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MOLECULAR MECHANISMS GOVERNING CONTRACTION-INDUCED METABOLIC RESPONSES AND SKELETAL MUSCLE REPROGRAMMING

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To my family

ABSTRACT

Physical exercise enhances skeletal muscle responsiveness to insulin and regulates metabolism by an insulin-independent mechanism. Elucidation of contraction-mediated molecular mechanisms is imperative for a better understanding of skeletal muscle metabolism and function, and may lead to the identification or validation of possible drug targets for the prevention or treatment of metabolic disorders. This thesis focuses on the role of AMPK and Interleukin (IL)-6 in skeletal muscle metabolism, because AMPK activity and skeletal muscle IL-6 release are increased during skeletal muscle contraction.

Contraction-mediated AMPK activity in white glycolytic extensor digitorum longus (EDL) muscle was inversely coupled to skeletal muscle glycogen content in wild-type and transgenic mice expressing a mutated form of the AMPKγ3 isoform (*Tg-Prkag3*^{225Q}), but not AMPKγ3 knockout (KO) mice, highlighting a role for the AMPKγ3 isoform in energy sensing during muscle contraction. Isolated skeletal muscle from *Tg-Prkag3*^{225Q} and AMPKγ3 KO mice were fatigue-resistant and fatigue-prone, respectively; and work performance was coupled to glycogen content in all mouse models, highlighting a role for AMPKγ3 in skeletal muscle ergogenics by controlling glycogen levels. Hypoxia-mediated glucose transport was partly reduced in skeletal muscle from AMPKγ3 KO mice. AICAR and contraction-mediated phosphorylation of the Akt substrate, AS160, was dependent on functional AMPK signaling, providing direct genetic evidence that AS160 is a phosphorylation target of AMPK.

IL-6 is released from contracting human skeletal muscle. We provide evidence that IL-6 release is greater from oxidative, compared to glycolytic skeletal muscle. Basal IL-6 release was increased from oxidative soleus muscle of AMPKα1 KO and AMPKα2 kinase-dead mice. Thus, we provide evidence for a role of AMPK in the basal regulation of IL-6 release from isolated oxidative skeletal muscle. Furthermore, AICAR-mediated suppression of basal IL-6 mRNA production and release was independent of functional AMPK signaling. Autocrine mechanisms may play a role in basal IL-6 release from isolated skeletal muscle.

IL-6 concentrations in the contracting skeletal muscle may exceed serum levels. We therefore investigated the direct effect of IL-6 on human skeletal muscle by exposing primary human skeletal muscle cells and isolated human skeletal muscle strips to IL-6. IL-6-exposure directly influences glucose metabolism, as determined by increased glucose transport and glucose incorporation into glycogen. In primary human skeletal muscle cells, IL-6-exposure activated components of the canonical insulin signaling cascade. Glucose incorporation into glycogen was sensitive to phosphatidylinositol (PI) 3-kinase inhibition. In contrast, IL-6-exposure was without effect on insulin signaling in isolated human skeletal muscle, and increased glucose metabolism was observed, concomitant with a trend for increased phosphorylation of AMPK. In primary human muscle cells, the IL-6-mediated enhancement of fatty acid oxidation was attenuated by silencing AMPKα isoforms. Long-term IL-6-exposure of primary myotubes enhanced growth and differentiation and increased the expression of genes involved in muscle metabolism.

In conclusion, this thesis work provides evidence that AMPK and IL-6 are central players in the regulation of contraction-mediated effects on metabolic responses in skeletal muscle. In addition to the involvement in acute regulation of metabolism, IL-6 may also participate in skeletal muscle adaptation to exercise.

Key words: AMP-activated protein kinase, interleukin-6, exercise, glucose metabolism, lipid metabolism, adaptation to exercise.

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III. **Glund, S.**, Treebak, J.T., Long, Y.C., Barres, R., Viollet, B., Wojtaszewski, J. F, Zierath, J. R. Role of AMPK in basal and AICAR-mediated IL-6 release from isolated mouse skeletal muscle. *(Submitted)*

IV. **Glund, S.,** Deshmukh, A., Long, Y. C., Moller, T., Koistinen, H. A., Caidahl, K., Zierath, J. R., and Krook, A. Interleukin-6 directly increases glucose metabolism in resting human skeletal muscle. *Diabetes* 56, 1630-1637, 2007.

V. Al-Khalili, L., Bouzakri, K., **Glund, S.,** Lonnqvist, F., Koistinen, H. A., and Krook, A. Signaling specificity of interleukin-6 action on glucose and lipid metabolism in skeletal muscle. *Molecular Endocrinology* 20, 3364-3375, 2006.

CONTENTS

LIST OF ABBREVIATIONS

a.u.	Arbitrary units
ACC	Acetyl-CoA carboxylase
AICAR	5-Aminoimidazole-4-carboxamide-1-b-D-ribonucleoside
AMP	Adenosine monophosphate
AMPK	5'-AMP-activated protein kinase
AS160	Akt substrate of 160 kDa
ATP	Adenosine triphosphate
BMI	Body mass index
CaMKK	Ca^{2+}/calmodulin dependent protein kinase kinase
CPT	Carnitine palmitoyltransferase
EDL	Extensor digitorum longus
GAPDH	Glyceraldehyd-3-phosphate dehydrogenase
GLUT	Glucose transporter
IKK	IkappaB kinase
IL-6	Interleukin-6
IMTG	Intramuscular triglyceride
IRS	Insulin receptor substrate
Jak	Janus kinase
JNK	c-Jun N-terminal kinase
KD	Kinase dead
KO	Knockout
MAP kinase	Mitogen activated protein kinase
MEF	Myocyte enhancer factor
mTOR	Mammalian target of rapamycin
NIDDM	Non-insulin dependent (type 2) diabetes mellitus
NUO	NADH ubiquinol oxidoreductase
PBS	Phosphate buffered saline
PGC	PPARγ coactivator
PI3-kinase	Phosphatidylinositol-3-kinase
PIP	Phosphoinositide-phosphates
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
R_a	Rate of appearance
R_d	Rate of disappearance
SDS	Sodium dodecyl sulfate
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
TBST	Tris-buffered saline containing 0.02% Tween 20
Tg-Prkag3^{225Q}	Transgenic mice expressing mutant R225Q AMPKγ3
TNF	Tumor necrosis factor
UCP	Uncoupling protein

1 INTRODUCTION

Glucose and lipid metabolism are tightly regulated processes in human physiology. Imbalances in substrate use and storage, resulting from genetic defects or inappropriate lifestyle habits, often result in obesity and metabolic disorders, including non-insulin dependent (type 2) diabetes mellitus (NIDDM). The number of people with obesity and NIDDM is increasing dramatically worldwide, thus efficient treatment and prevention strategies need to be developed.

Skeletal muscle is the largest organ in the body and is a major tissue involved in energy balance, accounting for ~20% of total energy expenditure. Skeletal muscle accounts for ~75% of insulin-stimulated glucose disposal. Roughly 90% of glucose transported into skeletal muscle is stored as glycogen. Skeletal muscle insulin resistance is a hallmark feature of NIDDM and related metabolic disorders. Insulin resistance affects all metabolic fates of glucose, including glucose transport, glycogen synthesis and glucose oxidation. Intriguingly, the acute and adaptive metabolic response of skeletal muscle to exercise and contraction remains functional in NIDDM, even if insulin action is impaired. Thus, a greater understanding of molecular and endocrine mechanisms that improve glucose and lipid homeostasis in response to muscle contraction is warranted, to reveal new entry points for therapies designed to prevent and treat metabolic disorders.

1.1 OBESITY, NIDDM AND INSULIN RESISTANCE

People with a body mass index (BMI) of more than 30 (kg/m^2) are considered to be obese. The incidence of obesity is growing dramatically in the United States, Europe and Worldwide (Li *et al.*, 2006; WHO, 2006), which in turn increases the incidence of NIDDM in an epidemic-like fashion (Zimmet *et al.*, 2001). NIDDM is a metabolic disorder primarily characterized by insulin resistance, impaired insulin secretion, and hyperglycemia. NIDDM is incurable with currently available medications (Moller, 2001). People with NIDDM display reduced whole body glucose utilization under hyperinsulinemic euglycemic clamp conditions, mainly due to reduced insulin-mediated glucose uptake into skeletal muscle (DeFronzo, 1988). Therefore, restoring normal insulin-mediated skeletal muscle glucose transport and improving glucose homeostasis are primary goals for the development of drugs to treat insulin resistance in NIDDM (Moller, 2001).

Obesity and NIDDM are associated with a low grade inflammation in adipose tissue (Wellen & Hotamisligil, 2005), which can be linked to the development of insulin resistance (Pradhan *et al.*, 2001; Hotamisligil, 2006). A characteristic feature of inflammation is the infiltration of inflamed tissue by immune cells, including macrophages. Macrophage infiltration of adipose tissue has been observed in obesity (Weisberg *et al.*, 2003) and is linked to the secretion of inflammatory cytokines (Hotamisligil *et al.*, 1995; Kern *et al.*, 1995; Weisberg *et al.*, 2003). Among others, the pro-inflammatory cytokine tumour necrosis factor (TNF) α has been strongly implicated in the development of insulin resistance (Saghizadeh *et al.*, 1996; Kiortsis *et al.*, 2005; Gonzalez-Gay *et al.*, 2006; Krogh-Madsen *et al.*, 2006), and thus bridges inflammation with modulation of insulin sensitivity. Exercise has been shown to dampen the whole body inflammatory state (Geffken *et al.*, 2001; Pischon *et al.*, 2003;

Petersen & Pedersen, 2005), and contracting skeletal muscle may directly contribute to this effect (Starkie *et al.*, 2003; Petersen & Pedersen, 2005).

1.2 SKELETAL MUSCLE PROPERTIES AND CLASSIFICATION

Mammalian skeletal muscle is composed of individual fibers that display marked differences in their contractile, metabolic and biochemical properties. The relative number of fibers with distinct metabolic and contractile characteristics constitutes the properties of a given muscle. Fiber-type composition is regulated by neuronal input, which itself depends on recruitment patterns and exercise training. Muscle fibers can be classified into two major groups depending on contractile and metabolic properties: namely white fast-twitch glycolytic fibers and red slow-twitch oxidative fibers. Contractile classification is based on the expression of the myosin heavy chain gene isoform. Four major myosin heavy chain genes have been identified in skeletal muscle, namely type I, IIA, IIX/IID and IIB (Schiaffino *et al.*, 1989; Schiaffino & Reggiani, 1994). The fibers differ in their contraction rate along a continuous spectrum. Type I muscle fibers maintain slow, but long-lasting contractions due to an enrichment in mitochondria, which consequently increases the oxidative capacity of the fiber. In contrast, type II fibers maintain fast, quickly-fatiguing contractions, due to a high level of glycolytic enzymes, and they mostly rely on glucose from glycogen as energy source. During neonatal development, fiber-type specific gene programs exist (DiMario *et al.*, 1993). Nevertheless in adults, type I and type II fibers adapt to changes in physiological requirements (Holloszy & Coyle, 1984), in particular to changes in frequency and intensity of muscle work (Kraemer *et al.*, 1995). Intriguingly, muscle fibers can contribute to whole body metabolic regulation through the release of proteins into the blood stream (Febbraio & Pedersen, 2005; Pedersen *et al.*, 2007). Because of their muscle origin, these proteins have been named “myokines” (Febbraio & Pedersen, 2005). The production and release of myokines is linked to contraction-mediated processes (Pedersen *et al.*, 2007), and an outstanding question is whether myokine-release is dependent on skeletal muscle fiber-type.

1.3 FIBER-TYPE AND INSULIN SENSITIVITY

Oxidative muscle fibers are more sensitive to insulin stimulation than glycolytic fibers, thus whole body insulin sensitivity is correlated with fiber-type composition (James *et al.*, 1985). Reduced proportion of type I fibers and increased proportion of type II fibers has been noted in NIDDM patients (Marin *et al.*, 1994), normoglycemic subjects with abdominal obesity and insulin resistance (Marin *et al.*, 1994), and first degree relatives of NIDDM patients (Nyholm *et al.*, 1997). Although these changes may occur secondary to elevated insulin concentrations (Marin *et al.*, 1994), they provide correlative evidence that whole body insulin sensitivity is dependent on the expression of oxidative fibers.

Skeletal muscle adaptations to endurance exercise training include increased glycogen stores and enhanced capacity for oxidation of fatty acids (Holloszy & Booth, 1976; Phillips *et al.*, 1996b), while the dependency on glucose and glycogen as substrate is attenuated (Crampes *et al.*, 1986). This is mainly achieved through increases in transcription rate of genes relevant for lipid and glucose metabolism by

acute exercise (Pilegaard *et al.*, 2000), as well as regular training (Pilegaard *et al.*, 2000; Pilegaard *et al.*, 2003). These exercise-mediated adaptations may either be directly coupled to contractile mechanisms or may result from autocrine or paracrine stimulation by muscle-released myokines. Consequently, the question as to whether exposure of human skeletal muscle and primary muscle cells to myokines resembles similar effects requires attention.

1.4 AMP-ACTIVATED PROTEIN KINASE

Skeletal muscle quickly responds to energy-depleting metabolic challenges, such as exercise or fasting. These stimuli reduce the AMP to ATP ratio, which signals that the energy status of the cell is compromised. AMPK senses variations in the AMP to ATP ratio. Activation of AMPK facilitates acute metabolic responses to restore normal energy status, and activates gene transcriptional programs along oxidative pathways. Thus, AMPK is proposed to play a central role in insulin-independent regulation of skeletal muscle metabolism and may contribute to skeletal muscle adaptation to exercise.

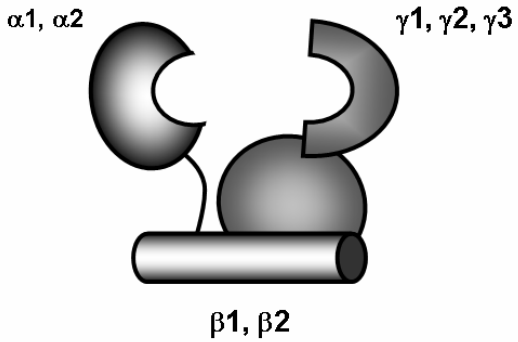


Figure 1: AMPK consists of three subunits. The expression of AMPK isoforms is tissue specific.

The ubiquitously expressed AMPK is a heterotrimeric complex consisting of a catalytic α and regulatory β and γ subunits (Carling, 2004; Hardie, 2004). There may be up to 12 different AMPK heterotrimers, due to the expression of two α (α1 and α2), two β (β1 and β2) and three γ (γ1, γ2 and γ3) isoforms (Carling, 2004; Hardie, 2004) and their tissue-specific expression pattern. AMPK senses energy depletion (i.e. reduction in the AMP to ATP ratio), by binding of AMP to the γ subunit of AMPK (Hardie *et al.*, 1999; Hardie *et al.*, 2006). This results in mild activation of AMPK, but more importantly, induces a conformational change turning AMPK into a better substrate for its upstream kinase LKB1. LKB1 phosphorylates the AMPKα subunit on Thr^{172} and thereby increases AMPK activity (Hawley *et al.*, 2003). At the same time, the increased concentration of AMP inhibits the activity of protein phosphatases that dephosphorylate AMPK, further increasing the AMPK-activating effect of AMP (Hardie *et al.*, 1999; Hardie *et al.*, 2006). LKB1 is constitutively active and is not subject to direct activation or inhibition (Lizcano *et al.*, 2004; Sakamoto *et al.*, 2004). Another AMPK kinase, the Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK)β, integrates Ca^{2+}-signaling with AMPK activation (Hawley *et al.*, 2005; Woods *et al.*, 2005), apparently independent of changes in the AMP to ATP ratio (Hawley *et al.*, 2005). This suggests a time-dependent relative role of AMPK kinases in the activation of AMPK during exercise, whereby CaMKKβ plays an important role at

the onset of skeletal muscle contraction, compared to a minor role at later stages (Jensen *et al.*, 2007).

AMPK has been implicated in numerous metabolic and mitogenic processes, including increased glucose uptake, increased lipid oxidation, regulation of metabolic gene expression and mitochondrial biogenesis. Distinct metabolic effects may be linked to the action of specific AMPK heterotrimers. For example, three AMPK heterotrimers ($\alpha2\beta2\gamma3$, $\alpha2\beta2\gamma1$ and $\alpha1\beta2\gamma1$) have been found in human skeletal muscle (Wojtaszewski *et al.*, 2005), whereby AICAR mediates glucose transport only in the presence of functional AMPK$\alpha2$ (Jorgensen *et al.*, 2004b) and AMPK$\gamma3$ (Barnes *et al.*, 2004) isoforms.

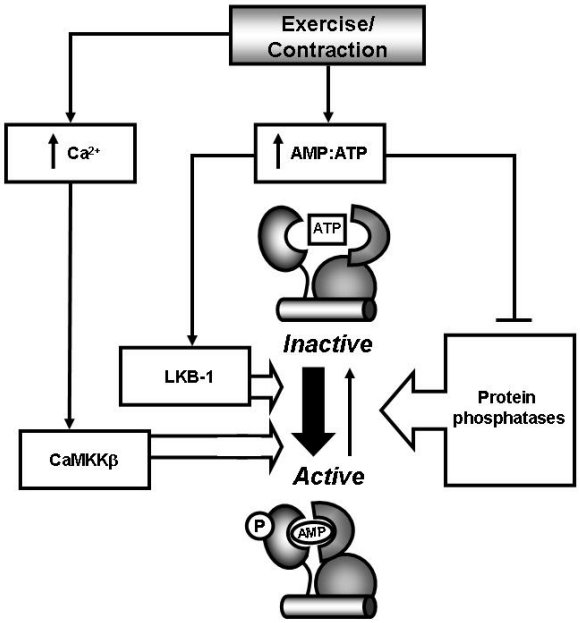


Figure 2: Mechanisms involved in contraction-mediated AMPK activation.

1.5 AMPK ACTIVITY AND MUSCLE GLYCOGEN CONTENT

There is considerable evidence suggesting an inverse relationship between AMPK activity and skeletal muscle glycogen levels. The AMPKβ subunit comprises a glycogen binding domain (Polekhina *et al.*, 2003), and basal and AICAR-stimulated AMPK activity are depressed when skeletal muscle glycogen is plentiful (Wojtaszewski *et al.*, 2002). Because glycogen addition does not change the activity of AMPK *in vitro* (Polekhina *et al.*, 2003), the glycogen binding domain of the AMPKβ subunit may be relevant for subcellular localization of AMPK rather than modulating AMPK kinase activity (Polekhina *et al.*, 2003).

