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NUTRIENT AND ENERGY SENSING IN SKELETAL MUSCLE

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To my family

ABSTRACT

Nutrient overload and physical inactivity often leads to the development of obesity and type 2 diabetes. Acute over-nutrition can induce insulin resistance, while physical exercise enhances skeletal muscle insulin sensitivity. Like every living cell, skeletal muscle senses nutrient and energy signals and to adjust metabolic flux. This thesis focuses on some of the key nutrient and energy sensing (exercise/contraction-induced) pathways in skeletal muscle that regulate metabolism.

AMPK is a key energy sensing enzyme, composed of three different subunits, with several isoforms existing for each subunit. The role of the different AMPK subunits in the regulation of mTOR signaling was investigated. In EDL muscle from wild-type mice, AICAR (a chemical AMPK activator) completely inhibited insulin-mediated phosphorylation of S6K1 (Thr^{389}), rpS6 ($Ser^{235/23}6$) and 4E-BP1 ($Thr^{37/46}$). Thus, AMPK is negative regulator of mTOR signaling. The inhibitory effects of AICAR were partially blocked in skeletal muscle from α2 AMPK depleted (KO) and γ3 AMPK KO mice, functional α2 AMPK and γ3 AMPK subunits are required for the AICAR-mediated inhibition of mTOR signaling.

Excessive amino acid availability impairs insulin action in skeletal muscle. In primary human myotubes, supra-physiological leucine concentrations reduced insulin-stimulated Akt phosphorylation, glucose uptake and glucose incorporation into glycogen. These results indicate nutrient overload induced insulin resistance. Depletion of S6K1 using siRNA enhanced basal glucose uptake and protected against the development insulin resistance in response to leucine. Study II highlights a direct role for S6K1 plays in insulin action and glucose metabolism.

Several proteins are phosphorylated in skeletal muscle in response to acute exercise. The effect of cycling or resistance exercise on the phosphorylation of Akt substrates was determined using an antibody that recognizes a consensus Akt phosporylation motif (PAS). Proteins of 160 and 300 kDa were indentified as AS160 (TBC1D4) and filamin A, respectively. Acute endurance exercise increased phosphorylation of TBC1D4 and filamin A, with concomitant increase in phosphorylation of Akt Ser^{473}, whereas acute resistance exercise was without effect. TBC1D4 and filamin A may provide link between acute exercise and metabolism in muscle.

Hypoxia is useful model to study effects of exercise/muscle contraction. In paper III, hypoxia-induced glucose transport was partially impaired in EDL muscle from γ3 AMPK KO mice, indicating a role for the γ3 AMPK subunit in glucose metabolism. These effects were uncoupled from AMPK and TBC1D1/D4 signaling, suggesting that an AMPK-and TBC1D1/D4-independent mechanism contributes to glucose transport in skeletal muscle. An interaction between AMPK and CaMK is implicated, since the CaMK inhibitor KN-93 had a more potent effect to reduce hypoxia-induced glucose transport in γ3 AMPK KO mice.

Nitric oxide (NO) is implicated in exercise-induced signaling networks. Exposure of human skeletal muscle to an NO donor increased glucose uptake, with a concomitant increase in cGMP levels and α1-associated AMPK activity. Thus, NO/cGMP signaling may be part of a novel pathway that regulates skeletal muscle glucose uptake.

In conclusion, AMPK and mTOR signaling play important roles in regulation of skeletal muscle metabolism. AMPK appears to have a heterotrimer-specific action on skeletal muscle metabolism. Furthermore, contraction/exercise responsive signaling pathways including CaMK, NO-cGMP and Akt are important in the regulation of skeletal muscle glucose uptake.

Keywords: Skeletal muscle, AMPK, mTOR, glucose metabolism, exercise, Akt, TBC1D1/D4, CaMK, NO-cGMP.

LIST OF PUBLICATIONS

I. **Deshmukh AS**, Treebak JT, Long YC, Viollet B, Wojtaszewski JF, and Zierath JR. Role of AMPK subunits in skeletal muscle mTOR signaling. *Molecular Endocrinology*, 2008, 22(5), 1105-12.

II. **Deshmukh AS**, Salehzadeh F, Metayer-Coustard S, Fahlman R, Nair KS, and Lubna Al-Khalili. Post-transcriptional gene silencing of ribosomal protein S6 kinase 1 restores insulin action in leucine-treated skeletal muscle. *Cell Molecular Life Sciences*, 2009, 66(8):1457-66.

III. **Deshmukh AS**, Coffey VG, Zhong Z, Chibalin AV, Hawley JA, and Zierath JR. Exercise-induced phosphorylation of the novel Akt substrates AS160 and filamin A in human skeletal muscle. *Diabetes*, 2006, 55, 1776-1782.

IV. **Deshmukh AS***, Glund S*, Tom RZ and Zierath JR. Role of the AMPK γ3 isoform in hypoxia-stimulated glucose transport in glycolytic skeletal muscle. *American Journal of Physiology-Endocrinology and Metabolism,* 2009, In press.
*These authors have contributed equally to this work.

V. **Deshmukh AS**, Long YC, Karlsson HK, Glund S, Barbosa TD, Zavadoski WJ, Gibbs ME, Koistinen HA, Wallberg-Henriksson H and Zierath JR. Nitric oxide increases cGMP levels, AMPKα1-isoform-specific activity and glucose transport in human skeletal muscle. *Diabetologia* (Submitted)

LIST OF THE PUBLICATIONS THAT ARE NOT INCLUDED IN THIS THESIS

I. Treebak JT*, Glund S*, **Deshmukh AS***, Klein DK, Long YC, Jensen TE, Jørgensen SB, Viollet B, Anderson L, Neuman D, Wallimann T, Richter EA, Chibalin AV, Zierath JR and Wojtaszewski JF. AMPK-mediated AS160 phosphorylation in skeletal muscle is dependent on AMPK catalytic and regulatory subunits. *Diabetes*, 2006, 55(7):2051-8.*These authors have contributed equally to this work.

II. Chadt A, Leicht K, **Deshmukh AS**, Jiang LQ, Scherneck S, Bernhardt U, Dreja T, Vogel H, Schmolz K, Kluge R, Zierath JR, Hultschig C, Hoeben RC, Schürmann A, Joost HG, Al-Hasani H.Tbc1d1 mutation in lean mouse strain confers leanness and protects from diet-induced obesity. *Nature Genetics*, 2008, 40(11):1354-9.

III. Glund S, **Deshmukh AS,** Long YC, Moller T, Koistinen HA, Caidahl K, Zierath JR and Krook A. Interleukin-6 directly increases glucose metabolism in resting human skeletal muscle. *Diabetes,* 2007, 56(6):1630-7.

IV **Deshmukh AS**, Hawley JA, and Zierath JR. Exercise-induced Phospho-proteins in Skeletal Muscle. *International Journal of Obesity*, 2008, 32, S18 - S23 (Review).

CONTENTS

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ACC	Acetyl-CoA carboxylase
AICAR	5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AS160	Akt substrate of 160 kDa
ATP	Adenosine triphosphate
BCAAs	Branched chain amino acids
BMI	Body mass index
CaMK	Ca2+-calmodulin-dependent protein kinase
CAMKK	calmodulin-dependent protein kinase kinase
CBD	Ca^{2+}/CaM binding domain
cGMP	Cyclic guanosine monophosphate
DMEM	Dulbecco's minimal essential media
4E-BP1	Eukaryotic translation initiation factor 4E binding protein 1
EDL	Extensor digitorum longus
eIF4E	Eukaryotic translation initiation factor 4E
eIF2	Translation elongation factor 2
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT	Glucose transporter
GSV	Glucose storage vesicles
GTP	Guanosine triphosphate
IR	Insulin receptor
IRS	Insulin receptor substrate
KO	Knockout
mTOR	Mammalian target of rapamycin
NO	Nitric oxide
NOS	Nitric oxide synthase
PAS	Phospho-Akt-Substrate
PIP3	phosphotidylinositol-3, 4,5-phosphate
PIP2	phosphotidylinositol-4,5-phosphate
PI3K	Phosphatidylinositol 3-kinase
PKB/Akt	Protein kinase B
PTEN	Phosphatase and tensin homolog
Rheb	Ras homolog enriched in brain
rpS6	Ribosomal protein S6
S6K1	Ribosomal S6 kinase 1
SIRT	Protein deacetylase sirtuins
Spermine NONOate	N-(2-Aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine
TBST	Tris-buffered saline containing 0.02% Tween 20
TSC	Tuberous sclerosis complex

1 INTRODUCTION

Every living cell has a fundamental ability to harmonize nutrient supply with energy expenditure. The term 'nutrient and energy sensing' can be defined and interpreted in different ways. In a simplistic view, nutrient and energy sensors are the proteins that modulate their functions in response to nutrient availability (nutrient sensor) or cellular energy status (energy sensor), which may trigger the downstream activation of signaling cascades. Nutrient- and energy-mediated signaling cascades regulate specific aspects of fuel and energy metabolism, ultimately influencing cell function, growth, proliferation and survival. In mammals, these signal transduction pathways appear to operate both independently from, and coordinately with, the hormone sensitive pathways. Since several of these pathways have remained evolutionary conserved from single cell yeast to mammals, they must have originally evolved independent of hormonal control.

1.1 NUTRIENT AND ENERGY SENSING IN SKELETAL MUSCLE

Skeletal muscle is the largest organ in the body and is a major tissue involved in energy metabolism. This is particularly important because of its classical features. Skeletal muscle accounts for ~75% of insulin stimulated glucose uptake and impairment in insulin-stimulated skeletal muscle glucose uptake is hallmark feature of type 2 diabetes (DeFronzo, 1985). Excessive nutrients, especially, branched chain amino acids, impair insulin action on glucose metabolism in skeletal muscle (Traxinger, 1989, Krebs, 2002). Skeletal muscle has high degree of flexibility, which adapts to various physiological demands (like exercise, nutrient overload etc.) by shifting energy substrate metabolism. Exercise-induced improvement in skeletal muscle remain functional in insulin resistant rats (Cortez, 1991), diabetic rats (Wallberg-Henriksson, 1984) and human with insulin resistance and type 2 diabetes (Christ-Roberts, 2003), even if insulin action is impaired. Thus skeletal muscle plays an important role in whole body substrate metabolism.

Skeletal muscle, like all eukaryotic cells, must continuously maintain a high, non-equilibrium ratio of ATP to ADP to survive. Catabolism increases energy production in the cells by converting ADP and phosphate to ATP, whereas almost all other cellular processes tend to consume energy by directly or indirectly converting ATP to ADP and phosphate (or AMP and pyrophosphate). The fact that the ATP:ADP ratio in cells usually remains almost constant indicates that the mechanism that maintains these process are efficiently balanced (Hardie, 2004). In healthy individuals, energy intake and expenditure are tightly controlled at a whole body level to maintain constant body weight. Changes in nutrient availability are sensed at a cellular level in multiple tissues, including skeletal muscle. For example, caloric overfeeding in rodents, which results in a positive energy balance, rapidly induces skeletal muscle insulin resistance (Wang, 2001), while calorie restriction in humans and rodents enhances skeletal muscle insulin sensitivity (Kelley, 1993, Cartee, 1994).

Skeletal muscle experiences major modifications in energy homeostasis during exercise. Acute physical exercise initiates a number of beneficial effects in skeletal muscle, including improvements in glucose homeostasis (Christ-Roberts, 2003, Wojtaszewski, 2000), lipid metabolism (Ezell, 1999, Goodpaster, 2002), and insulin sensitivity (Wojtaszewski, 2000), all of which are especially relevant for diabetic

patients (Christ-Roberts, 2003, Holloszy, 2005). Exercise training can also promote skeletal muscle hypertrophy and improve age-related sarcopenia and osteopenia (Hurley, 2000). Thus improvements in skeletal muscle metabolism and insulin action can occur with small perturbations in energy balance. This raises some obvious conceptual questions, such as why and how skeletal muscle so sensitive to nutritional and exercise/stress/energy responses?

1.1.1 Molecular nutrient and energy sensors

Each cell has several intrinsic pathways that can acutely detect or sense changes in energy status or nutritional availability and transduce signals from external stimuli to trigger process such as nutrient uptake, gene transcription and mRNA translation. In most cases, the transduction network consists of a group of protein kinases that direct a signal, or signals, to an appropriate end point(s). Indeed, previous studies provided evidence that number of sensors respond to perturbations in nutrient overload, including the serine/threonine protein kinase mTOR (mammalian target of rapamycin) (Um, 2004, Tzatsos, 2006, Krebs, 2007), the regulatory subunits of phosphatidylinositol 3-kinase (PI3-K i.e., p85, p55, p50) (McCurdy, 2005), and protein deacetylase sirtuins 1 (SIRT1) (Sun, 2007). Exercise or muscle contraction is one of the major modulators of skeletal muscle energy status. Depending on the intensity of exercise, phosphocreatine and ATP concentration can dramatically decrease. Exercise/contraction-induced effects on skeletal muscle have been attributed to several signaling nodes including AMP-activated protein kinase (AMPK) (Mu, 2001), Ca2+-calmodulin-dependent protein kinase (CaMK) (Wright, 2004, Wright, 2005), Nitric oxide-cyclic guanosine monophosphate (NO-cGMP) pathway (Bradley, 1999) and PKB/Akt (protein kinase B) (Sakamoto, 2002, Sakamoto, 2004). Each of these homeostatic regulators somehow directly or indirectly sense nutrient levels and/or cellular energy status and modulate either anabolic and/or catabolic pathways to achieve cellular homeostasis. In several cases, the responsive pathways have been fairly well delineated, but the actual sensor remains a mystery. Moreover, crosstalk between these pathways and their clinical implications in metabolic disease, like diabetes or cancer, is poorly understood. Therefore investigation of nutrient and energy sensing pathways in skeletal muscle is crucial.

1.2 MAMMALIAN TARGET OF RAPAMYCIN (MTOR): A NUTRIENT SENSOR

Target of rapamycin (TOR) protein kinase, originally identified in *Saccharomyces cervisiae*, is evolutionary conserved nutrient sensor (Heitman, 1991, Abraham, 2005). This serine/threonine kinase is centrally involved in the coordination of cell growth and proliferation in response to growth factors and nutrients. TOR controls many aspects of cell growth including translation initiation and elongation, ribosome biogenesis, autophagy, protein kinase C signaling, cell-cycle progression and transcription (Figure 1) (Abraham, 2005, Harris, 2003). After its discovery in yeast, the homologous mammalian TOR protein (mTOR) was identified (Sabatini, 1994, Brown, 1994). mTOR is ubiquitously expressed in all tissues with the highest levels in skeletal muscle and brain (Harris, 2003) and found to be activated by nutrient-rich conditions, particularly by high levels of amino acids and insulin (Bolster, 2004). Rapamycin

inhibits TOR signaling in all species and closely related homolog's of TOR have been identified in all eukaryotes (Crespo, 2002). A defect in mTOR signaling has been implicated in several diseases like diabetes and cancer (Um, 2004, Aoki, 2001, Dann, 2007).

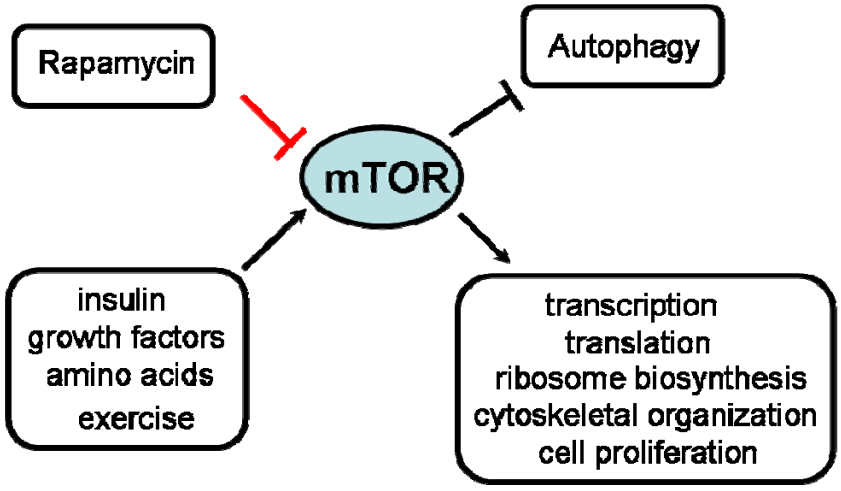

Figure 1. Overview of role of mTOR signaling.

mTOR exist in two protein complexes; mTORC1 consist of mTOR, the G-protein beta-like protein (GβL/LST8) and raptor, and is responsible for cell growth, whereas mTORC2 contains mTOR, GβL and rictor, and is important in cytoskeletal organization (Abraham, 2005). Ribosomal S6 kinase 1 (S6K1) and eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) are well-characterized substrates of mTOR (Abraham, 2005, Harris, 2003).

1.2.1 Regulation of mTOR

mTOR plays a pivotal role in mediating nutrients, growth factor, and energy sensing signals. Most upstream regulators of mTOR appear to function through the tumor suppressors tuberous sclerosis complex 1 (TSC1) and tuberous sclerosis complex 2 (TSC2). TSC1/TSC2 regulates mTOR activity via the RAS-like GTPase, Rheb (Ras homolog enriched in brain). TSC2 has GTPase-activating protein (GAP) activity towards the Rheb, and TSC1/TSC2 antagonizes the mTOR signaling pathway via stimulation of hydrolysis of Rheb (Tee, 2003, Li, 2004). The regulation of mTOR is complex because it is point of convergence for multiple signaling pathways, thereby integrating several signals (Proud, 2006, Proud, 2007, Abraham, 2005, Harris, 2003). For example, mTOR is independently regulated by amino acids, cellular energy levels, and growth factors (Figure 2).

1.2.1.1 Amino acids

Amino acids are positive regulators of mTOR signaling (Figure 2). Most of the studies using mammalian cells provide evidence to suggest that leucine is the most effective stimulator of the mTOR pathway (Buse, 1975, Hong, 1984, Li, 1978). Amino acids signal to mTOR independent of insulin and growth factor pathways (Harris, 2003). However, the basic mechanism by which mTOR senses amino acid levels still

remains unknown. mTOR has been proposed to be regulated directly or indirectly by intracellular amino acids, their metabolites, or amino acid-activated second messengers (Beugnet, 2003).

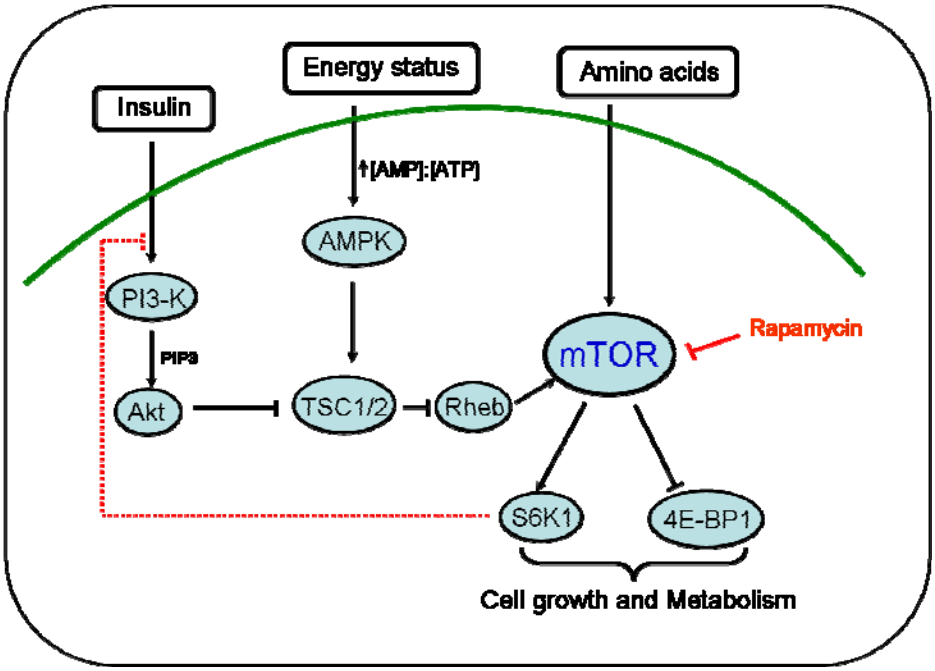

Figure 2. Model depicting the regulation of mTOR signaling in response to insulin, nutrients and cellular energy status.

1.2.1.2 Cellular energy status

mTOR senses energy sufficiency, in addition to its role as a nutrient-sensor (Figure 2). Previous studies provide evidence that a reduction of cellular ATP levels by 2-deoxyglucose inhibits mTOR signaling (Dennis, 2001). Moreover, mTOR has been shown to have an apparent K_m for ATP greater than 1 mM, which is higher than the K_m of most protein kinases (10-20 μM). This indicates that the requirement for a high K_m may allow mTOR activation only when cellular energy is high. mTOR also senses cellular energy levels by another mechanism, which is dependent on the AMP-activated protein kinase (AMPK) and described subsequently.

1.2.1.3 Hormonal stimulation

Growth factors such as insulin, stimulate mTOR signaling via canonical PI 3-K/Akt pathway (Figure 2) (Harris, 2003). Insulin activates the insulin receptor and phosphorylates the insulin receptor substrate (IRS) protein. Phosphorylated IRS-1 recruits and activates PI3-K. Other growth factors that stimulate PI3-K enter the pathway at this point. Activated PI3-K converts phosphotidylinositol-4,5-phosphate (PIP2) in the cell membrane to phosphotidylinositol-3,4,5-phosphate (PIP3). Phosphatase and tensin homolog (PTEN), the PIP3 phosphatase, is negative regulator of PI3-K signaling. The Ser/Thr kinase Akt (also called as protein kinase B) and PDK1 are recruited to the PIP3-containing membranes where PDK1 phosphorylates and activates Akt. Akt phosphorylates and inactivates TSC2, which increases mTOR kinase activity (Harris, 2003, Bolster, 2004). Conversely, AMPK phosphorylates and activates TSC2, which in turns inhibit mTOR signaling.

The cells must be able to integrate information coming from hormonal signals with those coming from nutrient and energy signals to appropriately phosphorylate key sensors. Cell-autonomous inputs seem to be at least partially dominant, because starved cells do not properly respond to hormonal signals (Dennis, 2001). The networks involved in integrating the signals from hormones and nutrients to mTOR are complex and need further clarification. Based on published literature, a proposed network is illustrated (Figure 2).

1.2.2 Downstream effects of mTOR activation

Experiments whereby rapamycin has been used to block mTOR signaling have revealed that mTOR plays a role in the regulation of a variety of cellular functions and process. mTOR controls protein synthesis, cell growth, and proliferation through multiple downstream targets. The major targets of mTOR appear to be components of the translation machinery, particularly those responsible for ribosome recruitment to mRNA (Abraham, 2005, Harris, 2003).

The two best characterized targets of mTOR signaling include S6K1 and 4E-BP1, key players that control protein synthesis. The phosphorylation state of these substrates reflects the activity of the mTOR-raptor branch of the pathway. Phosphorylation of 4E-BP1 inhibits the binding of 4E-BP1 with mRNA cap binding protein, eIF4E, thereby allowing the association of eIF4E with eIF4G to initiate translation (Brunn, 1997, Burnett, 1998). Upon activation, S6K1 phosphorylates ribosomal protein S6 (rpS6), a component of the 40S ribosomal subunit complex, and increases ribosomal biogenesis through the translation of a subclass of mRNAs containing a short oligopyrimidine sequence (Jefferies, 1997). However, this mechanism has been challenged (Tang, 2001). Among other reported substrates of mTOR relevant for translation, the translation elongation factor eEF2 has been studied in some detail. The eEF2 is inactivated upon phosphorylation by a specific kinase, termed eEF2 kinase. Extracellular stimuli induce the dephosphorylation of eEF2, which is inhibited by rapamycin (Browne, 2004). However role of eEF2 and eEF2 kinase in mTOR induced translation elongation is unclear.

1.2.2.1 mTOR pathways and skeletal muscle hypertrophy

Skeletal muscle hypertrophy is defined as an increase in muscle mass that comes as a result of an increase in protein synthesis and hence the size of the muscle fiber. Growth and maintenance of skeletal muscle mass is critical for long-term health and quality of life. This is evident by atrophic muscle observed in multiple clinical settings including cancer, AIDS and sepsis (Glass, 2003). Skeletal muscle is a highly adaptable tissue with a remarkable ability to sense different environmental cues like growth factor, nutrients, cytokines and mechanical stimuli. Amino acids increase skeletal muscle protein synthesis via direct activation of mTOR pathways, while growth factors increase protein synthesis via the PI3-K/Akt-mTOR pathway (Bolster, 2004). Unlike insulin, resistance exercise/skeletal muscle loading does not alter PI3-K activity, but increases mTOR activity via direct activation of Akt (Bodine, 2001). For example chronic overloading of the plantaris muscle leads to increased phosphorylation of Akt and proteins downstream of mTOR, such as 4E-BP1 and S6K1. The central role of mTOR in mediating a hypertrophic response under these loading conditions has been verified through *in vivo* treatment with rapamycin (Bodine, 2001).

Insulin, amino acids, and resistance exercise all enhance protein synthesis in skeletal muscle via mTOR signaling. Thus, investigating the interaction between multiple signaling pathways controlling protein synthesis is crucial to identify the role of mTOR in the regulation of skeletal muscle hypertrophy.

1.2.2.2 mTOR and insulin resistance

Insulin, like any other growth factor, is critical for the regulation of cell growth, survival, glucose uptake, and metabolism. Insulin exerts its effect on the cell via activation of the canonical PI3-K/Akt pathway. Full activation of the mTOR pathway requires signals from nutrients (e.g. amino acids) and growth factors (Hara, 1998) however nutrient overload often leads to obesity, insulin resistance, and type 2 diabetes (Um, 2006). In adipocytes and myocytes, an excess of amino acids has been shown to attenuate the insulin response on glucose uptake through an mTOR-dependent effect on IRS-1 (Tremblay, 2001, Takano, 2001). Hyperactivation of mTOR was associated with changes in phosphorylation, localization, and/or degradation of IRS-1 (Takano, 2001, Tremblay, 2001). A critical role for S6K1 in desensitizing the cell to insulin is suggested by studies whereby small interfering RNA knockdown (Harrington, 2004) and overexpression approaches have been taken in different experimental system (Shah, 2004). Moreover, S6K1-deficient mice are hypersensitive to insulin and are protected against diet-induced obesity (Um, 2004). These studies also provide evidence to suggest that S6K-1 mediates a negative feedback loop and impairs insulin signaling via changes in phosphorylation and/or degradation of IRS-1 (Fig 2).

Increased plasma amino acids in animals (Crozier, 2005, O'Connor, 2003)and humans (Rennie, 2002, Liu, 2002) have been shown to enhance translation initiation and protein synthesis and inhibit insulin-stimulated glucose transport in skeletal muscle (Traxinger, 1989, Pisters, 1991, Schwenk, 1987). Therefore, further investigation is required to determine whether circulating levels of amino acids and other nutrients affect insulin sensitivity in skeletal muscle through an mTOR-mediated regulation of IRS-1 function. Thus, mTOR is involved in multiple cellular processes and might function as a multichannel processor with different inputs eliciting different output. However the mechanism by which mTOR harmonizes signals from nutrients and growth factors is unclear.

1.3 AMP-ACTIVATED PROTEIN KINASE (AMPK): AN ENERGY SENSOR

AMP-activated protein kinase is an evolutionary conserved sensor of cellular energy status that contributes to the maintenance of energy balance within the cell. (Carling, 2004, Hardie, 2004). Recent work suggests that AMPK is involved in the fundamental regulation of energy balance at the whole body level by responding to hormonal and nutrient signals in the central nervous system and peripheral tissues that modulate food intake and energy expenditure. This pivotal role of AMPK places it in an important position for regulating whole-body energy metabolism (Hardie, 2004, Carling, 2004).

