX I), and ATP synthase α subunit (ATP syn α) were from Molecular Probes (Eugene, OR). AMPK subunit antibodies were generated, as described previously (Mahlapuu et al. 2004). Phospho-AMPK (Thr-172) and phospho-ACC (acetyl-CoA carboxylase, Ser-227) antibodies were from Cell Signalling Technology (Beverly, MA, USA) and Upstate Biochemicals (Waltman, MA, USA). Membranes were incubated in 5% fat-free milk in Tris-buffered saline containing 0.02% Tween 20 (TBST) and probed with the indicated antibodies. Thereafter, membranes were washed in TBST (6 × 10 min), incubated in appropriate secondary antibodies, and washed again in TBST. Proteins were visualized by chemiluminescence and quantified by densitometry.

3.12 Exercise protocol

Overnight fasted (16 h) wild-type, *Tg-Prkag3*^{225Q}, or *Prkag3*$^{-/-}$ mice were divided into sedentary and swimming group. Six mice swam in the same plastic container and water temperature was maintained between 32 and 33°C. The swimming protocol consists of four 30 min swim interval, separated by 5 min rest interval for a total swimming time of 2 h. After the last swim interval, mice were dried and either studied immediately or allowed to recover from the exercise for 2.5 h (recovery). At the onset of the recovery period, mice received an intraperitoneal glucose injection (0.5 mg/g body weight) and were allowed free access to chow and water.

3.13 Statistical analysis

Data are expressed as means ± S.E.M. Differences among groups were determined by two-way ANOVA followed by Fisher's least significant differences post-hoc analysis. Differences between two groups were determined by unpaired student's t-test. Significance was accepted at $p < 0.05$.

4 RESULTS AND DISCUSSION

Adult skeletal muscle is composed of a heterologous mix of myofibres, with distinctive contractile and metabolic properties. Skeletal muscle has a central role in the regulation of glucose homeostasis and thermogenesis, and displays metabolic flexibility in response to functional demands. AMPK and calcineurin signaling pathways have been shown to shape the skeletal muscle metabolic system. Investigation into the biochemical pathways governing the skeletal muscle metabolic events is critical for the understanding of physiology under normal or disease states, and may conceivably lead to novel preventive and therapeutic measures to treat or prevent metabolic or muscular diseases.

4.1 Fasting-induced coordinated expression of skeletal muscle metabolic genes

Skeletal muscle possesses substantial flexibility in the utilization of energy substrates. Under fasting conditions, there is a shift from glucose to lipid metabolism in skeletal muscle (Andres et al. 1956), as part of the whole-body adaptations to conserve glucose. The ability to shift carbon sources for energy production is critical for survival, as the living organism is frequently challenged with an irregular food supply. Such metabolic flexibility is also conserved in yeast (*Saccharomyces cerevisae*), whereby a highly coordinated transcriptional activation of metabolic enzymes forms the metabolic network (Hardie et al. 1998; Johnston 1999). Control of metabolic events in mammalian cells involves multiple mechanisms, including hormonal control of substrate availability, covalent modifications and allosteric interactions of enzymes. However, studies have provided evidence for a central role of transcriptional regulation in the metabolic response to specific perturbations (Kacser and Acerenza 1993; Ihmels et al. 2004).

Although fasting increases skeletal muscle lipid oxidation, whether fasting conditions would induce a coordinated transcriptional response for the lipid metabolic enzymes in white skeletal muscle is unknown. Paper I was designed to elucidate changes in mRNA abundance of lipid metabolic genes in mouse white gastrocnemius muscle in response to a 16-h fast. The transport of free fatty acid into the cytoplasm involves LPL which cleaves fatty acid from lipoprotein in the vascular space for facilitative transport across the plasma membrane into the cytoplasm via CD36, a long-chain fatty acid transporter (Coburn et al. 2000). The mRNA levels of *Lpl* and *Cd36* are coordinatedly up-regulated in skeletal muscle from wild-type mice in response to fasting (*Paper I, Figure 2*). Distal steps of lipid oxidation include transfer of fatty acyl-CoA into the mitochondria by CPT1 and β-oxidation, which involves HAD. There was an adaptive elevation in the expression of *Cpt1* and a non-significant trend of increase for *Had* ($p= 0.07$) in wild-type mice. Fasting also elicited marked increases in the expression of *Cs and Ucp3*, two genes that are essential for TCA cycle and lipid oxidation.

4.1.1 Coexpression of genes along metabolic pathways

The fasting-induced transcriptional response of genes essential for lipid metabolism in wild-type mice is highly coordinated (*Paper I, Figure 2*). A similar pattern of skeletal muscle lipid metabolic gene expression was also reported earlier in human skeletal muscle under fasting conditions, where a coordinated induction of *Lpl*, *Cpt1* and *Ucp3* was observed (Patti et al. 2003). One of the principles of transcriptional

control of metabolism in the yeast is that genes along a linear metabolic pathway are coexpressed, to favor metabolic flow towards linearity (Ihmels et al. 2004). Such tight transcriptional coordination is also observed in skeletal muscle from streptozotocin-induced diabetic mice, in which mRNA expression for all of the enzymes investigated for the fatty acid β-oxidation pathway were increased (Yechoor et al. 2002). In type 2 diabetic people, a coordinated reduction of genes essential for oxidative metabolism was also observed (Patti et al. 2003). Data presented in this thesis (*Paper I, Figure 2*), is consistent with findings of other laboratories (Yechoor et al. 2002; Patti et al. 2003) suggest that such coordinated control of metabolic genes in a linear fashion is conserved in eukaryotic cells such as skeletal muscle (Figure 5). Thus, a systematic investigation or analysis of candidate genes along metabolic pathways and branch points will undoubtedly provide a useful framework for gene expression profiling in normal physiology and disease states such as diabetes and skeletal muscle atrophy.

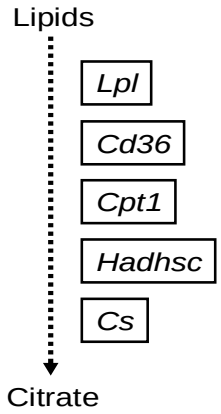

Figure 5: Coexpression of genes along lipid metabolic pathway in response to fasting. Such a coherent expression of genes (fasting-responsive genes as investigated in Paper I) supports an efficient linear metabolic flow for energy production. Similar coordinated expression of genes was reported in other studies.

4.2 The role of AMPK in skeletal muscle metabolic gene expression

The yeast analogue of AMPK, sucrose non-fermenting protein kinase (SNF1) is involved in the regulation of transcriptional responses during shifts in substrate utilization (Hardie et al. 1998; Johnston 1999). Although the flexibility in carbon source utilization is conserved in skeletal muscle, whether AMPK is essential for the fasting-induced transcriptional response is unknown. A role of the AMPK γ3 subunit in the regulation of skeletal muscle metabolism was proposed in earlier studies whereby skeletal muscle from pigs that carry a naturally occurring mutation of γ3 subunit (R225Q), have increased glycogen content (Estrade et al. 1993; Milan et al. 2000) and oxidative enzyme activities (Estrade et al. 1994; Lebret et al. 1999). Although the γ subunit includes γ1, γ2, and γ3 isoforms, the γ3 isoform was identified as the predominant isoform expressed in fast-glycolytic muscle, as assessed by real-time PCR and immunoblot analysis (Mahlapuu et al. 2004). Given the diverse contractile and metabolic properties between slow-oxidative and fast-glycolytic muscle, part of the aim in Paper I is to investigate whether AMPK is essential for the expression of skeletal muscle metabolic genes under fed or fasting conditions.

4.2.1 The effects of mutant γ3 (R225Q) on skeletal muscle gene expression

Most previous studies that investigated the role of AMPK in skeletal muscle gene expression involved chronic treatment of rodents with AICAR (Holmes et al.

1999; Winder et al. 2000; Stoppani et al. 2002). In Paper I, transgenic mice that express the mutant activating AMPK γ3 subunit (R225Q) in fast-glycolytic muscle (Barnes et al. 2004) were utilized to study the effect of AMPK activation on skeletal muscle gene expression. The mRNA expression and protein content of CD36 was elevated in skeletal muscle from *Tg-Prkag3*^{225Q} mice (*Paper I, Figure 1 & 2*), providing evidence that AMPK has a role in the regulation of skeletal muscle CD36 expression. A critical role of CD36 in skeletal muscle lipid metabolism was supported by the observation of a marked reduction in the transport and metabolism of long chain fatty acid in skeletal muscle of CD36-null mice (Coburn et al. 2000). Consistent with the increase in CD36 expression, mRNA level of *Cycs* and protein content of UCP3 in the mutant transgenic mice were increased. These findings are consistent with earlier reports in which pharmacological activation of AMPK induces expression of *Cycs* (Winder et al. 2000; Bergeron et al. 2001) and *Ucp3* (Stoppani et al. 2002) in skeletal muscle.