Glycogen repletion is positively correlated with glycogen synthase activity under strenuous exercise conditions (Conlee *et al.*, 1978). Overexpression of constitutively active glycogen synthase in skeletal muscle positively increases skeletal muscle glycogen levels (Fogt *et al.*, 2004), while genetic loss of glycogen synthase results in complete loss of glycogen (Pederson *et al.*, 2005). Glycogen synthase is phosphorylated by (Carling & Hardie, 1989), and co-immunoprecipitates with (Chen *et al.*, 1999b), AMPK *in vitro*, providing a molecular linkage between AMPK activation and glycogen synthesis rate. Phosphorylation of glycogen synthase on up to 9 residues results in a progressive inactivation and decreased sensitivity to allosteric activators. Conversely, binding of glucose 6-phosphate to glycogen synthase causes unfolding of the enzyme and allosteric activation that overrides inhibition by phosphorylation, and

causes glycogen synthase to translocate to glycogen particles (Lawrence & Roach, 1997; Nielsen *et al.*, 2001; Prats *et al.*, 2005). Thus, in addition to regulation of glycogen synthase activity by phosphorylation, AMPK may regulate glycogen synthesis by modulating glucose-6-phosphate levels. AMPK α2 kinase dead (KD) mice (Mu *et al.*, 2001), AMPKα2 knockout (KO) mice (Jorgensen *et al.*, 2004b), and mice lacking muscle specifically LKB1 (Koh *et al.*, 2006) display reductions in glycogen content of glycolytic skeletal muscle, providing evidence that AMPK activity is necessary to maintain normal muscle glycogen. The AMPKγ3 isoform is rather specifically expressed in glycolytic muscle. However, whether this isoform is necessary to link AMPK activity with glycogen content is unresolved. Furthermore, muscle fatigue is coupled to glycogen content (Layzer, 1990), raising the question whether the AMPKγ3 isoform plays a role in regulating muscle performance.

1.6 FUEL SELECTION AND METABOLIC FLEXIBILITY

Skeletal muscle utilizes glucose and lipids as fuels. In healthy lean subjects, skeletal muscle quickly switches from lipid oxidation and high rates of lipid uptake during fasting conditions (Andres *et al.*, 1956) to glucose metabolism in response to feeding or insulin-stimulation (Kelley *et al.*, 1990) This "metabolic flexibility" is achieved by a complex regulatory network (Kelley & Mandarino, 2000). Skeletal muscle from insulin resistant subjects displays an impairment of metabolic flexibility, whereby skeletal muscle does not appropriately increase lipid oxidation when challenged by fasting or exercise (Kelley & Mandarino, 2000) and conversely fails to shut off lipid oxidation during insulin-stimulation (Felber *et al.*, 1987). One of the proposed causal mechanisms is the ectopic (intramuscular) deposition of triglycerides (IMTG). There is compelling evidence that increased levels of IMTG have the capacity to impair insulin signal transduction (Storlien *et al.*, 1991; Phillips *et al.*, 1996a; Pan *et al.*, 1997; Kelley & Mandarino, 2000). During contraction, skeletal muscle oxidizes lipids, including IMTG, as fuel. Infusion of the myokine IL-6 into humans increases whole body lipid metabolism (van Hall *et al.*, 2003). Whether *in vitro*-exposure of skeletal muscle to the myokine IL-6 directly increases lipid metabolism remains to be determined.

1.7 SKELETAL MUSCLE LIPID METABOLISM

During exercise, skeletal muscle metabolism greatly depends on lipid oxidation. Lipid oxidation is regulated by the availability of fatty acids for β-oxidation in the mitochondria (Ruderman *et al.*, 1999). Carnitine palmitoyl transferase 1 (CPT1) mediates the transfer of long-chain fatty acids into the mitochondria (McGarry, 1995). High concentrations of malonyl-CoA, a precursor for fatty-acid synthesis, allosterically inhibit CPT1 (Ruderman *et al.*, 1999). Activated AMPK phosphorylates and inhibits its downstream-target acetyl-CoA carboxylase (ACC) (Winder & Hardie, 1996), which as a result blocks the synthesis of malonyl-CoA. At the same time, activated AMPK may (Saha *et al.*, 2000) or may not (Habinowski *et al.*, 2001) facilitate the decarboxylation of malonyl-CoA by stimulating malonyl-CoA decarboxylase. Thus malonyl-CoA levels, and consequently fatty acid oxidation, are highly sensitive to AMPK activation. The central role of ACC2 in the regulation of lipid oxidation in skeletal muscle is

highlighted by the phenotype of ACC2 KO mice, which are characterized by elevated muscle fatty acid oxidation rates that are insensitive to suppression by insulin (Abu-Elheiga *et al.*, 2001). Thus, targeting AMPK and thereby inhibiting ACC2 may be a rewarding strategy to reduce IMTG content, and consequently increase insulin sensitivity on glucose transport.

1.8 INSULIN-STIMULATION OF GLUCOSE TRANSPORT

Binding of insulin to the insulin receptor results in receptor autophosphorylation (White, 2003) and subsequent initiation of a complex signaling cascade. There are at least three pathways that convey the signal generated by the insulin receptor: the insulin receptor substrate (IRS)/PI3-kinase pathway, the retrovirus-associated DNA sequences (RAS)/mitogen-activated protein kinase (MAPK) pathway, and the Cbl-associated protein (CAP)/Cbl pathway. The RAS/MAPK cascade is important for gene-regulatory responses after insulin-stimulation, but not the acute regulation of glucose transport (Long *et al.*, 2004). The CAP/Cbl pathway has been reported to contribute to insulin-stimulated glucose transport (Watson *et al.*, 2001), however this is challenged by studies in 3T3-L1 adipocytes (Mitra *et al.*, 2004) and c-Cbl$^{-/-}$ mice (Molero *et al.*, 2004). The signal for metabolic actions of insulin is mainly transmitted and amplified through IRS molecules (IRS1 through 4), of which IRS1 and IRS2 are most relevant for insulin signaling in skeletal muscle (White, 2003). Studies in human skeletal muscle cells reveal IRS1 is required for glucose metabolism, while IRS2 has a key role in lipid metabolism (Bouzakri *et al.*, 2006). Tyrosine-phosphorylation of IRS1 increases binding and activity of PI3-kinase, which positions PI3-kinase in close proximity to the plasma membrane, and increases the availability of lipid substrates for PI3-kinase to generate phosphoinositide-phosphates (PIP), including PIP3. Subsequently, phosphoinositide-dependent kinase (PDK) 1 binds to PIP3, which in turn triggers PDK1-mediated Thr^{308}-phosphorylation of the serine-threonine kinase Akt (also called protein kinase B) (Jiang *et al.*, 2003). Ser^{473}-phosphorylation of Akt by the rictor-mTOR complex (Sarbassov *et al.*, 2005) facilitates Thr^{308} phosphorylation by PDK1, and is necessary for full activation of the kinase. Activated Akt promotes glucose transport, presumably through phosphorylation and inactivation of AS160. AS160 is a Rab GTPase-activating protein that has been strongly implicated in insulin-mediated glucose transport in adipocytes (Sano *et al.*, 2003; Zeigerer *et al.*, 2004) and is also present in human and rodent skeletal muscle (Bruss *et al.*, 2005; Karlsson *et al.*, 2005). Through its activity in the basal state, AS160 inactivates Rab proteins, small proteins involved in vesicle trafficking, thus preventing them from participation in the translocation of GLUT4 containing vesicles to the plasma membrane (Sano *et al.*, 2003; Zeigerer *et al.*, 2004). There are at least eight different phosphorylation sites on AS160 (Geraghty *et al.*, 2007), whereby AS160 phosphorylation reduces its activity; consequently increases the activity of target Rab proteins, and eventually increases glucose uptake via increasing the number of GLUT4 transporters in the plasma membrane.

1.9 INSULIN SIGNALING IN NIDDM

Skeletal muscle insulin signaling, and consequently insulin-mediated glucose transport, is impaired in NIDDM patients. Defects have been observed at different levels of the canonical insulin signaling cascade (Caro *et al.*, 1987; Garvey *et al.*, 1998; Kim *et al.*, 1999; Cusi *et al.*, 2000; Krook *et al.*, 2000; Arner *et al.*, 1987; Krook *et al.*, 1998; Karlsson *et al.*, 2005). The master-control of insulin-signaling towards glucose transport, however, appears to be IRS1. Insulin-signaling through IRS1 can be modulated by differential phosphorylation, as well as alterations in protein expression (White, 2003). IRS1 protein levels are unaltered in skeletal muscle from NIDDM patients (Krook *et al.*, 2000; Huang *et al.*, 2002), but IRS1 serine phosphorylation is increased, preventing normal tyrosine phosphorylation by the insulin receptor and thus down-regulating insulin signaling (reviewed in (Draznin, 2006)). Kinases for IRS1 serine phosphorylation include protein kinase C (PKC) θ (Kim *et al.*, 2004), c-Jun N-terminal kinase (JNK) (Hirosumi *et al.*, 2002), IkappaB kinase (IKK) β (Gao *et al.*, 2002) and p70S6 kinase (Tremblay & Marette, 2001). Triggers for IRS1 serine phosphorylation in metabolic disorders are manifold and include increased IMTG accumulation, exposure to pro-inflammatory cytokines, and importantly, physical inactivity (White, 2003).

1.10 EXERCISE ENHANCES INSULIN SIGNALING

Exercise training improves insulin sensitivity (Mikines *et al.*, 1988; Cartee *et al.*, 1989), lowers blood triglycerides (Thompson *et al.*, 1980; Schriewer *et al.*, 1983) and reduces whole body inflammatory state (Geffken *et al.*, 2001; Pischon *et al.*, 2003). Skeletal muscle insulin-sensitivity is increased after an acute bout of endurance exercise (Garetto *et al.*, 1984; Wallberg-Henriksson *et al.*, 1988). The increase in skeletal muscle insulin sensitivity is linked to the energy state, as insulin sensitivity remains enhanced until glycogen stores are restored (Fell *et al.*, 1982). While the acute effects of exercise on insulin sensitivity appear to be independent of changes in protein expression (Fisher *et al.*, 2002), changes in protein expression are central part of the long-term adaptation to exercise. In particular, increases in glucose transporter (GLUT)4 protein content (Kern *et al.*, 1990) have been attributed to an improved insulin-mediated glucose transport after exercise (Friedman *et al.*, 1990; Ploug *et al.*, 1990). This is observed along with an increased capacity for insulin signaling, including enhanced insulin-mediated IRS1 tyrosine phosphorylation (Chibalin *et al.*, 2000), IRS1-associated PI3-kinase activity (Chibalin *et al.*, 2000; Kirwan *et al.*, 2000) and Akt phosphorylation (Chibalin *et al.*, 2000). A proposed mechanism for this effect is the opposite regulation of the mammalian target of rapamycin (mTOR)-p70S6 kinase signaling pathway by insulin and AMPK via tuberous sclerosis complex 2 (TSC2) (Jozwiak *et al.*, 2005), whereby AMPK activation reduces p70S6 kinase-mediated IRS1 serine phosphorylation and consequently increases insulin signal transduction (Wang *et al.*, 2007). Moreover, AMPK has been shown to participate in the regulation of PPARγ coactivator (PGC)-1α (Winder *et al.*, 2006), a transcription factor involved in mitochondrial biogenesis, and thus further contributes to enhance insulin sensitivity. Taken together, exercise enhances insulin signal transduction through acute and long-term adaptations. Whether myokines can directly improve skeletal muscle insulin signal transduction is currently unknown.

1.11 EXERCISE STIMULATES MUSCLE GLUCOSE TRANSPORT

Exercise/muscle contraction potently stimulates skeletal muscle glucose transport (Wallberg-Henriksson & Holloszy, 1984; Nesher *et al.*, 1985). The effect of exercise/contraction and insulin on GLUT4 translocation to the plasma membrane, as well as glucose transport, are additive in isolated skeletal muscle (Nesher *et al.*, 1985; Constable *et al.*, 1988), suggesting the two stimuli signal through distinct pathways. Release of Ca^{2+} from the sarcoplasmatic reticulum is a fundamental process in skeletal muscle contraction, and Ca^{2+}-mediated signaling pathways have been implicated in mediating glucose transport in response to contraction (Youn *et al.*, 1991; Wright *et al.*, 2004). Conversely, skeletal muscle contraction reduces the energy level, and consequently activates AMPK (Hutber *et al.*, 1997). Pharmacological activation of AMPK stimulates glucose transport (Merrill *et al.*, 1997). Contraction-mediated glucose transport is partly abolished in isolated muscle from mice lacking LKB1 (Sakamoto *et al.*, 2005). Furthermore, *in vitro* exposure of skeletal muscle from AMPKα2 KD mice to contraction or hypoxia results in a partial or complete suppression of glucose transport, respectively (Mu *et al.*, 2001). In contrast, contraction-mediated glucose transport is unaltered in another mouse model with transgenic expression of inactivated AMPKα2 (Fujii *et al.*, 2005), as well as in mouse models lacking AMPK isoforms, such as the AMPKα2 and AMPKα1 KO mice (Jorgensen *et al.*, 2004b) or the AMPKγ3 KO mouse (Barnes *et al.*, 2004). Taken together, the role of AMPK in contraction-mediated glucose transport is incompletely resolved. However, contraction, similarly to insulin, induces AS160 phosphorylation (Bruss *et al.*, 2005), a process implicated in insulin-mediated glucose transport in adipocytes (Sano *et al.*, 2003). Thus the insulin signaling cascade and contraction-mediated processes may converge at the level of AS160 phosphorylation. If so, stimulation of contraction-related mechanisms may overcome signaling impairments in the insulin signaling cascade. An unresolved question is whether AMPK can directly target AS160 and whether this process is of relevance for contraction-mediated AS160 phosphorylation.

1.12 INTERLEUKIN-6

IL-6 is a cytokine that is involved in many biological processes, including inflammation and immune response (Hodge *et al.*, 2005; Burkhard & Peter, 2006), brain function (Balschun *et al.*, 2004) and fatigue (Spath-Schwalbe *et al.*, 1998). Several tissues may contribute to basal circulating IL-6 levels, whereby adipose tissue contributes the largest amount (up to 35%) (Mohamed-Ali *et al.*, 1997; Kern *et al.*, 2001). Two subunits make up the receptors for members of the IL-6 family of cytokines: gp130, a non-ligand binding protein responsible for intracellular signal transduction, and the ligand-specific alpha chain, IL-6Rα (also called CD126) in the case of IL-6 (Kamimura *et al.*, 2003). Stimulation of the IL-6 receptor activates several signaling pathways, including the Janus kinase (Jak), signal transducer and activator of transcription (STAT), Akt (Chen *et al.*, 1999a; Jee *et al.*, 2002; Jee *et al.*, 2004) and mitogen activated protein (MAP) kinase (Yang *et al.*, 2003; Lentzsch *et al.*, 2004) dependent signaling pathways. STATs are known to induce gene expression, including SOCS (suppressor of cytokine signaling) proteins (Murray, 2007). SOCS3 serves as a negative feedback for IL-6 signaling by inhibiting at the level of Jak (Murray, 2007),

but may also negatively affect insulin signaling via serine phosphorylation of IRS1 (Rieusset *et al.*, 2004). Consequently, IL-6 has been implicated in the development of insulin resistance, but this remains to be directly proven.

1.13 IL-6 AND INSULIN RESISTANCE

In line with the proposal of low grade inflammation as a causal event in obesity-driven insulin resistance, population based studies have provided evidence for a positive correlation between IL-6, obesity and insulin resistance (Kern *et al.*, 2001; Vozarova *et al.*, 2001; Bastard *et al.*, 2002; Spranger *et al.*, 2003), leading to the hypothesis of a causal relationship between elevated IL-6 and insulin resistance.

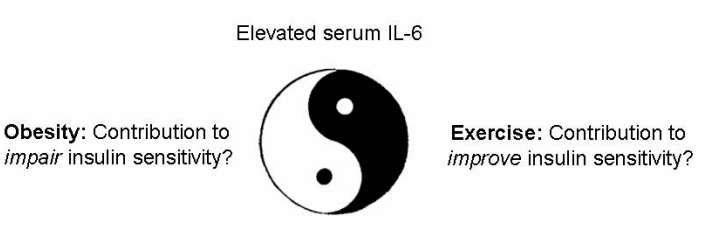


Figure 3: The yin and yang of elevated serum IL-6 concentrations with respect to whole body insulin sensitivity.

In lean first degree relatives of NIDDM patients, however, insulin resistance is evident in the absence of any elevation of IL-6 levels as compared to age, fitness and weight-matched controls (Petersen *et al.*, 2004). Also, serum IL-6 levels tend to increase with age (for review see (Maggio *et al.*, 2006)). Insulin resistance and NIDDM also increase with age, possibly explaining some of the observed correlations. Thus, an indirect association between metabolic disorder and IL-6 has been suggested, but causation has yet to be proven, and the causal relationship between IL-6, obesity and NIDDM remains a matter of debate today (Kristiansen & Mandrup-Poulsen, 2005; Maggio *et al.*, 2006; Pedersen *et al.*, 2006).

1.14 CONTRACTING SKELETAL MUSCLE RELEASES IL-6

Serum concentrations of IL-6 increase dramatically during prolonged endurance exercise (Ostrowski *et al.*, 1998; Steensberg *et al.*, 2000; Steensberg *et al.*, 2002; Febbraio *et al.*, 2003). Contracting skeletal muscle is the predominant source of serum IL-6 during exercise (Ostrowski *et al.*, 1998; Hiscock *et al.*, 2004; Banzet *et al.*, 2005), and the effects of IL-6 on non-muscle target-tissues may increase the flow of nutrients towards skeletal muscle, to enhance work performance (Fischer, 2006). Various molecular mechanisms have been implicated in the control of IL-6 production

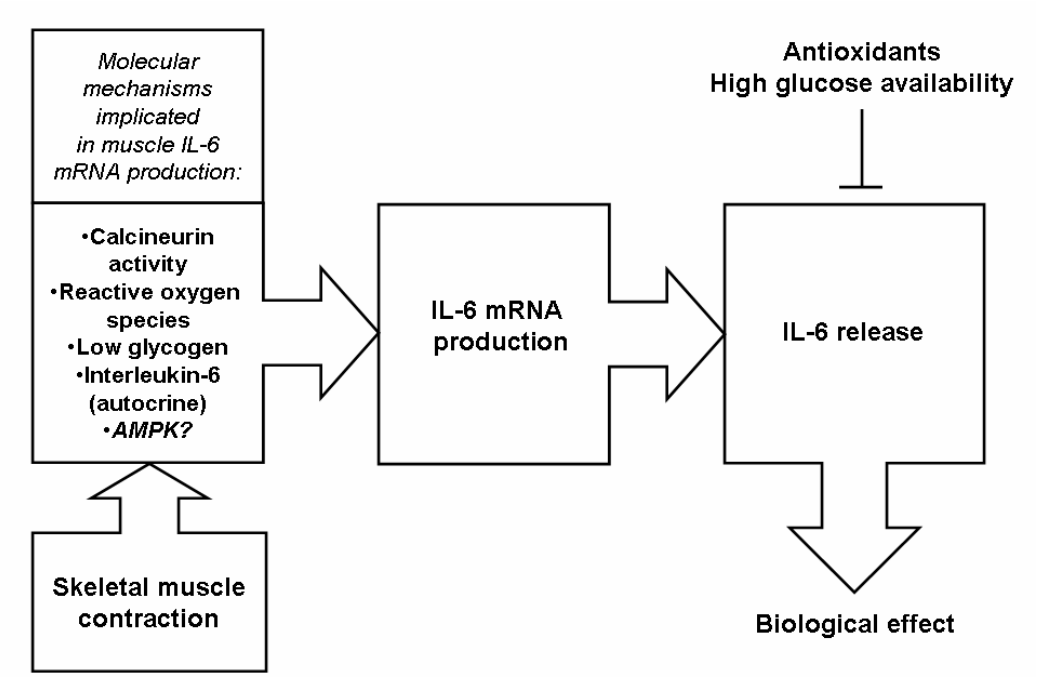


Figure 4: Overview of proposed mechanisms involved in contraction-mediated skeletal muscle IL-6 release.

in skeletal muscle, including Ca^{2+}-dependent pathways mediated via calcineurin activation (Keller *et al.*, 2006; Banzet *et al.*, 2007), increased reactive oxygen species (Kosmidou *et al.*, 2002), autocrine stimulation by IL-6 (Keller *et al.*, 2003; Weigert *et al.*, 2007), and impaired glucose availability (low glycogen levels) (Steensberg *et al.*, 2001; Chan *et al.*, 2004b). AMPK activation has also been implicated in IL-6 release, as AMPK activity and IL-6 release correlate during exercise (MacDonald *et al.*, 2003), but studies determining the direct effect of AMPK activation on isolated skeletal muscle IL-6 release are lacking.