1.3.1 Structure and activation of AMPK

AMPK exists as a heterotrimeric complex comprising catalytic α, and regulatory β and γ subunits (Figure 3). Each α and β subunits are encoded by distinctive

genes (α1, α2 and β1, β2), whereas the γ subunit is encoded by three genes (γ1, γ2 and γ3), giving the possibility to form 12 different heterotrimers. AMPK is activated by an increase in the ratio of AMP:ATP within the cell, and therefore it functions as an efficient sensor of the cellular energy charge. AMP binds to the γ subunit and promotes allosteric activation of AMPK by stimulating phosphorylation of the kinase domain at a critical threonine residue (Thr-172), by the upstream kinase, the tumor suppressor LKB1 (Carling, 2004, Kemp, 2003, Hardie, 2004). AMP binding also inhibits dephosphorylation of Thr^{172} by a protein phosphatase (Davies, 1995). AMPK essentially monitors changes in the cellular AMP:ATP ratio because all effects of AMP on the AMPK system (allosteric activation, phosphorylation, and inhibition of dephosphorylation) are antagonized by high concentration of ATP. AMPK is also activated by calmodulin-dependent protein kinase kinase (CaMKK) via Ca^{2+} - dependent pathway (Hawley, 2005, Hurley, 2005). Activation of AMPK by CaMKK appears to be independent of changes in AMP:ATP ratio.

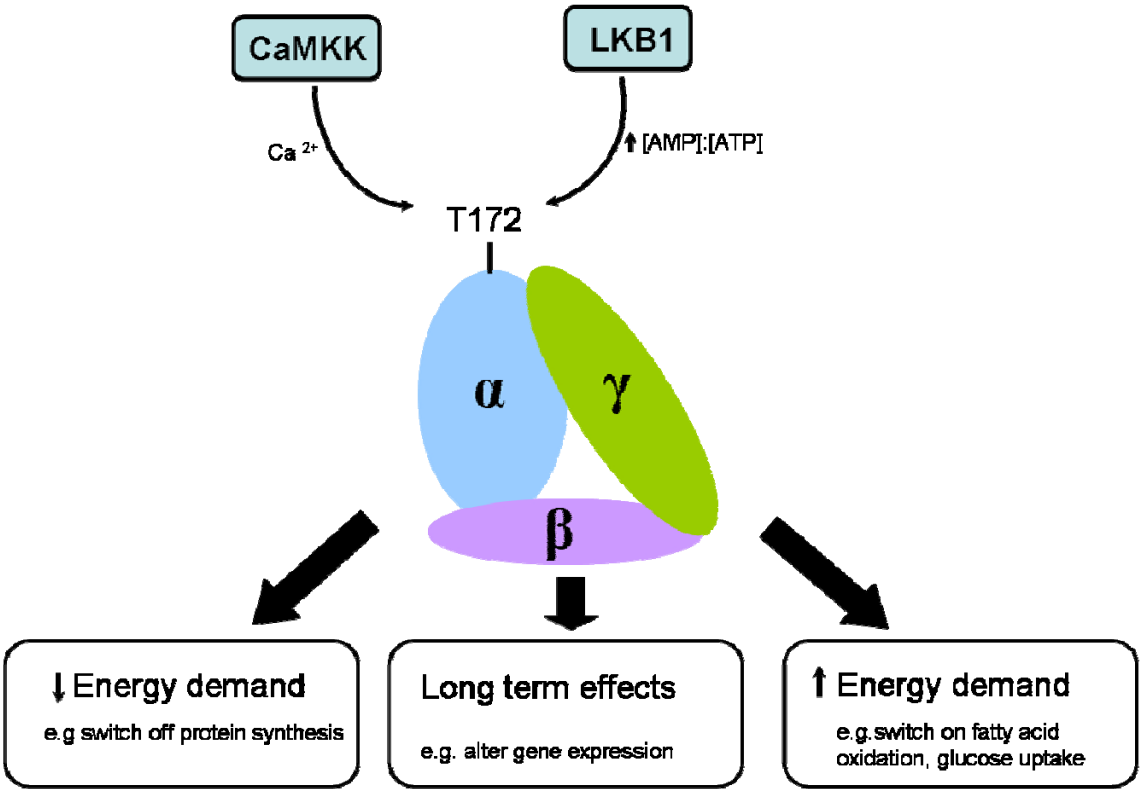

Figure 3. Schematic representation of the structure, activation and role of AMPK.

AMPK is a ubiquitously expressed serine/threonine protein kinase. Particularly, expression and activation of AMPK in liver, skeletal muscle, adipose tissues, pancreas, hypothalamus and heart is crucial for the regulation of whole body energy metabolism. AMPK is activated in all tissues during states of energy stress such as hypoxia, starvation or physical exercise, and it restores the energy depleted status by concomitantly inhibiting anabolic and stimulating catabolic pathways (Kahn, 2005, Long, 2006).

1.3.2 Roles of AMPK in skeletal muscle metabolism

Over the last few years, numerous workers have investigated the potential role of AMPK signaling in the regulation of multiple metabolic, protein synthetic, and transcriptional process in skeletal muscle.

1.3.2.1 AMPK and glucose uptake

In skeletal muscle, glucose transport can be activated by distinct pathways mediated by insulin and muscle contraction/exercise (Holloszy, 1996). AMPK regulates GLUT4 (glucose transporter) dependent glucose transport in response to diverse forms of cellular stress including contraction, hypoxia (Mu, 2001), and agents that disrupts the intracellular ATP:AMP ratio (Koistinen, 2003).

Early evidence suggesting a role for AMPK in the regulation of glucose transport came from studies using non-specific pharmacological activators One example is AICAR, a compound that is taken up into skeletal muscle and metabolized by adenosine kinase to form 5-aminoimidazole-4-carboxamide-1-β-$_D$-ribofluranotide, the monophosphorylated derivative that mimics the effect of AMP on AMPK (Merrill, 1997). Treatment of skeletal muscle with AICAR *in vitro* increased glucose uptake and this effect was unaffected by inhibition of the insulin-dependent PI3-kinase pathway (Merrill, 1997, Hayashi, 1998), thereby mimicking the effect of muscle contraction to increase glucose transport. The use of transgenic and knockout mouse models improved our understanding of the role of AMPK in the regulation of glucose uptake. Expression of a dominant negative α2 AMPK construct in skeletal muscle suppresses α2 and α1 isoform specific AMPK activity and completely prevents AICAR induced glucose transport (Mu, 2001). Moreover, genetic knockout of either the catalytic α2 (but not the α1 AMPK isoform) or the regulatory γ3 AMPK subunit completely abolishes AICAR-induced glucose transport (Barnes, 2004, Jorgensen, 2004). These data provide evidence to suggest that α2 and γ3 containing AMPK heterotrimeric complexes are involved in AICAR-induced glucose transport. AICAR-induced glucose transport in skeletal muscle-specific LKB1 knockout mice is also impaired (Sakamoto, 2005), providing additional evidence that AMPK is a positive regulator of skeletal muscle glucose uptake.

1.3.2.2 AMPK and fatty acid oxidation

During exercise, lipid oxidation in skeletal muscle is increased to meet energy demands. AMPK plays a central role in mediating exercise-induced fatty acid oxidation in skeletal muscle (Winder, 1996). The rate of fatty acid oxidation in skeletal muscle is controlled by the level of carnitine palmitoyltransferase-1 (CPT-1) (Ruderman, 1999), an enzyme which inhibits malonyl-CoA. Malonyl CoA acts as an allosteric inhibitor of CPT-1(Ruderman, 1999). Exercise/muscle contraction-induced activation of AMPK phosphorylates and inhibits acetyl CoA carboxylase (ACC) (Winder, 1996), an enzyme that controls synthesis of malonyl-CoA. This in turns facilitates the entry of long-chain acyl-CoA into mitochondria for oxidation (Ruderman, 1999). Several lines of evidence suggest that an accumulation of intracellular lipid induces insulin resistance in skeletal muscle, probably because of interference between the accumulated lipids and insulin signaling (Bonen, 2006). In this regard, the ability of AMPK to oxidize lipids provides

evidence to suggest that AMPK-activating pharmaceuticals may be useful in prevention and treatment of type 2 diabetes.

1.3.2.3 AMPK and protein synthesis

Protein synthesis comprises a large share of cellular energy expenditure (Rolfe, 1997); therefore it is unsurprising that activation of AMPK decreases protein synthesis. Since mTOR integrates the environmental signals and drives skeletal muscle protein synthesis, AMPK and mTOR signals appear to be inversely linked. The first evidence linking AMPK with the repression of mTOR signaling indicated that subcutaneous injection of AICAR in rats, increased skeletal muscle AMPK activity, concomitant with dephosphorylation of 4E-BP1 and S6K1 (Bolster, 2004). Since both 4E-BP1 and S6K1 are phosphorylated by mTOR, activation of AMPK appears to promote inactivation of mTOR. Further evidence indicates that AMPK activation increases phosphorylation of eEF2 with a concomitant decrease in protein synthesis (Horman, 2002), indicating a clear role for AMPK in the inhibition of mTOR signaling. Activation of AMPK by AICAR overrides the amino acid-induced activation of mTOR, providing evidence that negative regulation of mTOR caused by activation of AMPK is dominant to the positive input from amino acids (Dubbelhuis, 2002, Krause, 2002).

AMPK activation has been reported to repress mTOR signaling and protein synthesis through at least two mechanisms; 1) AMPK phosphorylates and activates TSC2 (Thr^{1227} and Ser^{1345}), which in turn inhibits mTOR signaling (Inoki, 2003). 2) AMPK directly phosphorylates mTOR on Thr^{2446} (Cheng, 2004). Whether phosphorylation of mTOR on Thr^{2446} by AMPK alters its catalytic activity or its interaction with regulatory proteins such as raptor or Rheb is unclear. However, phosphorylation of mTOR on Thr^{2446} is inversely correlated with phosphorylation of Ser^{2448} (Akt phosphorylation site) (Cheng, 2004). Although the role of AMPK isoforms in lipid and glucose metabolism is well studied, their role in protein metabolism is less explored.

1.3.2.4 AMPK and gene expression

The importance of chronic exercise for people with type 2 diabetes has been clearly established. AMPK has been proposed as one of the key molecules mediating exercise-induced skeletal muscle adaptations. These adaptations might occur as a consequence of changes in the gene expression pattern in response to chronic activation of AMPK (Zierath, 2002, Holloszy, 2005). Exercise and chronic administration of AICAR (Holmes, 1999) increases GLUT4 and hexokinase II protein content (Jorgensen, 2007), which has an impact on glucose transport. Similar to exercise training (Holloszy, 1967), AICAR also has been shown to increase mitochondrial markers (Winder, 2000). AMPK has been speculated to increase mitochondrial biogenesis via increased nuclear respiratory factor 1 (NRF-1) activity and peroxisome proliferator-activated receptor co-activator 1 (PGC1) content (Bergeron, 2001), two critical transcriptional regulators for mitochondrial gene expression. Collectively, these data suggests that AMPK is a critical energy sensor involved in the expression of key genes controlling the metabolic adaptation in skeletal muscle.

1.4 REGULATION OF GLUCOSE TRANSPORT IN SKELETAL MUSCLE

Insulin resistance of skeletal muscle glucose transport is a primary defect in the development of glucose intolerance and type 2 diabetes (Henriksen, 1994, Vaag, 1992). Insulin and physical exercise are the most physiologically relevant stimulators of glucose transport in skeletal muscle (Figure 4) (Wallberg-Henriksson, 1988). GLUT4 is the major glucose transporter isoform expressed in skeletal muscle and translocation of GLUT4 from an intracellular location to plasma membrane is a major mechanism through which insulin and exercise increase glucose transport. In the insulin resistant state, stimulation of glucose transport in response to exercise and contraction is normal (King, 1993, Brozinick Jr, 1998, Brozinick, 1994). Moreover, a single bout of exercise leads to a subsequent increase in insulin-stimulated glucose transport (Cartee, 1990, Cartee, 1989) and this is attributed to increased surface GLUT4 content (Roy, 1996). Strong epidemiological evidence suggests that regular exercise prevents or delays the onset of type 2 diabetes (Knowler, 2002, Tuomilehto, 2001). Despite the physiological importance of exercise in regulating glucose transport in skeletal muscle, the molecular mechanisms that mediate this phenomenon are still incompletely understood.

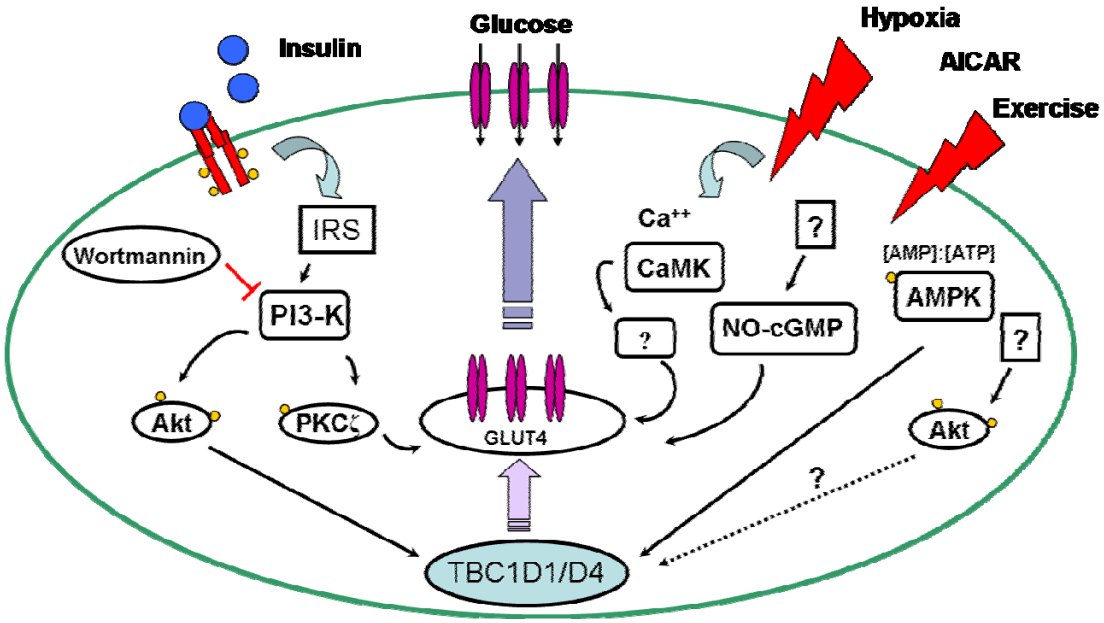

Figure 4. Overview of the insulin-dependent and insulin-independent regulation of glucose transport in skeletal muscle.

1.4.1 Insulin-dependent pathway

In skeletal muscle, insulin promotes glucose uptake by activating canonical PI 3-K/Akt pathway (Lee, 1995). Binding of insulin to the insulin receptor leads to downstream tyrosine phosphorylation of protein substrates that then engage and activate PI3-K. This leads to downstream signaling through PKB/Akt (Lee, 1995) and protein kinase C-λ/ζ (PKC-λ/ζ) (Bandyopadhyay, 1997), which results in GLUT4 translocation from its intracellular pool to the plasma membrane and glucose transport into the cell. Inhibition of PI3-kinase by pharmacological blockade using wortmanin reveals PI3-kinase as an essential molecule for insulin-stimulated GLUT4 translocation (Lee, 1995). Recently, the Rab GTPase activating proteins (Rab GAP) TBC1D4

(AS160; Akt substrate of 160 kDa) and TBC1D1 have been proposed to be the most distal signaling protein controlling insulin-mediated GLUT4 translocation (Kane, 2002, Roach, 2007). These proteins contain a GAP domain for Rabs, which are small G proteins required for vesicle trafficking process (Sano, 2003).

Skeletal muscle insulin resistance in type 2 diabetic humans is not associated with decreased level of GLUT-4 protein (Eriksson, 1992, Henriksen, 1994), however insulin stimulation of insulin receptor, IRS-1 tyrosine phosphorylation and IRS-1 immunoprecipitable PI3-kinase activity is reduced in skeletal muscle from insulin resistant subjects compared with insulin-sensitive controls (Björnholm, 1997). Moreover, insulin-stimulated Akt kinase activity is reduced in skeletal muscle from insulin resistant type 2 diabetic subjects (Krook, 1998), indicating defects in specific signaling proteins and GLUT4 protein translocation.

1.4.2 Exercise-induced signaling pathways

During exercise/contraction, skeletal muscle experiences major changes in cellular energy status mainly because ATP turnover is greatly increased. The signaling mechanism by which exercise or muscle contraction increases glucose uptake involves a highly regulated process whereby GLUT4 undergoes sorting and translocation from an intracellular storage site to the plasma membrane and t-tubules (Bryant, 2002, Dugani, 2005). This process may involve several kinases that sense changes in the intracellular environment during contraction (i.e., higher Ca^{2+}, AMP concentrations) and transduce signals to other unidentified proteins involved in GLUT4 translocation. Wortmannin, a PI3-kinase inhibitor does not inhibit contraction-induced glucose transport, indicating a role for different signals leading to glucose transport by insulin and exercise in skeletal muscle (Lee, 1995). Exercise/muscle contraction induced glucose transport has been attributed to several signaling nodes including AMPK (Mu, 2001), CaMKII (Wright, 2005, Wright, 2004), Akt (Sakamoto, 2002, Sakamoto, 2004), and NO-cGMP (Bradley, 1999); however substrates linking these events are elusive.

1.4.2.1 AMPK

Skeletal muscle contraction alters fuel status and depending on the intensity of the contraction, phosphocreatine and ATP levels can be significantly decreased. Therefore it is unsurprising that AMPK is activated during muscle contraction/exercise (Musi, 2001). Nevertheless, the role of AMPK in contraction-induced glucose transport is controversial. Contraction-induced glucose transport is partly reduced in mouse muscle overexpressing a dominant negative form of AMPK (kinase dead) (Mu, 2001). This finding suggests that contraction-induced glucose transport is partly dependent on AMPK. However, knockout of either catalytic α2 or the regulatory γ3 AMPK subunit does not inhibit contraction-stimulated glucose transport (Jorgensen, 2004, Barnes, 2004). Moreover, in LKB1 knockout mice, AMPK activation during contraction is virtually abolished, but glucose uptake is markedly inhibited (Sakamoto, 2005). Thus the role of AMPK in contraction-induced glucose transport is uncertain.

1.4.2.2 Calmodulin dependent protein kinase (CaMK)

Skeletal muscle contraction triggers the release of Ca^{2+} from sarcoplasmic reticulum. This facilitates an interaction of actin and myosin filaments and the

development of tension within the fibers (Holloszy, 1996). An increased concentration of myocellular Ca^{2+} has been proposed to be involved in mediating contraction-induced glucose transport (Holloszy, 1996, Holloszy, 1986). Early evidence that intracellular Ca^{2+} helps to enhance glucose transport in skeletal muscle comes from studies where a caffeine-exposure of isolated skeletal muscle induced an elevation in the myoplasmic Ca^{2+} concentration increased glucose transport (Holloszy, 1967). Conversely, dantrolene, which prevents the release of Ca^{2+} from the sarcoplasmic reticulum, attenuated hypoxia-mediated glucose transport (Cartee, 1991), providing evidence for an important role of Ca^{2+} in mediating energy status signaling. Studies based on inhibition of CaMK with KN92 and KN93 suggested that activation of CaMKII during muscle contraction play an essential role in the stimulation of glucose transport (Wright, 2005, Wright, 2004). In similar studies, this signaling pathway was proposed to be distinct from the AMPK pathway, however, recent data suggest that AMPK may in fact by directly activated by Ca^{2+}–signaling via CaMKK. Thus the cross-talk between CaMK and AMPK signaling has yet to be defined.

1.4.2.3 Akt (Protein kinase B)

Akt is activated by numerous growth factors and cellular stress (Vanhaesebroeck, 2000). Several lines of evidence suggest that Akt is critical in insulin-stimulated glucose uptake (Hajduch, 2001) however the role of Akt activation in contraction-stimulated glucose uptake is unknown. In humans, cycling exercise increases Akt activity in an intensity-dependent manner (Sakamoto, 2004). Akt is also phosphorylated in rat skeletal muscle after electrical stimulation (Sakamoto, 2002, Sakamoto, 2005), *in situ* contraction (Nader, 2001, Sakamoto, 2002) and treadmill running (Sakamoto, 2003). Contrary, several investigations provide evidence against contraction-induced activation or phosphorylation of Akt in human (Widegren, 1998), or rodent (Wojtaszewski, 1999, Markuns, 1999, Nader, 2001) skeletal muscle in response to endurance exercise. Likewise, with resistance exercise (Krisan, 2004), or *in vitro* or *in situ* contraction (Lund, 1998, Sherwood, 1999, Brozinick Jr, 1998) Akt is not phosphorylated or activated. The PI3-kinase inhibitors wortmannin and LY294002 completely inhibit contraction-stimulated Akt phosphorylation and activity (Sakamoto, 2002), but do not decrease contraction-stimulated glucose transport (Lee, 1995). These conflicting results may be due in part to differences arising from the various models and intensity of exercise employed, fiber type differences between the species and tissue sampling time points. Using a phospho-specific Akt substrate antibody (PAS), several Akt substrates (including TBC1D1/D4) have been detected after insulin stimulation (Kane, 2002, Roach, 2007). Whether similar proteins are phophorylated in response to exercise is unknown. Moreover, the identity of many of these putative substrates has yet to be confirmed.

1.4.2.4 NO-cGMP pathways

Nitric oxide (NO) is implicated in a wide array of signaling networks (McConell, 2006). NO is produced by different isoforms of NO synthase (NOS) (Moncada, 1993), of which neuronal-type (nNOS) and endothelial-type (eNOS) isoforms are expressed in skeletal muscle (McConell, 2006). In rodent and human, exercise increases skeletal muscle NO production, concomitant with increased glucose uptake (Balon, 1994, Roberts, 1997, Bradley, 1999). Moreover, exogenously administered NO, which is

generated from an NO donor, stimulates glucose transport in isolated rodent skeletal muscle (Balon, 1997, Young, 1997). Acute administration of the NOS inhibitor NG-monomethyl-L-arginine (L-NMMA) during exercise in humans reduces leg muscle glucose uptake (Bradley, 1999), providing additional evidence that NO signaling controls exercise-induced glucose uptake. The intracellular mechanism by which NO increases skeletal muscle glucose uptake is incompletely understood. Studies in rodent skeletal muscle have shown that NO exerts its action on glucose transport via activation of guanylate cyclase, leading to elevation of cGMP levels (Young, 1997, Ohlstein, 1982). Indeed, the cGMP analogue 8-bromo-cGMP also increases glucose uptake in isolated rodent skeletal muscle (Young, 1997). Crosstalk between NO-cGMP and the AMPK pathway has also been reported (Higaki, 2001, Lira, 2007, Chen, 1999, Chen, 2000), but whether it has any implication for contraction-induced glucose transport has yet to be determined. Thus, NO-cGMP may be part of a novel pathway that regulates skeletal muscle glucose uptake.

2 AIMS

Skeletal muscle is principal site for insulin-stimulated glucose uptake. In patients with type 2 diabetes, insulin-mediated glucose utilization in skeletal muscle is markedly impaired. Nutrient overload often leads to the development insulin resistance in obesity and type 2 diabetes. Like every living cell, skeletal muscle has several sensory systems that detects nutrient and energy signals and adjust the flux through metabolic pathways accordingly. Perturbations in the nutrient and energy sensing pathways may lead to the development of metabolic disorders. A vast number of environmental impositions (nutrient overload, contractile activity) can be overcome by means of an adaptive response from skeletal muscle. Therefore, the overall aim of this thesis was to study key nutrient and energy sensing pathways that regulate skeletal muscle metabolism.

Specific questions that were raised are-

- Do the AMPK α2 and γ3 subunits play a role in regulation of skeletal muscle mTOR signaling?

- Does the branched-chain amino acid leucine impair the acute action of insulin on signal transduction in primary human myotubes via negative feedback mechanism involving S6K1?

- Does resistance and endurance exercise phosphorylate Akt and Akt substrates in human skeletal muscle?

- Is the AMPK γ3 subunit involved in hypoxia-induced glucose uptake in glycolytic skeletal muscle?

- Does the nitric oxide (NO) donor increase glucose uptake in isolated human skeletal muscle? What are the underlying mechanisms?

3 EXPERIMENTAL PROCEDURES

3.1 EXPERIMENTAL MODEL: MOUSE SKELETAL MUSCLE

Mouse extensor digitorum longus (EDL) and/or soleus muscles were used in *Paper I* and *IV*. The AMPKγ3 KO and their respective wild-type littermates (*Paper I and IV*) were bred in-house, while AMPKα2 KO (*Paper I*) and their respective wild-type littermates were bred at the Copenhagen Muscle Research Center. All animals used in these studies were maintained on a 12-h light-dark cycle and allowed free access to water and standard rodent chow. Animals were fasted for ~ 4hr before the experiments. The regional ethical committee in Stockholm, Sweden and/or the Danish Animal Experimental Inspectorate approved all experimental procedures.

3.1.1 Animal models

EDL and/or soleus muscles from C57/Bl6, AMPKγ3 KO and AMPKα2 KO were used in this thesis. C57/Bl6 mice were purchased from SCANBUR AB (Sweden). The generation of AMPKγ3 KO (*Prkag3*$^{-/-}$) mice has been described previously (Barnes, 2004). Traditional gene targeting techniques were used to generate *Prkag3*$^{-/-}$ mice, with a knockout targeting construct that caused a major frameshift and premature stop codon. The generation of AMPKα2 KO has been described previously (Viollet, 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 phosphogycerol kinase promoter-driven neomycin selection cassette flanked by an additional *loxP* site. Germline-transmitting chimeric mice were mated with C57/Bl6 mice. Breeding of the heterozygous offspring with transgenic mice expressing Cre in germ cells resulted in heterozygous AMPKα2 KO mice.

3.1.2 Skeletal muscle incubation

Mice were anesthetized via an intraperitoneal injection of 2.5% Avertin (2,2,2-tribromo ethanol 99% and Tertiary amyl alcohol) (0.02 ml/g of body weight)), and EDL, soleus muscles were removed for analysis. A basal incubation buffer was prepared from pre-gassed (95% O_2, 5% CO_2) Krebs-Henseleit buffer (KHB) supplemented with 5 mM glucose, 15 mM mannitiol, 5 mM HEPES and 0.1% bovine serum albumin (radioimmunoassay grade). The muscles were incubated at 30°C in a shaking water bath under a constant gas phase of 95% O_2 and 5% CO_2, unless stated otherwise. For the hypoxia experiments (*Paper IV*), a separate basal hypoxia buffer was prepared and continuously gassed with 95% N_2, 5% CO_2. Muscles were incubated at 30°C for 30 min in basal incubation buffer to recover from the surgery.

3.1.2.1 Insulin and AICAR stimulation

In *Paper I,* after recovery, muscles were transferred to new vials containing incubation buffer. AICAR, insulin or AICAR and insulin together, were added as indicated in Table 1. The total time for the muscle incubation (including recovery) period was 90 min, where the media was changed after every 30 min. Thereafter, the

muscles were trimmed of non-muscle tissues, immediately frozen in liquid nitrogen, and stored at -80°C for later signaling analysis.

Conditions	(Inc) 30 min	(Inc) 30 min
Basal	Recovery	Recovery
AICAR	AICAR	AICAR
Insulin	Recovery	Insulin
AICAR + Ins	AICAR	AICAR + Ins

Table 1: Incubation protocol to test the effect of insulin or AICAR on mTOR signaling

3.1.2.2 Hypoxia stimulation

In *Paper IV,* after recovery, EDL and/or soleus muscles were transferred to new vials containing either normoxic incubation buffer (normoxia) or hypoxic incubation buffer (hypoxia), and continuously gassed with 95% O_2 and 5% CO_2 or 95% N_2, 5% CO_2, respectively (Cartee, 1991). KN-93 and/or KN-92 (25 μM, Calbiochem) were added to the media. After a 45 min incubation, the 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.