In contrast to previous findings of an AMPK-induced *Glut4* (*Slc2A4*) and *Hk2* expression in skeletal muscle, *Glut4* expression at the mRNA and protein level was unaltered by the expression of R225Q γ3 subunit in skeletal muscle (*Paper I, Figure 2*). Moreover, an unexpected down-regulation of *Hk2* in mutant transgenic mice was observed, with a similar pattern of reduction for *Pfkm* and *Gys* under fasting conditions. Such discrepancies could be due to the chronic use of AICAR in the treatment of rodents, which is known to activate other AMP-regulated enzymes (Kemp et al. 1999; Hardie 2003). The enhanced expression of lipid metabolic genes and a reciprocal reduction in glucose metabolic gene expression suggests a shift towards lipid utilization in skeletal muscle following activation of AMPK in the *Tg-Prkag3*^{225Q} mice. Consistent with this notion, the mutant transgenic mice showed a higher rate of lipid oxidation and a concomitant lower IMTG deposition under the challenge of a high-fat diet (Barnes et al. 2004). The mutant AMPK-induced reciprocal coordination between lipid and glucose metabolic gene expression in skeletal muscle was also associated with a glucose sparing effect in the *Tg-Prkag3*^{225Q} mice (*Paper I, Figure 6*). Under either fed or fasting conditions, there was a marked elevation of glycogen content in white gastrocnemius muscle from *Tg-Prkag3*^{225Q} mice when compared to wild-type mice, which is consistent with previous findings (Barnes et al. 2004).

4.2.2 AMPKγ3 is required for skeletal muscle metabolic gene expression

The skeletal muscle AMPK γ3-null mice were utilized to further validate the role of AMPK in fasting-induced skeletal muscle metabolic gene expression. While fasting induced a coordinated expression of genes for *Lpl, Cd36, Cpt1 and Cs* in wild-type mice, these transcriptional responses were essentially impaired in *Prkag3*$^{-/-}$ mice (*Paper I, Figure 2*). Similarly, fasting-induced expression of skeletal muscle *Gys* and *Ldh* was also impaired in the absence of AMPK γ3 subunit. The expression of *Ucp3* and *Pdk4* under fasting conditions was not impaired in the *Prkag3*$^{-/-}$ mice, suggesting that AMPK is not required for all fasting-induced transcriptional responses. In a follow-up oligonucleotide microarray study, a similar opposite expression profile was observed in skeletal muscle from *Tg-Prkag3*^{225Q} and *Prkag3*$^{-/-}$ mice for a myriad of genes, including glucose and lipid metabolic genes (Nilsson et al. 2006). Therefore, the AMPK γ3 mutant transgenic and knockout mice clearly display a gain- and loss-of-function phenotype, respectively, underscoring the role of AMPK in the transcriptional regulation of metabolic genes.

4.2.3 AMPK and PPAR isoform expression in skeletal muscle

The expression of *Lpl*, *Cd36*, *Cpt1, Cs* and *Ucp3* in skeletal muscle is regulated by the transcription factors PPARα (Mandard et al. 2004; Finck et al. 2005) and/or PPARδ (Muoio et al. 2002; Takahashi et al. 2006). Consistent with the fasting-induced expression of these genes, a trend of increase in PPARα and PPARδ expression was observed in wild-type mice in response to fasting (*Paper I, Figure 4*). The up-regulation of these genes however was completely blunted in the AMPK γ3-null mice.

In the *Tg-Prkag3*^{225Q} mice, PPARγ expression was increased. Although PPARγ was previously thought to play a more prominent role in adipocytes and liver based on its relatively low expression in skeletal muscle, the data obtained from skeletal muscle-specific PPARγ-null mice yielded surprising results. The mice developed glucose intolerance and insulin resistance in the absence of any dietary perturbations, and were unresponsive to the therapeutic effects of thiazolidinediones, a PPARγ agonist in clinical use for the treatment of type 2 diabetes (Hevener et al. 2003). When the *Tg-Prkag3*^{225Q} mice were fed on a high-fat diet, insulin stimulated glucose transport was preserved in skeletal muscle, with a superior response compared to wild-type mice treated in the same manner (Barnes et al. 2004). Whether the increased expression PPARγ in the AMPK mutant transgenic mice mediates any of the protective effects against the development of diet-induced skeletal muscle insulin resistance is unknown. At this point, whether the down-regulation of PPARδ in *Tg-Prkag3*^{225Q} mice is a compensatory response for the increased PPARγ expression is unclear. Further investigation is essential to delineate the link between the AMPKγ3 subunit and PPAR expression in skeletal muscle and the effects on skeletal muscle metabolism (Figure 6).

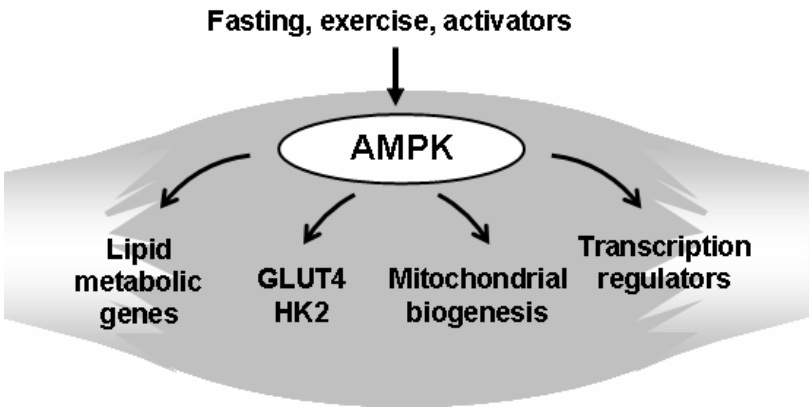

Figure 6: Canonical role of AMPK in metabolic gene expression. *Activation of AMPK by nutrient deprivation, exercise or chemical activators (e.g. AICAR) resulted in increased expression of genes essential for glucose and lipid metabolism. AMPK was also shown to activate transcription regulators such as MEF and PGC1α.*

4.3 Role of AMPK in exercise-induced metabolic and transcriptional events

The energy demand on skeletal muscle is enormously high during contraction (Sahlin et al. 1998), and several metabolic events are elicited during exercise to fuel the myofibers. By sensing the decrease in skeletal muscle energy stores, AMPK activates

energy-producing reactions such as glucose uptake and fatty acid oxidation while inhibiting energy-consuming events including protein synthesis (Hardie 2003; Kahn et al. 2005; Long and Zierath 2006). These AMPK-induced acute metabolic responses aim to rapidly restore energy balance in skeletal muscle. Long-term effects of AMPK activation include alteration in gene expression for enhanced glucose and lipid-oxidative metabolism, as well as mitochondrial biogenesis (Winder 2001; Long and Zierath 2006). The major aims of Paper II were to address the question of whether the AMPKγ3 subunit is involved in exercise-stimulated skeletal muscle glucose transport, and if genetic ablation or activation of the γ3 subunit would alter exercise-induced transcriptional responses.

4.3.1 AMPKγ3 is not essential for exercise-induced glucose uptake

A previous report from our laboratory demonstrated that *in vitro* contraction-induced glucose transport is unaltered in skeletal muscle from *Tg-Prkag3*^{225Q} and *Prkag3*$^{-/-}$ mice (Barnes et al. 2004). This experiment provided evidence that AMPKγ3 is not required for contraction-induced glucose uptake in EDL muscle, and the mutant AMPKγ3 subunit did not enhance such a response. Whether genetic alteration of AMPKγ3 subunit would affect exercise-induced glucose uptake is unknown, since differences exist between contraction induced by electrical-stimulation or exercise. *In vitro* electrical-stimulated muscle contraction recruits all motor units in the muscle simultaneously to generate force (Pette and Vrbova 1999). During exercise, however, alternate motor units are asynchronously recruited. Furthermore, glucose distribution in an *in vitro* system depends on diffusion, whereas delivery of glucose to myofibers is actively facilitated by the muscle vasculature. Activation of axons *in vitro* by electricity is extremely robust, leading to generation of force that far exceeds that of a normal muscle contraction during exercise. Therefore, in Paper II (*Figure 1*), fast-twitch EDL muscles from wild-type, *Tg-Prkag3*^{225Q} and *Prkag3*$^{-/-}$ mice were removed and studied for glucose transport immediately after a 2-h exercise bout.