A role for IL-6 in whole body glucose and lipid metabolism in humans has been demonstrated using infusion experiments (Stouthard *et al.*, 1995; Tsigos *et al.*, 1997; Lyngso *et al.*, 2002; van Hall *et al.*, 2003; Febbraio *et al.*, 2004; Petersen *et al.*, 2005; Watt *et al.*, 2005; Carey *et al.*, 2006). Lipid metabolism appears to be more sensitive to IL-6-stimulation than glucose metabolism. For example, whole body oxygen consumption and carbon dioxide production increases in response to IL-6 infusion (Tsigos *et al.*, 1997; Carey *et al.*, 2006). IL-6 stimulates fatty acid turnover (van Hall *et al.*, 2003; Petersen *et al.*, 2005), consistent with *in vitro* findings that IL-6 increases lipolysis in adipocytes (Trujillo *et al.*, 2004; Petersen *et al.*, 2005) and increases fatty acid oxidation in skeletal muscle cells (Petersen *et al.*, 2005; Carey *et al.*, 2006).

In vitro and *in vivo* studies in animals provide evidence that IL-6 interferes with insulin signal transduction in hepatocytes or liver (Kanemaki *et al.*, 1998; Senn *et al.*, 2002; Klover *et al.*, 2003; Senn *et al.*, 2003; Klover *et al.*, 2005). In contrast, an acute increase in the IL-6 concentration has little effect on the rate of glucose appearance (R_a) or disappearance (R_d) in humans (Steensberg *et al.*, 2003; Petersen *et al.*, 2005). Conversely, low levels of IL-6 increase glucose R_a and R_d during exercise (Febbraio *et*

al., 2004) and the existence of an exercise "cofactor" has been proposed (Febbraio *et al.*, 2004). However, interstitial IL-6 concentrations in the exercising muscle might be 5-100 fold higher than levels found in the circulation (Langberg *et al.*, 2002; Rosendal *et al.*, 2005) and the direct effect of IL-6 on skeletal muscle remains to be determined.

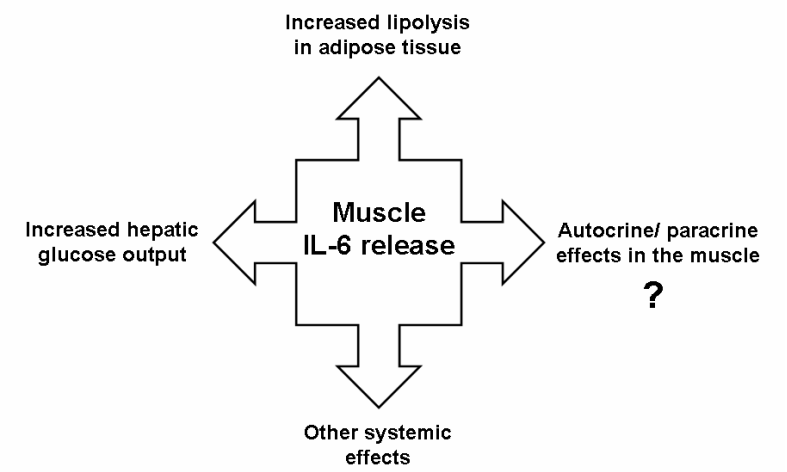


Figure 5: Proposed biological effects elicited by IL-6.

2 AIMS

Skeletal muscle is the body's most important tissue for glucose disposal in response to insulin stimulation, and furthermore displays a large capacity to oxidize lipids as energy substrate. In NIDDM and the metabolic syndrome, insulin action on skeletal muscle glucose uptake and metabolism is dysfunctional. Thus, investigations to identify and characterize molecular mechanisms that increase insulin sensitivity or offer insulin-independent regulation of skeletal muscle metabolism are the basis for the development of new treatment strategies to overcome insulin resistance. The overall aim of this thesis is to identify and validate molecular mechanisms governing contraction-mediated metabolic and mitogenic responses in skeletal muscle. In particular, the role of AMPK in skeletal muscle metabolic regulation was determined by applying genetic approaches. Studies were conducted to explore the relationship between AMPK and the myokine IL-6, as well as to investigate the effect of paracrine stimulation of skeletal muscle with IL-6.

The following specific questions were addressed:

- Does the AMPKγ3 subunit play a role in regulating skeletal muscle endurance, and what are underlying molecular mechanisms?
- Is AS160 an AMPK target in skeletal muscle?
- Does AMPK play a role in basal and AICAR-mediated IL-6 release from isolated skeletal muscle?
- Does IL-6 directly affect glucose metabolism in isolated human skeletal muscle?
- What are the effects of IL-6-exposure of primary human skeletal muscle cells on glucose and lipid metabolism, as well as growth and differentiation? What are underlying molecular mechanisms?

3 EXPERIMENTAL PROCEDURES

3.1 MOUSE SPECIFIC TECHNIQUES

3.1.1 Animal care

All animals utilized in this thesis were cared for in accordance with regulations for the protection of laboratory animals and complied with the European Convention for the protection of Vertebrate Animals used for Experiments and Other Scientific Purposes (Council of Europe 123, Strasbourg, France, 1986). The regional animal ethical committee in Stockholm, Sweden (*Paper I, II, III* and supplementary data presented in this thesis), or the Danish Animal Experimental Inspectorate (*Paper II and III*), approved all experimental procedures. Mice were maintained in a temperature and light controlled environment and had free access to water and standard rodent chow. Animals were studied in fed (*Paper II*) and fasted condition (overnight, ~16 h in *Paper I*, and 4 h in *Paper III*). All mice were bred in-house, except for AMPKα1 KO mice, AMPKα2 KO mice, AMPKα2 KD mice, and their respective wild-type littermates, which were bred at the Copenhagen Muscle Research Center (*Paper II* and *III*).

3.1.2 Mouse models

The generation of mutant transgenic *Tg-Prkag3*^{225Q} (AMPKγ3 R225Q) and AMPKγ3 KO mice were previously described (Barnes *et al.*, 2004). The mutant AMPKγ3 R225Q transgene is under the control of the skeletal muscle myosin light chain (MLC) promoter, with SV40 poly A signal and MLC1 enhancer inserted in 3' UTR. Traditional gene targeting techniques were applied to generate AMPKγ3 KO mice. The knockout targeting construct caused major frameshift with premature stop codon.

The AMPKα1 KO mouse and its phenotype have been described (Jorgensen *et al.*, 2004b). A mouse 129-strain genomic library (Stratagene, La Jolla, CA) was screened with a specific mouse AMPK catalytic α1-subunit 500-bp fragment. One genomic clone encompassing a 14.5-kb genomic fragment was used to generate the targeting construct.

Generation of the AMPKα2 KO mouse and its phenotype has been published (Viollet *et al.*, 2003). AMPKα2 genomic clones were isolated after screening a mouse 129-strain genomic library. The targeting construct was generated by flanking exon C, which encodes the AMPKα2 catalytic domain, with *loxP* sites for the Cre recombinase and inserting a phosphoglycerol kinase promoter-driven neomycin selection cassette flanked by an additional *loxP* site. Germline-transmitting chimeric mice were mated with C57/Bl6 mice. Breeding the heterozygous offspring with transgenic mice expressing Cre in germ cells resulted in heterozygous AMPKα2 KO mice.

The AMPKα2 KD mouse model (Mu *et al.*, 2001) was kindly provided by Morris J. Birnbaum, Howard Hughes Medical Institute and University of Pennsylvania School of Medicine, Philadelphia, PA, USA. To generate AMPKα2 KD mice, a rat AMPKα2 construct was mutated *in vitro* at a single site (lysine 45 was changed to arginine). This lysine residue is critical for ATP binding and hydrolysis, thus rendering the construct in a cDNA encoding a kinase-dead protein. The transgene is under the control of the muscle creatine kinase promoter.

3.1.3 Swimming exercise

Fed mice performed swim exercise as previously described (Ryder *et al.*, 1999), with modifications. Mice were assigned to rest or exercise group. Six mice swam together in plastic barrels filled with water to a depth of ~15 cm for four 30 min intervals separated by 5 min rest periods. Water temperature was maintained at 29°C. After the last swim interval, mice were immediately anaesthetized with Avertin (2,2,2-Tribromo ethanol 99% and Tertiary amyl alcohol (0.015-0.017 ml/g body weight i.p.)), and trunk blood was collected.

3.1.4 Skeletal muscle isolation

Basal incubation buffer was prepared from a stock solution of Krebs-Henseleit bicarbonate buffer (KHB) supplemented with 5 mmol/l glucose, 15 mmol/l mannitol, 5 mmol/l HEPES and 0.1% bovine serum albumin (BSA, radioimmunoassay grade), and continuously gassed with 95% O_2/ 5% CO_2. For the hypoxia experiments, a separate basal hypoxia buffer was prepared and continuously gassed with 95% N_2/ 5% CO_2. Mice were anesthetized with either Avertin (AMPK γ3 KO mice, *Tg-Prkag3*^{225Q} mice and their respective wild-type littermates (*Paper I, II, III*)), or pentobarbital (6 mg /100 g body wt i.p. for AMPKα1 KO, AMPKα2 KO and AMPKα2 KD mice (*Paper II and III*)), and extensor digitorum longus (EDL) and/or soleus muscles were excised. Muscles were incubated at 30˚C for 10 or 40 min (*Paper II*), 30 min (*Paper III*), or 60 min (*Paper I*) in basal incubation buffer to recover from the surgery. When appropriate, inhibitors were added to recovery media to ensure muscle penetration (*Paper II* and hypoxia experiments).

3.1.5 Pharmacological muscle stimulation and incubation

After recovery, muscles were transferred to new vials containing basal incubation buffer. AICAR (Sigma), insulin (Actrapid, Novo), ionomycin (Sigma), or AICAR and insulin together, were added as indicated in the respective studies. In some experiments, wortmannin (500 nmol/l, Sigma) (*Paper II*) or KN-93 (25 μmol/l, Calbiochem) (hypoxia experiments) was added to the media. Muscles were incubated at 30˚C for 30, 40 or 120 min (*Paper I, II or III*, respectively). For the AICAR time course experiments, EDL muscle was incubated for 10, 20, 40, or 60 min (*Paper II*). Thereafter, muscles were either trimmed of non-muscle tissue, immediately frozen in liquid nitrogen and stored at -80˚C for later signaling analysis, or incubated further to determine glucose transport. In *Paper III*, an aliquot of incubation media was stored at -20˚C for later determination of IL-6 concentration.

3.1.6 Hypoxia stimulation

After recovery, EDL muscle was transferred to new vials containing either basal incubation buffer (normoxia) or basal hypoxia buffer (hypoxia), and continuously gassed with 95% O_2/ 5% CO_2 or 95% N_2 /5% CO_2, respectively (Cartee *et al.*, 1991). After 45 min incubation, muscles were either directly frozen in liquid nitrogen and stored at -80˚C for later signaling analysis, or further incubated for the assessment of 2-deoxy-glucose transport.

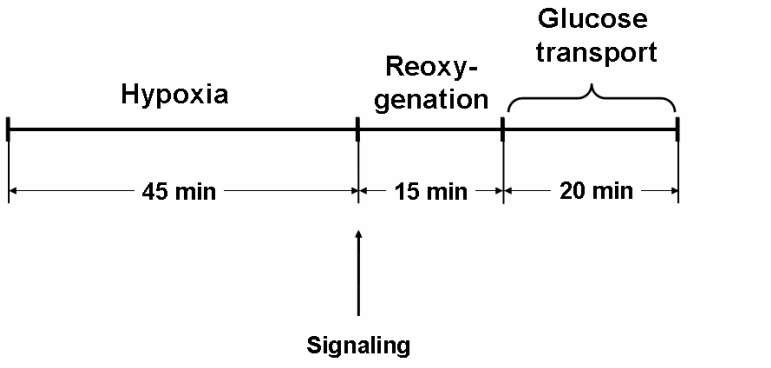


Figure 6: Timeline for muscle incubation to study the effect of hypoxia on glucose transport and signaling in AMPKγ3 KO mice.

3.1.7 Electrical stimulation *in vitro* and skeletal muscle fatigue

Isolated EDL muscle was placed between two platinum electrodes in a temperature controlled (30˚C) incubation chamber, containing oxygenized basal incubation buffer. The distal tendon was fixed to the bottom of the chamber, while the proximal tendon was connected to an isometric force transducer (Harvard Apparatus, South Natick, MA). Resting tension was set to 0.5 g. Electrical impulses were applied (*Paper I:* 0.2 ms pulse duration, amplitude 20 V, frequency 100 Hz for 0.2 sec every 2 sec, for 10 min; *Paper II:* 0.2 ms pulse duration, amplitude 30 V, frequency 100 Hz, 10 s per min for 10 min). A Student Oszillograph (Harvard Apparatus) was used to record force generation during each contraction. Peak force was determined from the first contraction recorded. Time (min) elapsed before force reached 50% of peak force was determined as a measure of muscle fatigue (*Paper I*). After electrical stimulation, muscles were immediately frozen for further analysis (glycogen content or protein phosphorylation), or further incubated for the determination of glucose oxidation (*Paper I*).

3.1.8 Muscle IL-6 release

IL-6 release from isolated mouse skeletal muscle was measured as the concentration of IL-6 in the media at the end of the 2 h incubation period. IL-6 concentration was determined by ELISA (R&D Systems, Abingdon, UK).

3.1.9 2-Deoxy-glucose transport

Glucose transport was determined as described (Wallberg-Henriksson *et al.*, 1987) using 2-deoxy-glucose (Hansen *et al.*, 1994). Following stimulation, muscles

were transferred to new vials containing glucose-free basal incubation buffer supplemented with 20 mmol/l mannitol with or without IL-6 or insulin, and incubated for 10 min. In the case of hypoxia stimulation, muscles were incubated for 15 min in glucose-free basal incubation buffer supplemented with 18 mmol/l mannitol and 2 mmol/l pyruvic acid. Thereafter muscles were transferred to new vials containing 1 mmol/l 2-deoxy-[1,2,^{3}H]glucose (2.5 μCi/ml) and 19 mmol/l [^{14}C]mannitol (0.7μCi/ml) and incubated for 20 min with or without IL-6 or insulin. Muscle was trimmed of non-muscle tissues, snap-frozen in liquid nitrogen and stored at -80°C until further analysis. Glucose transport was determined by the accumulation of intracellular 2-deoxy-[1,2,^{3}H]glucose. Data are expressed as nmol glucose per mg of protein per 20 min.

3.1.10 Muscle glucose oxidation and glycogen synthesis

After electrical stimulation or swim exercise, muscles were incubated at 30˚C for 60 min in basal or IL-6-containing incubation media containing 5 mmol/l [^{14}C]-glucose (0.2 μCi/ml). Vials were sealed air-tight, 0.2 ml of SolvableTM (2% Sodium Hydroxide, Dupont) was added into a center well and 0.5 ml of 15% perchloric acid was added to acidify the media. After collecting liberated [^{14}C]O_2 for 60 min, center wells were removed for scintillation counting. Results were expressed as nmol of oxidized glucose per g of wet tissue weight per hour. Muscle glucose incorporation into glycogen was determined as described (Cuendet *et al.*, 1976). Muscles were weighed and dissolved in 0.5 ml 1 N sodium hydroxide (NaOH) for 30 min. Trichloric acid (0.5 ml of a 20% solution) was added, samples were mixed and subjected to centrifugation (3500 g at 10°C for 15 min). Thereafter, 200 μl of a glycogen solution (20 mg/ml) and 2 ml of 95 % ethanol were sequentially added to the supernatant, and the mixture was incubated at -20°C for 1 h. After centrifugation (15 min at 2000 g), the resulting pellet was dissolved in water and an aliquot was used for scintillation counting.

3.1.11 Cell-free AMPK kinase assay.

Recombinant functional AMPK heterotrimers (α1β1γ1 or α2β2γ1), or GST fusion protein of CaMKKβ, were expressed in *E. coli* and purified (Tokumitsu *et al.*, 2001; Neumann *et al.*, 2003). Recombinant WT AMPK (α1β1γ1 or α2β2γ1) was incubated for 60 min at 30^{o}C with 0.5 mg recombinant CaMKKβ in activation buffer (10 mmol/l Hepes (pH 7.5), 5 mmol/l $MgCl_2$, 1 mmol/l AMP, 2 mmol/l ATP). EDL muscle lysates (100 mg) were incubated (30 min at 30˚C) in assay buffer (10 mmol/l Hepes (pH 7.5), 5 mmol/l $MgCl_2$, 1 mmol/l EGTA, 0.5 mmol/l Na_3VO_4, 40 μmol/l P^1,P^5-di(adenosine-5') pentaphosphate, 100 μmol/l AMP, 200 μmol/l ATP) with or without recombinant activated WT AMPK, as indicated (*Paper II*). The reaction was stopped by addition of sample buffer (6X) and heating (96˚C for 4 min). Samples were subjected to SDS-PAGE, followed by Western blot analysis with an antibody that was raised against specific Akt phosphorylation sites (PAS antibody).

3.1.12 RNA purification and real-time PCR

Muscles were homogenized in 1 ml Trizol reagent (Sigma). RNA was isolated according to manufacturer's protocol. Purified RNA was treated with DNAseI (DNA-free kit, Ambion, Huntingdon, UK) and used as template for cDNA synthesis (SuperScript first strand synthesis system (Invitrogen) with random hexamer primers). cDNA synthesis was performed according to the manufacturer's recommendation. cDNA was quantified by real-time PCR with the ABI PRISM 7000 sequence detector system and fluorescence-based SYBR-green technology (Applied Biosystems). All samples were analyzed in duplicates and normalized against the expression of acidic ribosomal phosphoprotein P0 (36B4) (Akamine *et al.*, 2007), using relative quantification method. The forward and reverse primer sequences for IL-6 and 36B4 are provided (Materials and Methods, *Paper III*).