3.1.3 Glucose transport

Hypoxia-induced glucose transport was determined using 2-deoxy-glucose (Hansen 1994). Following the incubation period, the muscles were transferred to new vials containing glucose-free KHB buffer supplemented with 18 mM mannitiol and 2 mM pyruvic acid. Thereafter, the muscles were transferred into vials containing KHB buffer with 1 mM 2-deoxy-[1,2,^{3}H] glucose (2.5 mCi/ml) and 19 mM [^{14}C]mannitol (0.7 mCi/ml). KN-93 or KN-92 was added at concentrations identical to pre-incubation conditions. Muscles treated with insulin for 30 min were used as an internal control. Transport of 2-deoxyglucose into the muscle was assessed for 20 min at 30°C. After incubation, the muscles were trimmed of non-muscle tissues, snap-frozen in liquid nitrogen, and stored at -80°C for later analysis. Glucose transport was determined by measuring the accumulation of intracellular 2-deoxy-[1,2,^{3}H] glucose. Data are expressed as nmol glucose per mg of protein per 20 min.

3.2 EXPERIMENTAL MODEL: PRIMARY HUMAN SKELETAL MUSCLE CULTURE

Primary human skeletal muscle cultured myotubes were used in *Paper II* and *Paper III.* Muscle biopsies were obtained with informed consent of the donors during scheduled abdominal surgery. The subjects enrolled in *Paper II* (4 male and 4 female) and *Paper III* (3 male) had no known metabolic disease. Mean age of the subjects for *Paper II* and *Paper III* was 55 ± 5 yrs, (BMI was 25.6 ± 1.5 kg/m^2) and 55 ± 4 yrs, (BMI was 25.0 ± 1.5 kg/m^2) respectively. Informed consent was received and the ethical committee at Karolinska Institutet and the regional ethical committee approved all protocols.

3.2.1 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 as described (Al-Khalili, 2003b). Cells were seeded in 6-well plates, petri dishes, or 25 cm^2 flasks (~1000 cells per cm^2). Upon reaching 70% confluence, myotube differentiation was initiated by changing to a differentiation media (DMEM supplemented with 4% FBS, 1% PeSt, 1% Fungizone). Two days later, serum levels were further decreased to 2% FBS for an additional three days, giving a total differentiation time of five days. The day before each experiment, cells were serum-starved overnight (more than 6 hr).

3.2.2 Giemsa/Wright staining

Giemsa/Wright staining was performed on a regular basis to asses the extent of differentiation of the human muscle cultured cells. Cells were washed once with PBS, fixed in methanol for 10 min and incubated in 1:10 Giemsa for 15 min and 1:10 Wright for 20 min. Cells were washed with double distilled H_2O and mono- or multinucleated cells were observed under phase contrast invert light microscope.

3.2.3 Leucine and insulin stimulation

In *Paper II*, time- and dose-response course experiments were performed to investigate the effect of leucine on glucose metabolism and intracellular signaling. Differentiated myotubes were incubated for 20 min or 2 h with 0.05, 0.5 or 5 mM leucine alone, or in combination with 60 nM insulin. Thereafter, a leucine concentration of 5 mM was chosen to study glucose metabolism in cells transfected with siRNA against S6K1. Insulin was added (for the insulin-stimulated condition) during the last 20 min for the protein phosphorylation analysis, during the last 60 min for the glucose uptake analysis or during the last 90 min for the glycogen synthesis analysis. For *Paper III*, myotube cultures were incubated for 30 min in presence or absence of 120 nM of insulin.

3.2.4 S6K1 siRNA transfection

siRNA transfection was performed as described previously (Al-Khalili 2003a) using Lipofectamine 2000 (Invitrogen) as the transfection agent. siRNA against S6K1 or a scramble sequence (Dharmacon, USA) (1 µg/ml) was mixed in serum/antibiotic-free DMEM (final volume 50 µl/ml) for 5 min and 1 µl of the transfection agent, lipofectamine 2000 (Invitrogen, Sweden) was mixed and incubated with 49 µl DMEM in a separate tube for 5 min. The two solutions were combined and mixed gently with agitation at room temperature for 30 min. Myocytes were freshly seeded 4-5 days before differentiation initiation. Differentiated myotubes (2 days) were washed with sterile PBS twice and thereafter, 1 ml of serum/antibiotic-free DMEM was added to each well and samples were incubated at 37°C. siRNA transfection complexes (100 µl) were added to each well and incubated for >16 h. Myotubes were washed with sterile PBS and 2 ml/well 2% FBS supplemented DMEM was added. Myotubes were used 4 days after transfection. Less than 5% cell death was observed in cultures exposed to siRNA/Lipofectamine 2000 as determined by the cell death ratio using reversed light microscopy. All data are presented from scramble and S6K1 transfected myotubes.

3.2.5 Glucose uptake

Myotube glucose uptake was determined by using radiolabeled 2-deoxy-D-glucose as previously described (Al-Khalili, 2003a). Overnight serum starved myotubes were stimulated with or without 5 mM leucine for 2 h in 5 mM glucose-DMEM and insulin (60 nM) was added during the last 60 min. Cells were washed in PBS and incubated with 10 μM 2-deoxy glucose and [^{3}H]2-deoxy glucose (2 μCi/ml) for 15 min. Cells were washed, harvested and subjected to scintillation counting. Each experiment was performed on duplicate wells.

3.2.6 Glycogen synthesis

Myotube glycogen synthesis was determined as the amount of [^{14}C] labelled glucose incorporated into glycogen, as described (Al-Khalili, 2003b). Overnight serum starved myotubes in 6-well dishes, were treated for 2 h with 0.5 or 5 mM leucine in the absence or presence of 60 nM insulin for 2 h. During the last 90 min, cells were incubated with 5 mM glucose-DMEM, supplemented with D-[U-^{14}C] glucose (final specific activity, 1 μCi/ml). Each experiment was performed on duplicate wells.

3.2.7 RNA purification and quantitative real-time RT-PCR

mRNA expression was measured by real-time polymerase chain reaction using TaqMan technology. Myoblasts were cultured in 100-mm dishes, and the differentiation was initiated at >80% confluence. Five days after differentiation, myotubes were FBS-starved for > 16 h and then incubated with leucine 5 mM for 2 h. At the end of the incubation, cells were washed three times with RNase-free phosphate-buffered saline and then harvested directly for RNA extraction (RNAeasy minikit, Qiagen, Crawley, UK). All RNA was DNase-treated before reverse transcription (RQ1 RNase-free DNase, Promega, Southampton, UK). The total RNA concentration was measured, and cDNA was generated using a reverse-transcription reaction with random hexamer primers (Invitrogen, Sweden). Real-time PCR (ABI-PRISMA 7000 Sequence Detector, Perkin-Elmer Life Sciences) was performed for quantification of specific mRNA content, and the data was collected and analyzed by ABI Prism 7000 SDS software version 1.1. mRNA content was normalized for β-actin or 18S mRNA. All TaqMan primer/probes were purchased from Applied Biosystems (Sweden).

3.3 EXPERIMENTAL MODEL: ISOLATED HUMAN SKELETAL MUSCLE

Isolated human skeletal muscle preparations were used in *Paper III* and *Paper V*. Muscle biopsies were obtained using either a Needle biopsy (*Paper III*) or Open biopsy (*Paper V*) procedure.

3.3.1 Subject characteristics

In *Paper III,* six male cyclists (age 28.7 ± 6.1 yr, body mass (BM) 74.7 ± 7.6 kg, peak oxygen uptake *[Vo_{2peak}]* 65.2 ± 6.4 ml·kg-1·min-1) with a prolonged history (8.5 ± 2.7 yr) of endurance training, volunteered to participate. At the time of the investigation, the subjects were riding 425 ± 292 km/wk (range 200-800 km) and did not participate in any form of resistance or strength training. The experimental procedures and possible risks associated with the study were explained to each subject who gave their written informed consent. All investigations were undertaken in the

morning after an overnight fast. The study was approved by the Human Research Ethics Committee of RMIT University and Ethical Committee of Karolinska Institutet.

In *Paper V*, seven healthy male volunteered to participate. The clinical characteristics of these subjects are presented in *Paper V* (*Paper V, table 1*)). Glucose, insulin, and HbA1C values were within the normal range, and serum aminotransferase levels were not elevated. None of the subjects used tobacco products or reported taking any medication. The subjects were asked to refrain from strenuous exercise for 48 h before the study and to report to the laboratory after an overnight fast. Informed consent was received from all subjects before participation. The study protocol was approved by the regional ethical committee at Karolinska Institutet.

3.3.2 Muscle biopsies and preparations

3.3.2.1 Needle biopsy

For the exercise study (*Paper III*), subjects reported to the laboratory in the morning, after 10-12 h overnight fast. After resting quietly in a supine position for 10 min, local anesthesia (2-3 ml of 1% Xylocaine (lignocaine)) was administered to the skin, subcutaneous tissue, and fascia of the *vastus lateralis* muscle, in preparation for the muscle biopsy procedure. A resting biopsy was taken using a 6-mm Bergström needle with suction applied (Evans, 1982). Approximately ~100 mg of muscle was removed and immediately frozen in liquid N_2. Immediately upon completion of an exercise testing session, a second biopsy was taken from the same leg (~5 cm distal from the resting sample) and frozen within 10-15 s of exercise cessation. Every attempt was made to extract tissue from approximately the same depth in the muscle. Samples were stored at –80°C until subsequent analysis. Muscle biopsies (40-50 mg) were freeze-dried overnight and subsequently dissected under a microscope to remove visible blood, fat and connective tissue. Muscles were homogenized in ice-cold homogenization buffer and used for further analysis.

3.3.2.2 Open biopsy

For *Paper V*, 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 plexiglass clips (9 mm in width), and incubated for 30 min in individual flask containing oxygenated KHB buffer to recover from the surgery procedure. All investigations were undertaken in the morning after an overnight fast.

3.3.3 Human exercise : design

The *Paper III* consisted of a crossover approach in which each subject performed two different exercise testing sessions, separated by a minimum of 7 days. One exercise session was undertaken in the habitual training discipline (i.e. cycling), while the other session comprised a bout of unfamiliar exercise (intense resistance-exercise, described subsequently). Muscle biopsies were taken at rest an immediately after the exercise session.

3.3.3.1 Peak oxygen uptake [Vo_{2peak}]

Predicted Vo_{2peak} was determined during an incremental maximal cycling test (Lode bicycle ergometer, Groningen, The Netherlands) to volitional fatigue, as

described previously (Hawley, 1992). The results from this maximal test were used to determine the power output that corresponded to ~70% of predicted Vo_{2peak} for each subject (to be employed in the subsequently described exercise testing sessions).

3.3.3.2 Maximal voluntary strength

Maximal concentric and eccentric strength for each subject was determined using seated leg extensions performed on a Kin-Com isokinetic dynamometer (Chattanooga, TN). Quadriceps strength was determined during a series of 3-repetition leg extension sets. Individual 1-RM was defined as the peak torque recorded during the concentric and eccentric contraction phases of the test protocol.

3.3.3.3 Diet and exercise control

Before each experimental trial (described subsequently), subjects refrained from vigorous physical activity for 24 h, and were provided with standardized pre-packed meals that consisted of 3 g CHO·kg-1 BM (body mass), 0.5 g protein·kg-1 BM, and 0.3 g fat·kg-1 BM, to be consumed as the final meal the evening prior to an experiment.

3.3.3.4 Exercise testing sessions: Resistance exercise

Maximal voluntary isokinetic leg extensions were performed on a Kin-Com dynamometer. Following a 2 × 5 repetition sub-maximal warm-up, subjects performed 8 sets of 5 repetitions at maximal effort. Each set was separated by a 3 min recovery period. Peak and mean torque were recorded for each leg extension set. Verbal encouragement and real time visual feedback was given to subjects during each repetition.

3.3.3.5 Exercise testing sessions: Cycling exercise

Subjects performed 60 min of continuous cycling at a power output that elicited ~70% of individual predicted $Vo_{2peak.}$

3.3.4 *In vitro* skeletal muscle incubations

In *Paper V*, after the open muscle biopsy procedure, smaller skeletal muscle strips were dissected from the biopsy specimen, mounted on Plexiglass clamps (9 mm in width), and incubated *in vitro* in pre-gassed (95% O_2 and 5% CO_2) KHB in a shaking water bath at 35°C for 30 min. The gas phase in the vials was maintained during the incubation procedure. Thereafter, skeletal muscle strips were incubated for 30 min at 35°C in KHB in the absence (basal) or presence of either 5 mM Spermine NONOate (N-(2-Aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine) (Calbiochem) or 120 nM insulin (Insulin Actrapid, Novo Nordisk). The concentrations of Spermine NONOate and insulin were maintained throughout all remaining incubation procedures.

3.3.5 Glucose transport

To assess the rate of glucose transport (*Paper V*), skeletal muscle strips were transferred to fresh KHB containing 20 mM mannitol and incubated at 35°C for 10 min. Thereafter, muscles were incubated for 20 min in KHB containing 5 mM 3-O-methyl [^{3}H]glucose (800 μCi/mM) and 15 mM [^{14}C]mannitol (53 μCi/mM). Thus, muscle strips were exposed to either Spermine NONOate or insulin for a total of 60 min. At the

end of the incubation protocol, skeletal muscle specimens were 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. Muscle lysates were stored at -80°C for subsequent signal transduction analysis.

3.3.6 cGMP measurement

Skeletal muscle strips were incubated in the absence or presence of Spermine NONOate for 60 min and then rapidly clamp frozen in liquid N_2 cooled tongs. Frozen muscles were homogenized on ice in 1 ml 10% trichloroacetic acid (TCA) using a polytron. Homogenates were subjected to centrifugation for 10 min at 14,000 g. Thereafter, the TCA was extracted from the supernatant with dH_2O saturated diethyl ether. Samples were then immediately frozen in liquid N_2 and concentrated in a speed vacuum. The concentrated samples were resuspended in assay buffer, acetylated, and then cGMP levels were measured by Amersham cGMP BioTRAK immunoassay according to manufacturer's instructions (Amersham RPN 226). cGMP levels were expressed as pmol/g wet weight.

3.4 GENERAL METHODOLOGY

3.4.1 Muscle homogenization

In *Paper I, III, IV* and *V*, muscles were homogenized in 0.3 ml of ice-cold lysis buffer (20 mM Tris (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 10 mM NaF, 1 mM MgCl, 1 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, 10% glycerol, 1% Triton X 100, 1 µg/ml aprotinin, 1 µg/ml leupeptin and µM microcystin) for 20 s using a motor-driven pestle. Homogenates were solubilized by end-over-end mixing at 4°C for 60 min and subjected to centrifugation for 10 min at 12000 g and 4°C. Protein concentration was determined using a commercially available kit (Pierce, Rockford, IL). The supernatant was frozen in liquid nitrogen and stored at -80°C for later immunoprecipitation, immunoblott analysis, glucose transport measurement or AMPK kinase activity assay.

3.4.2 Myotube protein extraction

In *Paper II, III* and *V*, in order to assess protein expression and phosphorylation, myotubes were grown in 100 mm dishes. After appropriate stimuli, myotubes were scraped into 400 µl ice-cold homogenization buffer. Homogenates were solubilized by end-over-end mixing at 4°C for 60 min and subjected to centrifugation for 10 min at 12000 g and 4°C. Protein concentration was determined using a commercially available kit (Pierce, Rockford, IL). The supernatant was frozen in liquid nitrogen and stored at -80 °C for later immunoprecipitation and immunoblot analysis.

3.4.3 Western blot analysis

Proteins (20 - 50 µg) solubilized in Laemmli sample buffer were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Phosphorylation and expression of various proteins were determined by using following antibodies against phospho AMPK Thr^{172}, phospho CaMK Thr^{286}, phospho Akt Ser^{473}, phospho Akt Thr^{308}, GSK3α/β Ser$^{21/9}$; phospho (Ser/Thr) Akt substrate (PAS), phospho-filamin A Ser^{2152}, phospho p70S6K Thr^{389}, phospho rpS6 Ser$^{235/236}$, phospho rpS6 $^{ser240/244}$, phospho 4E-BP1 Thr$^{37/46}$, phospho IRS-1 Ser^{1101} (Cell Signaling Technology), phospho ACCβ Ser^{227}, ACC, AMPK and TBC1D4 , phospho IRS-1 Ser^{307}, phospho IRS-1 Ser^{318}, and phospho IRS-1 Ser$^{636/639}$ (Upstate Biotechnology),

and GLUT4 (Chemicon), filamin A (Santa Cruz Biotechnology), filamin C or phopsho-filmain C (Ser^{2213}) (generous gift from Sir Philip Cohen, University of Dundee, Scotland). In *Paper III*, total AS160 protein expression was determined using an antibody kindly provided by Prof. Gustav E. Lienhard (Dartmouth Medical School, Hanover, NH). Anti-α1-AMPK and anti α2-AMPK antibodies were kindly provided by Prof. D. G. Hardie (University of Dundee, UK). Antibodies against pan-actin or GAPDH (Cell Signaling Technology) were used to confirm equal loading.

Membranes were incubated in 5-7.5% fat-free milk in Tris-buffered saline containing 0.02% Tween 20 (TBST) and probed with the indicated antibodies. Thereafter, membranes were washed in TBST (6 × 10 min), incubated in appropriate secondary antibodies, and washed again in TBST. Immuno-reactive proteins were visualized by enhanced chemiluminescence (ECL or ECL plus; Amersham, Arlington Heights, IL) and quantified by densitometry using Molecular Analyst Software (Bio-Rad).

3.4.3.1 Gradient SDS-PAGE

To achieve a broad molecular weight range of protein separation, gradient SDS-PAGE gels were used as specifically stated. Light (e.g. 5%) and heavy (e.g. 15%) acrylamide solutions were mixed in a chamber containing a stir-bar. Thereafter, a mixed acrylamide solution was pumped into the gel unit using a peristaltic pump. The peristaltic pump was used to smoothly control the flow rate. Protein bands, particularly in the low-molecular-weight range, are much sharper using this technique. Unlike the single-concentration gels, the gradient gels separate proteins in a way that can be easily represented to give a linear plot of proteins in the molecular weight range from 10 to 300 kDa (*Paper III*). This also facilitates molecular weight estimation.

3.4.3.2 Membrane stripping and reblotting

When specified, the membrane were stripped in buffer (62.5 mM Tris-Hcl pH 6.8, 100 mM β-mercaptoethanol and 2% (w/v) SDS) for 40 min at either 40°C (phospho-protein reblotting) or at 60°C (total protein reblotting) to remove the primary and secondary antibodies. The same membranes were blocked in TBST containing 5-7.5% milk for 2 h and incubated overnight with the fresh antibody of interest.

3.4.4 Immunoprecipitation

AS160 (TBC1D4) was immunoprecipitated from human (*Paper III*) and mouse (*Paper IV)* skeletal muscle lysate, while filamin was immunoprecipitated from human skeletal muscle lystae in *Paper III*. Homogenized muscle lysate (300-400 µg of protein) was subjected to immunoprecipitation with 3.5 µg of anti-AS160 antibody/filamin A at 4°C with gentle rotation overnight. The samples were incubated with an equal mixture of protein A sepharose (Amersham, Uppsala, Sweden) and protein G agarose (Sigma-Aldrich, St. Louis, MO) for 3 h at 4°C and subsequently washed three times with homogenization buffer and four times with phosphates buffered saline. The immunocomplex was suspended in Laemmli buffer containing β-mercaptoethanol. All the samples were heated at 95°C for 5 min and subjected to SDS-PAGE.

3.4.5 AMPK kinase assay

Isoform-specific, α1- and α2-associated AMPK activity was determined in *Paper IV* and *V*. Muscle homogenate (200-400 μg protein) were incubated overnight 4°C with antibodies against α1- and α2-AMPK subunits (provided by Prof. D. G. Hardie, University of Dundee, UK). Human muscle lysates from rested and exercised conditions were also incubated overnight at 4°C with antibodies against α1- and α2-AMPK and used as positive control for the assay. The immunoprecipitates were washed once in lysis buffer, once in 480 mM HEPES (pH 7.0) and 240 mM NaCl, and twice in 240 mM HEPES (pH 7.0) and 120 mM NaCl, leaving 10 μl of buffer with the Sepharose after the last wash. The immunoprecipitates were incubated for 30 min at 30°C in a total volume of 30 μl containing 833 μM DTT, 200 μM AMP, 100 μM AMARA-peptide (Upstate), 5 mM $MgCl_2$, 200 μM ATP and 2 μCi of [γ-^{32}P]-ATP to determine isoform-specific AMPK activity. The reaction was stopped by spotting 25 μl reaction mixtures onto a piece of P81 filter paper, which was then washed four times for 15 min in 1% phosphoric acid. The dried filter paper was analyzed for activity using liquid scintillation counting. AMPK activity was expressed as pmol/min/mg.

3.4.6 Statistical analysis

Data are expressed as mean ± SEM. Statistical evaluation between the groups was performed by a one-way ANOVA, two-way ANOVA, or student's t-test, as appropriate and as further specified in each study. The one-way ANOVA was followed by Fishers least significant test for *post hoc* determination. The two-way ANOVA was performed with or without repeated measures, as indicated, followed by Tukey's *post hoc* analysis to identify significant differences between groups, when appropriate. Equality of variance was ensured by Leven's test. In the case of a statistical difference, the data was log-transformed. Log transformation was sufficient to obtain equal variance in all cases. $p<0.05$ was considered significant.

4 RESULTS AND DISCUSSIONS

4.1 AMPK HETEROTRIMERS AND SKELETAL MUSCLE METABOLISM

AMPK is a heterotrimeric complex consisting of three subunits with seven isoforms (2α, 2β, 3γ), giving possibility to form 12 different heterotrimers (Hardie, 2004, Carling, 2004). The existence of 12 possible AMPK heterotrimers complicates the understanding of the physiological role of the AMPK system. Multiple mammalian isoform of three subunits exist and all subunits are differentially expressed in rodent and human skeletal muscle (Birk, 2006, Treebak, 2009a). Although all subunits of AMPK are expressed in human skeletal muscle tissue, only three AMPK heterotrimers (α1β2γ1, α2β2γ1, α2β2γ3) seem to exist in human *vastus lateralis* muscle (Birk, 2006). Mouse EDL (glycolytic) and soleus (oxidative) muscle express five AMPK heterotrimers (α2β2γ1, α2β2γ3, α1β2γ1, α2β1γ1, α1β1γ1) and the expression pattern is clearly fiber-type dependent (Treebak, 2009a). It is tempting to propose that each of these heterotrimers acts as an individual protein that is differentially activated in response to a specific stimulus (exercise, hypoxia, stress).

In humans, high intensity exercise increases the activity of the α2β2γ3 AMPK heterotrimeric complex, while the activity of the other two complexes is either unchanged or decreased (Birk, 2006), indicating that α2β2γ3 is the major complex activated under these conditions. Moreover, the activity of the α2β2γ3 AMPK complex was correlated with ACCβ Ser^{221} indicating that this complex mediates the regulation of ACCβ activity (Birk, 2006). Long-term (90 min) low intensity exercise increases the activity of the α2β2γ1 AMPK complex with a much slower time course than that of the α2β2γ3 AMPK complex (Treebak, 2007). In same study, phosphorylation of AS160 (TBC1D4) was correlated with the activation pattern of the α2β2γ1 AMPK complex, but not the α2β2γ3 AMPK complex. This indicates that particular heterotrimers and possible downstream targets of AMPK respond differently under different stimuli. In rodents, as in humans, the γ3 isoform is also predominantly associated with the α2 catalytic subunit (Treebak, 2009a, Mahlapuu, 2004, Yu, 2004). The predominant AMPK heterotrimeric complex expressed in glycolytic EDL skeletal (EDL) muscle contains the α2/β2/γ3 subunits (Mahlapuu, 2004, Treebak, 2009a). Functional α2 and γ3 AMPK subunits are required for AICAR-induced glucose transport in glycolytic skeletal muscle (Barnes, 2004, Jorgensen, 2004), indicating a subunit-specific physiological role. Moreover, the role of the AMPK γ3 subunit in the regulation gene expression and mitochondrial biogenesis has been addressed in AMPKγ3 knockout (*Prkag3*$^{-/-}$*)* mice, as well as in mice expressing an activating mutant form of AMPKγ3 (R225Q) (*Tg-Prkag3*^{225Q}) (Garcia-Roves, 2008). Collectively, these studies provide evidence to suggest that the diverse physiological responses of AMPK activation is heterotrimer/subunit dependent. Accordingly, isoform specific effects of AMPK were investigated in Paper I (mTOR signaling), Paper IV and Paper VI (glucose metabolism). The role of the AMPK subunits are well studied in respect to glucose and lipid metabolism, while the role of these subunits in the regulation of mTOR signaling and protein synthesis is less explored. More specifically, Paper I was designed to investigate the dependency of the AMPK α2 and γ3 subunits in the regulation of mTOR signaling.

4.2 INTERACTION BETWEEN AMPK AND mTOR SIGNALING

Skeletal muscle mTOR signaling is upregulated in response to resistance exercise, growth factor stimulation, and high-protein diet, all of which promote adaptive changes in skeletal muscle mass that correlate with increased mTOR activity (Glass, 2003, Glass, 2005). AMPK is a negative regulator of mTOR signaling (Kimball, 2006). This is obvious because protein synthesis is an energy consuming process and AMPK repress this process to maintain cellular energy homeostasis. In several model systems, the AICAR-induced activation of AMPK is associated with reduction in protein synthesis and a repression of mTOR signaling (Bolster, 2002, Horman, 2002). While the major support for repressive effects of AMPK on mTOR signaling comes from experimental approaches where AICAR has been used as a pharmacological activator of AMPK, the possibility that AICAR may act independently of AMPK activation through intracellular P(i) depletion and ZMP accumulation cannot be excluded (Guigas, 2007). In adults, skeletal muscle hypertrophy is characterized by an increase in the size of the individual myofibers, which develops in response to an enhanced rate of protein synthesis (Glass, 2005, Glass, 2003). Since AMPK is a negative regulator of mTOR signaling, it may play a role in the regulation of skeletal muscle mass (hypertrophy).

4.2.1 AMPK and insulin signaling in α2 and γ3 AMPK KO mice

In Paper I, the role of AMPK in mTOR signaling was determined in EDL muscle from α2 AMPK KO and γ3 AMPK KO mice after *in vitro* treatment with insulin and/or the AMPK activator, AICAR. AICAR-induced activation of AMPK in EDL muscle from α2 AMPK KO and γ3 AMPK KO mice was markedly impaired compared to the wild-type littermates, indicating the α2 and γ3 subunits are critical for the activation of AMPK (*Paper I, figure 1-2*). The insulin-induced increase in Akt phosphorylation indicates that insulin signaling was not impaired (*Paper I, figure 1-2*). AICAR and insulin did not interfere with signaling through their respective substrates of Akt or AMPK, respectively. Collectively, this result provides evidence that AMPK signaling is impaired, while insulin signaling is unaltered in EDL muscle from α2 AMPK KO and γ3 AMPK KO mice.