The rate of glucose transport was similar among *Tg-Prkag3*^{225Q}, *Prkag3*$^{-/-}$ and wild-type mice immediately after exercise (*Paper II, Figure 1*). This finding provided evidence that either an activating mutation or genetic ablation of the AMPKγ3 subunit did not affect exercise-induced glucose transport. This finding is consistent with previous findings in the AMPKα1-, α2- (Jorgensen et al. 2004) and γ3-null mice (Barnes et al. 2004). Namely, that AMPK is not required for *in vitro* contraction-stimulated skeletal muscle glucose uptake. Nonetheless, there is conflicting data that support a role of AMPK in contraction-stimulated glucose uptake. Skeletal muscle-specific overexpression of a dominant-negative form of AMPKα2 (Mu et al. 2001) or genetic ablation of LKB1 (Sakamoto et al. 2005), (an upstream kinase of AMPK) partially impaired contraction-induced glucose uptake. One possible explanation for such observations is that there are multiple contraction-induced signaling pathways that elicit skeletal muscle glucose transport. Calcium-dependent pathways that involve calcium/calmodulin kinase have been proposed to play a role in stimulation of glucose uptake in response to calcium release from the sarcoplasmic reticulum during muscle contraction (Chin et al. 1998; Ryder et al. 2001). Therefore, skeletal muscle contraction-activated signaling pathways may share intermediaries in the system for stimulation of glucose uptake, such that defects along a particular pathway may be masked by the full-stimulatory effects of other inputs at the points of convergence.

4.3.2 Decreased glucose uptake after recovery in Tg-Prkag3^{225Q} mice

The metabolic response of skeletal muscle after exercise is greatly influenced by the pre-exercise nutritional state and carbohydrate intake during exercise or recovery. Glucose transport was determined after a 2.5-h of recovery period during which the mice received an intraperitoneal glucose injection (0.5 mg/g body weight), as well as free access to chow and water. Under these conditions, the stimulatory effect of exercise on glucose transport was reversed after 2.5-h of recovery. Furthermore, glucose uptake in skeletal muscle from *Tg-Prkag3^{225Q}* mice was lower than in the wild-type mice after the recovery, with a similar non-significant trend for decrease in the presence of insulin (*Paper II, Figure 1*). This observation may be explained by elevated glycogen content in skeletal muscle of *Tg-Prkag3^{225Q}* mice after recovery (Barnes et al. 2004). Although muscle glycogen content of *Tg-Prkag3^{225Q}* mice was comparable to the wild-type mice immediately after exercise, the *Tg-Prkag3^{225Q}* mice had a dramatic elevation in skeletal muscle glycogen content during recovery (Barnes et al. 2004). An inhibitory effect of glycogen content on insulin-stimulated glucose uptake was previously described in glycogen-supercompensated muscles of exercised rats (Host et al. 1998), and impaired GLUT4 translocation was proposed as one of the underlying mechanism (Kawanaka et al. 1999; Kawanaka et al. 2000). However, the link between insulin-stimulated GLUT4 translocation and glycogen content in EDL muscles from *Tg-Prkag3^{225Q}* mice remains to be defined.

4.3.3 Enhanced lipid utilization in skeletal muscle from Tg-Prkag3^{225Q} mice

In addition to glycogen, lipids represent a major fuel for skeletal muscle, as evident by a marked increase in lipid utilization is observed during exercise. The lipid metabolic pathway was investigated in wild-type, *Tg-Prkag3^{225Q}* and *Prkag3$^{-/-}$* mice immediately after exercise or recovery, to determine the role of AMPK in exercise-induced lipid metabolism. Inhibitory phosphorylation of ACC (a negative regulator of lipid oxidation) was markedly elevated in skeletal muscle from *Tg-Prkag3^{225Q}* mice immediately after exercise (*Paper II, Figure 3*). This result suggests that there is an increased reliance on lipid oxidation during exercise in the *Tg-Prkag3^{225Q}* mice. Consistently, a lower level of IMTG was detected in skeletal muscle of *Tg-Prkag3^{225Q}* mice after exercise (*Paper II, Figure 3*), relative to wild-type mice. The preference for lipid-oxidative metabolism was also observed in skeletal muscle of *Tg-Prkag3^{225Q}* mice when subjected to a high-fat diet challenge (Barnes et al. 2004), suggesting that the mutant AMPKγ3 subunit enhances the ability of skeletal muscle to utilize lipid under dietary and exercise perturbations.

The activating mutant AMPKγ3 subunit did not affect the rate of glucose oxidation in skeletal muscle from the *Tg-Prkag3^{225Q}* mice immediately after exercise. However, glucose oxidation in skeletal muscle from the *Prkag3$^{-/-}$* mice was lower after exercise. Glycogen accumulation during exercise has been reported to be severely impaired in skeletal muscle of *Prkag3$^{-/-}$* mice after recovery (Barnes et al. 2004). Therefore, genetic knockout of the AMPKγ3 subunit results in a severe metabolic derangement in skeletal muscle. The precise molecular mechanism for such an impairment remains to be investigated.

4.3.4 Decreased lactate release in EDL muscles from Tg-Prkag3^{225Q} mice

To determine the rate of lactate release under fasted conditions, EDL muscles from wild-type and *Tg-Prkag3*^{225Q} mice were incubated in the absence or presence of insulin. Lactate release from the skeletal muscle provides evidence for glucose utilization in the muscle. Insulin increased lactate release 2.9-fold from EDL muscles of wild-type mice, and this effect was impaired in the *Tg-Prkag3*^{225Q} mice (Figure 7). This finding is consistent with a previous report showing reduced insulin-stimulated glucose uptake in EDL muscles from fasted *Tg-Prkag3*^{225Q} mice (Barnes et al. 2004). Taken together, these results provided evidence for a decreased reliance on glucose utilization in *Tg-Prkag3*^{225Q} mice.

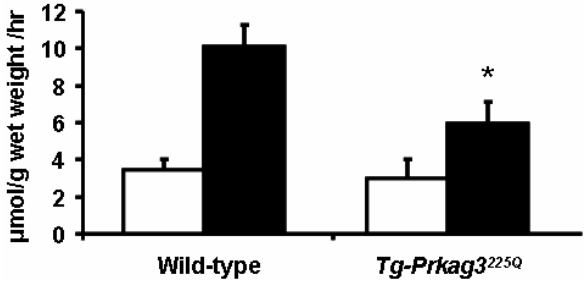

Figure 7: The rate of lactate release is reduced in EDL muscles from Tg-Prkag3^{225Q} *mice. EDL muscles from wild-type or Tg-Prkag3*^{225Q} *mice were incubated in the absence (open bars) or presence (closed bars) of insulin and lactate released into the incubation media was assessed. Data are means ± SEM for n=6 muscles. *, p < 0.05 compared to wild-type.*

4.3.5 Role of AMPKγ3 subunit in exercise-induced gene expression

Apart from the acute metabolic responses, AMPK has been proposed to mediate exercise-induced gene regulatory adaptations (Winder 2001; Long and Zierath 2006). An elevated mRNA expression of *Lpl*, *Cpt1*, *Had* and *Cycs* was observed in white gastrocnemius muscle from *Tg-Prkag3*^{225Q} mice immediately after exercise, while a lower expression of Cpt1 and Cycs was observed in the *Prkag3*$^{-/-}$ mice (*Paper II, Figure 4*). Therefore, the AMPKγ3 subunit plays a role in modulating the exercise-induced expression of genes essential for lipid metabolism.

The mRNA expression of genes involved in glycogen storage including *Glut4*, *Hk2* and *Gs* were coherently increased in *Tg-Prkag3*^{225Q} mice after exercise, when compared to wild-type mice (*Paper II, Figure 5*). This finding is consistent with the observation of enhanced glycogen supercompensation in white gastrocnemius muscles from *Tg-Prkag3*^{225Q} mice after recovery from exercise (Barnes et al. 2004). In white gastrocnemius from wild-type mice, the expression of *Glut4* and *Hk2* was up-regulated after recovery from exercise (*Paper II, Figure 5*). The expression profile is consistent with the carbohydrate load that the mice received during recovery, which included a bolus injection of glucose and free access to chow. These responses were essentially

blunted in *Prkag3*$^{-/-}$ mice (*Paper II, Figure 5*), supporting the notion that the AMPKγ3 subunit plays a role in gene regulatory events during and after exercise (Figure 8).