3.1.13 Glycogen content

Glycogen content was determined fluorometrically on HCl extracts as described (Lowry & Passonneau, 1972). Muscle tissue (4-10 mg) was placed in 500 µl of 1 mol/l HCl. Samples were boiled for 1-2 h with periodically shaking followed by centrifugation at 2000 g for 10 min. An aliquot of the supernatant (10 µl) was mixed with 2 ml of assay buffer (50 mmol/l Tris buffer (pH 8.1), 300 µmol/l ATP, 2 mmol/l $MgCl_2$, 0.02 % BSA, 40 µmol/l NADP, 1 µg/ml glucose-6-phosphate dehydrogenase), and initial fluorescence was measured. Hexokinase (50-100 µl) was subjected to centrifugation for 5 min at 4000 g. The pellet was re-suspended with an equal volume of enzyme diluting buffer (20 mmol/l Imidazol-HCl (pH 7.1), 0.02 % BSA). The resulting solution was added (2 µl) to the sample mix, and fluorescence was measured again after 30 min incubation at room temperature. A serial dilution of a 1 mmol/l glucose solution was used as standard.

3.1.14 Muscle homogenization

Muscles were pulverized over liquid nitrogen and homogenized in microcentrifuge tubes in ice-cold buffer (20 mmol/l Tris (pH 8.0), 137 mmol/l NaCl, 2.7 mmol/l KCl, 10 mmol/l NaF, 1 mmol/l $MgCl_2$, 1 mmol/l Na_3VO_4, 0.2 mmol/l phenylmethylsulfonyl fluoride (PMSF), 10% glycerol, 1% Triton X-100, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 1 µmol/l microcystin; or as specified (*Paper II*)) for 20 sec using a motor-driven pestle. Homogenates were rotated end-over-end for 1 h at 4˚C, followed by centrifugation at 12000 g for 10 min at 4˚C. After determining the protein content using the bicinchoninic acid method (Pierce, Rockford, IL), the supernatant was frozen in liquid nitrogen and stored at -80˚C for later immunoprecipitation, immunoblot analysis or AMPK kinase activity assay.

3.1.15 AS160 immunoprecipitation

EDL muscle lysate (350 µg of protein) was subjected to immunoprecipitation overnight with 3.5 µg of C-terminal anti-AS160 antibody at 4°C with gentle rotation. Samples were incubated for 3 h at 4°C with an equal mixture of protein A sepharose

(Amersham, Uppsala, Sweden) and protein G agarose (Sigma-Aldrich, St. Louis, MO), washed for three times with homogenization buffer and four times with phosphate buffered saline (PBS). Subsequently, the immunocomplex was boiled in Laemmli buffer containing β-mercaptoethanol and subjected to SDS-PAGE.

3.2 HUMAN SKELETAL MUSCLE TECHNIQUES

3.2.1 Human subjects

All study protocols were approved by the regional ethical committee Karolinska Institutet, Stockholm. Informed consent was received from all subjects before participation. The clinical characteristics of the healthy male volunteers (n=22) for *Paper IV* are presented (*Paper IV*, Table 1). None of the subjects were smokers or reported taking any medication. The subjects were asked to refrain from strenuous exercise for 48 h prior to the study and to report to the laboratory after an overnight fast. Study subjects for *Paper III* (4 male and 3 female) had no known metabolic disorders. Mean age was 52.5 ± 8 yr (BMI 25.3 ± 3.0 kg/m^2) and fasting blood glucose 5.6 ± 0.5 mmol/l.

3.2.2 Human muscle biopsy procedure

Skeletal muscle (~1 g) was obtained by means of an open biopsy. Biopsies were taken under local anesthesia (mepivakain chloride 5 mg/ml) from the *vastus lateralis* portion of the *quadriceps femoris* (Zierath, 1995). Muscle specimens (10-20 mg) were dissected from the biopsy material, mounted on Plexiglas clips (9 mm in width), and incubated for 30 min in individual flasks containing oxygenated basal incubation buffer to recover from the procedure.

3.2.3 Human muscle incubation and glucose transport

Skeletal muscle strips were incubated in basal incubation buffer. Muscles were pre-incubated for 30 min in the absence or presence of 120 ng/ml IL-6 (human recombinant, Roche). The IL-6 concentration was maintained throughout all subsequent incubation steps. Muscles were incubated in KHB supplemented with 18 mmol/l mannitol, 2 mmol/l pyruvate, 0.1% BSA and incubated for 30 min in the absence or presence of 0.36 nmol/l or 60 nmol/l insulin (Insulin Actrapid, Novo Nordisk). The concentration of insulin was maintained throughout all subsequent incubation steps.

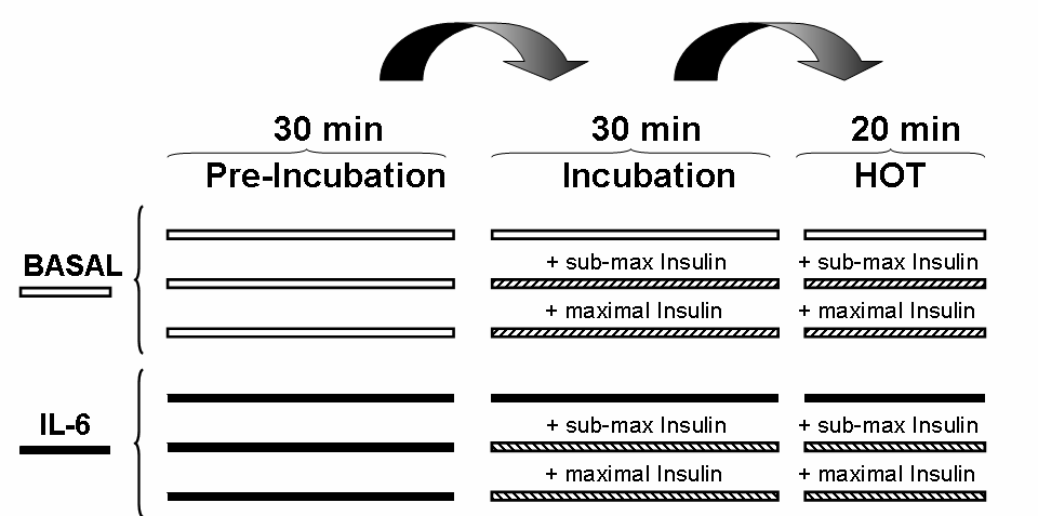


Figure 7: Protocol for the measurement of glucose transport in human skeletal muscle strips.

To assess the rate of glucose transport, skeletal muscle strips were incubated for 20 min in KHB containing 5 mmol/l 3-O-methyl-[^{3}H]glucose (800 µCi/mmol) and 15 mmol/l [^{14}C]mannitol (53 µCi/mmol), blotted of excess fluid, snap-frozen in liquid nitrogen and stored at -80°C until further analysis. Glucose transport was determined by the accumulation of intracellular 3-O-methyl-[^{3}H]glucose, and muscle lysate was stored at -80^{o}C for subsequent signal transduction analysis.

3.2.4 IL-6 time-course for signal transduction

A time-course for IL-6 effects on signal transduction was established by incubating human muscle strips in the presence or absence of 120 ng/ml IL-6 for 15 or 30 min in basal incubation media. Specimens were trimmed of non-muscle tissues, blotted to remove excess fluid, snap frozen in liquid nitrogen and stored (-80°C) until further analysis. Homogenates from muscles incubated under the glucose transport protocol were also analyzed for signal transduction. The time course therefore comprises 15, 30 and 80 min of IL-6 exposure.

3.2.5 Glucose oxidation and glycogen synthesis

Human muscle strips were incubated in sealed vials containing 2 ml of oxygenated (95 % O_2, 5 % CO_2) basal incubation media containing 5 mmol/l [^{14}C]glucose (0.3 µCi/ml). Muscles were incubated under basal, insulin (120 nmol/l), or IL-6 (120 ng/ml) stimulation, and oxygenated for the first 15 min of the incubation period. After 60 min, vials were placed on ice for 3 min. Muscles were quickly removed, frozen between tongs cooled to the temperature of liquid nitrogen and stored for later determination of glucose incorporation into glycogen. Glucose oxidation measurements were performed as described (Zierath, 1995). Vials were sealed and a center well was introduced. 200 µl Protosol (PerkinElmer Life Sciences) was injected into the center well. The incubation media was acidified with 0.5 ml of 15% perchloric acid, and liberated [^{14}C]O_2 was collected for 60 min. Thereafter, center wells were

removed and subjected to scintillation counting. Glucose incorporation into glycogen was determined as described for mouse muscle (3.1.10).

3.2.6 Restriction fragment-length polymorphism analysis

IL-6 polymorphism at -174 was determined as described (Fernandez-Real *et al.*, 2000). The promoter region surrounding -174 was amplified by polymerase chain reaction using primers as described (Fernandez-Real *et al.*, 2000). The reaction was carried out in a final volume of 50 µl containing 1.5 mmol/l $MgCl_2$, 0.2 mmol/l of each dNTP, 0.2 mmol/l of sense and anti-sense primer, and 2.5 U Taq polymerase. DNA was amplified by an initial denaturation of 10 min at 94°C, 35 cycles consisting of 1 min denaturation at 94°C, 1 min and 35 s annealing at 55°C, and 1 min extension at 72°C, and a final extension of 10 min at 72°C. PCR products were digested with SfaNI restriction enzyme at 37°C overnight. After separation on a 2% agarose gel, SfaNI restriction fragment-length polymorphism was detected by ethidium bromide staining. G/G, G/C, and C/C genotypes were classified according to the presence or absence of the SfaNI restriction sites. G/G, G/C, and C/C are homozygous for the presence of the site (140/58 bp), heterozygous for the presence and absence of the site (198/140/58 bp), and homozygous for the absence of the site (198 bp), respectively.

3.3 MUSCLE CELL CULTURE TECHNIQUES

3.3.1 Cell culture and differentiation

Muscle biopsies (*rectus abdominus*, ~1-3 g) were collected in cold PBS supplemented with 1% PeSt (100 units/ml penicillin/ 100 µg/ml streptomycin). Satellite cells were isolated and cultured as described (Al-Khalili *et al.*, 2003b). Myotubes were treated for 20 min, 3 h or 8 d with or without 5, 25, or 100 ng/ml IL-6. Media (including IL-6) was changed every day and prior to each experiment. Myotubes were incubated with serum free DMEM for 6 h before use.

3.3.2 AMPKα1 and α2 *si*RNA design and transfection

siRNA design. Constructs of AMPKα1 and AMPKα2 *si*RNA oligos (*Paper V*, Table 2) were designed with 3′ overhanging thymidine dimers. Unspecific targeting was prevented by eliminating sequences with significant homology to other genes in an alignment with human genome database using BLAST.

siRNA transfection. *si*RNA transfection was performed as described (Al-Khalili *et al.*, 2003a), with modifications. Transfection of myoblasts impaired myocyte differentiation, thus the method was applied to differentiated myocytes. Myoblasts were grown in six-well plates and differentiated into myotubes (2 days). Individual *si*RNA oligos were mixed in serum/antibiotic free DMEM for 5 min. The transfection agent (1 µl), Lipofectamine® 2000 (Invitrogen, Sweden), was separately mixed and incubated with 49 µl DMEM for 5 min. The two mixtures were combined and mixed by gentle agitation at room temperature for 30 min. The resultant (100 µl) was added to each well and incubated for >16 h. Myotubes were washed twice with sterile PBS, followed by addition of 1 ml of serum/ antibiotic free DMEM and 100 µl of the *si*RNA transfection

mixture to each well and incubation for >16h. Control cultures were prepared without *si*RNA oligos or with scramble *si*RNA oligos. No further cell death was observed in *si*RNA transfected cells compared to Lipofectamine 2000 treatment alone. Myotubes were washed with sterile PBS and 2 ml/well 4% FBS supplemented DMEM was added. Myotubes were used 4 days after transfection to determine glycogen synthesis and beta-oxidation as described below.

3.3.3 Giemsa/Wright staining

Myotubes used for long term (8 d) incubation with 25 ng/ml IL-6 were grown on six-well plates. To assess the extent of differentiation, myotubes were fixed in methanol (10 min), 1:10 Giemsa (15 min) and 1:10 Wright (20 min). Cells were washed with double distilled H_2O and mono- or multinucleated cells were observed under phase contrast invert light microscope. Cells were placed over Bürker-chamber and total number of myotubes counted in 20 squares (0.04 mm^2) using the 40X objective. Myotube formation and fusion rate after IL-6 stimulation was measured as percentage >5 nucleus/total myotubes/μm^2 over basal.

3.3.4 Myotube glucose uptake

Glucose uptake was determined as described (Somwar *et al.*, 1998) (15). Over night serum starved myotubes were stimulated with or without 25 ng/ml IL-6 for 60 min with 0, 6 or 60 nmol/l insulin in KREBS buffer (20 mmol/l HEPES, pH 7.4, 140 mmol/l NaCl, 5 mmol/l KCl, 2.5 mmol/l $MgSO_4$, 1 mmol/l $CaCl_2$). Thereafter, 2-deoxy[^{3}H] glucose was added to a final concentration of 10 μmol/l (1 μCi/ml). After further 10 min incubation, cells were washed, harvested and subjected to scintillation counting.

3.3.5 Myotube glycogen synthesis

Glycogen synthesis was determined as the amount of [^{14}C]glucose incorporated into glycogen, as described (Al-Khalili *et al.*, 2003b). Myotubes were treated as indicated and serum starved for 6 h prior to the assay. Thereafter, myotubes were stimulated with or without 25 ng/ml IL-6 for 90 min with addition of 0, 6 or 60 nmol/l insulin during the final 30 min before adding the radioactive glucose. Subsequently, cells were incubated with 5 mmol/l glucose DMEM, supplemented with [^{14}C]glucose (final specific activity, 0.18 μCi/μmol) for another 90 min. Each experiment was carried out on duplicate wells.

3.3.6 Myotube lactate release

Media was collected from day 7 differentiated myotubes stimulated with 25 ng/ml IL-6 with or without insulin for 0, 2, 24, 48 and 72 h in serum free DMEM. Lactate concentration in the media was determined using a kit (Biochemical Research Service Center, University of Buffalo, NY, USA).

3.3.7 Myotube free fatty acid oxidation

The determination of free fatty acid oxidation was performed as described (Petersen *et al.*, 2005), with modifications. Myoblasts were grown on 25 cm^2 cell culture flasks in growth medium (Ham-10 medium supplemented with 20% fetal bovine serum (FBS)) and differentiated to myotubes at >80% confluence. A hole was punctured into the lid of each flask. 2 sheets of 24 mm Whatman filter were encircled with a gauze bandage compass and pressed into the culture flask lid. Day 8 differentiated myotubes were serum starved over-night and treated for 180 min with 0.4 µCi of [^{14}C]palmitate or [^{14}C]oleate in 2 ml serum free DMEM in the presence or absence of IL-6 (25 ng/ml), insulin (60 nmol/l), or both. Thereafter, 200 µl Solvable reagent (benzethonium hydroxide, Packard) was added drop-wise to soak the filter, and 300 µl of 70% perchloric acid was injected through the hole and filter. After sealing the lid, flasks were incubated for 1 h at room temperature with slight agitation. The filter-compass was subjected to scintillation counting. As an indication of free fatty acid uptake, intracellular accumulation of palmitate was determined in the same cells. For this purpose, cells were washed 5 times with Tris-buffered saline containing 0.02% Tween 20 (TBST) and then lysed with 2 ml of 0.03% SDS for 2 h at room temperature with slight agitation. Accumulation of [^{14}C]palmitate was determined by liquid scintillation counting.

3.3.8 Myotube stimulation for immunoblot analysis

Myotubes were grown on 100 mm dishes and exposed to 5, 25, and 100 ng/ml IL-6 for 20 min, 3 h or 8 d. Insulin (60 nmol/l) was added to some dishes from each condition during the last 20 min of incubation time. Myotubes were scraped into 400 µl ice-cold homogenization buffer and rotated for 60 min at 4°C. After centrifugation (20000 x g for 10 min at 4°C) and determination of protein concentration, the supernatant was stored at -80°C for immunoblot analysis.

3.3.9 Myotube gene expression

Myoblasts were cultured in 100 mm dishes. At >90% confluence, differentiation was initiated in the presence or absence of IL-6. After 8 days, myotubes were washed with RNase free PBS, and RNA was extracted. After DNAse treatment, cDNA was prepared from total RNA samples using TaqMan reverse transcription reagent. mRNA expression was determined by Real-Time PCR (TaqMan, Applied Biosystems, Foster City, CA, USA) using a standard curve method. 18s served as a housekeeping gene. Primers and probes were designed either based on published literature, by Primer Express software (Perkin Elmer), or acquired by assays-on-demands (Applied Biosystem, Foster City, CA). Detailed information on primers and probes is given in *Paper V*.

3.4 GENERAL METHODOLOGY

3.4.1 Immunoblot analysis

Muscle/cell lysates were adjusted to equal protein concentration, diluted in Laemmli buffer and separated by SDS-PAGE. Subsequently, proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were incubated in 7.5% low fat milk in TBST and incubated overnight at 4˚C with primary antibodies (see below). The next morning, membranes were washed for 6 times 10 min with TBST, and incubated with appropriate horse-radish peroxidase-conjugated secondary antibody for 1 h at room temperature. Proteins were visualized by chemiluminescence (ECL or ECL plus, Amersham) and quantified by densitometry. Results are expressed as arbitrary units. Western blot analysis was performed using the following phosphospecific antibodies: AMPK on Thr-172 (Cell Signaling Technology, MA), ACCβ on Ser-227 (Upstate Biotechnologies), AS160 with a phospho-(Ser/Thr) Akt substrate (PAS) antibody (Cell Signaling Technology), PKB/Akt (Ser-473) (Cell Signaling), PKB/Akt (Thr-308) (Cell Signaling), STAT3 on Y705 (Upstate Biotechnologies), GSK3 alpha/beta (Cell Signaling), p38 MAPK Thr-180/Tyr-182 (Cell Signaling), S6 ribosomal protein (Ser-235/236) (Cell Signaling) Equal loading was ensured using a NADH ubiquinol oxidoreductase (NUO) protein specific antibody (Molecular Probes, Eugene, OR) (*Paper IV*) and using an affinity-purified rabbit antibody prepared against a GST fusion protein with a portion of mouse AS160 corresponding to amino acids 584-833 of human AS160 (*Paper II*, kindly provided by Gustav E. Lienhard, Dartmouth Medical School, Hanover, NH).

3.4.2 PI3-kinase activity

An aliquot (300 μg) of protein from myoblasts or myotube grown on 100 mm dishes and exposed to 25 ng/ml IL-6 for 8 d or 3 h, respectively; or 800 μg of protein from human skeletal muscle was subjected to immunoprecipitation overnight (4°C) with anti-IRS1 antibody coupled to protein A sepharose (Sigma). PI3-kinase activity was assessed directly on the protein A sepharose beads as described (Krook *et al.*, 1997) The immuno-precipitates were washed 3 times at 4°C with buffer A (homogenization buffer), 2 times in buffer B (500 mmol/l LiCl, 100 mmol/l Tris-HCl, pH 8.0), once in buffer C (150 mmol/l NaCl, 10 mmol/l Tris-HCl, 1 mmol/l EDTA, pH 7.6), and once in buffer D (20 mmol/l Hepes, 1 mmol/l dithiothreitol (DTT), 5 mmol/l $MgCl_2$, pH 7.6). After re-suspending the beads in 40 μl of buffer E (10 mmol/l β-glycerophosphate, 5 mmol/l $Na_4P_2O_7$, 30 mmol/l NaCl, 1 mmol/l DTT, pH 7.2), 20 μl of phosphatidylinositol/cholate solution (3 mg/ml in 1% (w/v) sodium cholate) was added to each tube. The reaction was started by the addition of 5 μCi of [γ-^{32}P]ATP in 40 μl of reaction mix (3 μmol/l Na_2ATP, 7.5 mmol/l $MgCl_2$) and incubated at 37 °C for 15 min. To terminate the reactions, 450 μl of $CHCl_3$:CH_3OH (1:2 v/v) was added. The product was then extracted by the addition of 150 μl of $CHCl_3$ and 150 μl of 0.1 mol/l HCl and then again by the addition of 300 μl of $CHCl_3$ and 300 μl of 0.1 mol/l HCl. Extracted lipid was dried under vacuum before re-dissolving in 25 μl of $CHCl_3$, CH_3OH, 0.1 mol/l HCl (200:100:1). Reaction products were separated by thin layer chromatography (run in a pre-equilibrated tank containing methanol:chloroform:ammonia:water, 300:210:45:75) and quantified using a

PhosphoImager Bio-Rad Laboratories (Richmond, CA). Results were normalized to a standard consisting of insulin-stimulated mouse liver.