4.2.2 AMPK: a negative regulator of mTOR signaling

To determine whether AMPK and mTOR signaling pathways intersect, skeletal muscle was exposed to a combination of insulin and the AMPK activator, AICAR and signal transduction at the level of mTOR, S6K1, rpS6 and 4E-BP1 was studied. In wild-type mice, AICAR inhibited insulin-mediated mTOR signaling (*Paper I, figure 1 and 3*), giving proof-of-principle that AMPK is a negative regulator of mTOR signaling. The insulin-induced activation of mTOR signaling was correlated with phosphorylation of Akt (*Paper 1, figure 1-3*), indicating the canonical insulin-Akt-mTOR pathways were involved. Similarly, the inhibitory effect of AICAR on insulin-mediated mTOR signaling was correlated with phosphorylation of AMPK (*Paper 1, figure 1-3*); clearly indicating that AMPK-mediated inhibitory signals are integrated at the level of mTOR signaling. In rats injected with AICAR, phosphorylation of mTOR, S6K1, and 4E-BP1 was decreased on insulin sensitive sites (Bolster, 2002). However in our experiments, AICAR alone was without effect on phosphorylation of these proteins

(*Paper I, figure 1 and 3*). Previous studies in rat hepatocytes and HEK293 cells provide evidence that AMPK activation inhibits protein synthesis via phosphorylation of eEF2 (eukaryotic elongation factors) (Horman, 2002). However we did not observed any changes in phosphorylation of eEF2K and its substrate eEF2 (eukaryotic elongation factors) in response to AICAR (Figure 5A-D). The varied results between these studies may be explained by the different experimental design employed.

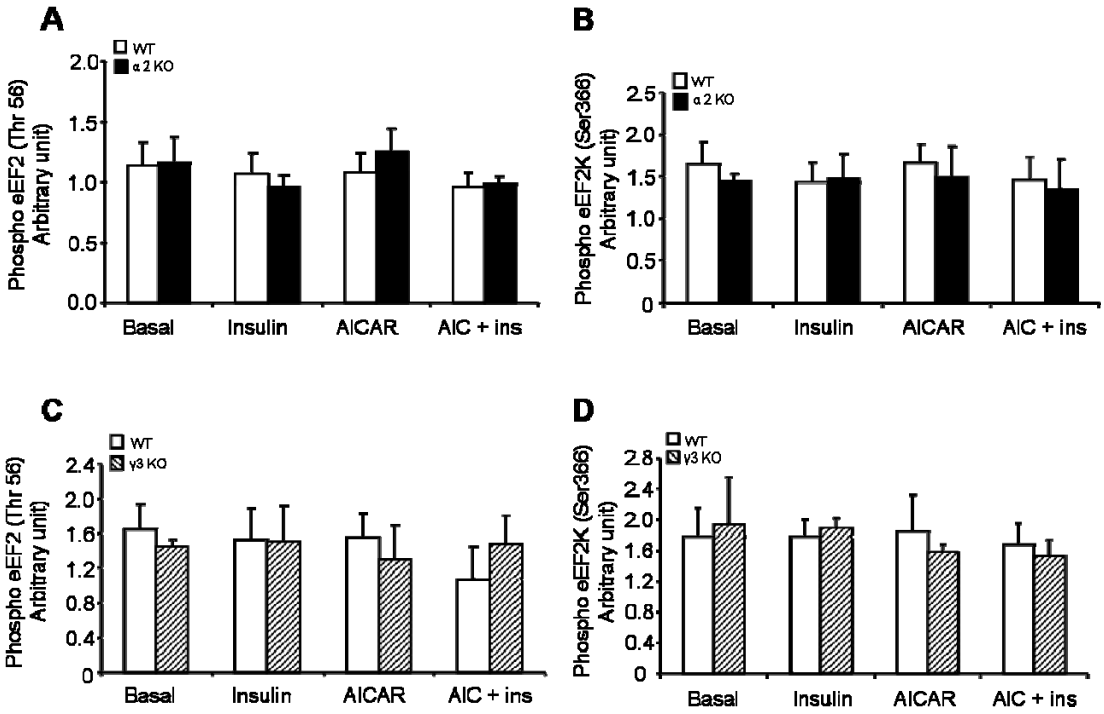

Figure 5. Effect of AICAR and insulin on phosphorylation of eEF2 and eEF2K. Phosphorylation of eEF2 Thr^{56} (A and C) and eEF2K Ser^{366} (B and D) in EDL muscle from α2 AMPK KO (closed bars), γ3 AMPK KO (hatched bars) and corresponding wild-type (open bars) littermates was determined. Results are mean ± SEM, n = 4 muscle for each genotype.

4.2.3 Role of AMPK subunits in the regulation of mTOR signaling

In α2 AMPK KO and γ3 AMPK KO mice, AICAR-mediated effects on glucose transport (Barnes, 2004, Jorgensen, 2004) and gene expression (Jorgensen, 2007, Vieira, 2008) are impaired, indicating the importance of these subunits in skeletal muscle metabolism. In Paper II, the activation of the Akt-S6K1 pathway is independent of AMPK α2 and γ3 subunits, since insulin signaling was intact in α2 AMPK KO and γ3 AMPK KO mice (*Paper I, figure 1-3*). AICAR repressed insulin-induced phosphorylation of mTOR, S6K1, rpS6 and 4E-BP1 in wild-type mice, while these effects were partially blocked in α2 AMPK KO and γ3 AMPK KO mice (*Paper I, figure 1 and 3*), indicating that the AICAR-mediated inhibition of mTOR signaling is dependent on α2 AMPK and γ3 AMPK subunits (Figure 6). Even though 4E-BP1 phosphorylation was not significantly increased (under AICAR + insulin treatment) in either knockout models, the phosphorylation pattern was strikingly similar to that observed for S6K1 and rpS6 phosphorylation.

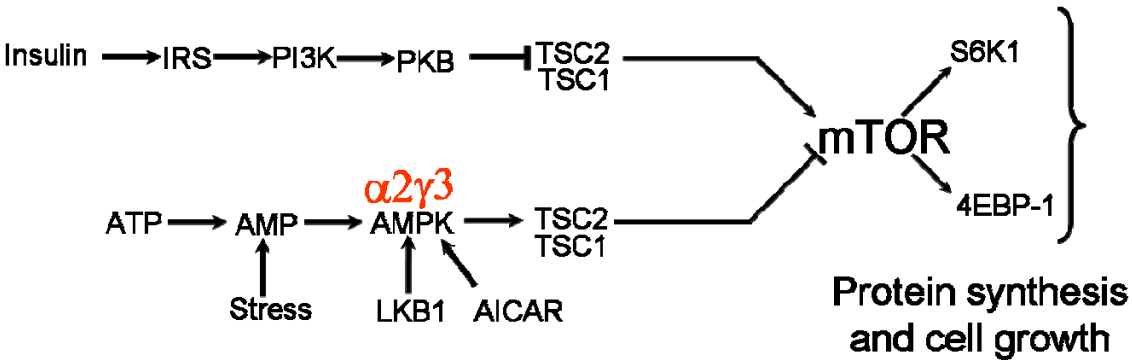

Figure 6. Schematic model representing the dependency of the α2 AMPK and γ3 AMPK subunits in the AICAR-mediated inhibition of mTOR signaling.

The inhibitory effect of AMPK on insulin-induced mTOR signaling has physiological relevance, since the γ3 isoform is mainly found in complexes containing α2 and β2 subunits in mouse glycolytic skeletal muscle (Mahlapuu, 2004, Treebak, 2009a). However, the inhibitory effects of AMPK on insulin-induced mTOR signaling are incompletely prevented in α2 AMPK KO and γ3 AMPK KO mice, suggesting a role for other AMPK subunits (*Paper I, figure 3*). We have previously reported that in γ3 AMPK KO mice, the expression of other AMPK subunits is unaltered (Mahlapuu, 2004). Conversely, in α2 AMPK KO mice, the expression of β1, β2 and γ3 subunits is decreased and the α1 expression is increased (Jorgensen, 2007). Expression of the α1 subunit in α2 AMPK KO mice does not restore total AMPK activity in α2 AMPK KO mice (Jorgensen, 2007), indicating a severe impairment in AMPK signaling. This may possibly explain why the inhibitory effects of AICAR on insulin-induced mTOR signaling are almost completely prevented in α2 AMPK KO mice compared to γ3 AMPK KO mice. Glycolytic EDL muscle also expresses α1 and γ1 subunits of AMPK (Mahlapuu, 2004, Treebak, 2009a), which might contribute to the AICAR-induced inhibition of mTOR signaling in the α2 AMPK KO and γ3 AMPK KO mice. Collectively these results indicate that functional α2 and γ3 subunits of AMPK are required for AICAR-induced inhibition of mTOR signaling. This also support the hypothesis that cross-talk at the level of AMPK and mTOR is critical for cellular energy homeostasis in health and disease. Future studies using either compounded AMPK KO mice, whereby α2 and γ3 subunits are deleted or β2-specific AMPK KO mice, may show a prominent rescue-effect of AICAR on insulin-induced mTOR signaling.

4.2.4 Role of AMPK in the regulation of skeletal muscle mass

The maintenance of skeletal muscle mass is a dynamic property, which can be changed with various types of exercise training. Resistance exercise and increased mechanical load can each independently increase skeletal muscle hypertrophy via Akt-induced activation of TSC2-mTOR pathway (Glass, 2003). Conversely, AMPK suppress mTOR signaling possibly via phosphorylation and activation of TSC2 (Thr^{1227} and Ser^{1345}) (Inoki, 2003) and/or phosphorylation and inactivation of mTOR on Thr^{2446} (Cheng, 2004). During endurance exercise, the AMP:ATP ratio progressively increases, which leads to the activation of AMPK (Holloszy, 2005), this in turns might inhibit mTOR signaling and reduce skeletal muscle mass. Indeed, an increased AMPK activity

was associated with decreased muscle hypertrophy (Thomson, 2005). Moreover, skeletal muscle-specific knockout of LKB1 is associated with a decrease in cell size (Sakamoto, 2005), highlighting a role of AMPK in the regulation of skeletal muscle hypertrophy. Understanding the mechanism regulating skeletal mass is necessary, since skeletal muscle plays a critical role in locomotion and metabolism. Even though the exact role of AMPK in the regulation of skeletal muscle mass is unclear, the temporal relationship between Akt and AMPK might be central to the regulation of mTOR signaling.

4.3 NUTRIENT OVERLOAD AND SKELETAL MUSCLE INSULIN RESISTANCE

States of nutrient oversupply and the dysregulation of insulin signaling are key features of insulin resistance in obesity and type 2 diabetes (Um, 2006, Patti, 1999). At the cellular and tissue levels, the availability of substrates for cellular energy production, such as amino acids and free fatty acids, may play an important role in modulating insulin signaling and metabolic regulation. Indeed, infusion of amino acids or fatty acids decreases insulin-stimulated glucose disposal *in vivo* (Pisters, 1991, Patti, 1999). Plasma concentrations of amino acids, particularly BCAAs are elevated in obesity and have been implicated in development of peripheral insulin resistance (Felig, 1975, Felig, 1969, Krebs, 2002). However, the mechanisms by which amino acids reduce skeletal muscle glucose uptake remains unclear. The experiments in Paper II were designed to investigate the effect of a supra-physiological concentration of leucine on insulin-mediated glucose uptake and metabolism in primary human skeletal muscle cells.

4.3.1 Leucine: a positive regulator of mTOR signaling

Amino acids can independently function as nutritional signaling molecules. Amino acids, especially BCAAs, play a pivotal role in hormonal secretion and action, intracellular signaling, gene transcription, and translation (Kimball, 2006, Anthony, 2001, Nair, 2005). BCAAs consist of leucine, isoleucine, and valine. Of these BCAAs, leucine appears to be the most important stimulator of intracellular signaling. Numerous studies have provided evidence that leucine has a unique ability to initiate signal transduction in skeletal muscle (Nair, 2005, Anthony, 2001).

Amino acids are known to induce the phosphorylation of S6K1 and 4-EBP1, two downstream modulators of mTOR (Hara, 1998, Wang, 1998). Indeed, cells deprived of amino acids show a rapid decline in the phosphorylation of S6K1 and 4E-BP1, which is rapidly reversed after re-addition of amino acids in rapamycin sensitive manner (Wang, 1998, Hara, 1998). To choose the appropriate dose of leucine for the *in vitro* studies, the effect of 0.05, 0.5, and 5 mM leucine alone or in combination with 60 nM insulin (20 min or 2 h) was determined on intracellular signaling (*Paper II*). Treatment of myotubes for 20 min or 2 h, with lower concentrations of leucine (0.05 and 0.5 mM) was without effect on phosphorylation of S6K1 and rpS6, while treatment of myotubes with 5 mM leucine (only after 20 min) increased phosphorylation of S6K1 and rpS6 (*Paper II, figure 1A-D*). Supra-physiological concentrations of leucine (5 mM) appear to transiently activate mTOR signaling because leucine-induced activation of S6K1 returns to basal after 2 h treatment. Leucine did not have an additive effect

(with insulin) on S6K1. Collectively, these results indicate that leucine activates mTOR signaling in time and dose-dependent manner.

4.3.2 Leucine-induced insulin resistance in skeletal muscle cells

The fact that both insulin and amino acids induce activation of mTOR signaling (Hara, 1998, Patti, 1999) raises the important question of whether 'cross-talk' between these pathways exist. Even though there are some reports that amino acids do not interfere with insulin-stimulated PI3-K and Akt activity (Hara, 1998, Shigemitsu, 1999), others found that amino acids decreased insulin-stimulated PI3-K activation (Patti, 1999, Tremblay, 2001). Leucine supplements have been shown to increase energy expenditure and to increase insulin sensitivity (Flakoll, 1992, Fried, 2007), but these effects may be dose dependent. In C_2C_{12} myotubes, exposure to 1 mM leucine was without effect on glucose utilization, whereas exposure to 5–10 mM leucine increased 2-deoxyglucose uptake and intracellular glycogen content (Doi, 2003). Moreover, in human skeletal myotubes, the addition of a balanced mixture of amino acids, including leucine (in the range of 400-800 μM), increased glycogen synthesis and S6K1 phosphorylation, and this effect was inhibited by blocking mTOR with rapamycin (Armstrong, 2001). Thus results regarding the effects of amino acids on insulin-induced glucose uptake, metabolism and signaling are conflicting.

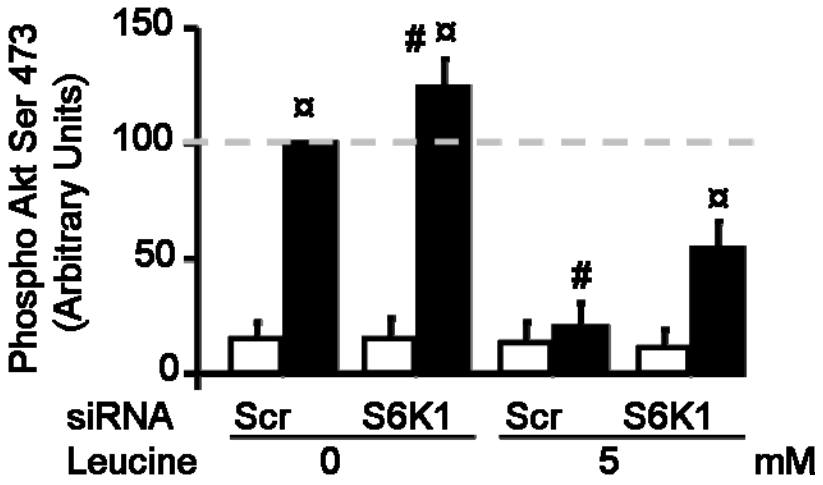

Figure 7: Effect of S6K1 silencing on phosphorylation of Akt Ser^{473}, after leucine stimulation. Human skeletal muscle myotubes were transfected with siRNA against S6K1 or a scrambled sequence. Cells were incubated for 2 h with 5 mM leucine; in the absence or presence of 60 nM insulin during the last 20 min. Results are expressed as percentage arbitrary unit over basal condition for myotubes transfected with a scramble sequence. Results are presented as mean ± SEM for n = 5-8 subjects. ¤ $p<0.05$ control/leucine vs insulin-stimulated, # $p<0.05$ leucine-insulin vs. control-insulin cells.

In primary human myotubes, a 2 h exposure to 0.5 mM leucine was without effect on basal or insulin-stimulated glycogen synthesis, while treatment with 5 mM leucine impaired basal and insulin-stimulated responses to glucose uptake and glycogen synthesis (*Paper II, figure 1E, figure 4A-B*). These results confirm previous studies, where leucine infusion in humans impaired insulin-stimulated whole body glucose uptake (Pisters, 1991, Schwenk, 1987). The differences in the effects of leucine on glucose metabolism between the present and earlier studies (Armstrong, 2001, Doi,

2003) are unclear, but might be due to experimental differences in the cell systems employed or the leucine concentration studied. Our analysis of intracellular signaling revealed that a 20 min treatment of primary human myotubes with leucine (5 mM) tended to reduce insulin-stimulated Akt (Ser^{473}) phosphorylation (40%, p = 0.13) (*Paper II, figure 3 C*), but the impairment in Akt phosphorylation was more clear after the 2 hr stimulation (Figure 7), indicating the cells developed insulin resistance. Since Akt has been implicated as an important target for mediating insulin action on glycogen synthesis, GLUT4 translocation, and glucose transport (Whiteman, 2002), leucine is likely to induce insulin resistance at the level of Akt. Thus, the leucine-induced impairment in basal and insulin-stimulated glucose uptake and glycogen synthesis was associated with a transient activation of S6K1 and impairment in insulin-stimulated Akt phosphorylation.

4.3.3 Role of S6K1 in metabolism

S6K1 is a member of the A, G, and C family of serine/threonine protein kinase. S6K1, an effecter of mTOR, is sensitive to hormones and nutrients, including insulin and amino acids (Um, 2006). However, full activation of the mTOR pathway requires signals from both nutrients and growth factors (Patti, 1999, Hara, 1998). Recently, amino acids have been proposed to induce insulin resistance via an S6K1-dependent effect on IRS-1 (Figure 8) (Patti, 1999, Tremblay, 2005, Baum, 2005). In adipocytes (Adochio, 2009) and L6 muscle cells (Tremblay, 2001), an excess of amino acids attenuates, whereas amino acids and starvation stimulates Akt activation and subsequent glucose uptake in response to insulin via S6K1 regulation. These effects occur in parallel with changes in the phosphorylation state, localization, and/or degradation of IRS-1. Studies in *Drosophila* provide evidence that the S6K1/2 ortholog, dS6K, is a negative regulator of dPKB (Radimerski, 2002). Since larval growth is extremely sensitive to nutrients (Oldham, 2000), the negative effects of amino acids on insulin signaling have been proposed to be mediated by S6K1. Studies in *S6K1*$^{-/-}$ mouse reveal that S6K1 also plays a role in a negative feedback loop to suppress insulin signaling (Um, 2004). *S6K1*$^{-/-}$ mice are protected against diet-induced obesity and remain insulin sensitive (Um, 2004). Moreover, phosphorylation of IRS-1 on Ser^{307} and Ser^{632} is reduced in either *S6K1*$^{-/-}$ mice fed a high fat-diet or in cultured cells whereby S6K1 has been silenced using siRNA-treatment to inhibit sites known to be elevated in animal models of obesity and in skeletal muscle from type 2 diabetic patients (Bouzakri, 2003). S6K1 activity is elevated in several animal models of diabetes and obesity, including genetic models such as ob/ob and K/K A(y) mice or dietary-induced insulin resistant mice (Um, 2004), indicating key role of S6K1 in metabolism.

4.3.4 Leucine-induced insulin resistance is mediated via S6K1

In Paper II, we used siRNA against S6K1 to test the hypothesis that leucine impairs insulin action on glucose uptake and metabolism due to a negative feedback regulation of S6K1. siRNA transfection against S6K1 reduced protein and mRNA expression in human myotubes (67% and 78%, respectively), compared to cells treated with a scramble siRNA control (*Paper II, figure 2, table 1*). siRNA transfection of S6K1 decreased basal and insulin-stimulated phosphorylation of S6K1 (*Paper II, figure 3A)*, indicating the transfection efficiency. Phosphorylation of the S6K1 substrate rpS6

on Ser$^{235/236}$ (*Paper II, figure 3B*) did not followed similar pattern as noted for S6K1 phosphorylation. This difference could be because the activity of rpS6 is regulated by phosphorylation on multiple sites (Ruvinsky, 2006).

Depletion of S6K1 increases basal glucose uptake and prevents the leucine-induced impairments in insulin action on glucose uptake and glucose incorporation into glycogen (*Paper II, figure 4A-B)*. The reductions in basal and insulin-stimulated glucose metabolism were not associated with changes in GLUT4 and GLUT1 expression (*Paper II, table 1*), probably because the leucine exposure was limited to 2 hours. We also provide evidence that siRNA against S6K1 enhances insulin-stimulated Akt phosphorylation and prevents against the leucine-induced impairments in insulin action (*Paper II, figure 3C*). The possible role of IRS-1 in mediating the leucine-S6K1 induced insulin resistance was also tested. Phosphorylation of IRS-1 at Ser^{318}, Ser^{302}, Ser^{1101,} Ser^{307} and Ser$^{636/639}$ was estimated. Our experiments provide no conclusive evidence to implicate a role for these sites in leucine action on IRS-1, possibly because we were studying the endogenous protein, rather than in cells, whereby IRS-1 was overexpressed.

Taken together, our results from Paper II provide direct evidence that the leucine-S6K1 axis participates in the negative regulation of insulin action on Akt phosphorylation and glucose metabolism. An illustrative model for the S6K1-mediated negative feedback loop promoting insulin resistance is shown (Figure 8).

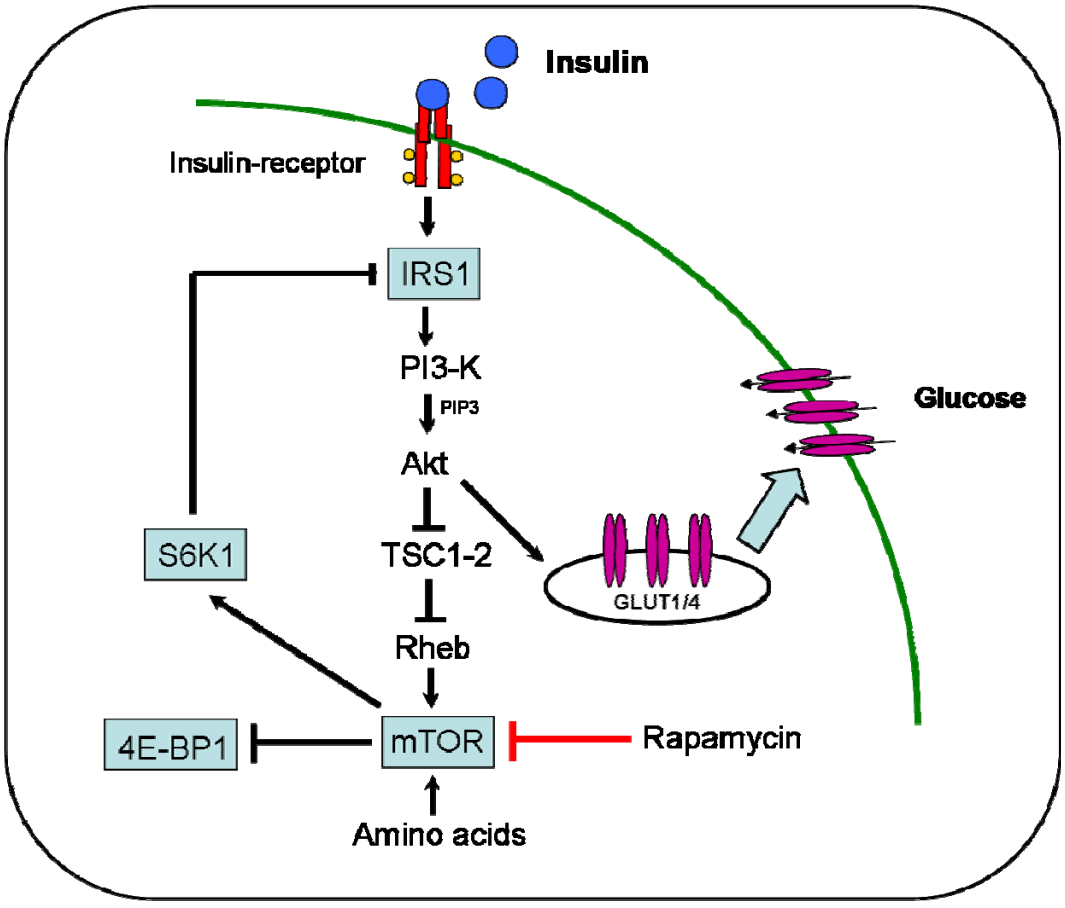

Figure 8. Proposed model for the regulation of a mammalian target of rapamycin/S6 protein kinase-1 mediated feedback loop that influences insulin sensitivity.

4.4 INSULIN-INDEPENDENT GLUCOSE TRANSPORT IN SKELETAL MUSCLE

The effects of insulin and muscle contraction/ cellular stress on glucose transport are independent (Lee, 1995, Holloszy, 1996). However, the point of convergence for insulin-dependent and insulin-independent signaling pathways is a mystery. More than one regulator is likely to be involved in the control of contraction-stimulated skeletal muscle glucose uptake and some redundancy may exist. A critical aspect that needs to be addressed is the cellular mechanism that bridges the gap between signal transducers and GLUT4 trafficking. The elucidation of these mechanisms will assist exercise physiologists in the design of the most effective regimens for the improvement of insulin action in insulin-resistant subjects with type 2 diabetes.

4.4.1 Role of Rab-GAPs in GLUT4 trafficking

4.4.1.1 GLUT4 trafficking

GLUT4 is responsible for mediating the facilitated transport of glucose across the plasma membrane and is primarily expressed in insulin sensitive tissues like fat, the heart and skeletal muscle (Liu, 1992, Bell, 1989). GLUT4 is found in many organelles like plasma membrane, sorting endosomes, recycling endosome, trans golgi network (TGN) and vesicle that mediate the transport of GLUT4 between these compartments (Bryant, 2002). Different models for GLUT4 cycling have been proposed, however according to the most widely accepted model, GLUT4 molecules are believed to cycle between intracellular compartments and the plasma membrane (Bryant, 2002, Dugani, 2005). Under basal conditions, GLUT4 appears to highly abundant in specialized compartments referred as 'GLUT4 storage vesicle (GSV). Insulin stimulates the movement of GLUT4 from GSV (Ploug, 1998, Bai, 2007), while exercise appears to translocate GLUT4 from a different (endosomal) pool to the plasma membrane (Cushman, 1998, Ploug, 1998). This could explain why insulin and exercise-induced have an additive effect on glucose uptake. GLUT4 cycling is regulated at the level of exocytosis, fusion, endocytosis and inter-endosomal transit. Several studies support the notion that there are multiple signaling pathways that converge on different aspect of GLUT4. Although the impact of specific signaling molecules on particular segments of GLUT4 traffic have been studied, some key remaining questions are: how do insulin (Akt/ PKCζ) and exercise/contraction communicate with GLUT4 containing compartments?; what is the primary recipient of these signals?; where do these two pathways converge?

4.4.1.2 Rab-GAPs TBC1D1 and TBC1D4

Rab proteins are involved in regulation of several steps in membrane transport processes, including vesicle motility, docking/fusion and transport of transport vesicles (Zerial, 2001, Pfeffer, 2004). Rab 4, 5 and 11 have been shown to play important role in GLUT4 translocation (Zerial, 2001). Rab proteins cycle between biologically "active" GTP-bound and "inactive" GDP-bound conformations, which is critical for their function. In their GTP-bound active state (Rab-GTPase), Rab proteins can directly interact with a variety of effector proteins and alter their function (Zerial, 2001, Pfeffer, 2004). Rab GTPase-activating proteins (Rab-GAPs) enhance the intrinsic GTPase activity of target Rab proteins and determine the lifetime of the bound GTP (Bernards,

2003). Recently, the TBC domain containing proteins TBC1D4 and TBC1D1 have been discovered as Rab GTPase-activating proteins and have been shown to be involved in GLUT4 mobilization to the plasma membrane in cell culture systems and in skeletal muscle (Sakamoto, 2008). Rab-GAP TBC1D4 is also termed 'AS160' – referring to an Akt substrate with a molecular weight of 160 kDa (Kane, 2002). Using an in vitro assay with recombinant TBC1D4 the GTPases activity towards Rab 2A, 8A, 10, and 14 was shown (Miinea, 2005), implicating a role of TBC1D4 in GLUT4 translocation. The Rab-GAP domain of TBC1D1 and TBC1D4 are quite similar (Roach, 2007), therefore it is unsurprising that GAP domain of TBC1D1 and TBC1D4 have activity towards similar Rabs (Roach, 2007).