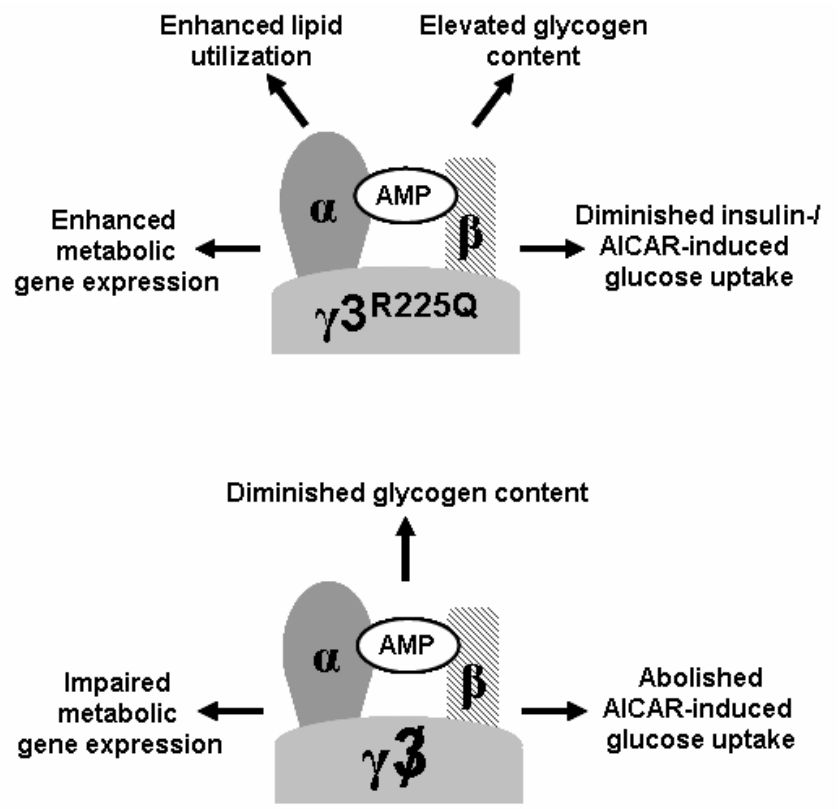

Figure 8: Effects of genetic manipulation of AMPKγ3 subunit. Activation of AMPK via γ3R225Q mutation resulted in enhanced metabolic and gene regulatory responses. The increase of glycogen content in skeletal muscle from Tg-Prkag3R225Q mice was associated with diminished AICAR- and Insulin-induced glucose uptake. Conversely, genetic ablation of AMPKγ3 subunit impaired AICAR-induced glucose uptake, as well as metabolic and gene regulatory responses.

4.4 Calcineurin activation and skeletal muscle glucose uptake

A role of AMPK in the molecular adaptations of skeletal muscle in response to several metabolic perturbations has been suggested (Winder 2001; Kahn et al. 2005; Long and Zierath 2006). However, whether AMPK-mediated metabolic effects are altered as part of skeletal muscle remodeling program, or if skeletal muscle reprogramming is associated with any changes in the expression of AMPK subunits is unknown. Calcineurin has been implicated in mediating fast-to-slow twitch skeletal muscle remodeling (Olson and Williams 2000). In Paper III, we sought to investigate whether calcineurin-mediated skeletal muscle remodeling leads to alterations in insulin-, AICAR- or contraction-stimulated glucose uptake. Furthermore, we investigated whether any of these changes could be explained by alteration in the expression of different isoforms of the AMPK subunits.

4.4.1 Impaired AICAR-induced glucose uptake in EDL muscle from MCK-CnA* mice

Transgenic mice that overexpressed an activated form of calcineurin (MCK-CnA*) specifically in fast-glycolytic muscles were utilized in this study. Skeletal muscle from the MCK-CnA* mice has previously been reported to display fast-to-slow twitch muscle remodeling (Naya et al. 2000). EDL muscles from these mice were incubated *in vitro* and the effects of insulin, AICAR or contraction on glucose transport was determined. Consistent with a previous report, an enhanced insulin-stimulated glucose uptake was observed in skeletal muscle from MCK-CnA* mice. As reported earlier, calcineurin-induced skeletal muscle reprogramming led to an enhanced expression of the IR, Akt and GLUT4 (Ryder et al. 2003). Calcineurin was proposed to activate the expression of these signaling intermediaries and this glucose transporter such that insulin-stimulated signaling and glucose transport is amplified.

Given that GLUT4 is involved in insulin-, AICAR- and contraction-induced skeletal muscle glucose transport (Winder 2001), Paper III aimed to investigate whether a similar enhancement in glucose uptake is induced by AICAR or contraction. In contrast to the enhanced insulin response, AICAR-induced skeletal muscle glucose uptake was impaired in EDL muscles from MCK-CnA* mice (*Paper III, Figure 1*). Therefore, the induction of fast-to-slow twitch skeletal muscle reprogramming resulted in an impaired response to the AMPK activator (Figure 9). This finding corroborates previous reports showing AICAR-induced glucose uptake is lower in the slow-twitch soleus muscles when compared to the fast-twitch EDL muscles (Jorgensen et al. 2004). The resistance to AICAR-stimulated glucose uptake is even more striking in the soleus muscles from rats, which is enriched in slow-twitch fiber and less heterogenous compared to the mouse soleus muscle. While there was a marked response in AICAR-stimulated glucose uptake in rat fast-twitch epitrochlearis muscles, such an effect was completely blunted in rat soleus muscle (Ai et al. 2002). Despite this, contraction-stimulated glucose uptake was unaltered in the MCK-CnA* mice when compared to wild-type mice (*Paper III, Figure 1*). Thus, skeletal muscle reprogramming via the activation of calcineurin improved insulin-stimulated glucose uptake, but impaired the response to AICAR. Furthermore, induction of the slow-twitch muscle program via calcineurin did not alter contraction-stimulated glucose uptake.

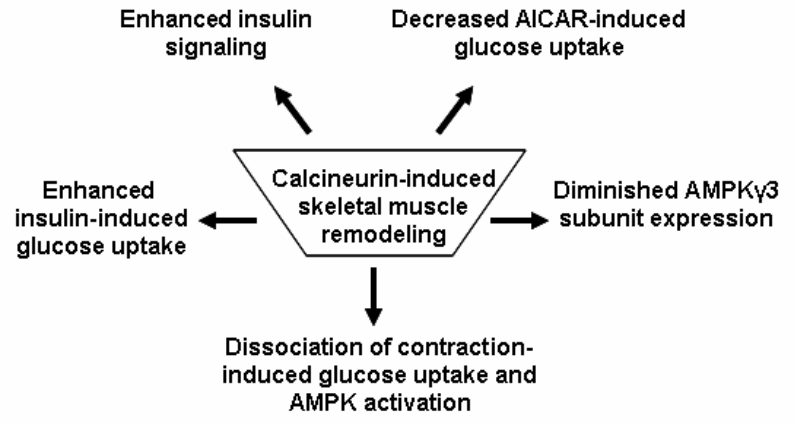

Figure 9: The effects of calcineurin activation on skeletal muscle glucose uptake. Activation of calcineurin enhanced insulin-induced signaling pathway and glucose uptake. Conversely, calcineurin-induced skeletal muscle remodeling decreased AICAR-stimulated glucose uptake, associated with a lower expression of AMPKγ3 subunit. Furthermore, activation of calcineurin did not alter contraction-stimulated glucose uptake, despite a diminished AMPK activation.

4.4.2 Unaltered AICAR-induced AMPK phosphorylation in EDL muscle from MCK-CnA* mice

A possible explanation for the reduction in AICAR-induced glucose uptake in EDL muscle of MCK-CnA* mice was an altered AMPK signal transduction. In response to AICAR treatment, phosphorylation of AMPK at Thr-172 was increased to a similar extent in wild-type and MCK-CnA* mice (*Paper III, Figure 2*). AICAR treatment also increased the phosphorylation of ACC (a downstream target of AMPK) to a similar magnitude in wild-type and MCK-CnA* mice (*Paper III, Figure 3*). Therefore, changes in AMPK phosphorylation could not explain the differences in AICAR-stimulated glucose transport in EDL muscle from MCK-CnA* mice.