3.4.3 Total AMPK activity

Antiserum raised against bacterially expressed AMPK α1, α2, β1, β2, and γ1 (pan-) AMPK protein was used to precipitate total AMPK protein to perform kinase assays as described (Barnes *et al.*, 2002). Skeletal muscle lysates (200 μg protein) were incubated for 3 h at 4°C with 5 μl pan-AMPK antiserum pre-bound to 20 μl protein A Sepharose (50 % slurry, Amersham Bioscience, Uppsala, Sweden). Immunoprecipitates were washed three times with lysis buffer and twice with 50 mmol/l HEPES (pH 7.5), 10% (vol/vol) glycerol, 1 mmol/l EDTA, and 1 mmol/l DTT. SAMS peptide (full sequence: HMRSAMSGLHLVKRR) was used as substrate to determine total AMPK activity in the washed immune complex. Kinase reactions were performed in reaction buffer (40 mmol/l HEPES buffer, pH 7.0, 0.2 mmol/l SAMS peptide, 0.2 mmol/l AMP, 80 mmol/l $NaCl_2$, 0.8 mmol/l DTT, 5 mmol/l $MgCl_2$, and 0.2 mmol/l ATP (containing 2 μCi [γ-^{32}P]ATP) for 60 min at 30°C, and terminated by centrifugation (9000 *g* for 30 s). Incorporation of [^{32}P]-ATP into the peptide was determined by liquid scintillation counting of sample aliquots spotted on P81 paper (Whatman International, Maidstone, UK).

3.4.4 Microarray

Gene expression analysis was performed with mRNA from primary human myotubes and human skeletal muscle biopsies using Human Genome Survey Microarray V2.0 according to protocols from the manufacturer Applied Biosystems (Foster City, CA). Microarray measurement and data analysis was performed by the respective core facility of the Karolinska Institutet.

3.4.5 Statistical analysis

Data are presented as mean ± SEM. Statistical difference between groups was determined by one-way ANOVA, two-way ANOVA, or student's *t*-test, as appropriate and further specified in each study. One-way ANOVA was followed by Fishers least significant test for post-hoc determination. Two-way ANOVA was performed with or without repeated measures, as indicated, followed by Tukey's post-hoc analysis to determine differences between groups, when appropriate. Equality of variances was ensured by Levene's test. In case of differences, data was log-transformed. Log-transformation was sufficient to obtain equal variances in all cases. Significance was accepted at $P<0.05$. Relationships were determined by linear correlation and regression analysis.

4 RESULTS

4.1 THE ROLE OF AMPK IN SKELETAL MUSCLE METABOLISM

4.1.1 AMPKγ subunit regulates muscle glycogen content

High skeletal muscle glycogen content in a large proportion of purebred Hampshire pigs, with great impact on meat yield, has been attributed to the dominant *RN*$^{-}$ mutation (Milan *et al.*, 2000). The *RN*$^{-}$ mutation is located in the γ3 isoform of AMPK (Milan *et al.*, 2000), an isoform that is predominantly and rather exclusively expressed in glycolytic skeletal muscle (Mahlapuu *et al.*, 2004). Transfection experiments in COS cells with heterotrimeric complexes containing either a wild-type or a mutated AMPKγ3 isoform revealed an increased AMPK activity and AMPK phosphorylation in the absence of AMP, with concurrent diminished AMP dependency (Barnes *et al.*, 2004). Similar results were obtained when an equivalent mutation was introduced into the γ1 isoform of AMPK (Hamilton *et al.*, 2001). Thus the AMPKγ3 R225Q mutation, the mouse analogue to the *RN*$^{-}$ (or R200Q) mutation in pigs, can be considered a gain-of-function mutation, as basal AMPK activity is increased. When looked at it from the viewpoint of its regulation, it may also be considered a loss-of-function mutation, as it looses its regulation by AMP. However, skeletal muscle from *RN*$^{-}$ pigs (Milan *et al.*, 2000), glycolytic muscle from fed and fasted *Tg-Prkag3*^{225Q} mice (*Paper I,* Table 2) or from mice with an equal mutation in the γ1 isoform (Barre *et al.*, 2007), as well as skeletal muscle from humans harboring a mutation in the γ3 isoform (Costford *et al.*, 2007) display increased muscle glycogen content. Moreover, mutations in the AMPKγ2 isoform in heart muscle as result of disease (Gollob *et al.*, 2001) or genetic manipulation (Luptak *et al.*, 2007) increase heart glycogen content. Taken together, this provides compelling evidence for an important role of the γ subunit of AMPK in the regulation of muscle glycogen content.

4.1.2 Glycogen feedback on AMPK activity: role of AMPKγ3

Transfection studies reveal the AMPKγ3 R225Q mutation (the mouse analogue of the *RN*$^{-}$ mutation) increases AMPK activity (Barnes *et al.*, 2004). In contrast, AMPK activity is diminished in skeletal muscle from fed *RN*$^{-}$ pigs (Milan *et al.*, 2000) and furthermore, AMPK activity is unaltered in glycolytic EDL muscle from fed and fasted *Tg-Prkag3*^{225Q} mice (*Paper I*, Table 1). This observation appears to be paradoxical. However, high glycogen levels have been shown to inhibit AMPK activity in human skeletal muscle (Wojtaszewski *et al.*, 2002; Wojtaszewski *et al.*, 2003; Jorgensen *et al.*, 2004a), thus normal or reduced AMPK activity in muscle harboring the mutation may result from a feedback mechanism of glycogen on AMPK activity. The 70% higher glycogen content in EDL muscle from *Tg-Prkag3*^{225Q} mice may balance the AMPK activity-increasing effect of the mutation, possibly by targeting the AMPK molecule to glycogen particles (Polekhina *et al.*, 2003).

Overnight fasting decreased muscle glycogen stores of *Tg-Prkag3*^{225Q} mice, AMPKγ3 KO mice and control mice ~50% (*Paper I,* Table 2). This reduction in glycogen was concomitant with a trend for an increase in AMPK activity in wild-type and *Tg-Prkag3*^{225Q} mice, but not AMPKγ3 KO mice (*Paper I*, Table 1), suggesting that glycogen depletion results in increased AMPK activation only in the presence of a

functional γ3 isoform. As expected, *in vitro* contraction resulted in depletion of glycogen stores in EDL muscle from fed and fasted wild-type mice (*Paper I*, Table 2), as well as in increased AMPK activity (*Paper I*, Table 1). Interestingly, when glycogen stores where pre-depleted by fasting, contraction-mediated AMPK activity in *Tg-Prkag3*^{225Q} mice was increased, compared to contraction-mediated AMPK activity without prior fasting (*Paper I*, Table 1). This provides further evidence that glycogen down-regulates AMPK activity in *Tg-Prkag3*^{225Q} mice. In skeletal muscle from AMPKγ3 KO mice, however, AMPK activity was unrelated to glycogen content. This further underscores that the regulatory relationship between AMPK activity and muscle glycogen content is only efficient in the presence of a functional γ3 isoform. The γ3 isoform is mainly detected in glycolytic muscle, which has a higher dependency on glycogen as energy fuel during muscle contraction. Thus the high sensitivity of AMPKγ3-containing heterotrimers to muscle glycogen levels may explain the recent finding that AMPKγ3-containing heterotrimers account for the major proportion of contraction-induced AMPK activation in response to exercise in humans (Birk & Wojtaszewski, 2006).

4.1.3 Hypoxia-mediated glucose transport

The maximal effect of insulin and exercise/muscle contraction on glucose transport are additive, suggesting two distinct and separate pathways contribute to the regulation of glucose transport (Nesher *et al.*, 1985; Constable *et al.*, 1988; Holloszy, 2003). The maximal effects of hypoxia and contraction are not additive, indicating, at least to some extent, the existence of a common signaling mechanism (Cartee *et al.*, 1991; Azevedo *et al.*, 1995). AMPK and Ca^{2+}-mediated signaling has been linked to contraction-mediated glucose transport in fast-twitch glycolytic muscle (Wright *et al.*, 2004). Dantrolene, an inhibitor of Ca^{2+}-release from the sarcoplasmatic reticulum, inhibits hypoxia-mediated glucose transport in rat muscle (Cartee *et al.*, 1991). In contrast, hypoxia-mediated glucose transport was completely abolished in skeletal muscle from AMPKα2 KD mice (Mu *et al.*, 2001). Consequently, and similar to contraction-mediated glucose transport, AMPK- and Ca^{2+}-dependent signaling mechanisms have been implicated in hypoxia-stimulated glucose transport (Cartee *et al.*, 1991; Mu *et al.*, 2001).

AICAR-mediated glucose transport is completely impaired in EDL muscle from mice lacking AMPKγ3, while contraction mediated-glucose transport is normal (Barnes *et al.*, 2004). This suggests that γ3-containing AMPK heterotrimers play an important role in the regulation of glucose transport in response to energy depleting stimuli, while additional signaling inputs regulate glucose transport in response to contraction. We exposed glycolytic EDL muscle from mice lacking the AMPKγ3 isoform to hypoxic media. Hypoxia-mediated glucose transport was partly prevented in AMPKγ3 KO mice (Fig. 8A), highlighting the important role of γ3 containing AMPK heterotrimers in the regulation of glucose transport in response to energy depleting stimuli. Thus, other signaling inputs also contribute to the regulation of glucose transport after hypoxia stimulation. We cannot exclude a role for other γ isoforms, in particular the γ1-containing AMPK heterotrimers. However, when EDL muscles from wild-type and AMPKγ3 KO mice were exposed to hypoxia in the presence or absence of the CaMKK inhibitor, KN-93, hypoxia-mediated glucose transport was reduced,

suggesting that Ca^{2+}-mediated processes participate in the regulation of hypoxia-stimulated glucose transport. A simplistic view is that Ca^{2+}-dependent kinases and AMPK control separate linear signaling pathways. Alternatively, Ca^{2+}-dependent kinases may lie upstream from AMPK (Jensen *et al.*, 2007). Indeed, CaMKKβ has been shown to regulate AMPK phosphorylation and glucose uptake in mouse skeletal muscle at the onset of titanic contraction in an intensity and time dependent manner (Jensen *et al.*, 2007). There is compelling evidence implicating AMPK, and particularly γ3-containing AMPK heterotrimers, in the regulation of glucose transport in response to hypoxia.

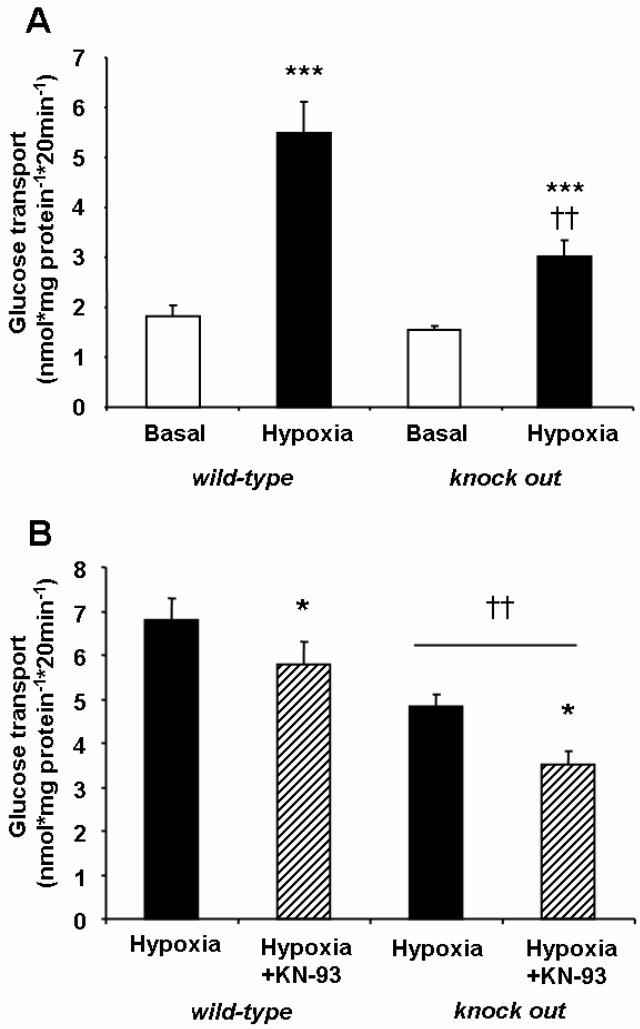


Figure 8: Hypoxia-mediated glucose transport. A) EDL muscle from AMPKγ3 KO mice and wild-type littermates were incubated under normoxic or hypoxic conditions for 45 min. 2-Deoxy-glucose transport (nmol x mg protein^{-1} x 20 min^{-1}) is reported as mean±SEM for n=8-11 muscles. ***P<0.001 for hypoxia effect, ††P<0.01 for genotype effect. B) EDL muscle from AMPKγ3 KO mice and wild-type littermates were incubated for 45 min under hypoxic conditions in the absence or presence of KN-93. 2-Deoxy-glucose transport is reported as mean±SEM for n=5 muscles. *P<0.05 for effect of KN-93, ††P<0.01 for effect of genotype.

4.1.4 AMPKγ3 R225Q mutation spares glucose for glycogen

Muscle contraction, energy depleting stimuli, as well as pharmacological activation of AMPK, result in increased glucose transport into skeletal muscle. The AMPKγ3 R225Q mutation is an AMPK-activating mutation, which would therefore be expected to result in increased glucose transport. However, basal and contraction-mediated glucose transport is normal in EDL muscle from *Tg-Prkag3^{225Q}* mice (Barnes *et al.*, 2004). Moreover, basal glycogen levels (*Paper I*, Table 2) and glycogen re-synthesis rates after swim exercise are elevated, despite normal glucose transport (Barnes *et al.*, 2004), suggesting that molecular mechanisms regulating the intra-

muscular fate of glucose may act to increase and maintain muscle glycogen levels. The fate of glucose in skeletal muscle can take two main paths, glycolysis and glucose storage as glycogen. *Tg-Prkag3*^{225Q} mice display reduced glucose oxidation after *in vitro* contraction (*Paper I*, Fig. 3) and increased utilization of muscle-triglycerides during swim exercise (Barnes *et al.*, 2005), thereby sparing glucose for storage as glycogen. In contrast, glucose oxidation is increased in AMPKγ3 KO mice after *in vitro* contraction (*Paper I*, Fig. 3). Thus, the AMPKγ3 R225Q-mutation promotes a metabolic shift towards glucose sparing for glycogen synthesis. This is further supported by the observation that expression of genes involved in lipid and glucose metabolism are oppositely regulated in *Tg-Prkag3*^{225Q} mice and AMPKγ3 KO mice after swim exercise, with the AMPKγ3 R225Q mutation promoting gene regulatory responses along lipid oxidative pathways (Barnes *et al.*, 2005). Moreover, AMPKα2 KD mice have lower glycogen content and defects in glycogen re-synthesis after treadmill running, compared with wild-type mice (Mu *et al.*, 2001; Mu *et al.*, 2003).

An additional or alternative proposed mechanism explaining the increased glycogen levels in muscle from *Tg-Prkag3*^{225Q} mice is based on equilibrium between the activity of γ3-containing AMPK heterotrimers and muscle glycogen levels. In normal mice, AMPK activity would drive glucose transport and subsequent storage as glycogen. At the same time, glycogen feedback on AMPK increases; reducing AMPK activity until a given basal AMPK activity and basal glycogen level are reached (*Paper I*, Table 1 and 2, data for wild-type mice). If the activity of AMPKγ3-containing heterotrimers is increased as a consequence of the AMPKγ3 R225Q mutation, glucose transport would be increased until sufficient additional glycogen is synthesized to "compensate" for the increases in AMPK activity. In contrast, lack of AMPK activity of γ3-containing AMPK heterotrimers reduces glucose transport and consequently reduces glycogen levels. Regardless of the exact mechanism, the findings presented here emphasize an important role for AMPK, and particularly γ3-containing AMPK heterotrimers, in the regulation of muscle glycogen levels.

4.1.5 Glycogen content determines muscle ergogenics

Elevated muscle glycogen levels have been linked to increased exercise performance (Karlsson & Saltin, 1971). AMPK has been implicated in exercise tolerance and performance (Mu *et al.*, 2001; Mu *et al.*, 2003). For example, AMPKα2 KD mice have reduced skeletal muscle glycogen content, concomitant with reduced spontaneous physical activity in voluntary wheel running experiments and increased fatigue in response to electrical stimulation of isolated skeletal muscle (Mu *et al.*, 2001; Mu *et al.*, 2003). Skeletal muscle glycogen levels are reduced in mice lacking the AMPKα2 (Jorgensen *et al.*, 2004b) and AMPKγ3 isoforms (*Paper I, Table 2*) (Barnes *et al.*, 2004), suggesting AMPK plays an important role in the control of glycogen content. Skeletal muscle work performance is directly influenced by glycogen content (Karlsson & Saltin, 1971). To investigate the role of AMPKγ3 in work performance, we electrically stimulated EDL muscle *in vitro* and measured time to 50% fatigue. Time to 50% fatigue was enhanced in EDL from *Tg-Prkag3*^{225Q} mice under fed and fasted conditions, while EDL muscle from AMPK γ3 KO mice were fatigue-prone (*Paper I*, Fig. 2A). This enhancement in muscle performance is independent of changes in fiber-type (Nilsson *et al.*, 2006) and thus provides evidence that genetic modification

of AMPK can increase work performance by altering glycogen levels, independent of capillary density or serum factors. In support of this, skeletal muscle work performance from wild-type, *Tg-Prkag3*^{225Q} and AMPKγ3 KO mice was positively correlated with glycogen content. Therefore, AMPK-mediated glycogen content, rather than AMPK *per se*, is a determinant of *in vitro* work performance.