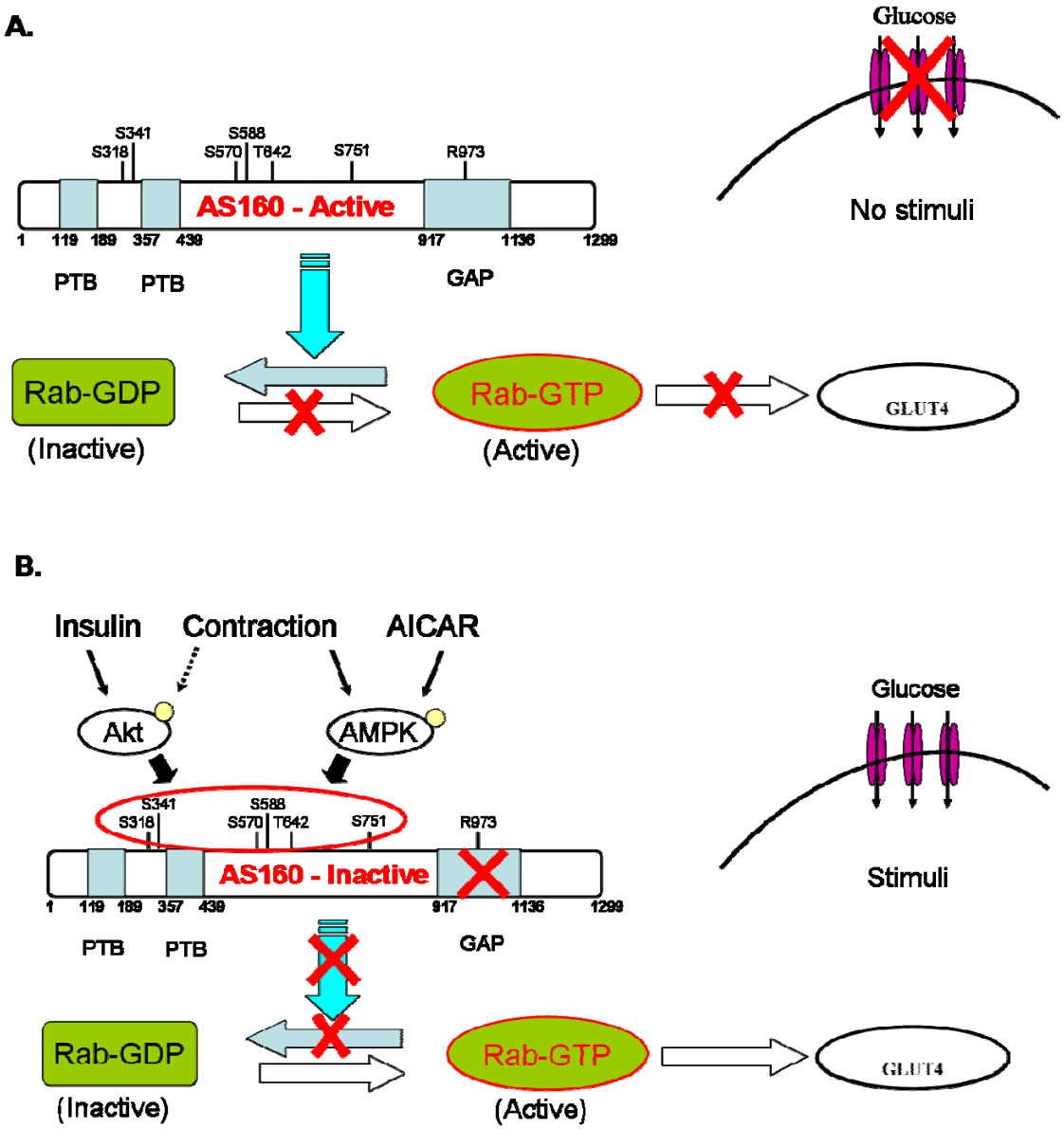

Figure 9. Working hypothesis for the regulation of AS160 (TBC1D4) under not-stimulated (A) and stimulated (insulin, AICAR, contraction) conditions.

The elucidation of the precise role of TBC1D1 and TBC1D4 in the regulation of GLUT4 trafficking is an ongoing work. Intriguingly, these Rab-GAPs appear to be modulated by insulin and/or skeletal muscle contraction (Sakamoto, 2008), suggesting this molecule acts as a point of convergence for insulin-dependent and –independent signaling pathways. One current hypothesis suggests that insulin and exercise promotes the phosphorylation of TBC1D4 and TBC1D1, leading to increased GTP loading of Rab proteins on GLUT4 vesicles and subsequently to an increased interaction with Rab effectors that control GLUT4 vesicle translocation (Sakamoto, 2008, Deshmukh, 2008). A working hypothesis for the regulation of AS160 (TBC1D4) in response to different stimuli is shown (Figure 9A and 9B, Deshmukh, 2008). TBC1D1 is also regulated in a somewhat similar fashion as TBC1D4, however there are some striking differences with respect to the phosphorylation pattern by upstream kinases. In Paper III, IV and V, we have addressed several aspects of TBC1D1 and TBC1D4 regulation.

4.4.2 Exercise-induced phosphoproteins in skeletal muscle

A single bout of prolonged aerobic exercise (30-60 min at ~ 60-70 % of maximal oxygen consumption) can lower plasma glucose levels, owing to normal contraction-induced stimulation of GLUT4 translocation and glucose transport activity in insulin-resistant skeletal muscle (Henriksen, 2002). The search for possible exercise-induced signaling intermediates is ongoing. Phosphorylation and activation of several signaling molecules improves glucose homeostasis after exercise (Deshmukh, 2008, Jessen, 2005). However further investigations are needed to elucidate the specific molecular mechanism underlying the beneficial effects of acute exercise.

While the acute effects of exercise on glucose metabolism are clearly insulin-independent, the canonical insulin signaling cascade has been used as a framework by investigators in an attempt to resolve the mechanisms by which muscle contraction governs glucose metabolism. We took a similar approach in Paper III, where our aim was to identify and characterize putative Akt-substrates in response to acute exercise. The effect of endurance and resistance exercise on phosphorylation of Akt and putative Akt-substrates is reported in this thesis (Paper III).

4.4.2.1 Exercise-induced activation of Akt

The effects of exercise on Akt are particularly important because it plays a critical role in glucose uptake in response to insulin (Hajduch, 2001). PDK-1 is an upstream kinase for Akt Thr^{308}, whereas Akt Ser^{473} is phosphorylated by the mTOR-rictor complex (Sarbassov, 2005). Ser^{473} phosphorylation precedes the phosphorylation of Thr^{308} and it is essential for the recognition of Akt by PDK1 (Alessi, 1996a). Some (Sakamoto, 2002, Sakamoto, 2003, Sakamoto, 2004, Bruss, 2005, Nader, 2001), but not all studies (Widegren, 1998, Wojtaszewski, 1999) studies have shown that muscle contraction increases Akt activity/or phosphorylation. In Paper III, Akt Ser^{473} phosphorylation was increased immediately after a single bout of cycling exercise, whereas Akt phosphorylation was unchanged after resistance exercise (*Paper III, figure 1A*). In contrast, Akt Thr^{308} phosphorylation was unaltered in response to either mode of exercise (*Paper III, figure 1B).* The lack of Thr^{308} phosphorylation may suggest that Akt is not activated during the exercise protocol. Alternatively there may be only a transient activation (if any) because full activation of Akt requires phosphorylation of

both Thr^{308} and Ser^{473} residues (Alessi, 1996a). These results are consistent with our previous studies, where acute moderate cycling exercise was shown to be without any measurable increase in Akt activity (Widegren, 1998).

4.4.2.2 Exercise and Phospho-Akt-Substrates (PAS)

Akt contributes to a variety of cellular responses. This multiplicity of Akt function might be due to the variation and specificity of its substrates. Efficient phosphorylation of Akt requires a minimum sequence motif 'RXRXXS/T', where R is arginine, X is any amino acid and S/T (serine/threonine) is the phosphorylation site (Alessi, 1996b). Up to now more that 50 proteins have been identified as putative substrates for Akt and all are reported to phosphorylate the above-mentioned Akt-phospho motif (Obata, 2000). Several of these substrates may link insulin signaling and metabolic or gene regulatory responses.

Due to the availability of a PAS (phospho-Akt substrate) antibody, it has recently become easier to study substrates that are specifically phosphorylated at 'RXRXXS/T'. Using a PAS antibody, several insulin-elicited phosphoproteins (pp250, pp160, pp105, pp75, pp47, pp43, and pp32) were detected in 3T3-L1 adipocytes (Kane, 2002). Since Akt is rapidly activated by insulin and known to be required for full insulin-stimulated GLUT4 translocation to the plasma membrane (Hill, 1997, Hajduch, 2001), the identity of one or more down-stream targets of Akt that could potentially provide link between Akt activation and GLUT4 translocation became important to resolve (Kane, 2002). AS160 (TBC1D4), a protein with molecular signature of Rab-GAP, was subsequently discovered to play a role in GLUT4 translocation (Kane, 2002, Sano, 2003). However, many of the physiologically relevant substrates for Akt remain to be identified.

In Paper III, the effect of cycling or resistance exercise on the phosphorylation of Akt substrates was determined in human skeletal muscle (*Paper III, figure 2*). Several exercise responsive proteins were detected in crude lysates after immunoblot analysis with the PAS antibody. The phosphoproteins (pp) of 300, 250, 180, 160, 150 42, and 34 kDa were among the most predominant immunoreactive proteins identified (*Paper III, figure 2A*). Four of these proteins (pp300, pp160, pp180, pp150) were clearly phosphorylated in response to exercise (*Paper III, figure 2B*). We further identified pp160 and pp300 as AS160 (TBC1D4) and filamin A, respectively.

4.4.2.3 Exercise phosphorylates AS160

Prior to the discovery of TBC1D1, the term 'AS160' was primarily used to describe TBC1D4. Earlier studies characterizing the role of Akt substrates were performed using the PAS antibody and molecular weight determinations. Since the molecular weight of TBC1D1 and TBC1D4 is relatively close, these proteins cannot easily be studied with the PAS antibody. Thus, when referring to studies where the investigators have used the PAS antibody to characterize the TBC1D1/4 proteins, the term 'AS160' will be used for this part of the discussion. When investigators have used specific antibodies to characterize TBC1D1/4, the specific molecule will be discussed.

As160 was first shown to be phosphorylated in adipocytes in response to insulin stimulation (Kane, 2002). Subsequently, AS160 was also shown to be phosphorylated in rat skeletal muscle in response contraction (Bruss, 2005). In an immunoprecipitation study, pp160 was indentified and characterized as AS160 (*Paper*

III, figure 3A). An acute bout of endurance cycling led to a robust increase in AS160 phosphorylation (*Paper III, figure 3B*), with a concomitant increase in phosphorylation of Akt Ser^{473} (*Paper III, figure 1A*). Conversely, resistance exercise was without effect on AS160 phosphorylation (*Paper III, figure 3B*). As contraction-induced AS160 phosphorylation in rat epitrochlearis muscle is completely abolished by wortmannin (Bruss, 2005), this observation implicates that AS160 is regulated via a PI-3K–Akt signaling pathway. However with the current exercise protocol, Akt Thr^{308} phosphorylation was not altered, providing evidence to suggest that the effect of exercise on AS160 phosphorylation is likely to be Akt-independent. The increase in exercise-induced AS160 phosphorylation is of clinical relevance because insulin-stimulated phosphorylation of AS160 is impaired in skeletal muscle from type 2 diabetic subjects (Karlsson, 2005b). Thus discovery of AS160 as potential intermediate of both insulin and exercise-induced signaling pathways provides a possible link between these two pathways and may explain how insulin sensitivity can be enhanced in response to exercise.

4.4.2.4 Exercise phosphorylates filamin A

The actin filament network plays an important role in GLUT4 trafficking (Saltiel, 2003, Tsakiridis, 1999). Disruption of the actin cytoskeleton impairs GLUT4 translocation to cell surface of skeletal muscle and adipocytes (Tsakiridis, 1999, Saltiel, 2003). The actin binding protein filamin A was identified and characterized as PAS (pp300) (*Paper III*). Like AS160 and Akt Ser^{473}, filamin A is phosphorylated in response to endurance exercise (*Paper III, figure 4*). Filamin is expressed as three highly related isoforms termed filamin A, filamin B and filamin C (Stossel, 2001). The skeletal muscle-specific isoform, filamin C, has been identified and characterized as an insulin-responsive Akt substrate (Murray, 2004). Using an immunoprecipitation approach, we revealed that filamin C is phosphorylated in human muscle in response to insulin, whereas filamin A is phosphorylated in response to endurance exercise, but not insulin (*Paper III, figure 4*).

Phosphorylation of filamin A may lead to actin remodeling and this might be involved in membrane ruffle formation (Carroll, 1982, Glogauer, 1998). Filamin A has more that 20 other binding partners, including actin, membrane receptors, small GTPases, and stress-activated protein kinase (Woo, 2004). Filamin isoforms may also act as scaffolding proteins or tethering components of signaling pathways to enhance or suppress activation by particular agonists (Murray, 2004, Stossel, 2001). Phosphorylation of filamin A may directly stimulate binding or cross-linking of actin filaments and regulate their binding with other partners to modulate signaling events mediating exercise-induced metabolic or gene regulatory responses.

Filamin A, as well as several other exercise-responsive phosphoproteins (pp), including pp250, pp180, pp150 pp42, pp34 kDa and AS160, was detected in crude lysates of skeletal muscle after immunoblott analysis with the PAS antibody (*Paper III, figure 2*). However, due to the limitation in the amount of human material available for this study, we were unable to characterize all of the phosphoproteins detected using the PAS antibody.

The PAS antibody recognizes the phosphorylation of Ser/Thr residues present in a conserved 'RXRXXS/T' motif. Therefore, the PAS antibody is likely to recognize phosphorylation of substrates that belong to the closely related kinases such as Akt, AMPK, PKC and p70S6 kinase (Alessi, 1996a, Obata, 2000). Exercise has multiple

effects on metabolic and gene regulatory responses and these responses are unlikely to be mediated by a singular pathway (Jessen, 2005). Therefore exercise-induced phosphorylation of AS160 and filamin A might be mediated by other exercise responsive kinases such as AMPK, aPKC and CaMK.

4.4.3 TBC1D1 and TBC1D4: similarity and differences

Since the discovery of TBC1D1 (Roach, 2007), several workers have revealed similarities and interesting differences between TBC1D4 and TBC1D1. For example, TBC1D4 and TBC1D1 are differentially expressed in different tissues. Cultured adipocytes (Roach, 2007, Chavez, 2008), human white adipose tissue (Stone, 2006), and rodent and human skeletal muscle (Treebak, 2009b, Treebak, 2009a, Taylor, 2008) have a differential expression of TBC1D4 and TBC1D1 at the mRNA and protein level. TBC1D1 is highly expressed in white adipose tissue and in mouse skeletal muscle containing primarily type II fibers, whereas AS160 is expressed highly in cultured adipocytes and type I mouse skeletal muscle. Differences in the expression pattern of TBC1D1 and TBC1D4 might account for tissue-specific machinery for GLUT4 translocation

4.4.3.1 TBC1D4 and TBC1D1 are multi-kinase substrates

Insulin stimulates phosphorylation of TBC1D4 (AS160) at 5 sites via Akt (Sano, 2003). Inactive forms of TBC1D4 (AS160), in which 4 phosphorylation sites (Ser^{318}, Ser^{588}, Thr^{642}, Ser^{751}) have been mutated to alanine (AS160-4P) abolish insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes (Sano, 2003). Although there is only 50% identity between TBC1D4 (AS160)and TBC1D1, the GAP domain of these proteins is 79% identical. Moreover, TBC1D1 has comparable predicted Akt phosphorylation sites at Thr^{596} and Ser^{507}, corresponding to Thr^{642} and Ser^{570} on TBC1D4 (AS160), respectively (Roach, 2007). Unlike TBC1D4 (4 phosphorylation sites), TBC1D1 is phosphorylated mainly at Thr^{596} (equivalent to Thr^{642} on TBC1D4) in response to insulin (Roach, 2007), indicating differential regulation of TBC1D4 and TBC1D1 in response to insulin.

TBC1D4 is phosphorylated by AICAR and is a substrate for AMPK (Treebak, 2006). Interestingly, in mouse EDL muscle, AICAR increased the phosphorylation of TBC1D4, as detected AS160 by the PAS antibody (Treebak, 2006, Kramer, 2006), but Thr^{642} phosphorylation was unaltered (Kramer, 2006). AICAR also phosphorylates Thr^{596} and PAS sites on TBC1D1 (Pehmoller, 2009). The PAS antibody primarily recognizes Thr^{596} on TBC1D1 and Thr^{642} on TBC1D4 (Sano, 2003, Ramm, 2005, Chen, 2008, Geraghty, 2007). In HEK-293 cells, AICAR also phosphorylates Ser^{588}, Thr^{642}, Ser^{751} on TBC1D4 (Geraghty, 2007). These results indicate that TBC1D4 and TBC1D1 are differentially regulated by AICAR. Similar to AICAR, contraction of mouse skeletal muscle leads to an increase in the phosphorylation on TBC1D1 at Thr^{596} and Ser^{237} in an AMPK dependent manner (Pehmoller, 2009). In humans, an acute bout of exercise increases the phosphorylation of TBC1D4 at Ser^{318}, Ser^{588}, Ser^{341}, Ser^{751}(Treebak, 2009b), indicating differential regulation of TBC1D1 and TBC1D4 in response to contraction/exercise. Contraction-induced TBC1D1 PAS phosphorylation is inhibited by compound C, whereas TBC1D4 (AS160) PAS phosphorylation was unaltered (Funai, 2009). Conversely, wortmanin inhibits contraction-induced TBC1D4 (AS160) PAS phosphorylation, while TBC1D1 PAS phosphorylation was unchanged

(Bruss, 2005). Collectively these results indicate that TBC1D4 and TBC1D1 are differentially regulated by different stimuli and primarily act as substrates for Akt and AMPK respectively.

Exercise-induced phosphorylation of AS160 (Paper III) is unlikely to be dependent on AMPK because in experiments performed using tissue collected from an identical exercise testing protocol, we have reported that AMPK signaling is not activated in response to an acute bout of cycling exercise performed by endurance trained individuals (Coffey, 2006). Since the molecular weight of TBC1D1 is ~150 kDa, it is likely that this exercise responsive phosphoprotein "pp150" (*Paper III, figure 2*) is TBC1D1. Interestingly, both forms of exercise increased phosphorylation of pp150 in human skeletal muscle (*Paper III, figure 2*).

Skeletal muscle contraction is initiated by an increase in intracellular Ca^{2+}, which has been reported to stimulate glucose uptake (Henriksen, 1989, Youn, 1991). In addition to the GAP domain, AS160 also contains a functional Ca^{2+}/CaM binding domain (CBD) (Kane, 2005). Contraction- but not insulin-induced glucose transport was inhibited in skeletal muscle expressing the CBD mutant (Kramer, 2007), providing a role for Ca^{2+}dependent kinases in regulation of AS160. In Paper IV, we have addressed role of CaMK in regulation of TBC1D4/D1.

TBC1D1 and TBC1D4 are phosphorylated at multiple sites by a variety of agonists that stimulate different protein kinases in intact cells. This indicates there is a complex regulation, with specialized roles for these proteins in skeletal muscle and adipose tissue. Further work is required to characterize the physiological roles of TBC1D4 and TBC1D1 in the regulation of GLUT4 trafficking.

4.4.4 Hypoxia-induced glucose transport in skeletal muscle

Hypoxia is useful model for exercise. The effect of insulin and exercise/muscle contraction on glucose transport are additive (Constable, 1988, Holloszy, 2003), while the maximal effect of hypoxia and contraction are not additive (Cartee, 1991, Azevedo, 1995), indicating hypoxia and muscle contraction may share common signaling mechanisms. Hypoxia is thought to activate glucose transport by mimicking contraction-induced calcium release from sarcoplasmic reticulum (Azevedo, 1995, Cartee, 1991). Stimulation of muscle with caffeine, which releases Ca^{2+} from the sarcoplasmic reticulum also increases muscle glucose uptake, thereby providing additional evidence for a role of Ca^{2+} signaling in regulation of glucose transport (Holloszy, 1967). The Ca^{2+}/calmodulin (CaM)-competitive inhibitors of CaM-dependent protein kinase (CaMK), KN-62 and KN-93, have been used to determine the role of Ca^{2+} in contraction-mediated glucose transport (Wright, 2005, Wright, 2004). Hypoxia- mediated effects on glucose transport appeared to be fiber-type dependent (Wright, 2005, Wright, 2004). In slow-twitch rat soleus muscle, contraction and hypoxia-stimulated glucose uptake were completely dependent on Ca^{2+}-sensitive pathways (Wright, 2005), whereas in rat fast-twitch glycolytic muscle, AMPK and Ca^{2+}-mediated signaling pathways were linked to contraction-mediated glucose transport (Wright, 2004). However, in skeletal muscle from transgenic mice overexpressing kinase dead (KD) AMPKα2, AICAR and hypoxia-mediated glucose transport was completely abolished (Mu, 2001), suggesting AICAR and hypoxia-stimulated glucose transport is largely AMPK-dependent. These finding suggest that in

glycolytic muscle, glucose uptake during hypoxia is mediated by CaMK and AMPK signaling.

In Paper IV, we investigated role of CaMK and AMPK signaling in hypoxia-induced glucose transport. Mouse models whereby AMPK signaling was impaired (AMPKγ3 KO) were used to address the possible role of AMPK. In addition, the Ca^{2+}/calmodulin competitive inhibitor, KN-93 was used to dissect role of Ca^{2+}-depending signaling pathways.

4.4.4.1 Dependency of the AMPK γ3 subunit

Previously, we have reported that AICAR-induced glucose transport in EDL muscle from mice lacking the AMPKγ3 subunit, is completely inhibited (Barnes, 2004). Thus provides evidence for an importance role of the γ3-containing AMPK subunits in the regulation of glucose transport under energy depleting conditions. Hypoxia-mediated glucose transport was partially reduced in EDL muscle from mice lacking the AMPKγ3 subunit (*Paper IV, figure 2A*). This finding highlights the role of the γ3-containing AMPK subunits in hypoxia-mediated glucose transport. These results also provide evidence that hypoxia-mediated glucose transport requires AMPKγ3-dependent, as well as AMPKγ3-independent mechanisms, since glucose transport in EDL muscle was partly reduced in AMPK γ3 KO mice. Contrary, in soleus muscle, where the AMPK γ3 subunit is undetectable (Mahlapuu, 2004), the effect of hypoxia on glucose transport was comparable between wild-type and AMPKγ3 KO mice (*Paper IV, figure 1B*). Collectively, these results indicate the AMPK γ3 subunit plays a role in hypoxia-mediated glucose transport.

4.4.4.2 Role of the AMPK 3 subunit in hypoxia-induced signaling

To further elucidate the signaling pathways contributing to hypoxia-mediated glucose transport, we assessed phosphorylation of AMPK, Akt, and other downstream targets including ACC and TBC1D1/D4. As expected, Akt was not activated in response to hypoxia (data not shown). Despite the reduction in hypoxia-induced glucose transport, AMPK signaling was not impaired in EDL muscle from AMPKγ3 KO mice. This was evident by the preserved effect of hypoxia on AMPK and ACC phosphorylation (*Paper IV, figure 2C-2D*). These results also provide evidence that the AMPKγ3 isoform is dispensable for hypoxia-induced AMPK activation (*Paper IV, figure 4*) and are consistent with previous studies, showing AICAR-mediated AMPK and ACC phosphorylation is maintained, despite a profound impairment in glucose transport (Barness 2004). However at earlier time points, the AICAR-mediated AMPK phosphorylation was reduced in AMPKγ3 KO mice (Treebak, 2006). In human skeletal muscle, high intensity exercise primarily activates γ3 containing AMPK complexes (Birk, 2006). However when exercise is performed at lower intensity for a longer period (90 min), activity of the α2β2γ1 AMPK complex was increased over a much slower time course than that of the α2β2γ3 AMPK complex (Treebak, 2007). Mouse EDL also contains γ1 associated AMPK heterotrimers (Treebak, 2009a), therefore it is possible that hypoxia activates heterotrimeric complexes containing the γ1 subunit in both genotypes, because α1- and α2- associated AMPK activity was unaltered in response to hypoxia (*Paper IV, figure 2E-2F*).

AICAR-induced phosphorylation of TBC1D4 (AS160) is severely impaired in EDL muscle from AMPKγ3 KO mice (Treebak, 2006), however hypoxia-mediated phosphorylation of TBC1D4 and TBC1D1 as determined using a PAS antibody was

similar between wild-type and AMPKγ3 KO mice (*Paper IV, figure 3*). Thus, γ1 associated AMPK complexes are likely to be responsible for phosphorylation of TBC1D4 and TBC1D1, since exercise-induced phosphorylation of TBC1D4 (AS160) in humans is correlated with the activation pattern of the α2β2γ1, but not the α2β2γ3 AMPK complex (Treebak, 2007). Signals detected by the PAS antibody reflect phosphorylation of both TBC1D1 from that of TBC1D4 (AS160). Thus, we attempted to differentiate phosphorylation of TBC1D1/D4. Immunoprecipitation with TBC1D4 followed by immunoblot analysis with PAS (*Paper IV, figure 3B*) showed a similar pattern as isoform specific phosphorylation of TBC1D1/D4 (*Paper IV, figure 3A-3B*). Moreover, we did not observed a PAS-TBC1D1 signal in the immunodepleted samples from any of the treatments (*Paper IV, figure 3B*), suggesting a role for TBC1D4 in hypoxia-induced glucose transport. Since the expression of TBC1D4 in EDL is very low (*Paper IV, figure 3B*, (Treebak, 2009a, Taylor, 2008), we cannot exclude the possibility that TBC1D1 may play a role, particularly because of its relatively high expression in EDL muscle (Taylor, 2008, Treebak, 2009a) and its regulation by AMPK (Pehmoller, 2009). Because commercial antibodies to detect TBC1D1 are poor, we have not performed any further detailed analysis of TBC1D1. Nevertheless, hypoxia-mediated TBC1D1/D4 phosphorylation is uncoupled from glucose transport in AMPKγ3 KO mice, similar to earlier results for contraction-mediated events in mice with impaired AMPK signaling (Treebak, 2006). This provides evidence that TBC1D1/D4 -independent mechanisms contribute to hypoxia-, as well as contraction-mediated glucose transport in skeletal muscle.

4.4.4.3 Interaction between CaMK and AMPK

The role of CaMK and AMPK signaling in hypoxia-induced glucose transport was studied using the Ca^{2+}/calmodulin competitive inhibitor, KN-93. The specificity of KN-93 was tested in EDL muscle from C57BL/6 mice. As expected, KN-93 reduced hypoxia-mediated glucose transport in EDL muscle, while KN-92 (a pseudo inhibitor of Ca^{2+}/calmodulin-dependent protein kinase) treatment was without effect (*Paper IV, figure 1B*). Neither KN-93 nor KN-92 had any effect on basal glucose transport (*Paper IV, figure 1B*). KN-93 reduced hypoxia-mediated glucose transport in EDL muscle from wild-type mice (*Paper IV, figure 2A*). In EDL muscle from AMPKγ3 KO mice, KN-93 lead to a greater impairment in hypoxia-mediated glucose transport (*Paper IV, figure 2A*). These results indicate that KN-93 has greater ability to inhibit hypoxia-induced glucose transport in skeletal muscle from AMPKγ3 KO mice, highlighting possible cross-talk between AMPK and CaMK signaling. Inhibition of Ca^{2+} signaling by KN-93 was evident by a reduction in CaMK II phosphorylation in EDL muscle from wild-type and AMPKγ3 KO mice (*Paper IV, figure 2B*).