4.4.3 Changes in AMPK α subunit isoform expression in EDL muscles from MCK-CnA* mice

Since AMPK is a heterotrimer composed of α, β and γ subunits, the altered AICAR-stimulated glucose uptake in MCK-CnA* mice may be due to changes in the isoform expression of various subunits of AMPK. For example, skeletal muscle from α2 subunit knockout mice displayed impaired AICAR-induced glucose uptake, supporting the essential role of α2 subunit in mediating the AICAR effect (Jorgensen et al. 2004). Activation of calcineurin induced an increase in α1 protein content in fast-twitch skeletal muscle, whereas α2 protein content remained unchanged (*Paper III, Figure 4*). In AMPK α1-knockout mice, AICAR-induced glucose uptake was unaltered in EDL and soleus muscles (Jorgensen et al. 2004). The α1 subunit is therefore dispensable for the AICAR response. Since the ablation of α1 subunit did not affect AICAR-induced glucose uptake in either EDL or soleus muscles, any fiber-type dependent responses to AICAR is unlikely to be linked to alterations in α1 expression. Taken together, the increase in α1 protein content could not account for the alteration in AICAR-induced glucose uptake in EDL muscle of MCK- CnA* mice.

4.4.4 Decreased AMPKγ3 subunit expression in EDL muscle from MCK-CnA* mice

The regulatory AMPKγ subunits contain CBS binding domains that are thought to be critical for the nucleotide binding and therefore activation of AMPK (Hardie et al. 1998). Among the three isoforms of the AMPKγ subunit, the expression of γ3 isoform showed a high specificity for white glycolytic muscle and appeared to be the predominant γ-isoform in this muscle type (Mahlapuu et al. 2004). In rats and mice, the mRNA expression and protein content of the γ3 subunit is highly enriched in white muscles when compared to red muscles. A similar high degree of skeletal muscle-specific γ3 subunit expression is also observed in human skeletal muscle. When the γ subunit isoform expression was assessed in EDL muscles from wild-type and MCK-CnA* mice, a decreased mRNA expression and protein content of the γ3 subunit was observed (*Paper III, Figure 5*). An earlier study revealed that the AMPKγ3 subunit is critical for the effect of AICAR-, but dispensable for the effect of contraction on glucose uptake (Barnes et al. 2004). Therefore, activation of calcineurin in fast-glycolytic muscle suppressed the expression of the AMPKγ3 subunit. Thus, calcineurin-mediated fast-to-slow twitch skeletal muscle remodeling suppressed the expression of the regulatory AMPKγ3 subunit, and this is associated with impaired AICAR-induced glucose uptake.

4.4.5 Dissociation between AMPK phosphorylation and contraction-induced glucose uptake

Although AMPK has been proposed as a signal transducer for contraction-induced skeletal muscle glucose uptake, its definite role remains largely unresolved. Genetic ablation of either the AMPKα2 or γ3 subunit abolishes AICAR- but is without effect on contraction-induced skeletal muscle glucose uptake (Jorgensen et al. 2004). Skeletal muscle from dominant-negative AMPKα2 transgenic mice showed a similar impairment in the AICAR response, although a modest decrease in contraction-stimulated glucose uptake was observed (Mu et al. 2001). More recently, result obtained from LKB1-deficient mice showed that LKB1-AMPK system was essential for AICAR and contraction-stimulated glucose uptake (Sakamoto et al. 2005). Although the results supported a link between AICAR- and contraction-induced glucose uptake, whether LKB1 mediates the contraction effect via downstream proteins other than AMPK is unknown. An additional aim of Paper III was to determine the effects of calcineurin-mediated skeletal muscle remodeling on contraction-stimulated glucose uptake. In contrast to AICAR stimulation, contraction-induced glucose uptake was comparable between wild-type and MCK-CnA* mice (*Paper III, Figure 1*), despite a reduction in AMPK phosphorylation (*Paper III, Figure 2*). Taken together with the results from AMPKα2-, AMPKγ3-null and AMPKα2 dominant-negative mice (Mu et al. 2001; Barnes et al. 2004; Jorgensen et al. 2004), the results from MCK-CnA* mice in Paper III underscored the mechanistic differences between AICAR- and contraction-stimulated glucose uptake.

Another observation in Paper III is that the impaired contraction-induced AMPK phosphorylation did not affect contraction-induced glucose uptake in the EDL muscle from MCK-CnA* mice. Calcineurin-mediated fast-to-slow twitch skeletal muscle remodeling clearly resulted in a dissociation between contraction-stimulated

glucose uptake and AMPK activation. Therefore, AMPK-signaling pathways seem to be fiber-type-dependent (Table 1). Indeed, contraction-induced glucose uptake in rat soleus muscle was not associated with activation of AMPK (Derave et al. 2000), but rather was dependent on activation of CaMK (Wright et al. 2005). Conversely, contraction-induced glucose uptake in fast-glycolytic rat epitrochlearis muscles was associated with the activation of AMPK, in addition to CaMKII (Wright et al. 2004). Calcium-dependent pathways have been suggested to be essential for contraction-stimulated glucose transport in slow- and fast-twitch muscles, whereas AMPK-dependent pathway is only required in fast-twitch muscles (Wright et al. 2005). The data of Paper III provided evidence that calcineurin-mediated fast-to-slow twitch skeletal muscle remodeling did not have a negative impact on contraction-induced glucose transport, but led to dissociation between contraction-induced glucose uptake and AMPK activation.

Table 1: Evidence for a fiber-type dependence of skeletal muscle AMPK-signaling pathway

Study/ model	**Brief description of findings**	**Reference**
Rat fast- and slow-twitch skeletal muscles	AICAR induced activation of AMPK in fast- and slow-twitch muscles, but stimulated glucose uptake only in fast-twitch muscle.	Ai, 2002; Wright 2004; Wright 2005
Mouse and rat white skeletal muscle	Selective expression of AMPKγ3 subunit in white skeletal muscle.	Mahlapuu, 2004
AMPKγ3 knockout mice (*Prkag3*$^{-/-}$*)*	Abolished AICAR-induced glucose uptake, impaired glycogen synthesis and gene expression.	Barnes, 2004, Paper I & II
Calcineurin-induced fast-to-slow twitch skeletal muscle remodeling (MCK-CnA* mice)	Impaired AICAR-induced glucose uptake associated with decreased AMPKγ3 subunit expression. Unaltered contraction-induced glucose uptake despite lower AMPK activation.	Paper III
Rat soleus muscles with high glycogen content	Unaltered contraction-induced glucose uptake without AMPK activation.	Derave, 2000
Chronic AICAR treatment in rats	Enhanced glucose uptake and GLUT4 expression limited to fast-glycolytic muscles, undetectable response in slow-oxidative muscles.	Buhl, 2001
Chronic AICAR treatment in mouse	AICAR-induced gene expression observed in white but not red gastrocnemius muscle. Ablation of AMPK α2 abolished effects in white gastrocnemius muscle.	Jorgensen, 2007

4.5 Calcineurin-induced skeletal muscle metabolic remodeling

Signals from motor neurons play a central role in the regulation of skeletal muscle metabolic properties. Chronic low frequency electrical stimulation that mimics the firing pattern of a slow motor neuron activates the expression of lipid metabolic genes including *Lpl* (Hamilton et al. 1998), *Cd36* (Bonen et al. 1999), *Had* (Theriault et al. 1994) and *Cs* (Theriault et al. 1994; Nuhr et al. 2003). In addition, the expression of *Glut4* and *Hk2* (Kong et al. 1994) are also augmented in response to chronic electrical stimulation to favor the storage of glycogen in skeletal muscle. Calcineurin has been implicated in the transduction of slow motor neuronal signals to the myofibers (Chin et al. 1998). A previous report from our laboratory provided evidence that calcineurin-induced skeletal muscle remodeling enhanced the expression of IR, Akt and GLUT4, in support of an enhanced insulin-stimulated glucose uptake (Ryder et al. 2003). In Paper IV, the impact of calcineurin-mediated skeletal muscle remodeling on fuel substrate metabolism and changes in metabolic gene expression program was investigated.