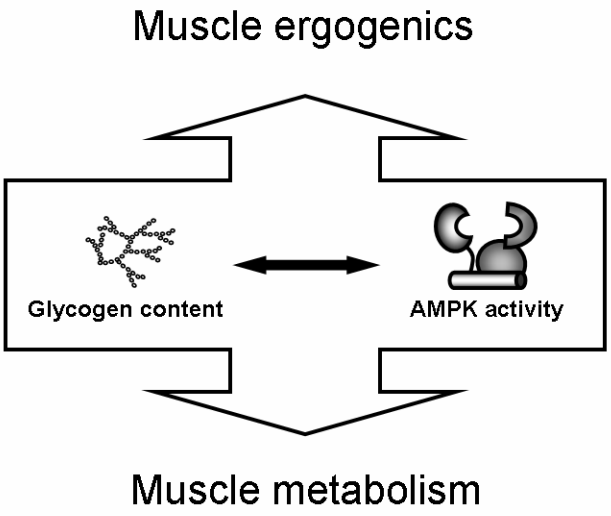


Figure 9: Interplay between AMPK activity and glycogen content determines muscle ergogenics and metabolism.

4.1.6 Is AMPK an AS160 kinase?

In skeletal muscle, AS160 phosphorylation may be stimulated by insulin, as well as contraction/exercise (Bruss *et al.*, 2005). This implicates that AS160 might be a point of converge of the insulin-signaling cascade and contraction-mediated signaling pathways (Bruss *et al.*, 2005; Kramer *et al.*, 2006b) (Fig. 10). A potential clinical relevance is underscored by the observation that insulin-mediated AS160 phosphorylation is reduced in skeletal muscle from people with NIDDM (Karlsson *et al.*, 2005). However, the molecular trigger for insulin-independent AS160 phosphorylation is incompletely understood. We therefore asked the question whether AMPK plays a role in mediating insulin-independent AS160 phosphorylation.

Similarly to findings in rat muscle (Bruss *et al.*, 2005), *in vitro* contraction increased the phosphorylation of AS160 in mouse EDL muscle (*Paper II*, Fig. 3C). Pharmacological activation of AMPK with AICAR resulted in a time-dependent increase in AS160 phosphorylation, concomitant with the expected increase in phosphorylation of AMPK and its downstream target, ACC (*Paper II*, Fig. 1C-E). Furthermore, addition of activated recombinant AMPK heterotrimers to EDL muscle lysate increased the phosphorylation of AS160 (*Paper II*, Fig. 2E and F), suggesting that AS160 is directly phosphorylated by AMPK. Ca^{2+}- and AMPK-dependent signaling mechanisms have been implicated in the regulation of glucose transport in response to skeletal muscle contraction (Wright *et al.*, 2004). However, the relative importance of these two signaling pathways might depend on timing and exercise intensity, and the mechanism is poorly understood. To further study the role of AMPK in mediating AS160 phosphorylation, we utilized mouse models with impaired AMPK signaling: AMPKα2 KO mice (Viollet *et al.*, 2003), AMPK α2 KD mice (Mu *et al.*,

2001) and AMPKγ3 KO mice (Barnes *et al.*, 2004). We determined AS160 phosphorylation after pharmacologically activating AMPK with AICAR in isolated EDL muscle. Glucose transport had been reported to be completely prevented under similar conditions (Mu *et al.*, 2001; Barnes *et al.*, 2004; Jorgensen *et al.*, 2004b), suggesting functional AMPK is necessary for AICAR-mediated stimulation of glucose transport. In line with impairments in glucose transport, AICAR-mediated AS160 phosphorylation was also prevented in the absence of functional AMPK (*Paper II*, Fig. 3A),

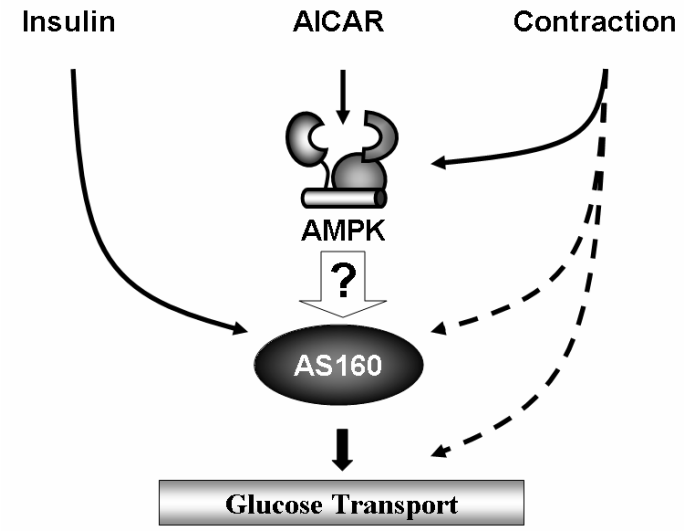


Figure 10: Is AS160 point of convergence of the insulin signaling cascade and contraction-mediated processes?

suggesting that functional AMPK is necessary for AICAR-mediated AS160 phosphorylation. Contraction-mediated glucose transport is normal (Barnes *et al.*, 2004; Jorgensen *et al.*, 2004b), or partly reduced (Mu *et al.*, 2001) in the mouse models studied in *Paper II*. When AMPK was activated by *in vitro* electrical stimulation, the increase in AS160 phosphorylation observed in wild-type mice was prevented in the absence of functional AMPKα subunits, but not AMPKγ3 subunits (*Paper II*, Fig. 3C), suggesting functional AMPK is necessary for contraction-mediated AS160 phosphorylation. However, γ3-containing AMPK heterotrimers might be dispensable. This is further supported by contraction experiments in transgenic mice with inactivated AMPKα2, whereby glucose transport is unaltered, despite partially reduced AS160 phosphorylation (Kramer *et al.*, 2006a). Thus, *Paper II* provides direct genetic evidence that AS160 is a downstream target of AMPK. Furthermore, in relation to previously reported glucose transport data, the findings discussed here challenge an important role of AMPK-mediated AS160 phosphorylation in mediating contraction-induced glucose transport. Moreover, the results challenge the requirement of AMPK in the regulation of contraction-mediated glucose transport.

4.1.7 Basal AS160 phosphorylation and glucose transport

Basal AS160 phosphorylation was impaired in AMPKα2 KD and AMPKα2 KO mice (*Paper II*, Fig. 3A and C and Fig. 4B). This would be predicted to reduce basal glucose transport, based on findings in 3T3-L1 adipocytes, in which AS160 expression is silenced (Eguez *et al.*, 2005; Larance *et al.*, 2005). However, basal glucose transport is unaltered in these mouse models (Mu *et al.*, 2001; Jorgensen *et al.*, 2004b), suggesting a threshold effect in which only a permissive level of AS160

phosphorylation may be necessary for basal glucose uptake. For *Paper II* we used the PAS antibody that recognizes proteins with serine or threonine residues that follow arginine at position P-5 (RXRXXS/T). The recognition of AS160 by anti-PAS was largely abolished in a recent cell study, when Thr^{642} was mutated to alanine, indicating that phosphorylated Thr^{642} is the prime anti-PAS recognition site on AS160 under serum, IGF-1 and insulin-stimulated conditions (Geraghty *et al.*, 2007). Therefore, an altered, but not detected, phosphorylation pattern might be present in the basal state in AMPKα2 KD and AMPKα2 KO mice. The recognition sites for AMPK and Akt differ (Weekes *et al.*, 1993; Hardie *et al.*, 1998; Kane *et al.*, 2002), thus AMPK might phosphorylate AS160 on other, unidentified phosphorylation sites, which could regulate AS160 activity or location. In contrast, basal AS160 phosphorylation was normal in AMPKγ3 KO mice, suggesting that γ3-containing AMPK heterotrimers might be dispensable for basal AS160 phosphorylation. Insulin-mediated Akt and AS160 phosphorylation (*Paper II*, Fig. 4B) and insulin-mediated glucose transport is normal in AMPKα2 KO (Viollet *et al.*, 2003) and AMPKα2 KD mice (Mu *et al.*, 2001), suggesting that the reduction in AS160 phosphorylation does not impair insulin signaling.

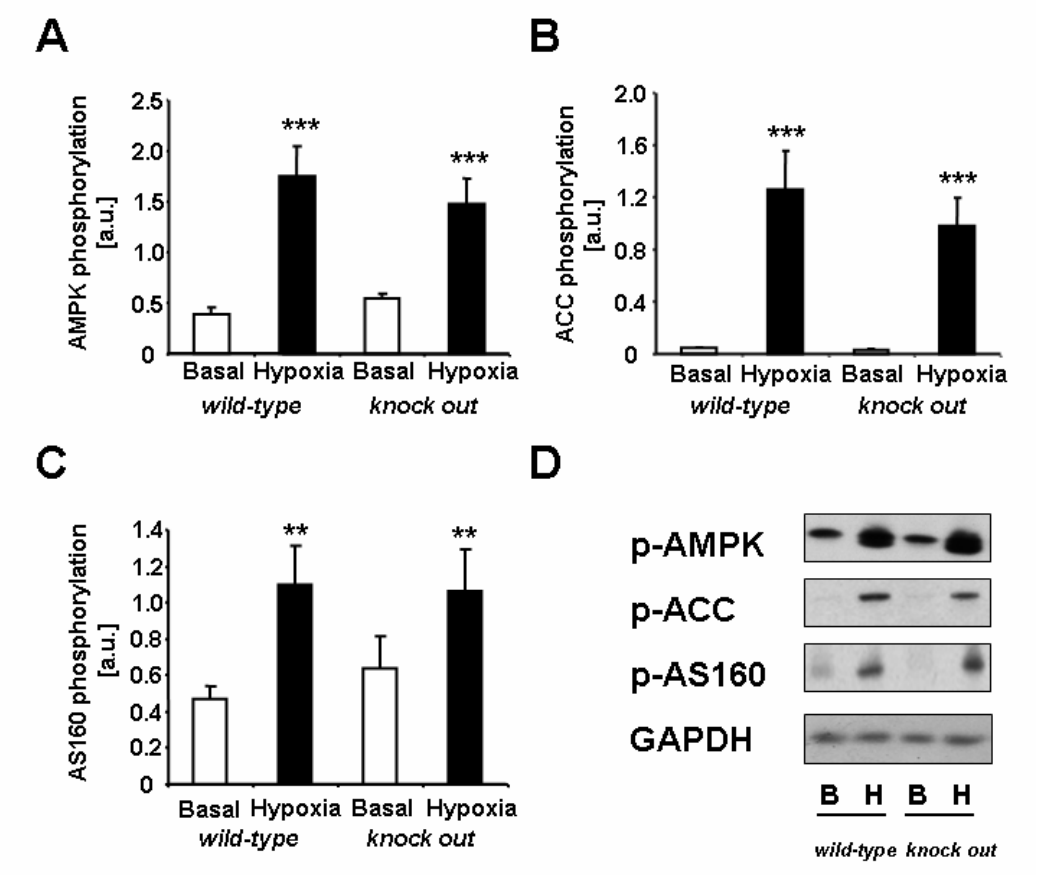


Figure 11: Hypoxia-mediated phosphorylation of AMPK, ACC and AS160 is unaltered in AMPKγ3 KO mice. EDL muscle from AMPKγ3 KO mice or wild-type littermates were incubated under normoxic or hypoxic conditions for 45 min. Phosphorylation of AMPK (A), ACC (B) and AS160 (C) is reported as mean±SEM arbitrary units (a.u.) for n= 6-9 muscles. Representative immunoblots are shown (D). Equal loading was ensured by immunoblot analysis for GAPDH protein expression (D). **P<0.01 and ***P<0.001 for hypoxia effect.

4.1.8 AMPKγ3 is dispensable for hypoxia-mediated signaling

Hypoxia-mediated glucose transport is partly reduced in AMPKγ3 KO mice (Fig. 8A), suggesting an important role of γ3-containing AMPK heterotrimers. To better understand the molecular signaling mechanisms, mouse EDL muscle from AMPKγ3 KO mice and wild-type littermates were incubated under hypoxic or normoxic conditions. Hypoxia-mediated AMPK and ACC phosphorylation was normal in AMPKγ3 KO mice, suggesting that the AMPKγ3 subunit is dispensable for hypoxia activation of AMPK (Fig. 11A and B). This finding is in agreement with previous experiments showing AICAR-mediated AMPK and ACC phosphorylation is maintained, despite profound impairments in glucose transport (Barnes *et al.*, 2004). However, at earlier time-points, AICAR-mediated AMPK phosphorylation is reduced in AMPKγ3 KO mice (*Paper II*, Tab. 1). In *Paper II* evidence that the AMPKγ3 isoform is necessary for AICAR-mediated AS160 phosphorylation, but dispensable for contraction-mediated responses was provided. Hypoxia-mediated AS160 phosphorylation was normal in AMPKγ3 KO mice (Fig. 11C), providing evidence for an uncoupling of glucose transport from AS160 phosphorylation in AMPKγ3 KO mice. Collectively, *Paper II* and previous glucose transport data (Mu *et al.*, 2001; Barnes *et al.*, 2004; Jorgensen *et al.*, 2004b) provide evidence for the contribution of AS160-independent signaling inputs to hypoxia-, as well as contraction-mediated glucose transport in skeletal muscle. For example Tbc1d1, a new Akt substrate, is an AS160 related protein which expresses a GAP domain with identical Rab specificity, and regulates GLUT4 translocation in 3T3-L1 adipocytes (Roach *et al.*, 2007 240). Whether Tbc1d1 participates in hypoxia- and contraction-mediated signaling in skeletal muscle remains to be determined.

4.1.9 AS160 phosphorylation: regulation by Ca^{2+}-signaling

Hypoxia-mediated glucose transport is partly reduced in AMPKγ3 KO mice (Fig. 8A). Moreover, glucose transport is further reduced in the presence of the CaMKK inhibitor, KN-93 (Fig. 8B). To investigate the role of Ca^{2+}-mediated signaling pathways, mouse EDL muscle from AMPKγ3 KO mice and wild-type littermates were incubated under hypoxic conditions in media containing KN-93 (Fig. 12). AMPK and ACC phosphorylation was normal in AMPKγ3 KO mice, suggesting Ca^{2+}-mediated signaling pathways do not participate in the regulation of AMPK and ACC activation in response to hypoxia. In contrast to hypoxic stimulation alone, AS160 phosphorylation was not increased by hypoxic stimulation in the presence of KN-93. Furthermore, while a trend for an increase was still present in the wild-type mice, this was not observed in KO mice (Fig. 12C). AS160 contains a calmodulin-binding domain (Kane & Lienhard, 2005) and a recent study utilizing transgenic expression of AS160 mutants in skeletal muscle provides evidence that this domain is important for contraction, but not insulin-mediated glucose transport (Kramer *et al.*, 2007). Taken together, this indicates a role of Ca^{2+}-mediated signaling pathways in the regulation of AS160 activity in response to hypoxia and contraction, however, further research is needed to confirm and delineate the molecular mechanisms for these effects.

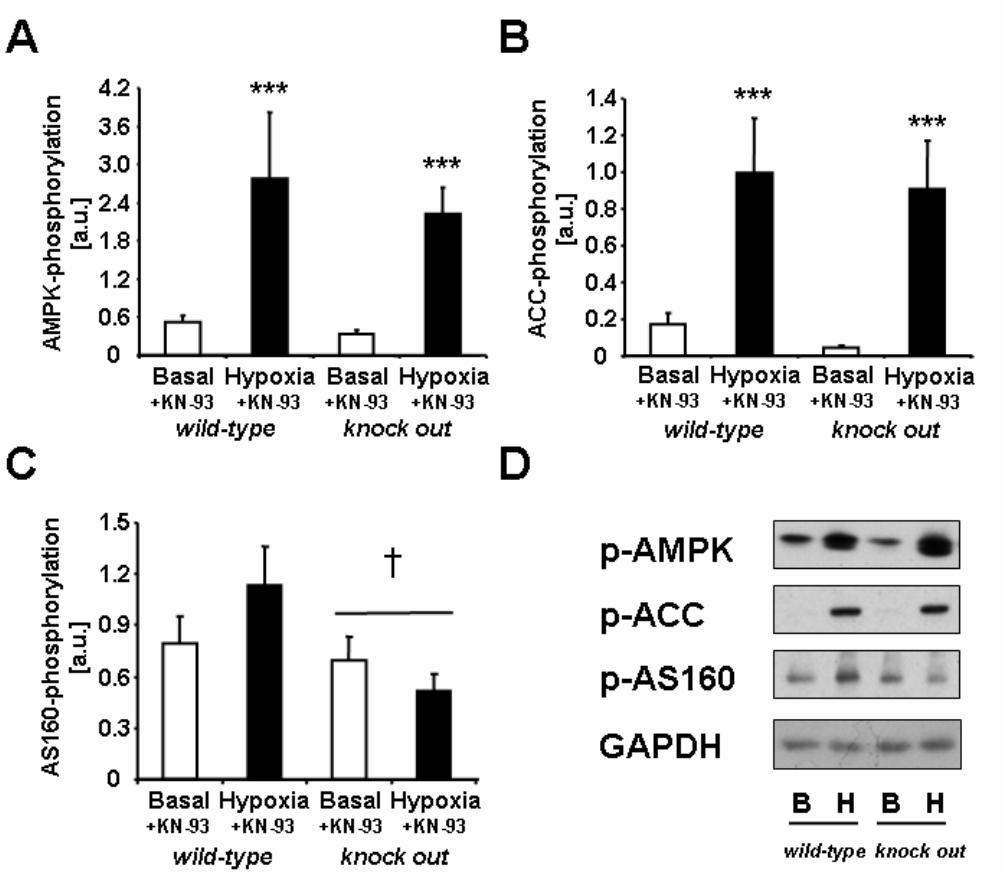


Figure 12: Hypoxia-mediated phosphorylation of AMPK, ACC and AS160 in the presence of the Ca^{2+}/calmodulin competitive inhibitor, KN-93. EDL muscle from AMPKγ3 KO mice or wild-type littermates were incubated under normoxic or hypoxic conditions for 45 min in the presence of KN-93. Phosphorylation of AMPK (A), ACC (B) and AS160 (C) is reported as mean±SEM for n= 7-9 muscles. Representative immunoblots are shown for basal (B) and hypoxic (H) conditions (D). Equal loading was ensured by immunoblotting for GAPDH protein expression (D). *P<0.05 and *** P<0.001 for the hypoxia effect, †P<0.05 and †† P<0.01 for genotype effect.

4.2 THE ROLE OF AMPK IN SKELETAL MUSCLE IL-6 RELEASE

4.2.1 Exercise increases IL-6 serum concentration in mice

There is compelling evidence from human studies that contracting skeletal muscle produces and releases IL-6 in large amounts, resulting in increased IL-6 serum concentrations (reviewed in (Fischer, 2006)). To verify the presence of a similar mechanism in mice, fed and fasted C57/Bl6 mice were subjected to a 2 h bout of swim exercise. Fed basal serum IL-6 concentrations were <10 pg/ml (Fig. 13). Exercise increased IL-6 serum concentrations ~7-fold (Fig. 13), providing evidence that exercise-mediated increases in IL-6 serum levels are similarly regulated as in mouse and humans.

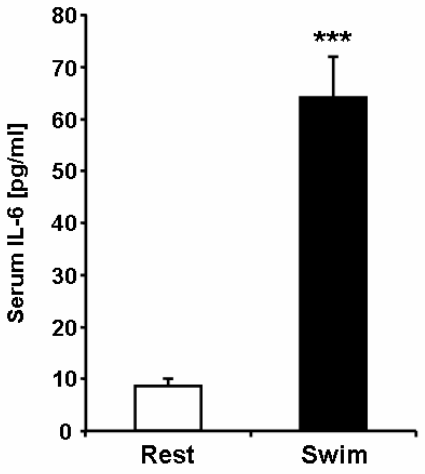


Figure 13: Exercise increases IL-6 serum concentration in mice. Fed C57/Bl6 mice were subjected to 2 h swim exercise. Trunk blood was collected and IL-6 concentration was measured in the serum. ***P<0.001; n=6 (swim) and 12 (rest) mice.