Earlier studies provided evidence that caffeine- and contraction-induced glucose transport is inhibited in presence of KN-93 (Wright, 2005, Wright, 2004). In similar studies, contraction-induced AMPK phosphorylation was unaltered by KN-93 treatment (Wright, 2005, Wright, 2004), suggesting that CaMKs regulate glucose transport by an AMPK-independent mechanism. Consistent with these earlier findings (Wright, 2004, Wright, 2005), KN-93 did not alter either hypoxia-induced phosphorylation of AMPK and ACC (*Paper IV, figure 2C-2D*) or isoform specific (α1 and α2) AMPK activity (*Paper IV, figure2E-2F*), regardless of genotype. Conversely, in rat soleus muscle, treatment with KN-93 potentially inhibits contraction-induced AMPK phosphorylation and activity, with a concomitant inhibition of glucose uptake (Jensen, 2007a). Furthermore, the CaMKK-inhibitor, STO-609, inhibits contraction-

induced AMPK activity and glucose transport similar to that observed with KN93 (Jensen, 2007a). In a parallel study, treatment of mouse soleus muscle with caffeine increased α1-AMPK activity, while treatment with KN-93, STO-609, or dantrolene reduced caffeine-induced activation of α1-AMPK and glucose uptake (Jensen, 2007b). Thus, Ca^{2+}/CaM-dependent signaling events appear to lie upstream of AMPK (Jensen, 2007a, Jensen, 2007b). In regard to the above mentioned studies, neither STO-609 nor KN-93 are specific inhibitors, as discussed earlier (Papers IV, (Jensen, 2007b)). Thus, further work is needed to understand the cross-talk between AMPK and Ca^{2+} signaling. Genetic models with deficiencies in CaMKK isoforms may clarify the interaction between these signaling events. Based on present body of literature and the results presented here, a schematic representation of hypoxia-induced glucose transport is depicted (Figure 10).

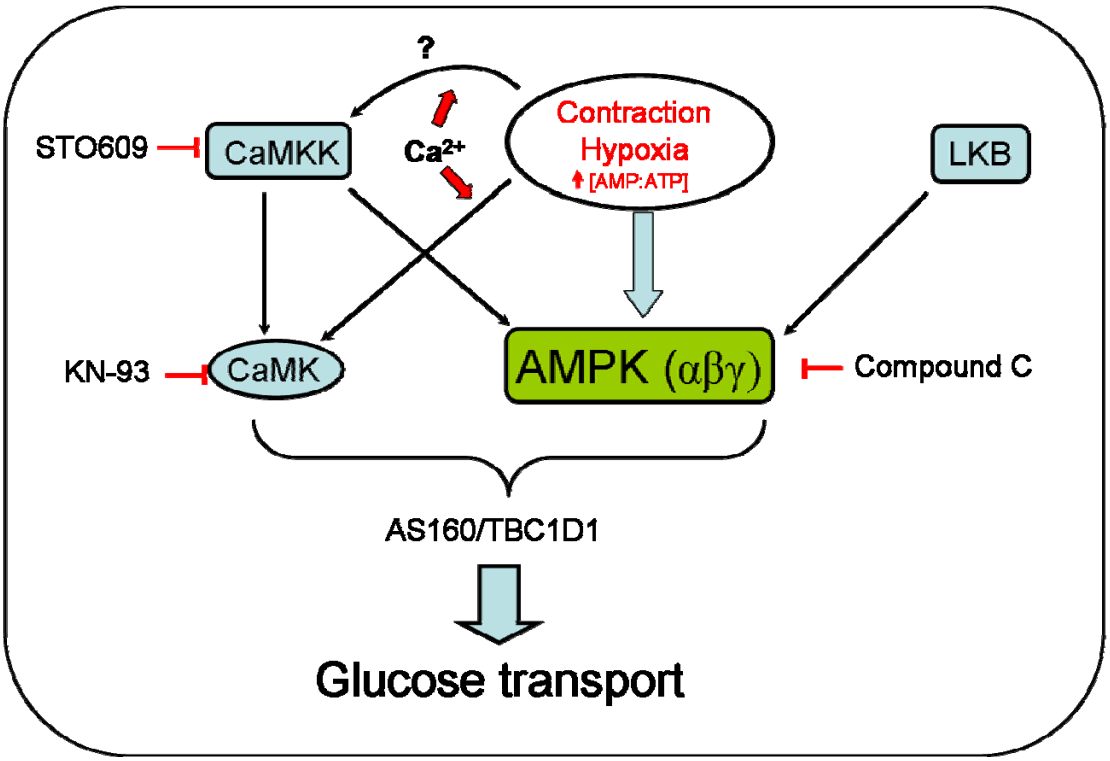

Figure 10. Schematic representation of hypoxia-induced glucose transport in skeletal muscle.

4.4.4.4 TBC1D1/D4 phosphorylation: role of Ca^{2+} signaling

Hypoxia-induced phosphorylation of TBC1D1/D4 is not impaired in EDL muscle from AMPK γ3 KO mice (*Paper IV, figure 3*). This contrasts the effects of AICAR on phosphorylation of TBC1D4 (AS160) (Treebak 2006). TBC1D4 contains a functional Ca^{2+}/CaM binding domain (CBD) (Kane, 2005) and contraction-induced glucose transport is inhibited in skeletal muscle expressing the CBD mutant (Kramer, 2007), suggesting a possible role for Ca^{2+} signaling in the regulation of TBC1D4. Treatment with KN-93 did not alter hypoxia-induced phosphorylation of TBC1D1/D4 in EDL muscle from wild-type mice (*Paper IV, figure 3*). Conversely, in AMPK γ3 KO mice, KN-93 dramatically reduced hypoxia-induced phosphorylation of TBC1D1/D4

(*Paper IV, figure 3*), indicating cross-talk between AMPK and CaMK signaling. Thus, a possible role of the CBD domain of TBC1D4 cannot be excluded. Similar to the effects of glucose transport (*Paper IV, figure 2A*), KN-93 has a pronounced inhibitory effect on hypoxia-induced phosphorylation on TBC1D1/D4, in EDL muscle from AMPK γ3 KO mice. Similar finding with respect to AMPK and CaMK signaling have been recently reported (Murgia, 2009) since inactivation of both AMPK- and CaMK-dependent pathways have a larger effect on transcriptional inhibition of proteins (such as GLUT4). Clearly, further work is needed to clarify the role of Ca^{2+}signaling in the regulation of TBC1D1/D4.

4.4.5 NO-cGMP pathways and skeletal muscle glucose transport

Nitric oxide (NO) has been implicated as an important signaling molecule in the regulation of insulin-independent, contraction-mediated glucose transport (Figure 11) (Balon, 1994, Bradley, 1999, Roberts, 1997). NO release is increased 50-200% during periods of repetitive isometric contraction (Balon, 1994). NO donor drugs increase glucose transport in isolated rat skeletal muscle (Balon, 1997, Etgen, 1997, Higaki, 2001) and variety of cell lines including mouse L929 fibroblasts (Van Dyke, 2003), mouse 3T3-L1 adopicytes (Tanaka, 2003) and rat cadiomyocytes (Jensen, 2003). In humans, an infusion of sodium nitroprusside (SNP) into the femoral artery increases basal leg glucose uptake (Durham, 2003), providing clinical evidence that NO signaling controls exercise-induced glucose uptake. Studies of the effect of an NO donor on glucose uptake in isolated human skeletal muscle are lacking. In Paper V, we investigated the effect of the NO donor Spermine NONOate (N-(2-Aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine) on glucose transport and intracellular signaling in isolated human skeletal muscle.

4.4.5.1 Spermine NONOate increase glucose transport via NO-cGMP pathway

NO function as an endogenous physiological modulator of skeletal muscle, at the tissue and cellular level (Reid, 1998, McConell, 2006). NO directly acts on skeletal muscle fibers to influence muscle metabolism and contractile function (McConell, 2006, Reid, 1998). In humans, an acute administration of the NOS inhibitor NG-monomethyl-L-arginine (L-NMMA) during exercise reduces leg muscle glucose uptake (Bradley, 1999). The effect of NO on glucose uptake in skeletal muscle appears to be mediated by cGMP (Young, 1997, Ohlstein, 1982). In Paper V, human skeletal muscle strips were treated with Spermine NONOate and insulin and glucose transport was assed. Consistent with earlier studies (Koistinen, 2003), insulin increased skeletal muscle glucose transport 5-fold (*Paper V, figure 1A*). Spermine NONOate treatment also increased skeletal muscle glucose uptake (*Paper V, figure 1A*), with a concomitant increase in cGMP levels (*Paper V, figure 1B*). Our results in human skeletal muscle are compatible with earlier studies in rodent muscle, and indicate NO donors increase cGMP levels and glucose transport (Young, 1997).

The mechanism by which cGMP regulates glucose uptake may involve two enzymes, namely guanylate cyclase and phosphodiesterase (Figure 11), but other mechanism are likely to be involved. Inhibition of guanylate cyclase prevents the SNP-induced increase in cGMP levels and glucose transport (Young, 1997). Conversely, treatment of isolated skeletal muscle with a phosphodiesterase inhibitor (Zaprinast) increases cGMP levels (Figure 11), with a concomitant increase in glucose uptake (Young, 1998). Due to limitations in the source of human material, we were unable to perform additional inhibitor studies. However, we are currently investigating role of cGMP in skeletal muscle glucose uptake using rat L6 muscle cells. Collectively, these

studies provide evidence that the NO/cGMP pathway is likely to be the important in the regulation of glucose transport.

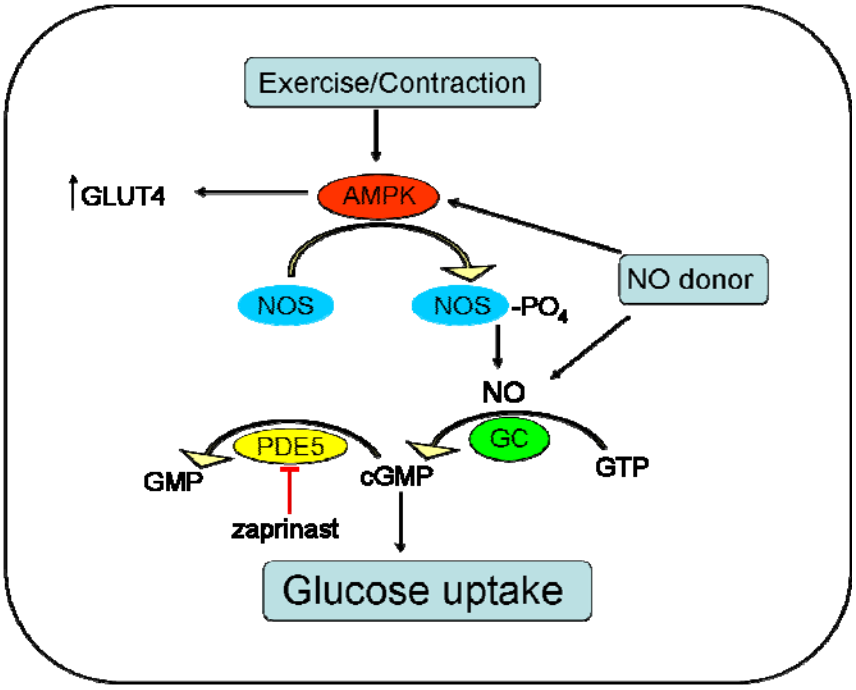

Figure 11. Role of NO-cGMP pathways in skeletal muscle glucose uptake.

4.4.5.2 Effect of spermine NONOate on signal transduction

To further determine the intracellular mechanism by which the NO donor Spermine NONOate increases glucose transport, components of the canonical insulin signaling cascade were assessed. Insulin exposure led to an expected increase in phosphorylation of pAkt, AS160 and GSK3, while exposure to the NO donor was without effect (*Paper V, figure 2A-D*), suggesting that NO signaling does not interfere with insulin signaling. These results are also consistent with previous finding that NO increases glucose transport through an insulin-independent translocation of GLUT4 (Tanaka, 2003). Whether a combined effect of an NO donor and insulin will have additive/synergetic effects on skeletal muscle glucose transport is unknown We are currently addressing this question using L6 muscle cells. Neuronal-type (nNOS) and endothelial-type (eNOS) isoforms of NOS bind to calmodulin in a calcium-dependent manner and these binding event are important for of NO synthesis (McConell, 2006). In human skeletal muscle, Spermine NONOate did not appear to interact with Ca^{2+}/calmodulin signaling, since exposure to either insulin or the NO donor was without effect on CaMKII phosphorylation (Figure 12A).

AMPK is considered to be an upstream kinase for NOS, since it phophorylates and activates endothelial and neuronal forms of NOS (Figure 11) (Chen, 1999, Chen, 2000). However, a chronic exposure of L6 muscle cells to SNP increases GLUT4 mRNA expression by an AMPK-dependent mechanism (Lira, 2007), positioning AMPK downstream of NO signaling. Exposure of human skeletal muscle to Spermine NONOate increased AMPKα1-specific activity, while AMPKα1-specific activity was unchanged (*Paper V, figure 1C-D*). Total protein abundance of the AMPKα1 and AMPKα2 subunits was unaltered during the muscle incubation procedure (*Paper V,*

figure 2A). Interestingly, in rodent skeletal muscle, varying concentration SNP increases AMPK-α1, but not AMPK-α2 associated activity (Higaki, 2001). Whether the NO pathways lie upstream or downstream of AMPK remains a matter of debate. A positive feedback interaction between AMPK and NOS in the control of skeletal muscle metabolism has been implicated (Lira, 2007). NO has also been linked to the positive (Lira, 2007) and negative (Bedard, 1997) modulation of GLUT4 expression. However, GLUT4 protein content was unaltered in response to the NO donor (Figure 12B), presumably because of the shorter incubation time and low concentration of the NO donor used during the incubation protocol. Our results provide evidence that the NO donor Spermine NONOate increases cGMP levels and promotes glucose transport, concomitant with AMPKα1-isoform-specific activation in human skeletal muscle. Further studies by which AMPKα1-isoform-specific signaling is directly linked to NO action are warranted.

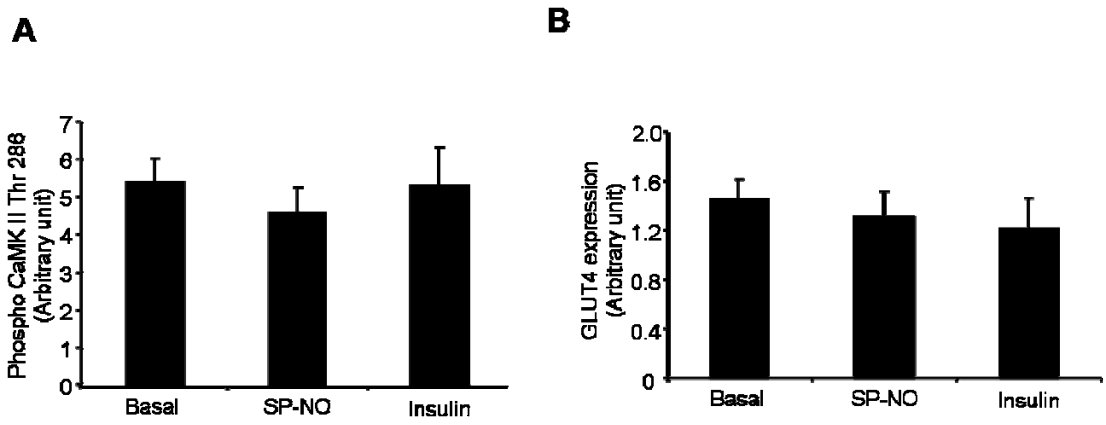

Figure 12. Effect of an NO donor on CaMKII phosphorylation (A) and GLUT4 expression (B). Skeletal muscle strips from seven healthy subjects were incubated in the absence (basal) or presence of either Spermine NONOate (NO donor; 5 mmol/l) or insulin (120 nmol/l). Quantification of mean±SEM arbitrary units for pCaMKII (Thr^{286}) (A) and GLUT4 protein expression.

Manipulation of the NO/cGMP signaling cascade may have clinical implications to enhance glucose uptake by an insulin-independent mechanism and potentially improve whole body glucose homeostasis in type 2 diabetic patients. NOS inhibition reduces glucose uptake during exercise in type 2 diabetic patients to a greater extent than in control subjects (Kingwell, 2002). Nevertheless, the NO donor-induced responses were identical in primary human skeletal muscle cells derived from healthy and type 2 diabetic subjects (Henstridge, 2009). Although skeletal muscle produces NO and endogenous NO modulates skeletal muscle function, the physiological importance of NO action and its role in glucose metabolism needs further attention.

5 SUMMARY

- We provide the evidence that AMPK is a negative regulator of mTOR signaling Moreover, the inhibitory effects of AMPK on insulin-induced mTOR signaling are mediated via functional α2 and γ3 subunits of AMPK.
- Supra-physiological concentrations of leucine induced insulin resistance via S6K1 in human muscle cells, suggesting role of S6K1 in the modulation by which nutrient overload causes insulin resistance.
- Exercise phosphorylates several proteins at a 'Phospho-Akt-substrate' motif. Proteins with a molecular weight of 160 and 300 kDa were indentified as AS160 (TBC1D4) and filamin A, respectively.
- AS160 (TBC1D4) and filamin A are phosphorylated in an exercise-dependent manner. Endurance exercise leads to a robust increase in Akt Ser^{473} phosphorylation, with a concomitant increase in AS160 (TBC1D4) and filamin A phosphorylation, while resistance exercise was without any effect. Filamin A and filamin C are stimulated in an exercise-and insulin-specific manner, respectively.
- Hypoxia-mediated glucose transport in EDL muscle requires the presence of a functional γ3 subunit of AMPK. Hypoxia-induced glucose transport is mediated via CaMK and AMPK pathways and cross-talk between AMPK and CaMK exit.
- The NO donor, Spermine NONOate promotes glucose transport in isolated human skeletal muscle, possibly via increases in cGMP levels and AMPKα1-isoform-specific activation.

6 CONCLUSION AND FUTURE PERSPECTIVES

In this thesis, the evolving evidence concerning environmental inputs, including nutrient availability, exercise, stress responses and pathways that sense nutrient and energy status on the canonical insulin signaling cascades is evaluated (Figure 13).

In primary human skeletal muscle cells, leucine activates mTOR signaling in time and dose-dependent manner. Supra-physiological concentration of leucine (5 mM) induces insulin resistance via a S6K1-mediated negative feedback mechanism. In response to excessive amino acids, hyperactivation of S6K1 induces insulin resistance via changes in the state of IRS-1 phosphorylation, localization, and/or degradation (Adochio, 2009, Tremblay, 2001). Our result, including the determination of IRS-1 expression and phosphorylation at several serine residues, does not provide conclusive evidence to implicate a role for IRS-1 in leucine action. Rather, our results suggest that the leucine-induced activation of S6K1 impairs insulin action at the level of Akt phosphorylation, glucose uptake and glycogen synthesis. Thus, S6K1 plays an direct and essential role in regulation of skeletal muscle glucose metabolism. A direct interaction between S6K1-IRS-1 in primary human skeletal muscle culture remains to be demonstrated. In C2C12 myoblast, leucine activates mTOR signaling via inhibition of AMPK (Du, 2007). Whether this also occurs in *in vivo* systems and in human skeletal muscle cultures has yet be determined.

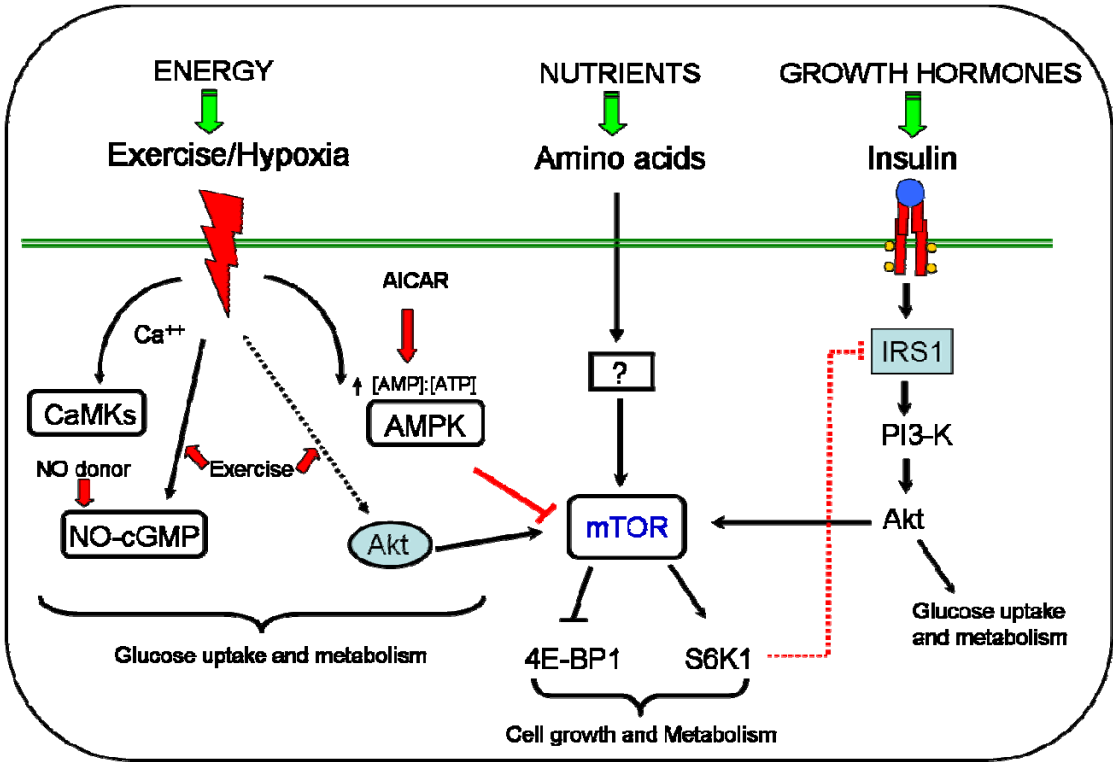

Figure 13. Summary of the signaling pathways activated in response to nutrients, energy-status and growth factors, as investigates in this thesis work.

In mouse EDL muscle, both hypoxia and AICAR activate AMPK. Complexes containing α2 and γ3 subunits of AMPK are required for the inhibitory effects on insulin-induced mTOR signaling, supporting the essential role of AMPK α2 and γ3

subunits in the regulation of mTOR signaling. However, the AICAR-induced inhibitory effect on insulin-mediated mTOR signaling was incompletely prevented in EDL muscle from α2 AMPK KO and γ3 AMPK KO. Thus AMPK heterotrimers containing α1 and γ1 subunits may play a role and require further investigation. Resistance exercise-induced hypertrophy is mediated in part via Akt (Leger, 2006).Whether endurance exercise also a lead to a decrease in skeletal muscle mass via AMPK is currently unknown. The temporal relationship between Akt and AMPK may be central to the regulation of mTOR signaling and the control of skeletal muscle mass. The expression the γ3 subunit of AMPK is important for hypoxia-induced glucose transport because this response is partially impaired in EDL muscle from γ3 AMPK KO mice. Unlike AICAR, hypoxia-induced AMPK signaling is not impaired in EDL muscle from γ3 AMPK KO mice, indicating a stimuli-specific activation of AMPK heterotrimers. Hypoxia-induced glucose transport was uncoupled from phosphorylation of TBC1D1/D4. Thus in response to hypoxia the γ1 associated AMPK complexes appear to be responsible for phosphorylation of TBC1D4 and TBC1D1, since the exercise-induced phosphorylation of TBC1D4 (AS160) in humans was correlated with the activation pattern of the α2β2γ1, but not the α2β2γ3 AMPK complex (Treebak, 2007). Hypoxia-responsive AMPK heterotrimers are yet to be identified. The physiological roles of AMPK heterotrimers containing γ1, β1 and β2 subunits have yet to be explored in depth. Compounded KO mice, where combinations of different catalytic and regulatory subunits of AMPK have been depleted will be required to reveal the definite roles of each heterotrimer in the regulation of skeletal muscle metabolism.

Hypoxia also increases glucose uptake via activation of CaMK signaling. This thesis work shows that there is a clear interaction between AMPK and Ca^{2+} signaling, since KN-93 treatment had a greater inhibition of hypoxia-induced glucose transport in skeletal muscle from γ3AMPK KO mice. Whether the hypoxia-induced activation of Ca^{2+} signaling lies upstream or downstream of AMPK is currently unknown. Interaction of CaMK with AMPK is likely to be heterotrimer specific. Through the use of mouse models where CaMK signaling is impaired, the cross-talk between AMPK and CaMK signaling can be further elucidated. Clearly further work is needed to investigate these mechanisms.

Whether exercise activates Akt is controversial issue. The PAS antibody is highly useful for the identification of novel phosphoproteins involved in insulin-and exercise mediated signaling cascades. Even though a role for AMPK is appreciated in exercise-induced signaling pathways, AMPK is not activated in skeletal muscle after acute endurance exercise in people with a history of endurance exercise training. Neither endurance nor resistance exercise resulted in activation of Akt; however endurance exercise increased phosphorylation of Akt Ser^{473}, AS160 (TBC1D4) and filamin A. PAS pp150 responded to both forms of exercise and this phosphorylation is likely to be TBC1D1. However, identity of pp150 and several other exercise responsive PAS proteins needs to be confirmed. Since the PAS antibody can recognize substrates that belong to a family of closely related kinases including Akt, AMPK, aPKC and CaMK, it is likely that the effect of exercise on phosphorylation of PAS proteins is mediated by more than one kinase. The identification of specific substrates for these novel protein kinases will provide a better understanding of exercise-induced metabolic and biological events in skeletal muscle.

The role of NO-cGMP activation in the effect of exercise-induced glucose transport is incompletely resolved. In human skeletal muscle, the NO-induced increase

in glucose transport was associated with an increase in cGMP levels and α1 associated AMPK activity. Whether the increased in cGMP levels is sufficient to activate α1 containing AMPK heterotrimer is an unresolved question. Through the use of mouse models where AMPK signaling is impaired the effect of NO on AMPK and the role of this signaling pathway in skeletal muscle glucose uptake can be better resolved. Another issue that needs to be addressed in future work is whether AMPK lies upstream or downstream of NO-cGMP signaling. Thus nutrient, energy, and growth factor sensing pathways form highly integrated networks that control several biological processes.

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8 REFERENCES

Abraham, R. T. 2005. TOR signaling: an odyssey from cellular stress to the cell growth machinery. *Curr Biol,* **15,** R139-141.

Adochio, R., Leitner, J. W., Hedlund, R. & Draznin, B. 2009. Rescuing 3T3-L1 adipocytes from insulin resistance induced by stimulation of Akt-mammalian target of rapamycin/p70 S6 kinase (S6K1) pathway and serine phosphorylation of insulin receptor substrate-1: effect of reduced expression of p85alpha subunit of phosphatidylinositol 3-kinase and S6K1 kinase. *Endocrinology,* **150,** 1165-1173.

Al-Khalili, L., Cartee, G. D. & Krook, A. 2003a. RNA interference-mediated reduction in GLUT1 inhibits serum-induced glucose transport in primary human skeletal muscle cells. *Biochem Biophys Res Commun,* **307,** 127-132.