4.5.1 The impact of calcineurin activation on glycogen synthesis and glucose oxidation

Although activation of calcineurin enhanced insulin-stimulated glucose uptake, the effects on glucose utilization by the myofibers is unknown. To determine whether calcineurin-mediated adaptations in skeletal muscle impinged upon the metabolic fate of glucose, the rate of glucose incorporation into glycogen and glucose oxidation were determined in skeletal muscle from wild-type and MCK-CnA* mice. Under insulin-stimulated conditions, the rate glucose incorporation into glycogen was increased in the EDL and soleus muscle from wild-type mice, with the greatest effect observed in soleus muscle (*Paper IV, Figure 1*). Activation of calcineurin in skeletal muscle enhanced insulin-stimulated glucose incorporation into glycogen in the EDL and soleus muscle from MCK-CnA* mice.

The rate of glucose oxidation in the EDL muscle from wild-type mice was increased in response to insulin treatment (*Paper IV, Figure 1*). However, the stimulatory effect of insulin on glucose oxidation was blunted in the EDL muscle from MCK-CnA* mice. Therefore, calcineurin-induced skeletal muscle remodeling reduced the partitioning of glucose for oxidation, but induced glucose sparing for glycogen synthesis.

4.5.2 Calcineurin coordinates changes in glucose metabolic genes

The expression of genes that are essential for glucose metabolism was determined to evaluate whether calcineurin induced a shift in glucose metabolism via gene expression reprogramming. The mRNA expression of glycolytic genes including *Pfkm*, *Aldoa* and *Gapdh* were consistently reduced in EDL muscle of MCK-CnA* mice (*Paper IV, Figure 2*). In a coherent fashion, there is an increase in the expression of *Ldh* and *Pdk4* (*Paper IV, Figure 2*), a potent negative regulator for glucose entry into the TCA cycle. Consistent with the mRNA result, the protein content of ALDOA and GAPDH were decreased, whereas HK2 and PDK4 content was increased in EDL muscle from MCK-CnA* mice (*Paper IV, Figure 3*). The increased HK2 expression is consistent with the previous finding of an increased expression of GLUT4 and GS (Ryder et al. 2003), in support of enhanced glucose incorporation into glycogen in the

EDL muscle from MCK-CnA* mice. Therefore, activation of calcineurin in fast-glycolytic skeletal muscle induced a highly coordinated change in at least three modules of glucose metabolic gene expression: activation of genes for glycogen storage (*Glut4*, *Hk2* and *Gs*), suppression of genes for glycolysis (*Pfkm*, *Aldoa* and *Gapdh*) and induction of genes for glucose oxidation (*Ldh* and *Pdk4*). The alteration in the glucose metabolic gene expression program was associated with a shift towards glycogen storage and repression of glucose oxidation.

4.5.3 Calcineurin increases skeletal muscle lipid oxidation and lactate release

Given the profound alteration in the metabolic fate of glucose that was induced by calcineurin, lipid oxidation, an important pathway for skeletal muscle energy production was also determined. Activation of calcineurin in EDL muscle resulted in an increased rate of lipid oxidation under insulin-stimulated conditions (*Paper IV, Figure 4A*), consistent with previous report of increased basal lipid oxidation (Ryder et al. 2003). The increased capacity for lipid oxidation and enhanced expression of PDK4 further supports the notion of a shift in the fuel utilization from glucose towards lipids (Figure 10). The marked increase in the rate of lactate release from EDL muscle of MCK- CnA* mice (*Paper IV, Figure 4B*) further provides evidence for a lower entry of glucose into the TCA cycle.

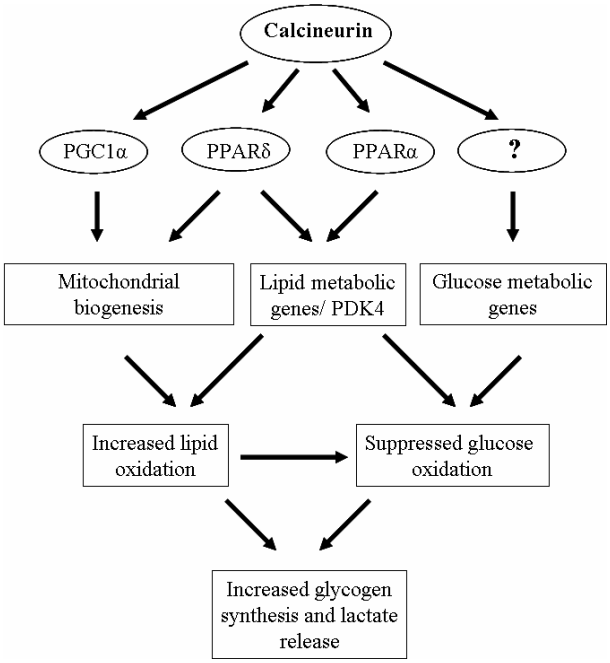

Figure 10: Putative mechanism for calcineurin-induced skeletal muscle metabolic remodeling. Calcineurin activates transcription regulators including PPARα, PPARδ and PGC1α, which induces coordinated expression of lipid and glucose metabolic genes, as well as mitochondrial biogenesis. The role of other transcription regulators in the suppression of glycolytic genes is unknown. The calcineurin-induced metabolic gene program elevates lipid oxidation, lactate release and glycogen synthesis but suppresses glucose oxidation.

4.5.4 Calcineurin activates genes critical for lipid-oxidative metabolism

To further define the calcineurin-induced alterations in the skeletal muscle metabolic gene expression program, expression of genes involved in lipid oxidation were determined by real-time PCR. The expression of *Lpl* and *Cd36*, two genes that are involved in the transport of free fatty acids into the myofiber were increased in EDL muscle from MCK-CnA* mice (*Paper IV, Figure 5*). In a concerted manner, the expressions of genes involved in the CPT system (*Cpt1*, *Cpt2* and *Slc25a20*) were up-regulated in the transgenic mice. Furthermore, activation of calcineurin in fast-glycolytic muscle activated a consistent increase in the expression of *Decr1*, *Acadvl* and *Hadhsc*, genes that are involved in lipid β-oxidation. The increase in lipid oxidation in EDL muscle from MCK-CnA* mice is therefore supported by activation of genes along the linear metabolic pathway of lipid utilization including transport into the myofiber and mitochondria, as well as β-oxidation.

The mitochondrial electron transport chain is critical for the production of ATP from NADH and $FADH_2$, which are derived from fatty acid β-oxidation. Consistent with the increase in lipid oxidation, protein content of the mitochondrial oxidative phosphorylation machinery (complex I through V) were co-ordinately increased (*Paper IV, Figure 6*) in EDL muscle from MCK-CnA* mice. Importantly, activation of calcineurin in skeletal muscle resulted in mitochondrial biogenesis, in support of a higher lipid-oxidative capacity.

4.5.5 Activation of calcineurin and PPARs in skeletal muscle

The profound coordinated pattern of gene expression induced by calcineurin suggests downstream transcription regulators are involved shaping the calcineurin-induced metabolic alterations. The expression of genes involved in the regulation of lipid metabolism such as *Lpl*, *Cd36*, *Cpt1*, *Cpt2*, *Acadvl*, *Hadhsc* and *Pdk4* are regulated by PPARα. (Mandard et al. 2004). Transduction of cultured C2C12 mouse myotubes with a constitutively active calcineurin induces the expression of *Pppar*α via promoter activation (Schaeffer et al. 2004). In skeletal muscle, overexpression of PPARα activates the expression of *Cd36*, *Cpt2*, *Slca25a20*, *Decr1*, *Acadvl*, *Hadhsc* and *Pdk4*, concomitant with an increase in lipid oxidation (Finck et al. 2005). Conversely, genetic ablation of PPARα in mice impairs palmitate oxidation in slow-twitch soleus, as well as fast-twitch epitrochlearis muscle, underlining the critical role of PPARα in skeletal muscle lipid oxidation. The result presented in Paper IV (Figure 7) provides evidence that in skeletal muscle, activation of calcineurin induces the expression of PPARα, which activates lipid metabolic gene expression, in support of enhanced lipid oxidation.

PPARδ has also been implicated in the regulation of skeletal muscle lipid metabolic gene expression. In cultured rat (Muoio et al. 2002; Tanaka et al. 2003) and human myotubes (Muoio et al. 2002), activation of lipid oxidation and regulatory genes has been observed after treatment with a selective PPARδ agonist. Similarly, pharmacological activation of PPARδ *in vivo* results in activation of lipid metabolic genes such as *Cpt1*, *Pdk4* and *Ucp3*, and this is associated with increased lipid oxidation (Tanaka et al. 2003). In EDL muscle from MCK-CnA* mice, protein content of PPARδ was increased (*Paper IV, Figure 7*). However, the protein content of PPARγ, a PPAR isoform that modulates insulin sensitivity was unaltered in the EDL muscle from MCK-CnA* mice (Figure 11). Thus, the result in Paper IV provided evidence for

the role of PPARα and PPARδ in calcineurin-mediated skeletal muscle lipid oxidation and gene expression program.