4.2.2 Basal muscle IL-6 release: role of fiber-type and AMPK

Skeletal muscle IL-6 release is evident from human studies (reviewed in (Fischer, 2006)) and from *in vitro* studies, whereby incubation of isolated rat (Holmes *et al.*, 2004) and mouse (*Paper III*, Fig. 1A-D) skeletal muscle increases the concentration of IL-6 in the incubation media, as well as the level of IL-6 mRNA. In agreement with previous findings in rat skeletal muscle (Banzet *et al.*, 2005), basal IL-6 release was higher from oxidative soleus muscle compared to glycolytic EDL muscle (*Paper III*, Fig. 1A and B). Oxidative skeletal muscle is more sensitive to insulin, when compared to glycolytic muscle (James *et al.*, 1985), raising the possibility that the higher basal IL-6 release contributes to the enhanced insulin sensitivity characteristic of oxidative fiber-type. To investigate the role of AMPK in basal IL-6 release, we studied skeletal muscle from mouse models with impaired AMPK signaling. Basal IL-6 release from glycolytic EDL muscle was normal from AMPKα2 KD mice, AMPKα1 KO mice and AMPKγ3 KO mice (*Paper III*, Fig 2A and 3A and C), suggesting that AMPK is not involved in the regulation of basal IL-6 release from isolated glycolytic muscle. In contrast, basal IL-6 release from soleus muscle of AMPKα2 KD and AMPKα1 KO mice was increased, compared with respective wild-type littermates (*Paper III*, Fig. 2B and 3B and D). This raises the possibility that AMPK plays a role in basal IL-6 release from oxidative muscle, at least under *in vitro* conditions. Incubation of skeletal muscle myotubes with IL-6 altered the transcriptional profile of selected genes (*Paper V*). Thus, an elevated basal IL-6 release from oxidative muscle of AMPKα2 KD mice may have contributed to alterations in their transcription profile (Mu *et al.*, 2003).

4.2.3 AMPK may regulate basal IL-6 release

There is accumulating evidence suggesting IL-6 activates AMPK in skeletal muscle (Kelly *et al.*, 2004) (*Paper IV* and *V*). In IL-6 KO mice, skeletal muscle and adipose tissue AMPK phosphorylation is dramatically reduced at rest and after exercise (Kelly *et al.*, 2004). In addition, IL-6 can stimulate its own production in skeletal muscle by an incompletely described autocrine mechanism (Keller *et al.*, 2003; Weigert *et al.*, 2007), which may be of relevance for the apparent exponential increase of IL-6

serum concentrations towards the end of exercise bouts (Fischer, 2006). An autocrine mechanism probably contributed to basal IL-6 release under conditions applied in *Paper III*, because the IL-6 media concentration in our experiments reached levels above normal fed resting serum levels (>50 pg/ml in basal media from soleus muscle after 2 h incubation (*Paper III*, Fig. 1B, 2B; and 3B and D) vs. <10 pg/ml in fed mice (Fig. 13)). Taken together, our observation that basal IL-6 release was increased in skeletal muscle from AMPKα2 KD and AMPKα1 KO mice indicates a regulatory mechanism, in which basal IL-6 release is dampened through an AMPK-dependent mechanism. Whether or not this process directly involves IL-6-mediated stimulation of AMPK phosphorylation by the means of a feedback mechanism remains to be determined. The finding that basal IL-6 release is unaltered in soleus muscle from AMPKγ3 KO mice may be explained by the finding that AMPKα subunit expression is also unaltered in this mouse model (Mahlapuu *et al.*, 2004). Moreover, AMPKγ3 protein and mRNA are undetectable in slow-twitch oxidative skeletal muscle (Mahlapuu *et al.*, 2004). AMPKα2 expression is increased 1.6-fold in AMPKα1 KO mice (Jorgensen *et al.*, 2004b), while it is reduced 82% in AMPKα2 KD mice. Exercise is associated with increased activity of α2β2γ3 heterotrimers in human skeletal muscle (Birk & Wojtaszewski, 2006), and exercise-mediated AMPKα2 activity correlates with IL-6 release (MacDonald *et al.*, 2003). Therefore, our findings implicate the AMPKα1 isoform in the regulation of basal IL-6 release from oxidative muscle; whereby exercise-mediated increases in AMPKα2 activity would not interfere with skeletal muscle IL-6 release.

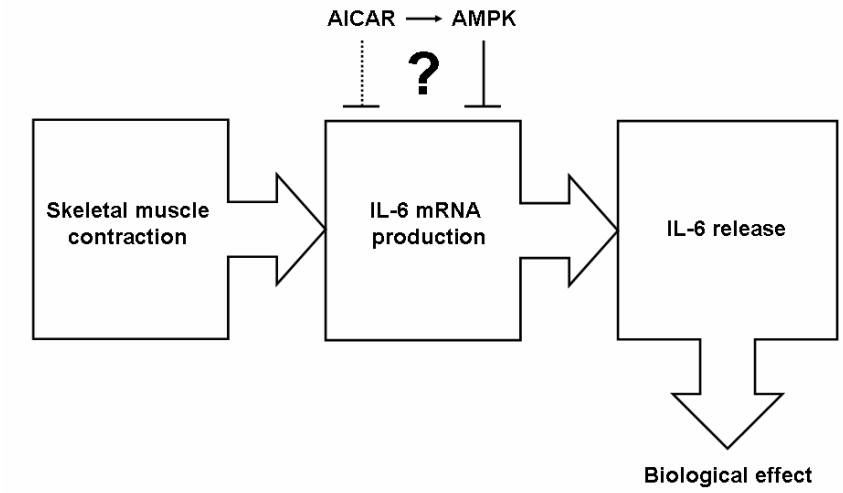


Figure 14: Is AICAR-mediated suppression of IL-6-release dependent on functional AMPK signaling?

4.2.4 AICAR-mediated IL-6 release is independent of AMPK

Pharmacological activation of AMPK with AICAR has been implicated in the regulation of muscle IL-6 release (Du *et al.*, 2005; Weigert *et al.*, 2007). For example, exposure of mouse cardiac fibroblasts to AICAR increases IL-6 production (Du *et al.*, 2005), while in cultured C2C12 cells *si*RNA-mediated silencing of AMPKα subunits prevents the effect of AICAR to increase IL-6 mRNA (Weigert *et al.*, 2007). In contrast, IL-6 protein release from human adipose tissue or adipocytes *in vitro* is

blocked in the presence of AICAR (Lihn *et al.*, 2004; Sell *et al.*, 2006). Similarly, AICAR-exposure prevents basal IL-6 release and mRNA production in isolated mouse skeletal muscle *in vitro* (*Paper III*). The discrepancy between the effects of AICAR on IL-6 release in some cultured cell systems versus isolated tissues is unapparent, but may be related to the cell type or differentiation state. AMPK activity is positively correlated with IL-6 release during exercise in humans (MacDonald *et al.*, 2003), and the suppression of IL-6 release by an activator of AMPK appears paradoxical. We therefore investigated the role of AMPK in AICAR-mediated suppression of IL-6 release by utilizing skeletal muscle from mouse models with impaired AMPK signaling. AICAR suppressed IL-6 release to a similar extent in skeletal muscle from AMPKα2 KD, AMPKα1 KO and AMPKγ3 KO mice, compared to respective wild-type littermates (*Paper III*, Fig. 2 and 3). These results provide evidence that suppression of basal IL-6 release by AICAR-exposure is independent of functional AMPK.

4.2.5 Ca^{2+}-signaling in the regulation of muscle IL-6 release

Ca^{2+}-signaling pathways are of great relevance for contraction-stimulated skeletal muscle IL-6 release (Banzet *et al.*, 2005; Keller *et al.*, 2006; Banzet *et al.*, 2007). The use of cyclosporin A or FK506, inhibitors of the Ca^{2+}-dependent protein phosphatase calcineurin in human skeletal muscle cells (Keller *et al.*, 2006) or in rats *in vivo* (Banzet *et al.*, 2007), provides evidence for an important role for calcineurin activation in skeletal muscle IL-6 release. Activation of Ca^{2+}-dependent signaling pathways by stimulation with the Ca^{2+}-ionophore, ionomycin, has been shown to increase IL-6 production in rat soleus muscle and human muscle cells (Holmes *et al.*, 2004; Keller *et al.*, 2006). In *Paper III*, however, ionomycin stimulation did not result in increased IL-6 release or mRNA production from isolated mouse skeletal muscle. A possible explanation is the longer duration of the muscle incubation period used in our experiments (2 h) versus the earlier (1 h) study (Holmes *et al.*, 2004). Conversely, autocrine regulation of IL-6 release is dependent on intracellular calcium (Weigert *et al.*, 2007), and thus autocrine Ca^{2+}-dependent signaling mechanisms stimulated by IL-6 may have masked the effect of ionomycin in *Paper III*.

4.3 EFFECT OF IL-6 ON SKELETAL MUSCLE METABOLISM AND GENE EXPRESSION

4.3.1 IL-6-mediated glucose transport

During exercise, skeletal muscle releases IL-6 into the circulation and consequently, IL-6 has been proposed as a mediator of whole body glucose metabolism during exercise (Febbraio *et al.*, 2004). In contrast, IL-6 is also known as an autocrine or paracrine effector (Yudkin *et al.*, 2000; Lauta, 2001), raising the possibility that in addition to its role in whole body metabolism, it elicits local effects directly in skeletal muscle. This is further supported by experiments applying microdialysis, which reveal local IL-6 concentrations in skeletal muscle may be 5-100-fold higher than circulating levels (Langberg *et al.*, 2002; Rosendal *et al.*, 2005). We therefore explored the effect of IL-6 on glucose metabolism in model systems of human skeletal muscle.

Short term exposure to IL-6 directly increased glucose uptake in primary human skeletal muscle cells (*Paper V*, Fig. 2A) and human skeletal muscle *in vitro* (*Paper IV*, Fig. 1). The effect of IL-6 on glucose transport in human muscle cells was similar to that achieved in response to insulin. Human skeletal muscle cells have relatively modest insulin-mediated glucose transport rates, presumably due to high GLUT1 and reduced GLUT4 expression (Al-Khalili *et al.*, 2003b). In adult human muscle incubated *in vitro*, the effect of IL-6 on glucose transport (~30% increase) was moderate when compared to the effect of insulin. Skeletal muscle is the predominant tissue responsible for glucose utilization under insulin-stimulated conditions. Our data is in agreement with the observed ~15% increase in glucose infusion rate and glucose oxidation in response to IL-6 infusion during hyperinsulinemic-euglycemic clamp studies (Carey *et al.*, 2006). Interestingly, an 8 day stimulation with IL-6 robustly increased glucose transport in primary human muscle cells (*Paper V*, Fig. 2A). In addition to the acute effects of IL-6 on glucose metabolism, this finding might result from mitogenic effects of IL-6, as well as effects of IL-6 on differentiation.

We tested whether exposure of skeletal muscle to IL-6 would alter insulin action on glucose uptake. An acute pre-exposure of human skeletal muscle to IL-6 was without effect on insulin-mediated glucose transport in muscle cells (*Paper V*, Fig. 2A) and muscle strips *in vitro* (*Paper IV*, Fig. 1), suggesting that IL-6 does not acutely modulate insulin sensitivity. However, increased sensitivity to insulin was found in rat muscle 3 h after IL-6 exposure (Geiger *et al.*, 2007). IL-6 has been observed to sensitize human skeletal muscle cells to insulin by increasing the Akt Ser^{473} phosphorylation (Weigert *et al.*, 2005), with a concomitant and augmented sub-maximal insulin-mediated Akt phosphorylation and glycogen synthesis (Weigert *et al.*, 2005). Acute stimulation with IL-6 may directly increase glycogen synthesis (*Paper IV*, Fig. 2A and *Paper V*, Fig. 1A and B), although this is not generally observed (Weigert *et al.*, 2005). IL-6 enhances the effect of insulin to increase glycogen synthesis, acutely (*Paper V*, Fig. 1B) (Weigert *et al.*, 2005), and also after long term stimulation (*Paper V*, Fig. 1C), suggesting that IL-6 stimulates mechanisms that spare glucose for storage into glycogen. This is underscored by the observation that IL-6 release from skeletal muscle is augmented when glycogen levels are low (Steensberg *et al.*, 2001). In addition to increased glycogen synthesis, IL-6-exposure directly increased glucose oxidation in adult human skeletal muscle (*Paper IV*, Fig. 2B). This may suggest a general increase in glucose metabolism elicited by acute IL-6 exposure.

4.3.2 IL-6 signaling

IL-6 signals through the IL-6 receptor. One of the main intracellular effects is the induction of Jak-STAT signaling (Lutticken *et al.*, 1994; Kamimura *et al.*, 2003), which is known to alter gene transcription of target tissues (Kamimura *et al.*, 2003). Exposure to IL-6 increased the phosphorylation of STAT3 in primary human skeletal muscle cells and intact human skeletal muscle incubated *in vitro* (*Paper V*, Fig. 4a and *Paper IV* Fig. 3A and B), while STAT3 phosphorylation was unaffected by insulin stimulation. In primary muscle cells, IL-6-stimulated STAT3 phosphorylation peaked after 20 min and returned to basal levels after 3 h. The transient effect of IL-6 on STAT3 phosphorylation may be explained by the induction of SOCS3 protein (Rieusset *et al.*, 2004), a known mediator of feedback control of STAT3 signaling. In

adult human muscle strips, STAT3 phosphorylation was unaltered after 15 min, but was increased after 30 and 80 min IL-6-exposure. IL-6-mediated STAT3 phosphorylation is usually a rather rapid event (Weigert *et al.*, 2005), thus the delayed increase in STAT3 phosphorylation in human skeletal muscle strips is possibly related to the reduced incubation temperature (30°C), compared to ~37°C usually applied in cell culture studies.

Akt (Chen *et al.*, 1999a; Jee *et al.*, 2002; Jee *et al.*, 2004) and MAP kinase signaling pathways have been reported to participate in IL-6 signaling events (Yang *et al.*, 2003; Lentzsch *et al.*, 2004). IL-6-exposure resulted in moderately increased Akt phosphorylation at Ser^{473} and Thr^{308} in primary human skeletal muscle cells (*Paper V*, Fig. 4c and d), though Thr^{308}-phosphorylation of Akt was inconsistently observed (Weigert *et al.*, 2005). Despite the increase in Akt phosphorylation, IL-6-exposure did not increase the phosphorylation of AS160 in primary human skeletal muscle cells (*Paper V*, Fig. 4e). No direct effect of IL-6 on phosphorylation of components of the insulin signaling cascade was noted in adult human skeletal muscle *in vitro* (*Paper IV*, Fig. 6). Moreover, IL-6 did not alter insulin-signaling in adult skeletal muscle (*Paper IV*, Fig. 6), indicating that IL-6 exerts a greater effect on insulin-signaling of primary human skeletal muscle cells compared to intact adult skeletal muscle strips.

To verify the effect of IL-6 on Akt phosphorylation, we determined upstream signaling events, namely IRS1-associated PI3-kinase activity. IL-6 induces a rapid recruitment of IRS1 to the IL-6 receptor complex in cultured skeletal muscle cells (Weigert *et al.*, 2006). Moreover, IL-6 mediates rapid and transient phosphorylation of Ser^{318} of IRS1, peaking after 5 min (Weigert *et al.*, 2006). IRS1 Ser^{312} phosphorylation was unaltered after IL-6-exposure (*Paper V*, Fig. 4f). Ser^{312} phosphorylation of IRS1 has been implicated in mediating insulin-resistance (Aguirre *et al.*, 2000; Taniguchi *et al.*, 2006), supporting the finding of increased insulin-mediated glucose metabolism after IL-6 exposure in primary human skeletal muscle cells. Consequently, IL-6-exposure increased IRS1-associated PI3-kinase activity in primary human skeletal muscle cells (*Paper V*, Fig. 5A). Unexpectedly, IL-6-mediated IRS1-associated PI3-kinase activity was increased compared to the insulin-stimulated state, underscoring an important role of PI3-kinase in mediating the effects of IL-6 (Jee *et al.*, 2002). This was further highlighted by our finding that inhibition of PI3-kinase abolished the effects of IL-6 on glucose metabolism (*Paper V*, Fig. 6). Unexpectedly, and in contrast to the findings in primary human skeletal muscle cells, IL-6 exposure was without effect on IRS1-associated PI3-kinase activity in human skeletal muscle strips (*Paper IV*, Fig. 6A). The reason for the discrepancy is not apparent, but the differentiation state of the adult skeletal muscle versus the primary muscle cultured cells may offer an explanation. Activation of MAP kinases has been implicated in the mitogenic effects of IL-6 (Yang *et al.*, 2003; Lentzsch *et al.*, 2004). Exposure to IL-6 increased ERK phosphorylation in primary human skeletal muscle cells, with a peak effect observed after 20 min stimulation (*Paper V*, Fig. 4g). IL-6 increased the phosphorylation of p38 MAP kinase, which was further elevated by additional stimulation with insulin (*Paper IV*, Fig. 5). Taken together, we identified molecular mechanisms by which IL-6 may increase glucose metabolism. Furthermore, a discrepancy between the effects of IL-6 on adult human muscle strips *in vitro* vs. the effect on primary human muscle cultured cells was unraveled.

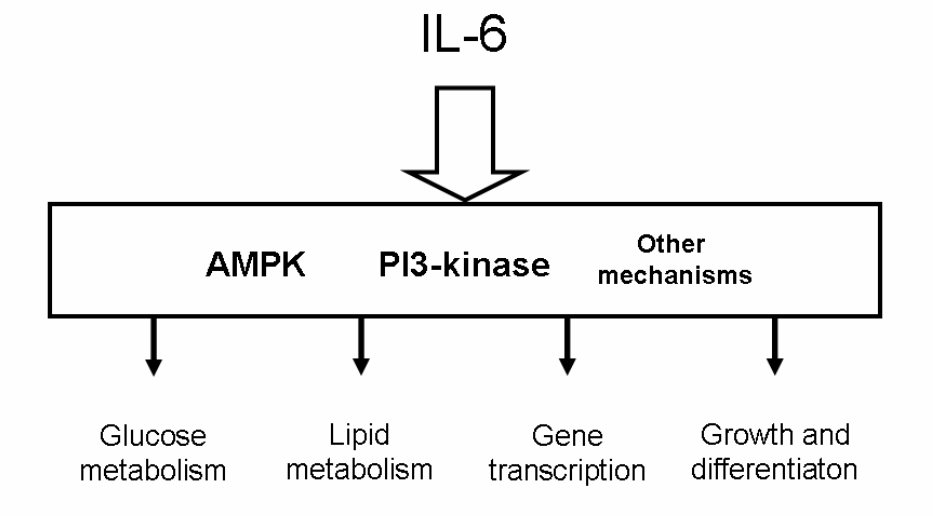


Figure 15: IL-6-stimulation affects skeletal muscle growth, differentiation and metabolism.