Al-Khalili, L., Chibalin, A. V., Kannisto, K., Zhang, B. B., Permert, J., Holman, G. D., E, E., Ding, V. D. H., Zierath, J. R. & Krook, A. 2003b. Insulin action in cultured human skeletal muscle cells during differentiation: Assessment of cell surface GLUT4 and GLUT1 content. *Cell Mol Life Sci,* **60,** 991-998.

Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P. & Hemmings, B. A. 1996a. Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J,* **15,** 6541-5651.

Alessi, D. R., Caudwell, F. B., Andjelkovic, M., Hemmings, B. A. & Cohen, P. 1996b. Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. *FEBS Lett,* **399,** 333-338.

Anthony, J. C., Anthony, T. G., Kimball, S. R. & Jefferson, L. S. 2001. Signaling pathways involved in translational control of protein synthesis in skeletal muscle by leucine. *J Nutr,* **131,** 856S-860S.

Aoki, M., Blazek, E. & Vogt, P. K. 2001. A role of the kinase mTOR in cellular transformation induced by the oncoproteins P3k and Akt. *Pro Natl Acad Sci U S A,* **98,** 136-141.

Armstrong, J. L., Bonavaud, S. M., Toole, B. J. & Yeaman, S. J. 2001. Regulation of Glycogen Synthesis by Amino Acids in Cultured Human Muscle Cells. *J. Biol. Chem.,* **276,** 952-956.

Azevedo, J. L., Jr., Carey, J. O., Pories, W. J., Morris, P. G. & Dohm, G. L. 1995. Hypoxia stimulates glucose transport in insulin-resistant human skeletal muscle. *Diabetes,* **44,** 695-698.

Bai, L., Wang, Y., Fan, J., Chen, Y., Ji, W., Qu, A., Xu, P., James, D. E. & Xu, T. 2007. Dissecting multiple steps of GLUT4 trafficking and identifying the sites of insulin action. *Cell Metab,* **5,** 47-57.

Balon, T. W. & Nadler, J. L. 1994. Nitric oxide release is present from incubated skeletal muscle preparations. *J Appl Physiol,* **77,** 2519-1521.

Balon, T. W. & Nadler, J. L. 1997. Evidence that nitric oxide increases glucose transport in skeletal muscle. *J Appl Physiol,* **82,** 359-363.

Bandyopadhyay, G., Standaert, M. L., Zhao, L., Yu, B., Avignon, A., Galloway, L., Karnam, P., Moscat, J. & Farese, R. V. 1997. Activation of protein kinase C (alpha, beta, and zeta) by insulin in 3T3/L1 cells. Transfection studies suggest a role for PKC-zeta in glucose transport. *J. Biol. Chem.,* **272,** 2551-2558.

Barnes, B. R., Marklund, S., Steiler, T. L., Walter, M., Hjalm, G., Amarger, V., Mahlapuu, M., Leng, Y., Johansson, C., Galuska, D., Lindgren, K., Abrink, M., Stapleton, D., Zierath, J. R. & Andersson, L. 2004. The 5'-AMP-activated protein kinase {gamma}3 isoform has a key role in carbohydrate and lipid metabolism in glycolytic skeletal muscle. *J. Biol. Chem.,* **279,** 38441-38447.

Baum, J. I., O'Connor, J. C., Seyler, J. E., Anthony, T. G., Freund, G. G. & Layman, D. K. 2005. Leucine reduces the duration of insulin-induced PI 3-kinase activity in rat skeletal muscle. *Am J Physiol Endocrinol Metab,* **288,** E86-91.

Bedard, S., Marcotte, B. & Marette, A. 1997. Cytokines modulate glucose transport in skeletal muscle by inducing the expression of inducible nitric oxide synthase. *Biochemical J,* **325,** 487-493.

Bell, G. I., Murray, J. C., Nakamura, Y., Kayano, T., Eddy, R. L., Fan, Y. S., Byers, M. G. & Shows, T. B. 1989. Polymorphic human insulin-responsive glucose-transporter gene on chromosome 17p13. *Diabetes,* **38,** 1072-1075.

Bergeron, R., Ren, J. M., Cadman, K. S., Moore, I. K., Perret, P., Pypaert, M., Young, L. H., Semenkovich, C. F. & Shulman, G. I. 2001. Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. *Am J Physiol Endocrinol Metab,* **281,** E1340-1346.

Bernards, A. 2003. GAPs galore! A survey of putative Ras superfamily GTPase activating proteins in man and Drosophila. *Biochimica et Biophysica Acta,* **1603,** 47-82.

Beugnet, A., Tee, A. R., Taylor, P. M. & Proud, C. G. 2003. Regulation of targets of mTOR (mammalian target of rapamycin) signalling by intracellular amino acid availability. *Biochem J.,* **372,** 555-566.

Birk, J. B. & Wojtaszewski, J. F. 2006. Predominant alpha2/beta2/gamma3 AMPK activation during exercise in human skeletal muscle. *J Physiol,* **577,** 1021-1032.

Bjornholm, M., Kawano, Y., Lehtihet, M. & Zierath, J. R. 1997. Insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activity in skeletal muscle from NIDDM subjects after in vivo insulin stimulation. *Diabetes,* **46,** 524-527.

Bodine, S. C., Stitt, T. N., Gonzalez, M., Kline, W. O., Stover, G. L., Bauerlein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J. C., Glass, D. J. & Yancopoulos, G. D. 2001. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nae Cell Biol,* **3,** 1014-1019.

Bolster, D. R., Crozier, S. J., Kimball, S. R. & Jefferson, L. S. 2002. AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. *J Biol Chem,* **277,** 23977-23980.

Bolster, D. R., Jefferson, L. S. & Kimball, S. R. 2004. Regulation of protein synthesis associated with skeletal muscle hypertrophy by insulin-, amino acid- and exercise-induced signalling. *Proc Nutr Soc,* **63,** 351-356.

Bonen, A., Dohm, G. L. & van Loon, L. J. 2006. Lipid metabolism, exercise and insulin action. *Essays Biochem,* **42,** 47-59.

Bouzakri, K., Roques, M., Gual, P., Espinosa, S., Guebre-Egziabher, F., Riou, J. P., Laville, M., Le Marchand-Brustel, Y., Tanti, J. F. & Vidal, H. 2003. Reduced activation of phosphatidylinositol-3 kinase and increased serine 636 phosphorylation of insulin receptor substrate-1 in primary culture of skeletal muscle cells from patients with type 2 diabetes. *Diabetes,* **52,** 1319-1325.

Bradley, S. J., Kingwell, B. A. & McConell, G. K. 1999. Nitric oxide synthase inhibition reduces leg glucose uptake but not blood flow during dynamic exercise in humans. *Diabetes,* **48,** 1815-1821.

Brown, E. J., Albers, M. W., Shin, T. B., Ichikawa, K., Keith, C. T., Lane, W. S. & Schreiber, S. L. 1994. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature,* **369,** 756-758.

Browne, G. J. & Proud, C. G. 2004. A novel mTOR-regulated phosphorylation site in elongation factor 2 kinase modulates the activity of the kinase and its binding to calmodulin. *Mol Cell Biol,* **24,** 2986-1997.

Brozinick Jr, J. T. & Birnbaum, M. J. 1998. Insulin, but Not Contraction, Activates Akt/PKB in Isolated Rat Skeletal Muscle. *J. Biol. Chem.,* **273,** 14679-14682.

Brozinick, J. T., Jr., Etgen, G. J., Jr., Yaspelkis, B. B., 3rd & Ivy, J. L. 1994. Glucose uptake and GLUT-4 protein distribution in skeletal muscle of the obese Zucker rat. *Am J Physiol,* **267,** R236-243.

Brunn, G. J., Hudson, C. C., Sekulic, A., Williams, J. M., Hosoi, H., Houghton, P. J., Lawrence, J. C., Jr. & Abraham, R. T. 1997. Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. *Science,* **277,** 99-101.

Bruss, M. D., Arias, E. B., Lienhard, G. E. & Cartee, G. D. 2005. Increased Phosphorylation of Akt Substrate of 160 kDa (AS169) in rat skeletal muscle in response to insulin or contractile activity. *Diabetes,* **54,** 41-50.

Bryant, N. J., Govers, R. & James, D. E. 2002. Regulated transport of the glucose transporter GLUT4. *Nat Rev Mol Cell Biol,* **3,** 267-277.

Burnett, P. E., Barrow, R. K., Cohen, N. A., Snyder, S. H. & Sabatini, D. M. 1998. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Pro Natl Acad Sci U S A,* **95,** 1432-1437.

Buse, M. G. & Reid, S. S. 1975. Leucine. A possible regulator of protein turnover in muscle. *J Clin Invest,* **56,** 1250-1261.

Carling, D. 2004. The AMP-activated protein kinase cascade--a unifying system for energy control. *Trends Biochem Sci,* **29,** 18-24.

Carroll, R. C. & Gerrard, J. M. 1982. Phosphorylation of platelet actin-binding protein during platelet activation. *Blood,* **59,** 466-471.

Cartee, G. D. & Dean, D. J. 1994. Glucose transport with brief dietary restriction: heterogenous responses in muscles. *Am J Physiol,* **266,** E946-952.

Cartee, G. D., Douen, A. G., Ramlal, T., Klip, A. & Holloszy, J. O. 1991. Stimulation of glucose transport in skeletal muscle by hypoxia. *J Appl Physiol,* **70,** 1593-1600.

Cartee, G. D. & Holloszy, J. O. 1990. Exercise increases susceptibility of muscle glucose transport to activation by various stimuli. *Am J Physiol,* **258,** E390-393.

Cartee, G. D., Young, D. A., Sleeper, M. D., Zierath, J., Wallberg-Henriksson, H. & Holloszy, J. O. 1989. Prolonged increase in insulin-stimulated glucose transport in muscle after exercise. *Am J Physiol,* **256,** E494-499.

Chavez, J. A., Roach, W. G., Keller, S. R., Lane, W. S. & Lienhard, G. E. 2008. Inhibition of GLUT4 translocation by Tbc1d1, a Rab GTPase-activating protein abundant in skeletal muscle, is partially relieved by AMP-activated protein kinase activation. *J Biol Chem,* **283,** 9187-9195.

Chen, S., Murphy, J., Toth, R., Campbell, D. G., Morrice, N. A. & Mackintosh, C. 2008. Complementary regulation of TBC1D1 and AS160 by growth factors, insulin and AMPK activators. *Biochem J,* **409,** 449-459.

Chen, Z. P., McConell, G. K., Michell, B. J., Snow, R. J., Canny, B. J. & Kemp, B. E. 2000. AMPK signaling in contracting human skeletal muscle: acetyl-CoA carboxylase and NO synthase phosphorylation. *Am J Physiol Endocrinol Metab,* **279,** E1202-1206.

Chen, Z. P., Mitchelhill, K. I., Michell, B. J., Stapleton, D., Rodriguez-Crespo, I., Witters, L. A., Power, D. A., Ortiz de Montellano, P. R. & Kemp, B. E. 1999. AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Lett,* **443,** 285-289.

Cheng, S. W., Fryer, L. G., Carling, D. & Shepherd, P. R. 2004. Thr2446 is a novel mammalian target of rapamycin (mTOR) phosphorylation site regulated by nutrient status. *J Biol Chem,* **279,** 15719-15722.

Christ-Roberts, C. Y., Pratipanawatr, T., Pratipanawatr, W., Berria, R., Belfort, R. & Mandarino, L. J. 2003. Increased insulin receptor signaling and glycogen synthase activity contribute to the synergistic effect of exercise on insulin action. *J Appl Physiol,* **95,** 2519-2529.

Coffey, V. G., Zhong, Z., Shield, A., Canny, B. J., Chibalin, A. V., Zierath, J. R. & Hawley, J. A. 2006. Early signaling responses to divergent exercise stimuli in skeletal muscle from well-trained humans. *FASEB J,* **20,** 190-192.

Constable, S. H., Favier, R. J., Cartee, G. D., Young, D. A. & Holloszy, J. O. 1988. Muscle glucose transport: interactions of in vitro contractions, insulin, and exercise. *J Appl Physiol,* **64,** 2329-2332.

Cortez, M. Y., Torgan, C. E., Brozinick, J. T., Jr. & Ivy, J. L. 1991. Insulin resistance of obese Zucker rats exercise trained at two different intensities. *Am J Physiol,* **261,** E613-619.

Crespo, J. L. & Hall, M. N. 2002. Elucidating TOR signaling and rapamycin action: lessons from Saccharomyces cerevisiae. *Microbiol Mol Biol Rev,* **66,** 579-591.

Crozier, S. J., Kimball, S. R., Emmert, S. W., Anthony, J. C. & Jefferson, L. S. 2005. Oral leucine administration stimulates protein synthesis in rat skeletal muscle. *J Nutr,* **135,** 376-382.

Cushman, S. W., Goodyear, L. J., Pilch, P. F., Ralston, E., Galbo, H., Ploug, T., Kristiansen, S. & Klip, A. 1998. Molecular mechanisms involved in GLUT4 translocation in muscle during insulin and contraction stimulation. *Adv Exp Med Biol,* **441,** 63-71.

Dann, S. G., Selvaraj, A. & Thomas, G. 2007. mTOR Complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer. *Trends Mol Med,* **13,** 252-259.

Davies, S. P., Helps, N. R., Cohen, P. T. & Hardie, D. G. 1995. 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC. *FEBS Lett,* **377,** 421-415.

DeFronzo, R. A., Gunnarsson, R., Bjorkman, O., Olsson, M. & Wahren, J. 1985. Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. *J Clin Invest,* **76,** 149-155.

Dennis, P. B., Jaeschke, A., Saitoh, M., Fowler, B., Kozma, S. C. & Thomas, G. 2001. Mammalian TOR: a homeostatic ATP sensor. *Science,* **294,** 1102-1105.

Deshmukh, A. S., Hawley, J. A. & Zierath, J. R. 2008. Exercise-induced phospho-proteins in skeletal muscle. *Int J Obes,* **32 Suppl 4,** S18-23.

Doi, M., Yamaoka, I., Fukunaga, T. & Nakayama, M. 2003. Isoleucine, a potent plasma glucose-lowering amino acid, stimulates glucose uptake in C2C12 myotubes. *Biochem Biophys Res Commun.,* **312,** 1111-1117.

Du, M., Shen, Q. W., Zhu, M. J. & Ford, S. P. 2007. Leucine stimulates mammalian target of rapamycin signaling in C2C12 myoblasts in part through inhibition of adenosine monophosphate-activated protein kinase. *J Anim Sci,* **85,** 919-927.

Dubbelhuis, P. F. & Meijer, A. J. 2002. Hepatic amino acid-dependent signaling is under the control of AMP-dependent protein kinase. *FEBS Lett,* **521,** 39-42.

Dugani, C. B. & Klip, A. 2005. Glucose transporter 4: cycling, compartments and controversies. *EMBO Reports,* **6,** 1137-1142.

Durham, W. J., Yeckel, C. W., Miller, S. L., Gore, D. C. & Wolfe, R. R. 2003. Exogenous nitric oxide increases basal leg glucose uptake in humans. *Metab Clin Experim,* **52,** 662-665.

Eriksson, J., Koranyi, L., Bourey, R., Schalin-Jantti, C., Widen, E., Mueckler, M., Permutt, A. M. & Groop, L. C. 1992. Insulin resistance in type 2 (non-insulin-dependent) diabetic patients and their relatives is not associated with a defect in the expression of the insulin-responsive glucose transporter (GLUT-4) gene in human skeletal muscle.*Diabetologia,* **35,** 143-147.

Etgen, G. J., Jr., Fryburg, D. A. & Gibbs, E. M. 1997. Nitric oxide stimulates skeletal muscle glucose transport through a calcium/contraction- and phosphatidylinositol-3-kinase-independent pathway. *Diabetes,* **46,** 1915-1919.

Ezell, D. M., Geiselman, P. J., Anderson, A. M., Dowdy, M. L., Womble, L. G., Greenway, F. L. & Zachwieja, J. J. 1999. Substrate oxidation and availability during acute exercise in non-obese, obese, and post-obese sedentary females. *Int J Obes Relat Metab Disord,* **23,** 1047-1056.

Felig, P. 1975. Amino Acid Metabolism in Man. *Annu Rev Biochem,* **44,** 933-955.

Felig, P., Marliss, E. & Cahill, G. F., Jr. 1969. Plasma amino acid levels and insulin secretion in obesity. *N Engl J Med,* **281,** 811-816.

Flakoll, P. J., Wentzel, L. S., Rice, D. E., Hill, J. O. & Abumrad, N. N. 1992. Short-term regulation of insulin-mediated glucose utilization in four-day fasted human volunteers: role of amino acid availability. *Diabetologia,* **35,** 357-366.

Fried, S. K. & Watford, M. 2007. Leucing weight with a futile cycle. *Cell Metab,* **6,** 155-156.

Funai, K. & Cartee, G. D. 2009. Inhibition of contraction-stimulated AMP-activated protein kinase inhibits contraction-stimulated increases in PAS-TBC1D1 and glucose transport without altering PAS-AS160 in rat skeletal muscle. *Diabetes,* **58,** 1096-1104.

Garcia-Roves, P. M., Osler, M. E., Holmstrom, M. H. & Zierath, J. R. 2008. Gain-of-function R225Q mutation in AMP-activated protein kinase gamma3 subunit increases mitochondrial biogenesis in glycolytic skeletal muscle. *J Biol Chem,* **283,** 35724-35734.

Geraghty, K. M., Chen, S., Harthill, J. E., Ibrahim, A. F., Toth, R., Morrice, N. A., Vandermoere, F., Moorhead, G. B., Hardie, D. G. & MacKintosh, C. 2007. Regulation of multisite phosphorylation and 14-3-3 binding of AS160 in response to IGF-1, EGF, PMA and AICAR. *Biochem J,* **407,** 231-241.

Glass, D. J. 2003. Molecular mechanisms modulating muscle mass. *Trends Mol Med,* **9,** 344-350.

Glass, D. J. 2005. Skeletal muscle hypertrophy and atrophy signaling pathways. *Int J Biochem Cell Biol,* **37,** 1974-1984.

Glogauer, M., Arora, P., Chou, D., Janmey, P. A., Downey, G. P. & McCulloch, C. A. 1998. The role of actin-binding protein 280 in integrin-dependent mechanoprotection. *J Biol Chem,* **273,** 1689-1698.

Goodpaster, B. H., Wolfe, R. R. & Kelley, D. E. 2002. Effects of obesity on substrate utilization during exercise. *Obes Res,* **10,** 575-84.

Guigas, B., Taleux, N., Foretz, M., Detaille, D., Andreelli, F., Viollet, B. & Hue, L. 2007. AMP-activated protein kinase-independent inhibition of hepatic mitochondrial oxidative phosphorylation by AICA riboside. *Biochem J,* **404,** 499-507.

Hajduch, E., Litherland, G. J. & Hundal, H. S. 2001. Protein kinase B (PKB/Akt)--a key regulator of glucose transport? *FEBS Lett,* **492,** 199-203.

Hara, K., Yonezawa, K., Weng, Q.-P., Kozlowski, M. T., Belham, C. & Avruch, J. 1998. Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J Biol Chem,* **273,** 14484-14494.

Hardie, D. G. 2004. The AMP-activated protein kinase pathway--new players upstream and downstream. *J Cell Sci,* **117,** 5479-5487.

Harrington, L. S., Findlay, G. M., Gray, A., Tolkacheva, T., Wigfield, S., Rebholz, H., Barnett, J., Leslie, N. R., Cheng, S., Shepherd, P. R., Gout, I., Downes, C. P. & Lamb, R. F. 2004. The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. *J Cell Biol,* **166,** 213-223.

Harris, T. E. & Lawrence, J. C., Jr. 2003. TOR signaling. *Science's Stke [Electronic Resource]: Signal Transduction Knowledge Environment,* **2003,** re15.

Hawley, J. A. & Noakes, T. D. 1992. Peak power output predicts maximal oxygen uptake and performance time in trained cyclists. *Eur J Appl Physiol Occup Physiol,* **65,** 79-83.

Hawley, S. A., Pan, D. A., Mustard, K. J., Ross, L., Bain, J., Edelman, A. M., Frenguelli, B. G. & Hardie, D. G. 2005. Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metab,* **2,** 9-19.

Hayashi, T., Hirshman, M. F., Kurth, E. J., Winder, W. W. & Goodyear, L. J. 1998. Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. *Diabetes,* **47,** 1369-1373.

Heitman, J., Movva, N. R. & Hall, M. N. 1991. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science,* **253,** 905-909.

Henriksen, E. J. 2002. Invited review: Effects of acute exercise and exercise training on insulin resistance. *J Appl Physiol,* **93,** 788-796.

Henriksen, E. J., Rodnick, K. J. & Holloszy, J. O. 1989. Activation of glucose transport in skeletal muscle by phospholipase C and phorbol ester. Evaluation of the regulatory roles of protein kinase C and calcium.[erratum appears in J Biol Chem 1990 Apr 5;265(10):5917]. *J Biol Chem,* **264,** 21536-21543.

Henriksen, J. E., Alford, F., Handberg, A., Vaag, A., Ward, G. M., Kalfas, A. & Beck-Nielsen, H. 1994. Increased glucose effectiveness in normoglycemic but insulin-resistant relatives of patients with non-insulin-dependent diabetes mellitus. A novel compensatory mechanism. *J Clin Invest,* **94,** 1196-1204.

Henstridge, D. C., Drew, B. G., Formosa, M. F., Natoli, A. K., Cameron-Smith, D., Duffy, S. J. & Kingwell, B. A. 2009. The effect of the nitric oxide donor sodium nitroprusside on glucose uptake in human primary skeletal muscle cells. *Nitric Oxide,* **21,** 126-131.

Higaki, Y., Hirshman, M. F., Fujii, N. & Goodyear, L. J. 2001. Nitric oxide increases glucose uptake through a mechanism that is distinct from the insulin and contraction pathways in rat skeletal muscle. *Diabetes,* **50,** 241-247.

Hill, M. M., Clark, S. F. & James, D. E. 1997. Insulin-regulatable phosphoproteins in 3T3-L1 adipocytes form detergent-insoluble complexes not associated with caveolin. *Electrophoresis,* **18,** 2629-2637.

Holloszy, J. O. 2003. A forty-year memoir of research on the regulation of glucose transport into muscle. *Am J Physiol Endocrinol Metab,* **284,** E453-467.

Holloszy, J. O. 2005. Exercise-induced increase in muscle insulin sensitivity. *J Appl Physiol,* **99,** 338-343.

Holloszy, J. O., Constable, S. H. & Young, D. A. 1986. Activation of glucose transport in muscle by exercise. *Diabetes Metab Rev,* **1,** 409-423.

Holloszy, J. O. & Hansen, P. A. 1996. Regulation of glucose transport into skeletal muscle. *Rev Physiol Biochem Pharmacol,* **128,** 99-193.

Holloszy, J. O. & Narahara, H. T. 1967. Enhanced permeability to sugar associated with muscle contraction. Studies of the role of Ca^{++}. *J Gen Physiol,* **50,** 551-562.

Holmes, B. F., Kurth-Kraczek, E. J. & Winder, W. W. 1999. Chronic activation of 5'-AMP-activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle. *J Appl Physiol,* **87,** 1990-1995.

Hong, S. O. & Layman, D. K. 1984. Effects of leucine on in vitro protein synthesis and degradation in rat skeletal muscles. *J Nutr,* **114,** 1204-1212.

Horman, S., Browne, G., Krause, U., Patel, J., Vertommen, D., Bertrand, L., Lavoinne, A., Hue, L., Proud, C. & Rider, M. 2002. Activation of AMP-activated protein kinase leads to the phosphorylation of elongation factor 2 and an inhibition of protein synthesis. *Curr Biol,* **12,** 1419-1423.

Hurley, B. F. & Roth, S. M. 2000. Strength training in the elderly: effects on risk factors for age-related diseases. *Sports Med,* **30,** 249-268.

Hurley, R. L., Anderson, K. A., Franzone, J. M., Kemp, B. E., Means, A. R. & Witters, L. A. 2005. The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases. *J Biol Chem,* **280,** 29060-29066.

Inoki, K., Zhu, T. & Guan, K. L. 2003. TSC2 mediates cellular energy response to control cell growth and survival. *Cell,* **115,** 577-590.

Jefferies, H. B., Fumagalli, S., Dennis, P. B., Reinhard, C., Pearson, R. B. & Thomas, G. 1997. Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k. *EMBO J,* **16,** 3693-3704.

Jensen, J., Sharikabad, M. N., Ostbye, K. M., Melien, O. & Brors, O. 2003. Evidence that nitroprusside stimulates glucose uptake in isolated rat cardiomyocytes via mitogen-activated protein kinase. *Arch Physiol Biochem,* **111,** 239-245.

Jensen, T. E., Rose, A. J., Hellsten, Y., Wojtaszewski, J. F. & Richter, E. A. 2007a. Caffeine-induced Ca(2+) release increases AMPK-dependent glucose uptake in rodent soleus muscle. *Am J Physiol Endocrinol Metab,* **293,** E286-292.

Jensen, T. E., Rose, A. J., Jorgensen, S. B., Brandt, N., Schjerling, P., Wojtaszewski, J. F. & Richter, E. A. 2007b. Possible CaMKK-dependent regulation of AMPK phosphorylation and glucose uptake at the onset of mild tetanic skeletal muscle contraction. *Am J Physiol Endocrinol Metab,* **292,** E1308-317.

Jessen, N. & Goodyear, L. J. 2005. Contraction signaling to glucose transport in skeletal muscle. *J Appl Physiol,* **99,** 330-337.

Jorgensen, S. B., Treebak, J. T., Viollet, B., Schjerling, P., Vaulont, S., Wojtaszewski, J. F. & Richter, E. A. 2007. Role of AMPKalpha2 in basal, training-, and AICAR-induced GLUT4, hexokinase II, and mitochondrial protein expression in mouse muscle. *Am J Physiol Endocrinol Metab,* **292,** E331-339.

Jorgensen, S. B., Viollet, B., Andreelli, F., Frosig, C., Birk, J. B., Schjerling, P., Vaulont, S., Richter, E. A. & Wojtaszewski, J. F. 2004. Knockout of the alpha2 but not alpha1 5'-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranosidebut not contraction-induced glucose uptake in skeletal muscle. *J Biol Chem,* **279,** 1070-1079.

Kahn, B. B., Alquier, T., Carling, D. & Hardie, D. G. 2005. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab,* **1,** 15-25.

Kane, S. & Lienhard, G. E. 2005. Calmodulin binds to the Rab GTPase activating protein required for insulin-stimulated GLUT4 translocation. *Biochem Biophys Res Commun,* **335,** 175-180.

Kane, S., Sano, H., Liu, S. C. H., Asara, J. M., Lane, W. S., Garner, C. C. & Lienhard, G. E. 2002. A Method to Identify Serine Kinase Substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. *J. Biol. Chem.,* **277,** 22115-22118.

Karlsson, H. K., Zierath, J. R., Kane, S., Krook, A., Lienhard, G. E. & Wallberg-Henriksson, H. 2005b. Insulin-stimulated phosphorylation of the Akt substrate AS160 is impaired in skeletal muscle of type 2 diabetic subjects. *Diabetes,* **54,** 1692-1697.

Kelley, D. E., Wing, R., Buonocore, C., Sturis, J., Polonsky, K. & Fitzsimmons, M. 1993. Relative effects of calorie restriction and weight loss in noninsulin-dependent diabetes mellitus. *J Clin Endocrinol Metab,* **77,** 1287-1293.

Kemp, B. E., Stapleton, D., Campbell, D. J., Chen, Z. P., Murthy, S., Walter, M., Gupta, A., Adams, J. J., Katsis, F., van Denderen, B., Jennings, I. G., Iseli, T., Michell, B. J. & Witters, L. A. 2003. AMP-activated protein kinase, super metabolic regulator. *Biochem Soc Trans,* **31,** 162-168.