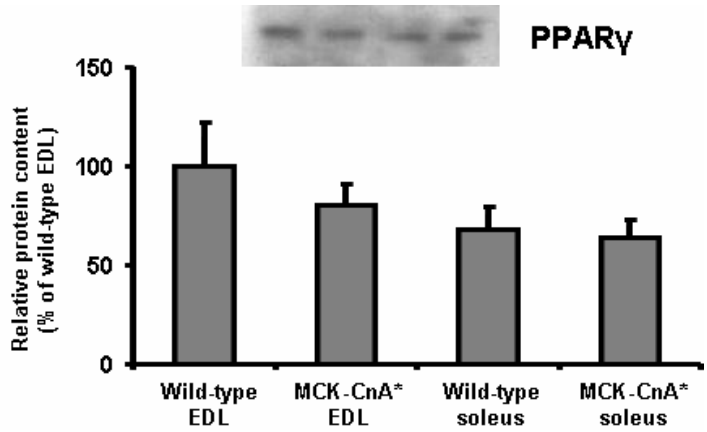

Figure 11: Protein content of PPARγ was unaltered in skeletal muscles from MCK-CnA mice. Protein content of PPARγ in EDL and soleus muscles of wild-type and MCK-CnA* mice was assessed by Western blot analysis. Data are means ± SEM for n=5-6 muscles.*

4.5.6 Calcineurin and transcription regulators of mitochondrial biogenesis

One of the major features in skeletal muscle is the adaptation response to increase oxidative capacity through mitochondrial biogenesis. Several transcription regulators including PGC1α and PPARδ have been implicated in mediating the induction of skeletal muscle mitochondrial biogenesis. Skeletal muscle-specific transgenic mice for PGC1α (Lin et al. 2002) or activated PPARδ (Wang et al. 2004) displayed an induction of slow-oxidative myofiber transformation. Conversely, impaired mitochondrial gene expression (Leone et al. 1999; Arany et al. 2005) and respiration (Leone et al. 1999) were observed in skeletal muscle from PGC1α knockout mice. Furthermore, genetic or pharmacological activation of PPARδ led to increase expression of mitochondrial genes including *Cpt1* and multiple *Cox* genes. In EDL muscle from MCK-CnA* mice, a marked increase in the expression of PGC1α was observed (Paper IV, Figure 7). This result provides a link between calcineurin and the transcription regulators, PGC1α and PPARδ in the induction of skeletal muscle mitochondrial biogenesis.

4.5.7 Calcineurin and exercise-induced skeletal muscle adaptations

The results in Paper IV provided evidence that calcineurin regulates metabolic pathways in skeletal muscle via coordinated changes in gene expression (Figure 10). Several studies have implicated calcineurin as a potential signal transducer for exercise-induced human skeletal muscle adaptations. In human subjects, exercise-induced skeletal muscle PGC1α (Pilegaard et al. 2003b; Norrbom et al. 2004; Garnier et al. 2005) or mitochondrial (Garnier et al. 2005) gene expression is associated with activation of calcineurin (Pilegaard et al. 2003b; Norrbom et al. 2004; Garnier et al. 2005). Inhibition of calcineurin activity in mice by cyclosporine treatment blunted the

exercise-induced activation of skeletal muscle MEF (Wu et al. 2001), supporting a role of calcineurin in exercise-induced transcriptional adaptations. Activation of MEF was also observed in human skeletal muscle after a single acute bout of exercise as evidenced by an increase in MEF nuclear abundance and binding capacity (McGee et al. 2005). However, most studies involving human subjects in general have provided evidence that the major adaptations of skeletal muscle to endurance exercise includes increases in oxidative enzyme activities, and less dramatic effects on the transition of the MHC (Pette and Staron 2001). Although Paper IV showed that activation of calcineurin in skeletal muscle increased glycogen synthesis, lipid oxidation and mitochondrial biogenesis, effects that mirrored adaptive response to exercise, a profound transformation in MHC expression has been reported in the same transgenic mouse model (Naya et al. 2000). The data therefore seems to contradict observations in human studies. However, increased duration and intensity of endurance training can drive transformation beyond the fast MHC population in rodents (Green et al. 1984; Demirel et al. 1999) and humans (Howald et al. 1985). On the other extreme, an opposite transition from slow-twitch to fast-twitch muscle remodeling was observed in spinal-cord injured patients (Aksnes et al. 1996). The precise role of calcineurin in the regulation of skeletal muscle contractile and metabolic adaptations to exercise or motor neuron activity in humans remains to be determined.

5 SUMMARY

- Skeletal muscle (mouse white gastrocnemius muscle) displayed a coordinated increase in the transcription of genes essential for lipid oxidation (*Lpl*, *Cd36*, *Cpt1* and *Cs*) and glucose metabolism (*Gs*, *Ldh*, *Pdk4*) in the adaptive response to fasting.
- These fasting-induced transcriptional responses were impaired in white gastrocnemius muscle from *Prkag3*$^{-/-}$ mice. Conversely, white gastrocnemius muscles from *Tg-Prkag3*^{225Q} mice (transgenic mice that harbor an activating mutant form of AMPKγ3), displayed an enhanced expression of several fasting-responsive genes involved in lipid-oxidative metabolism. Therefore, AMPKγ3 subunit plays a role in the coordinated expression of genes critical for glucose and lipid metabolism in mouse white skeletal muscle.
- Exercise increased glucose uptake in EDL muscle from wild-type mice, with similar responses observed in *Tg-Prkag3*^{225Q} and *Prkag3*$^{-/-}$ mice. Thus, the AMPKγ3 subunit is dispensable for exercise-induced skeletal muscle glucose uptake and transgenic expression of mutant AMPKγ3(R225Q) does not enhance the exercise response.
- White gastrocnemius muscles from *Tg-Prkag3*^{225Q} mice displayed an increase in ACC phosphorylation, concomitant with a reduction in intramuscular triglyceride content immediately after exercise, suggesting that the AMPKγ3(R225Q) mutation drives metabolism towards a greater reliance on lipid oxidation during exercise.
- Expression of AMPKγ3(R225Q) enhanced exercise-induced mRNA expression of *Lpl*, *Cpt1* and *Had* in white gastrocnemius muscle from *Tg-Prkag3*^{225Q} mice, with opposing trend observed in *Prkag3*$^{-/-}$ mice. In white gastrocnemius muscle from *Prkag3*$^{-/-}$ mice, induction of mRNA expression for *Glut4* and *Hk2* was impaired during the recovery period.
- Increased insulin-, but suppressed AICAR-stimulated glucose uptake was observed in EDL muscle from transgenic mice that overexpressed an activated form of calcineurin (MCK-CnA* mice). Contraction-stimulated skeletal muscle glucose uptake; however, was unaffected by activation of calcineurin.
- In EDL muscle from MCK-CnA* mice, contraction-induced AMPK phosphorylation was decreased, but the effects of AICAR was unaltered.
- Increased AMPKγ1, decreased AMPKγ3 and unaltered AMPKγ2 protein expression was observed in EDL muscle from MCK-CnA* mice.
- Lipid oxidation, glucose incorporation into glycogen and lactate release were elevated, whereas glucose oxidation was suppressed in EDL muscle from MCK-CnA* mice.
- In EDL muscle from MCK-CnA* mice, there was an induction of mitochondrial biogenesis, as well as the metabolic gene expression program that supported lipid oxidation, but repressed glucose oxidation.
- The protein expression of PPARα, PPARδ and PGC1α was increased in EDL muscle from MCK-CnA* mice.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

The works presented in this thesis focused on investigating the role of AMPK and calcineurin in the regulation of skeletal muscle metabolic and gene regulatory events. Fasting activated coordinated changes in the expression of regulatory genes for lipid metabolism in white gastrocnemius muscle from wild-type mice, as an adaptation to increase lipid oxidation. The fasting-induced transcriptional adaptations were impaired in the *Prkag3*$^{-/-}$ mice, supporting the essential role of AMPKγ3 in mediating the fasting-induced responses. Conversely, in the *Tg-Prkag3*^{225Q} mice (transgenic mice expressing an activating mutant form of the AMPKγ3 subunit), an enhanced expression of several fasting-responsive lipid metabolic genes was observed. Furthermore, a reciprocal down-regulation in mRNA expression of *Hk2* and *Pfkm* was observed in the transgenic mice, suggesting activation of AMPK resulted in a refinement in the reciprocity between lipid and glucose metabolic gene expression. Whether AMPK acts in a skeletal muscle autonomous fashion, or mediates changes in hormonal or central signals to the muscle during fasting remains to be determined.