4.3.3 IL-6 and AMPK

IL-6 is implicated in the regulation of AMPK phosphorylation (Kelly *et al.*, 2004; Carey *et al.*, 2006; Geiger *et al.*, 2007). In IL-6 KO mice, basal and exercise-mediated AMPK phosphorylation is dramatically reduced in skeletal muscle and adipose tissue (Kelly *et al.*, 2004). Incubation of rat muscle or F422A adipocytes with IL-6 *in vitro* increases AMPK phosphorylation (Kelly *et al.*, 2004; Geiger *et al.*, 2007). The effect of IL-6 to enhance glucose uptake in skeletal myotubes is abolished by expression of a dominant-negative construct of AMPK (Carey *et al.*, 2006). Accordingly, when human skeletal muscle strips were incubated in the presence or absence of IL-6, a transient elevation in AMPK phosphorylation was noted (*Paper IV*, Fig. 4A). This effect was of similar timing compared to a previous report (Kelly *et al.*, 2004), however this effect was not statistically significant (*Paper IV*, Fig. 4A). Interestingly, a significant effect of IL-6 on AMPK was noted by correcting for inter-individual variation via calculating the IL-6 effect over each individual subject's basal level, suggesting that the inter-individual variation between the subjects masks the IL-6-mediated AMPK phosphorylation.

Phosphorylation of S6 ribosomal protein, a downstream target of p70S6 kinase, was reduced after exposure of human muscle to IL-6. This is in agreement, but does not confirm the effect of IL-6 on AMPK activation, because activation of AMPK suppresses protein synthesis through concurrent repression of mammalian target of rapamycin (mTOR) signaling and activation of MAP kinase signaling (Krause *et al.*, 2002; Chan *et al.*, 2004a; Williamson *et al.*, 2006). AMPK phosphorylation was increased after exposure of primary human skeletal muscle cultured cells to IL-6 (*Paper V*, Fig. 4b). Therefore, accumulating evidence places IL-6 upstream of AMPK phosphorylation and signaling in skeletal muscle. IL-6-mediated AMPK phosphorylation has also been attributed to increases in basal- and insulin-mediated glucose uptake, as well as increased lipid oxidation in L6 myotubes (Carey *et al.*, 2006). Additional studies are warranted to better describe the link between IL-6-signaling and metabolic effects of IL-6 in human skeletal muscle.

4.3.4 IL-6 increases lipid metabolism through AMPK

IL-6 infusion results in increased whole body fatty acid turnover in young (van Hall *et al.*, 2003), elderly (Petersen *et al.*, 2005) and NIDDM subjects (Petersen *et al.*, 2005). While incubation of adipocytes with IL-6 increases lipolysis (Trujillo *et al.*, 2004; Petersen *et al.*, 2005), exposure to IL-6 of primary human skeletal muscle cells (*Paper V*, Fig. 3) or L6 myotubes (Petersen *et al.*, 2005; Carey *et al.*, 2006) increases palmitate oxidation. Furthermore, IL-6 increases lipid oxidation in isolated rat soleus muscle (Bruce & Dyck, 2004), providing evidence for an involvement of IL-6 in the regulation of skeletal muscle fatty acid metabolism. Importantly, IL-6-mediated lipid metabolism was sensitive to silencing of AMPKα isoforms by *si*RNA in primary human skeletal muscle cells (*Paper V*, Fig. 8B), providing direct evidence that the effects of IL-6 on skeletal muscle lipid oxidation are mediated by AMPK. IL-6 exposure also increased the intracellular accumulation of palmitate, an indication of fatty acid uptake into the cell (*Paper V*, Fig. 3A). Comparable to lipid oxidation, palmitate accumulation was sensitive to silencing of AMPKα isoforms (*Paper V*, Fig. 8A). Thus, IL-6 mediates lipid uptake and metabolism via an AMPK-dependent pathway in human skeletal muscle cells and may contribute to increased whole body fatty acid turnover during IL-6 infusion.

4.3.5 IL-6-mediated glycogen synthesis depends on PI3-kinase

AMPK silencing did not alter basal or IL-6-mediated glucose incorporation into glycogen (*Paper V*, Fig. 8C). This was an unexpected finding, because in L6 myotubes IL-6-mediated glucose uptake was inhibited in the absence of functional AMPK signaling (Carey *et al.*, 2006). We therefore tested the role of the canonical insulin-signaling cascade in mediating the effects of IL-6 on glucose transport by utilizing a specific inhibitor of PI3-kinase, LY294002 (Vlahos *et al.*, 1994). Inhibition of PI3-kinase suppressed the effect of insulin to increase glucose incorporation into glycogen, confirming the effect of the inhibitor to block insulin-signaling. Exposure to LY294002 also blunted the effect of IL-6 to increase glucose incorporation into glycogen (*Paper V*, Fig. 6), suggesting a PI3-kinase-dependent pathway. Consistent with our finding of a role for AMPK-dependent signaling in IL-6-mediated lipid metabolism, exposure to LY294002 did not affect IL-6-mediated lipid oxidation (*Paper V*, data not shown). Taken together, we provide evidence that IL-6 may stimulate at least two distinct signaling pathways, and that these pathways independently regulate glucose and lipid metabolism.

4.3.6 IL-6 promotes differentiation of primary muscle cells

IL-6 is released by skeletal muscle in response to acute exercise bouts. Interestingly, the magnitude of exercise-induced IL-6 mRNA expression in contracting human skeletal muscle is markedly reduced after a 10 week knee-extensor training interval (Fischer *et al.*, 2004), suggesting that adaptive processes related to long-term training reduce IL-6 mRNA production in response to muscle contraction. Moreover, IL-6 receptor mRNA concentration increases in response to acute and chronic exercise, suggesting that skeletal muscle is sensitized to IL-6 in response to training (Keller *et*

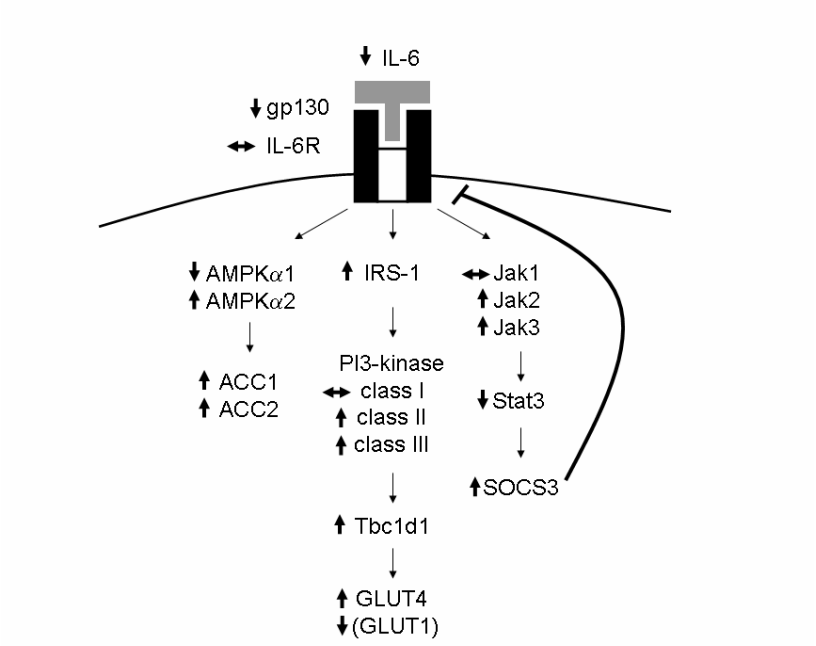
al., 2005). Exposure to IL-6 enhances skeletal muscle differentiation (*Paper V*, Fig. 9) (Okazaki *et al.*, 1996). This is in contrast to the effect of IL-6 on adipose tissue, whereby differentiation is impaired (Sopasakis *et al.*, 2004). In primary human skeletal muscle cultures exposed to IL-6 for 8 days, mRNA expression of key genes involved in metabolism and differentiation was increased (*Paper V*, Table 2 and Fig. 10). For example, the expression of GLUT4, peroxisome proliferator-activated receptor (PPAR)γ coactivator (PGC)-1α, and myocyte enhancer factor (MEF)2D was significantly increased (*Paper V*, Table 2 and Fig. 10). GLUT4 and PGC-1 have been linked to insulin sensitivity in skeletal muscle (Al-Khalili *et al.*, 2005), while MEF2D is a key myogenic transcription factor implicated in the regulation of GLUT4 (Silva *et al.*, 2005). Interestingly, the increase in PGC-1 mRNA after IL-6 exposure was sensitive to silencing of AMPKα isoforms, providing further evidence that AMPK is an important mediator of PGC-1 activity (Terada *et al.*, 2002; Lee *et al.*, 2006). Long-term exposure to IL-6 also increased the mRNA expression of uncoupling protein (UCP) 2, the PPAR isoforms (α,δ,γ), and FAT4, important genes for lipid metabolism. In contrast, the expression of UCP3 and GLUT1 mRNA was decreased. These alterations correlate to some extent with metabolic adaptations of skeletal muscle to exercise training, including increased oxidative metabolism, increased reliance on lipids and increased mitochondria content (Holloszy, 1975; Holloszy & Booth, 1976; Phillips *et al.*, 1996b) and may therefore suggest a role of IL-6 in skeletal muscle adaptation to exercise.

4.3.7 Hypothesis: IL-6 targets satellite cells

IL-6 increases growth and differentiation of primary human skeletal muscle cells (*Paper V*, Fig. 9) (Cantini *et al.*, 1995; Okazaki *et al.*, 1996). Moreover, IL-6 stimulates expression of genes implicated in mitochondrial biogenesis, as well as glucose and lipid metabolism; effects that can also be observed in response to exercise training. The exercise-mediated skeletal muscle IL-6 release may therefore be of relevance for skeletal muscle adaptation to exercise, by targeting satellite cells (Schultz & McCormick, 1994; Cantini *et al.*, 1995). Satellite cells are surrounding the muscle fibers and are rapidly activated to proliferate, differentiate and fuse (Schultz & McCormick, 1994), a process implicated in muscle growth and repair after injury (Schultz & McCormick, 1994). IL-6-exposure increased glucose and lipid metabolism in primary human skeletal muscle cells. In contrast, IL-6 had a moderate effect on glucose metabolism in human skeletal muscle *in vitro*. Moreover, IL-6-exposure of skeletal muscle strips *in vitro* did not stimulate the insulin signaling cascade, which is particularly evident from the lack of IL-6-mediated IRS1-associated PI3-kinase activity. Therefore, mature human muscle appears less sensitive to IL-6-stimulation compared to primary muscle cells. Human skeletal muscle cells are generated by isolation and culturing of satellite cells; and satellite cells are also present in human skeletal muscle strips. The moderate effect of IL-6 observed in *Paper IV* may possibly represent the effect of IL-6 on the satellite cells. Because IL-6 enhances muscle growth and differentiation of primary human myotubes (*Paper V*, Fig. 9) (Cantini *et al.*, 1995; Okazaki *et al.*, 1996), IL-6 released from skeletal muscle fibers during exercise may stimulate growth and differentiation of satellite cells, contributing to skeletal muscle repair and adaptation after exercise. Further support for this hypothesis comes from IL-6 KO mice. Basal AMPK phosphorylation is impaired in skeletal muscle from mice

lacking IL-6 (Kelly *et al.*, 2004). Moreover, exercise endurance is reduced in IL-6 KO mice (Faldt *et al.*, 2004), providing evidence that IL-6 is necessary for normal exercise capacity (Faldt *et al.*, 2004). IL-6 is also important for tendon healing (Lin *et al.*, 2006), raising the possibility that repair processes are IL-6-dependent. Taken together, IL-6 released by contracting skeletal muscle is likely to exert paracrine effects, in addition to a regulatory function on whole body metabolism.

Figure 16: mRNA expression of selected proteins implicated in IL-6 signaling in biopsies from human



skeletal muscle compared to human myotubes, as determined by microarray analysis. n=3 subjects. Greater than 2-fold differences are indicated (↓, ↑ and ↔ for 2-fold lower, higher or less than 2-fold different expressed, respectively).

4.3.8 Signaling in primary muscle cells versus mature muscle

The different response to IL-6 of primary human skeletal muscle cells and adult human skeletal muscle *in vitro* was unexpected. Additional studies are warranted to unfold the molecular mechanisms underlying the distinct response. As a first step, mRNA from differentiated adult skeletal muscle (skeletal muscle biopsies) and from primary myotubes generated from identical subjects, was subjected to microarray analysis. Our preliminary data indicates that genes implicated in IL-6 signaling are differentially expressed (Fig. 16). The reduced mRNA expression of gp130, in combination with increased SOCS3 expression may contribute to reduce IL-6-mediated effects in differentiated muscle (Fig. 16). However, more studies are needed to define parallels and differences between primary human skeletal muscle cells and adult human skeletal muscle to allow better prediction of effects in mature muscle through the study of primary muscle cells.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The aim of this study was to explore signaling pathways that control the insulin-dependent and -independent response of skeletal muscle to metabolic challenges. Because exercise elicits beneficial effects on skeletal muscle glucose and lipid metabolism, we focused on contraction-mediated molecular mechanisms.

Evidence from genetically modified mice has highlighted an important role of AMPK in the regulation of skeletal muscle glycogen levels. When AMPK activity is increased due to mutations in the AMPKγ subunit, glycogen levels are increased in muscle or heart. Conversely, when AMPK signaling is impaired, muscle glycogen levels are reduced. In *Paper I* we found an inverse relationship between contraction-mediated AMPK activity and glycogen content only in the presence of a functional AMPKγ3 isoform. We also provide evidence that the increase in glycogen concentration, rather than the mutation *per se*, directly increased *work* performance. Whether or not *in vitro* fatigue-resistance in *Tg-Prkag3*^{225Q} mice translates into increased spontaneous activity or increased exercise endurance *in vivo,* remains to be determined. Furthermore, glycogen levels are inversely correlated with skeletal muscle insulin sensitivity (Ivy *et al.*, 1985; Cartee *et al.*, 1989; Shulman *et al.*, 1990; Barnes *et al.*, 2004). Whether or not activating AMPK by targeting the AMPKγ3 isoform is promising as treatment for metabolic disorders needs to be more intensively determined by studying the phenotype of older *Tg-Prkag3*^{225Q} mice or by introducing the R225Q mutation into a genetic background that mimics features of NIDDM, such as in the *ob/ob* mouse (Lindstrom, 2007).

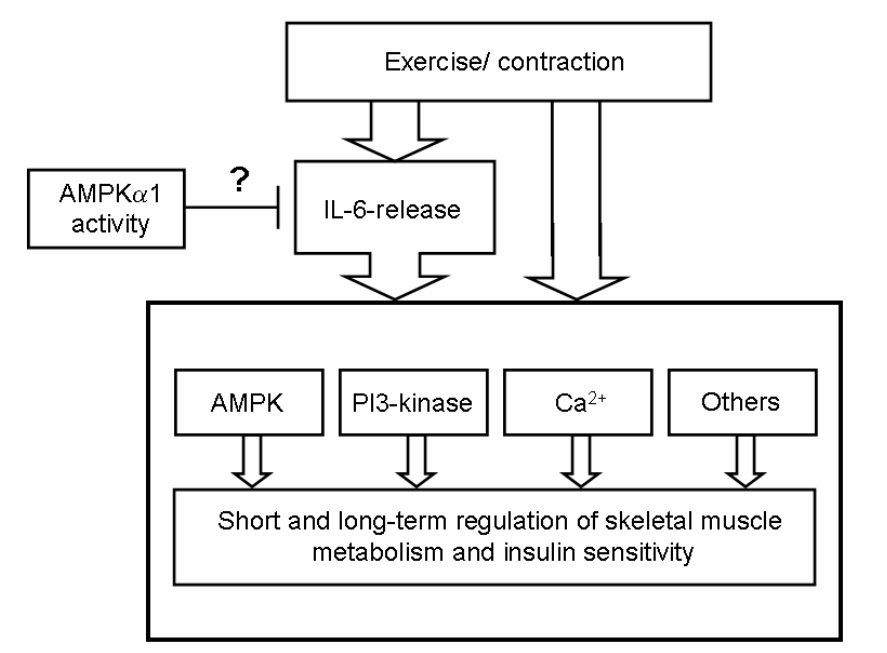


Figure 17: Contraction-mediated regulation of skeletal muscle metabolism.

Impaired AMPK signaling prevented AICAR and contraction-mediated AS160 phosphorylation in mouse EDL muscle, providing genetic evidence that AS160 is a

downstream target of AMPK. While these findings are consistent with impaired AICAR-mediated glucose transport under similar conditions, the normal or only partly reduced contraction-mediated glucose transport suggests other signaling inputs contribute to the regulation of glucose transport after electrical stimulation. Whether these signaling inputs comprise phosphorylation sites on AS160 that were not detected by the tools utilized in this thesis work, or involve other signaling molecules or pathways, remains to be determined.

Although acute activation of signaling pathways for skeletal muscle metabolism are apparent in response to contraction, skeletal muscle also functions as an endocrine organ and contributes to whole body metabolic regulation. Evidence is provided showing IL-6 is released from mouse skeletal muscle *in vitro* in a fiber-type specific manner. AICAR suppresses IL-6 release by down-regulating IL-6 mRNA, independent of functional AMPK signaling. IL-6 may up-regulate its own expression in skeletal muscle. Basal IL-6 release is higher from isolated soleus muscle with dysfunctional AMPK signaling. An AMPKα1-dependent feedback mechanism on *in vitro* IL-6 release that may be of relevance for the regulation of IL-6 release at later stages of exercise is proposed. However, to confirm and advance these findings, skeletal muscle IL-6 release needs to be studied in response to other stimuli, including pharmacological and physiological AMPK activators.

IL-6 exposure of isolated human skeletal muscle increases glucose metabolism, presumably through an AMPK dependent pathway. Stimulation of primary human skeletal muscle cells with IL-6 enhances glucose metabolism by a pathway sensitive to PI3-kinase inhibition, whereas silencing of AMPKα isoforms prevents IL-6-mediated increases in lipid metabolism. Furthermore, IL-6 exposure increases growth and differentiation of skeletal muscle cells, and enhances the expression of genes involved in metabolism. Thus in addition to its effects on whole body metabolism, IL-6 is proposed to elicit local paracrine effects in skeletal muscle.

Activation of AMPK and IL-6 release are central contraction-mediated responses in skeletal muscle. The results presented in this thesis provide evidence that targeting AMPK, e.g. by targeting AMPKγ3 specifically in the muscle, activates molecular mechanisms altering glucose metabolism and increasing muscle performance. Skeletal muscle releases IL-6 under basal conditions *in vitro*. Evidence is provided for an AMPK-dependent regulatory mechanism of skeletal muscle IL-6 release. Conversely, IL-6-exposure of skeletal muscle *in vitro* enhances glucose and lipid metabolism by pathways depending on AMPK and PI3-kinase. Furthermore, stimulation with IL-6 enhanced growth and differentiation of primary myotubes. This thesis work emphasizes the central role of AMPK in skeletal muscle metabolism and highlights that autocrine/paracrine mechanisms play an important role in skeletal muscle metabolic regulation.

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