The enhanced metabolic flexibility of white skeletal muscle of *Tg-Prkag3*^{225Q} mice was further supported by the observation of enhanced exercise-induced ACC phosphorylation and intramuscular triglyceride utilization. An enhanced metabolic gene expression profile was also observed in the *Tg-Prkag3*^{225Q} mice in response to acute exercise, with an opposing trend observed in the *Prkag3*$^{-/-}$ mice. Thus, the mutant AMPKγ3(R225Q) enhanced biochemical and gene regulatory pathways of skeletal muscle to adapt to the environmental demands.

Although the role of AMPK in skeletal muscle metabolism is apparent, the effect of the AMPK signaling pathway itself is dependent on muscle fiber-type. Activation of calcineurin induced fast-to-slow twitch muscle remodeling (Naya et al. 2000), and the remodeling suppressed AICAR-induced glucose uptake in EDL muscles from MCK-CnA* mice (transgenic mice for an activated form of calcineurin). The impaired AICAR response was associated with a decrease in the expression of AMPKγ3 subunit, a critical subunit required for AICAR-stimulated glucose uptake (Barnes et al. 2004). Contraction-induced glucose uptake in EDL muscles however was unaffected in the MCK-CnA* mice, despite a decrease in contraction-induced AMPK phosphorylation, suggesting AMPK is dispensable for the contraction response in the reprogrammed muscle. However, whether calcineurin-mediated muscle remodeling alters lipid and glucose metabolism in response to exercise or contraction remains to be demonstrated.

Besides AMPK signaling pathways, calcineurin controls the metabolic gene program in skeletal muscle. An enhanced glycogen synthesis and a reciprocal repression of glucose oxidation was observed in EDL muscle from MCK-CnA* mice. Consistently, calcineurin increased skeletal muscle lipid oxidation and lactate release. The repressed glucose oxidation and increased lactate release was supported by a coordinated down-regulation of glycolytic gene and elevation in *Pdk4* expression. In line with the increase in lipid oxidation, the expression of genes essential for lipid

utilization and mitochondrial biogenesis were activated. Consistent with the gene expression program, calcineurin induced the expression of PPARα, PPARδ and PGC1α, transcription regulators which are involved in metabolic and mitochondrial gene expression. Future work is needed to define whether the increased capacity to utilize fat and spare glycogen has an effect on skeletal muscle and exercise performance of the transgenic mice

These findings support a reciprocal pattern of regulation in the expression of lipid and glucose metabolic genes. Therefore, the flexibility of lipid and glucose utilization in skeletal muscle is not only regulated at the level of covalent and allosteric modifications, which has been referred to as the Randle cycle (Frayn 2003), but the reciprocity is also conserved at transcriptional level. The results are clinically relevant and pertinent to human muscle metabolism. A reduction in lipid-oxidative gene expression (Patti et al. 2003), impaired flexibility to increase lipid oxidation, and associated intramuscular triglyceride accumulation are linked to type 2 diabetes (Kelley and Mandarino 2000). Activation of AMPK or calcineurin to improve skeletal muscle metabolic inflexibility may offer novel therapeutic entry points for the treatment of insulin resistance and metabolic disease. Genetic activation of the AMPKγ3 subunit protected mice against diet-induced skeletal muscle insulin resistance (Barnes et al. 2004) and prevented skeletal muscle fatigue during anaerobic exercise (Barnes et al. 2005). Similarly, the increased lipid-oxidative capacity of activated calcineurin transgenic mice resulted in improved insulin responsiveness and protection against the development of diet-induced insulin resistance and glucose intolerance (Ryder et al. 2003). The AMPK and calcineurin pathways are critical for skeletal muscle metabolic flexibility, and may offer novel therapeutic and preventive strategies for metabolic diseases.

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7 ACKNOWLEDGEMENT

The compilation of this thesis marks the final phase of my doctoral program, a journey that would not have come this far without the contributions of the following individuals. My sincere gratitude goes out to:

Professor Juleen Zierath, my scientific advisor and research mentor, for invaluable guidance and the freedom to explore, for trust in my abilities and being ever understanding and encouraging.

Dr. Ulrika Widegren, my co-advisor, for excellent scientific discussion and support, for always being positive and trusting me.

Professor Harriet Wallberg-Henriksson, for invaluable advice and help in my scientific development, and for creating a tradition of excellence in our laboratory.

Professor Marc Gilbert, for sharing exceptional expertise in physiology, and for stimulating discussions.

Docent Alex Chibalin for helpful advice and discussions, and for excellent experimental expertise. Docent Anna Krook, for insightful input and discussions, and for always being approachable.

The dedicated senior research and post-doctoral fellows. Dr. Dana Galuska, for excellent help in human muscle biopsy experiments and ethical issues. Dr. Elaine Vieira, for fruitful collaboration and delightful moments that we shared. Dr. Håkan Karlsson, for constant help, especially during the early morning human muscle experiments. Dr. Jeff Ryder, for outstanding scientific accuracy and insightful discussions. Dr. Karim Bouzakri, for always being there for me, even when you are miles away. Dr. Lubna Al-Khalili, for your concern and sensitivity to my well-being, and for the warmth during the darker winter. Dr. Marie Björnholm, for tremendous effort in facilitating animal experiments. Dr. Megan Osler, for being always enthusiastic and helpful. Dr. Pablo Garcia-Roves, for insightful discussions and valuable support. Dr. Ying Leng for consistent help and collaborations. Dr. Zhihui Zhong, for always being supportive and helping me to settle down at Kungshamra. Docent Inger Kühn, Drs. Elisabet Suárez, Romain Barrés and Qunfeng Jiang for specialized skills and knowledge that greatly enhanced our research team.

The esteemed and distinguished scientists and collaborators whom generously lent their expertise: Professor Eric Olson, University of Texas Southwestern Medical Center; Dr. Jørgen Wojtaszewski, University of Copenhagen; Professor Leif Andersson, Swedish University of Agricultural Sciences; Dr. Margit Mahlapuu, Biovitrum AB; and Professor Stefano Schiaffino, University of Padova.

Margareta Svedlund for superb administrative assistance, for going the extra mile beyond the call of duty, and most importantly for heartwarming kindness. Emily Thorburn, for meticulous assistance in the maintenance of animal models.

The current and former students in our research group who have greatly enriched my experience in the laboratory. Anna Zachrisson, for countless help in timely completion of laboratory duty. Atul Deshmukh, for unflagging support and invaluable encouragement. Firoozeh Salehzadeh, for many interesting discussions that we shared. Maria Ahlsén, for invaluable help with the Swedish healthcare and administrative system. Maria Holmström, for special research interest that added value to our research group. Peter Sögård, for insightful discussion and rewarding collaboration. Reginald Austin, for constructive advice and consistent support. Dr. Brian Barnes and Stephan Glund, for collaborative efforts in numerous experiments. Dr. David Krämer, for your unique sense of humor that lightened up the days. Dr. Sebastio Perrini, for constant care and concern. Dr. Tatiana Steiler, for being there for me during the rainy seasons. Dr. Olga Kotova, for always being helpful whenever called upon.

Special thanks go out to Dr. Weng Onn Lui, for encouraging me to pursue a Ph.D. at Karolinska Institutet. To Colombo Mestrige, Jia Jing Lee, Keng Ling Wallin, Yin Choy Chuan, and all my friends, for great care and never-ending support.

Family back in Malaysia. Pa, for all the trips to the highlands, for your clarity in explanation, a gift that I could only hope for during my writing endeavors. Ma, for your love and care, and for your faith in me. Ko and Han, for the years filled with fun and laughter, we were lucky to have survived the experiments with flammables. Dorena, for always being there for me with tremendous support. Xuan and Rui, for remembering your uncle who is so far away.

Yu Han, my child, for reminding me the simplicity of joy. To my wife, Sue Chin, for always being by my side, and above all, for sacrifice and understanding that far exceed what I could ever reasonably ask for.

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