Kimball, S. R. 2006. Interaction between the AMP-activated protein kinase and mTOR signaling pathways. *Med Sci Sports Exerc,* **38,** 1958-1964.

King, P. A., Betts, J. J., Horton, E. D. & Horton, E. S. 1993. Exercise, unlike insulin, promotes glucose transporter translocation in obese Zucker rat muscle. *Am J Physiol,* **265,** R447-452.

Kingwell, B. A., Formosa, M., Muhlmann, M., Bradley, S. J. & McConell, G. K. 2002. Nitric oxide synthase inhibition reduces glucose uptake during exercise in individuals with type 2 diabetes more than in control subjects. *Diabetes,* **51,** 2572-2580.

Knowler, W. C., Barrett-Connor, E., Fowler, S. E., Hamman, R. F., Lachin, J. M., Walker, E. A., Nathan, D. M. & Diabetes Prevention Program Research, G. 2002. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med,* **346,** 393-403.

Koistinen, H. A., Galuska, D., Chibalin, A. V., Yang, J., Zierath, J. R., Holman, G. D. & Wallberg-Henriksson, H. 2003. 5-amino-imidazole carboxamide riboside increases glucose transport and cell-surface GLUT4 content in skeletal muscle from subjects with type 2 diabetes. *Diabetes,* **52,** 1066-1072.

Kramer, H. F., Taylor, E. B., Witczak, C. A., Fujii, N., Hirshman, M. F. & Goodyear, L. J. 2007. The calmodulin-binding domain of AS160 regulates contraction- but not insulin-stimulated glucose uptake in skeletal muscle. *Diabetes, **56,** 2854-2862*.

Kramer, H. F., Witczak, C. A., Taylor, E. B., Fujii, N., Hirshman, M. F. & Goodyear, L. J. 2006. AS160 regulates insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle. *J Biol Chem,* **281,** 31478-31485.

Krause, U., Bertrand, L. & Hue, L. 2002. Control of p70 ribosomal protein S6 kinase and acetyl-CoA carboxylase by AMP-activated protein kinase and protein phosphatases in isolated hepatocytes. *Eur J Biochem,* **269,** 3751-3759.

Krebs, M., Brunmair, B., Brehm, A., Artwohl, M., Szendroedi, J., Nowotny, P., Roth, E., Furnsinn, C., Promintzer, M., Anderwald, C., Bischof, M. & Roden, M. 2007. The Mammalian target of rapamycin pathway regulates nutrient-sensitive glucose uptake in man. *Diabetes,* **56,** 1600-1607.

Krebs, M., Krssak, M., Bernroider, E., Anderwald, C., Brehm, A., Meyerspeer, M., Nowotny, P., Roth, E., Waldhausl, W. & Roden, M. 2002. Mechanism of Amino Acid-Induced Skeletal Muscle Insulin Resistance in Humans. *Diabetes,* **51,** 599-605.

Krisan, A. D., Collins, D. E., Crain, A. M., Kwong, C. C., Singh, M. K., Bernard, J. R. & Yaspelkis, B. B., III 2004. Resistance training enhances components of the insulin signaling cascade in normal and high-fat-fed rodent skeletal muscle. *J Appl Physiol,* **96,** 1691-1700.

Krook, A., Roth, R. A., Jiang, X. J., Zierath, J. R. & Wallberg-Henriksson, H. 1998. Insulin-stimulated Akt kinase activity is reduced in skeletal muscle from NIDDM subjects. *Diabetes,* **47,** 1281-1286.

Lee, A. D., Hansen, P. A. & Holloszy, J. O. 1995. Wortmannin inhibits insulin-stimulated but not contraction-stimulated glucose transport activity in skeletal muscle. *FEBS Lett,* **361,** 51-54.

Leger, B., Cartoni, R., Praz, M., Lamon, S., Deriaz, O., Crettenand, A., Gobelet, C., Rohmer, P., Konzelmann, M., Luthi, F. & Russell, A. P. 2006. Akt signalling through GSK-3beta, mTOR and Foxo1 is involved in human skeletal muscle hypertrophy and atrophy. *J Physiol,* **576,** 923-933.

Li, J. B. & Jefferson, L. S. 1978. Influence of amino acid availability on protein turnover in perfused skeletal muscle. *Biochimica et Biophysica Acta,* **544,** 351-359.

Li, Y., Inoki, K. & Guan, K. L. 2004. Biochemical and functional characterizations of small GTPase Rheb and TSC2 GAP activity. *Mol Cell Biol,* **24,** 7965-7975.

Lira, V. A., Soltow, Q. A., Long, J. H., Betters, J. L., Sellman, J. E. & Criswell, D. S. 2007. Nitric oxide increases GLUT4 expression and regulates AMPK signaling in skeletal muscle. *American Journal of Physiology - Endocrinology & Metabolism,* **293,** E1062-1068.

Liu, M. L., Olson, A. L., Moye-Rowley, W. S., Buse, J. B., Bell, G. I. & Pessin, J. E. 1992. Expression and regulation of the human GLUT4/muscle-fat facilitative glucose transporter gene in transgenic mice. *J Biol Chem,* **267,** 11673-11676.

Liu, Z., Jahn, L. A., Wei, L., Long, W. & Barrett, E. J. 2002. Amino acids stimulate translation initiation and protein synthesis through an Akt-independent pathway in human skeletal muscle. *J Clin Endocrinol Metab,* **87,** 5553-5558.

Long, Y. C. & Zierath, J. R. 2006. AMP-activated protein kinase signaling in metabolic regulation. *J. Clin. Invest.,* **116,** 1776-1783.

Lund, S., Pryor, P. R., Ostergaard, S., Schmitz, O., Pedersen, O. & Holman, G. D. 1998. Evidence against protein kinase B as a mediator of contraction-induced glucose transport and GLUT4 translocation in rat skeletal muscle. *FEBS Lett,* **425,** 472-474.

Mahlapuu, M., Johansson, C., Lindgren, K., Hjalm, G., Barnes, B. R., Krook, A., Zierath, J. R., Andersson, L. & Marklund, S. 2004. Expression profiling of the gamma-subunit isoforms of AMP-activated protein kinase suggests a major role for gamma3 in white skeletal muscle. *Am J Physiol Endocrinol Metab,* **286,** E194-200.

Markuns, J. F., Wojtaszewski, J. F. P. & Goodyear, L. J. 1999. Insulin and Exercise Decrease Glycogen Synthase Kinase-3 Activity by Different Mechanisms in Rat Skeletal Muscle. *J. Biol. Chem.,* **274,** 24896-24900.

McConell, G. K. & Kingwell, B. A. 2006. Does nitric oxide regulate skeletal muscle glucose uptake during exercise? *Exerc Sport Sci Rev,* **34,** 36-41.

McCurdy, C. E., Davidson, R. T. & Cartee, G. D. 2005. Calorie restriction increases the ratio of phosphatidylinositol 3-kinase catalytic to regulatory subunits in rat skeletal muscle. *Am J Physiol Endocrinol Metab,* **288,** E996-E1001.

Merrill, G. F., Kurth, E. J., Hardie, D. G. & Winder, W. W. 1997. AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. *Am J Physiol,* **273,** E1107-1112.

Miinea, C. P., Sano, H., Kane, S., Sano, E., Fukuda, M., Peranen, J., Lane, W. S. & Lienhard, G. E. 2005. AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. *Biochem J,* **391,** 87-93.

Moncada, S. & Higgs, A. 1993. The L-arginine-nitric oxide pathway. *N Eng J Med,* **329,** 2002-2012.

Mu, J., Brozinick, J. T., Jr., Valladares, O., Bucan, M. & Birnbaum, M. J. 2001. A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Mol Cell,* **7,** 1085-1094.

Murgia, M., Jensen, T. E., Cusinato, M., Garcia, M., Richter, E. A. & Schiaffino, S. 2009. Multiple signalling pathways redundantly control glucose transporter GLUT4 gene transcription in skeletal muscle. *J Physiol,* **587,** 4319-4327.

Murray, J. T., Campbell, D. G., Peggie, M., Mora, A. & Cohen, P. 2004. Identification of filamin C as a new physiological substrate of PKBalpha using KESTREL. *Biochem J,* **384,** 489-494.

Musi, N., Fujii, N., Hirshman, M. F., Ekberg, I., Froberg, S., Ljungqvist, O., Thorell, A. & Goodyear, L. J. 2001. AMP-activated protein kinase (AMPK) is activated in muscle of subjects with type 2 diabetes during exercise. *Diabetes,* **50,** 921-927.

Nader, G. A. & Esser, K. A. 2001. Intracellular signaling specificity in skeletal muscle in response to different modes of exercise. *J Appl Physiol,* **90,** 1936-1942.

Nair, K. S. & Short, K. R. 2005. Hormonal and signaling role of branched-chain amino acids. *J Nutr,* **135,** 1547S-1552S.

O'Connor, P. M., Kimball, S. R., Suryawan, A., Bush, J. A., Nguyen, H. V., Jefferson, L. S. & Davis, T. A. 2003. Regulation of translation initiation by insulin and amino acids in skeletal muscle of neonatal pigs. *Am J Physiol Endocrinol Metab,* **285,** E40-53.

Obata, T., Yaffe, M. B., Leparc, G. G., Piro, E. T., Maegawa, H., Kashiwagi, A., Kikkawa, R. & Cantley, L. C. 2000. Peptide and protein library screening defines optimal substrate motifs for AKT/PKB. *J Biol Chem,* **275,** 36108-36115.

Ohlstein, E. H., Wood, K. S. & Ignarro, L. J. 1982. Purification and properties of heme-deficient hepatic soluble guanylate cyclase: effects of heme and other factors on enzyme activation by NO, NO-heme, and protoporphyrin IX. *Arch Biochem Biophys,* **218,** 187-198.

Oldham, S., Montagne, J., Radimerski, T., Thomas, G. & Hafen, E. 2000. Genetic and biochemical characterization of dTOR, the Drosophila homolog of the target of rapamycin. *Genes Dev,* **14,** 2689-2694.

Patti, M.-E. 1999. Nutrient Modulation of Cellular Insulin Action. *Ann NY Acad Sci,* **892,** 187-203.

Pehmoller, C., Treebak, J. T., Birk, J. B., Chen, S., Mackintosh, C., Hardie, D. G., Richter, E. A. & Wojtaszewski, J. F. 2009. Genetic disruption of AMPK signaling abolishes both contraction- and insulin-stimulated TBC1D1 phosphorylation and 14-3-3 binding in mouse skeletal muscle. *Am J Physiol Endocrinol Metab,* **297,** E665-675.

Pfeffer, S. & Aivazian, D. 2004. Targeting Rab GTPases to distinct membrane compartments. *Nat Rev Mol Cell Biol,* **5,** 886-896.

Pisters, P. W., Restifo, N. P., Cersosimo, E. & Brennan, M. F. 1991. The effects of euglycemic hyperinsulinemia and amino acid infusion on regional and whole body glucose disposal in man. *Metabolism,* **40,** 59-65.

Ploug, T., van Deurs, B., Ai, H., Cushman, S. W. & Ralston, E. 1998. Analysis of GLUT4 distribution in whole skeletal muscle fibers: identification of distinct storage compartments that are recruited by insulin and muscle contractions. *J Cell Biol,* **142,** 1429-1446.

Proud, C. G. 2006. Regulation of protein synthesis by insulin. *Biochem SocTrans,* **34,** 213-216.

Proud, C. G. 2007. Signalling to translation: how signal transduction pathways control the protein synthetic machinery. *Biochem J,* **403,** 217-234.

Radimerski, T., Montagne, J., Rintelen, F., Stocker, H., van der Kaay, J., Downes, C. P., Hafen, E. & Thomas, G. 2002. dS6K-regulated cell growth is dPKB/dPI(3)K-independent, but requires dPDK1. *Nature Cell Biology,* **4,** 251-255.

Ramm, G. & James, D. E. 2005. GLUT4 trafficking in a test tube. *Cell Metab,* **2,** 150-152.

Reid, M. B. 1998. Role of nitric oxide in skeletal muscle: synthesis, distribution and functional importance. *Acta Physiol Scand,* **162,** 401-409.

Rennie, M. J., Bohe, J. & Wolfe, R. R. 2002. Latency, duration and dose response relationships of amino acid effects on human muscle protein synthesis. *J Nutr,* **132,** 3225S-3227S.

Roach, W. G., Chavez, J. A., Miinea, C. P. & Lienhard, G. E. 2007. Substrate specificity and effect on GLUT4 translocation of the Rab GTPase-activating protein Tbc1d1. *Biochem J,* **403,** 353-358.

Roberts, C. K., Barnard, R. J., Scheck, S. H. & Balon, T. W. 1997. Exercise-stimulated glucose transport in skeletal muscle is nitric oxide dependent. *Am J Physiol,* **273,** E220-E225.

Rolfe, D. F. & Brown, G. C. 1997. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiolog Rev,* **77,** 731-758.

Roy, D. & Marette, A. 1996. Exercise induces the translocation of GLUT4 to transverse tubules from an intracellular pool in rat skeletal muscle. *Biochem Biophys Res Comm,* **223,** 147-152.

Ruderman, N. B., Saha, A. K., Vavvas, D. & Witters, L. A. 1999. Malonyl-CoA, fuel sensing, and insulin resistance. *Am J Physiol,* **276,** E1-E18.

Ruvinsky, I. & Meyuhas, O. 2006. Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. *Trends Biochem Sci,* **31,** 342-348.

Sabatini, D. M., Erdjument-Bromage, H., Lui, M., Tempst, P. & Snyder, S. H. 1994. RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell,* **78,** 35-43.

Sakamoto, K., Arnolds, D. E. W., Ekberg, I., Thorell, A. & Goodyear, L. J. 2004. Exercise regulates Akt and glycogen synthase kinase-3 activities in human skeletal muscle. *Biochem Biophys Res Commun,* **319,** 419.

Sakamoto, K., Aschenbach, W. G., Hirshman, M. F. & Goodyear, L. J. 2003. Akt signaling in skeletal muscle: regulation by exercise and passive stretch. *Am J Physiol Endocrinol Metab,* **285,** E1081-1088.

Sakamoto, K., Hirshman, M. F., Aschenbach, W. G. & Goodyear, L. J. 2002. Contraction Regulation of Akt in Rat Skeletal Muscle. *J. Biol. Chem.,* **277,** 11910-11917.

Sakamoto, K. & Holman, G. D. 2008. Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic. *Am J Physiol Endocrinol Metab,* **295,** E29-37.

Sakamoto, K., McCarthy, A., Smith, D., Green, K. A., Grahame Hardie, D., Ashworth, A. & Alessi, D. R. 2005. Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. *EMBO J,* **24,** 1810-1820.

Saltiel, A. R. & Pessin, J. E. 2003. Insulin signaling in microdomains of the plasma membrane. *Traffic,* **4,** 711-716.

Sano, H., Kane, S., Sano, E., Miinea, C. P., Asara, J. M., Lane, W. S., Garner, C. W. & Lienhard, G. E. 2003. Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *J Biol Chem,* **278,** 14599-14602.

Sarbassov, D. D., Guertin, D. A., Ali, S. M. & Sabatini, D. M. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science,* **307,** 1098-1101.

Schwenk, W. F. & Haymond, M. W. 1987. Decreased uptake of glucose by human forearm during infusion of leucine, isoleucine, or threonine. *Diabetes,* **36,** 199-204.

Shah, O. J., Wang, Z. & Hunter, T. 2004. Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies. *Curr Biol,* **14,** 1650-1656.

Sherwood, D. J., Dufresne, S. D., Markuns, J. F., Cheatham, B., Moller, D. E., Aronson, D. & Goodyear, L. J. 1999. Differential regulation of MAP kinase, p70S6K, and Akt by contraction and insulin in rat skeletal muscle. *Am J Physiol Endocrinol Metab,* **276,** E870-878.

Shigemitsu, K., Tsujishita, Y., Hara, K., Nanahoshi, M., Avruch, J. & Yonezawa, K. 1999. Regulation of translational effectors by amino acid and mammalian target of rapamycin signaling pathways. Possible involvement of autophagy in cultured hepatoma cells. *J Biol Chem,* **274,** 1058-1065.

Stone, S., Abkevich, V., Russell, D. L., Riley, R., Timms, K., Tran, T., Trem, D., Frank, D., Jammulapati, S., Neff, C. D., Iliev, D., Gress, R., He, G., Frech, G. C., Adams, T. D., Skolnick, M. H.*, et al.* 2006. TBC1D1 is a candidate for a severe obesity gene and evidence for a gene/gene interaction in obesity predisposition. *Hum Mol Gen,* **15,** 2709-2720.

Stossel, T. P., Condeelis, J., Cooley, L., Hartwig, J. H., Noegel, A., Schleicher, M. & Shapiro, S. S. 2001. Filamins as integrators of cell mechanics and signalling. *Nat Rev Mol Cell Biol,* **2,** 138-145.

Sun, C., Zhang, F., Ge, X., Yan, T., Chen, X., Shi, X. & Zhai, Q. 2007. SIRT1 improves insulin sensitivity under insulin-resistant conditions by repressing PTP1B.[see comment]. *Cell Metab,* **6,** 307-319.

Takano, A., Usui, I., Haruta, T., Kawahara, J., Uno, T., Iwata, M. & Kobayashi, M. 2001. Mammalian target of rapamycin pathway regulates insulin signaling via subcellular redistribution of insulin receptor substrate 1 and integrates nutritional signals and metabolic signals of insulin. *Mol Cell Biol,* **21,** 5050-5062.

Tanaka, T., Nakatani, K., Morioka, K., Urakawa, H., Maruyama, N., Kitagawa, N., Katsuki, A., Araki-Sasaki, R., Hori, Y., Gabazza, E. C., Yano, Y., Wada, H., Nobori, T., Sumida, Y. & Adachi, Y. 2003. Nitric oxide stimulates glucose transport through insulin-independent GLUT4 translocation in 3T3-L1 adipocytes. *Eur J Endocrinol,* **149,** 61-67.

Tang, H., Hornstein, E., Stolovich, M., Levy, G., Livingstone, M., Templeton, D., Avruch, J. & Meyuhas, O. 2001. Amino acid-induced translation of TOP mRNAs is fully dependent on phosphatidylinositol 3-kinase-mediated signaling, is partially inhibited by rapamycin, and is independent of S6K1 and rpS6 phosphorylation. *Mol Cell Biol,* **21,** 8671-8683.

Taylor, E. B., An, D., Kramer, H. F., Yu, H., Fujii, N. L., Roeckl, K. S., Bowles, N., Hirshman, M. F., Xie, J., Feener, E. P. & Goodyear, L. J. 2008. Discovery of TBC1D1 as an insulin-, AICAR-, and contraction-stimulated signaling nexus in mouse skeletal muscle. *J Biol Chem,* **283,** 9787-9796.

Tee, A. R., Manning, B. D., Roux, P. P., Cantley, L. C. & Blenis, J. 2003. Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. *Curr Biol,* **13,** 1259-1268.

Thomson, D. M. & Gordon, S. E. 2005. Diminished overload-induced hypertrophy in aged fast-twitch skeletal muscle is associated with AMPK hyperphosphorylation. *J Appl Physiol,* **98,** 557-564.

Traxinger, R. R. & Marshall, S. 1989. Role of amino acids in modulating glucose-induced desensitization of the glucose transport system. *J Biol Chem,* **264,** 20910-20916.

Treebak, J. T., Birk, J. B., Hansen, B. F., Olsen, G. S. & Wojtaszewski, J. F. 2009a. A-769662 activates AMPK beta1-containing complexes but induces glucose uptake through a PI3-kinase-dependent pathway in mouse skeletal muscle. *Am J Physiol Cell Physiol,* **297,** C1041-1052.

Treebak, J. T., Birk, J. B., Rose, A. J., Kiens, B., Richter, E. A. & Wojtaszewski, J. F. 2007. AS160 phosphorylation is associated with activation of alpha2beta2gamma1- but not alpha2beta2gamma3-AMPK trimeric complex in skeletal muscle during exercise in humans. *Am J Physiol Endocrinol Metab,* **292,** E715-722.

Treebak, J. T., Frosig, C., Pehmoller, C., Chen, S., Maarbjerg, S. J., Brandt, N., MacKintosh, C., Zierath, J. R., Hardie, D. G., Kiens, B., Richter, E. A., Pilegaard, H. & Wojtaszewski, J. F. 2009b. Potential role of TBC1D4 in

enhanced post-exercise insulin action in human skeletal muscle. *Diabetologia,* **52,** 891-900.

Treebak, J. T., Glund, S., Deshmukh, A., Klein, D. K., Long, Y. C., Jensen, T. E., Jorgensen, S. B., Viollet, B., Andersson, L., Neumann, D., Wallimann, T., Richter, E. A., Chibalin, A. V., Zierath, J. R. & Wojtaszewski, J. F. 2006. AMPK-mediated AS160 phosphorylation in skeletal muscle is dependent on AMPK catalytic and regulatory subunits. *Diabetes,* **55,** 2051-2058.

Tremblay, F., Jacques, H. & Marette, A. 2005. Modulation of insulin action by dietary proteins and amino acids: role of the mammalian target of rapamycin nutrient sensing pathway. *Curr Opin Clin Nutr Metab Care,* **8,** 457-462.

Tremblay, F. & Marette, A. 2001. Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. *J. Biol. Chem.,* **276,** 38052-38060.

Tsakiridis, T., Tong, P., Matthews, B., Tsiani, E., Bilan, P. J., Klip, A. & Downey, G. P. 1999. Role of the actin cytoskeleton in insulin action. *Microsc Res Tech,* **47,** 79-92.

Tuomilehto, J., Lindstrom, J., Eriksson, J. G., Valle, T. T., Hamalainen, H., Ilanne-Parikka, P., Keinanen-Kiukaanniemi, S., Laakso, M., Louheranta, A., Rastas, M., Salminen, V., Uusitupa, M. & Finnish Diabetes Prevention Study, G. 2001. Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. *N Eng J Med,* **344,** 1343-1350.

Tzatsos, A. & Kandror, K. V. 2006. Nutrients suppress phosphatidylinositol 3-kinase/Akt signaling via raptor-dependent mTOR-mediated insulin receptor substrate 1 phosphorylation. *Mol Cell Biol,* **26,** 63-76.

Um, S. H., D'Alessio, D. & Thomas, G. 2006. Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. *Cell Metab,* **3,** 393-402.

Um, S. H., Frigerio, F., Watanabe, M., Picard, F., Joaquin, M., Sticker, M., Fumagalli, S., Allegrini, P. R., Kozma, S. C., Auwerx, J. & Thomas, G. 2004. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature,* **431,** 200-205.

Vaag, A., Henriksen, J. E. & Beck-Nielsen, H. 1992. Decreased insulin activation of glycogen synthase in skeletal muscles in young nonobese Caucasian first-degree relatives of patients with non-insulin-dependent diabetes mellitus. *J Clin Invest,* **89,** 782-788.

Wallberg-Henriksson, H., Constable, S. H., Young, D. A. & Holloszy, J. O. 1988. Glucose transport into rat skeletal muscle: interaction between exercise and insulin. *J Appl Physiol,* **65,** 909-913.

Wallberg-Henriksson, H. & Holloszy, J. O. 1984. Contractile activity increases glucose uptake by muscle in severely diabetic rats. *J Appl Physiol,* **57,** 1045-1049.

Van Dyke, D. A., Walters, L., Frieswyk, D., Kokmeyer, D. & Louters, L. L. 2003. Acute effects of troglitazone and nitric oxide on glucose uptake in L929 fibroblast cells. *Life Sci,* **72,** 2321-2327.

Wang, J., Obici, S., Morgan, K., Barzilai, N., Feng, Z. & Rossetti, L. 2001. Overfeeding rapidly induces leptin and insulin resistance. *Diabetes,* **50,** 2786-2791.

Wang, X., Campbell, L. E., Miller, C. M. & Proud, C. G. 1998. Amino acid availability regulates p70 S6 kinase and multiple translation factors. *Biochem J,* **334,** 261-267.

Vanhaesebroeck, B. & Alessi, D. R. 2000. The PI3K-PDK1 connection: more than just a road to PKB. *Biochem J,* **346,** 561-576.

Whiteman, E. L., Cho, H. & Birnbaum, M. J. 2002. Role of Akt/protein kinase B in metabolism. *Trends Endocrinol Metab,* **13,** 444-451.

Widegren, U., Jiang, X. J., Krook, A., Chibalin, A. V., Bjornholm, M., Tally, M., Roth, R. A., Henriksson, J., Wallberg-henriksson, H. & Zierath, J. R. 1998. Divergent effects of exercise on metabolic and mitogenic signaling pathways in human skeletal muscle. *FASEB J.,* **12,** 1379-1389.

Vieira, E., Nilsson, E. C., Nerstedt, A., Ormestad, M., Long, Y. C., Garcia-Roves, P. M., Zierath, J. R. & Mahlapuu, M. 2008. Relationship between AMPK and the transcriptional balance of clock-related genes in skeletal muscle. *Am J Physiol* **295,** E1032-1037.

Winder, W. W. & Hardie, D. G. 1996. Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. *Am J Physiol,* **270,** E299-304.

Winder, W. W., Holmes, B. F., Rubink, D. S., Jensen, E. B., Chen, M. & Holloszy, J. O. 2000. Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle. *J Appl Physiol,* **88,** 2219-2226.

Viollet, B., Andreelli, F., Jorgensen, S. B., Perrin, C., Geloen, A., Flamez, D., Mu, J., Lenzner, C., Baud, O., Bennoun, M., Gomas, E., Nicolas, G., Wojtaszewski, J. F., Kahn, A., Carling, D., Schuit, F. C*., et al.* 2003. The AMP-activated protein kinase alpha2 catalytic subunit controls whole-body insulin sensitivity. *J Clin Invest,* **111,** 91-98.

Wojtaszewski, J. F., Hansen, B. F., Gade, Kiens, B., Markuns, J. F., Goodyear, L. J. & Richter, E. A. 2000. Insulin signaling and insulin sensitivity after exercise in human skeletal muscle. *Diabetes,* **49,** 325-331.

Wojtaszewski, J. F. P., Higaki, Y., Hirshman, M. F., Michael, M. D., Dufresne, S. D., Kahn, C. R. & Goodyear, L. J. 1999. Exercise modulates postreceptor insulin signaling and glucose transport in muscle-specific insulin receptor knockout mice. *J. Clin. Invest.,* **104,** 1257-1264.

Woo, M. S., Ohta, Y., Rabinovitz, I., Stossel, T. P. & Blenis, J. 2004. Ribosomal S6 kinase (RSK) regulates phosphorylation of filamin A on an important regulatory site. *Mol Cell Biol,* **24,** 3025-3035.

Wright, D. C., Geiger, P. C., Holloszy, J. O. & Han, D. H. 2005. Contraction- and hypoxia-stimulated glucose transport is mediated by a Ca2+-dependent mechanism in slow-twitch rat soleus muscle. *Am J Physiol Endocrinol Metab,* **288,** E1062-1066.

Wright, D. C., Hucker, K. A., Holloszy, J. O. & Han, D. H. 2004. Ca2+ and AMPK both mediate stimulation of glucose transport by muscle contractions. *Diabetes,* **53,** 330-335.

Youn, J. H., Gulve, E. A. & Holloszy, J. O. 1991. Calcium stimulates glucose transport in skeletal muscle by a pathway independent of contraction. *Am J Physiol,* **260,** C555-C561.

Young, M. E. & Leighton, B. 1998. Evidence for altered sensitivity of the nitric oxide/cGMP signalling cascade in insulin-resistant skeletal muscle. *Biochem J,* **329,** 73-79.

Young, M. E., Radda, G. K. & Leighton, B. 1997. Nitric oxide stimulates glucose transport and metabolism in rat skeletal muscle in vitro. *Biochem J,* **322,** 223-228.

Yu, H., Fujii, N., Hirshman, M. F., Pomerleau, J. M. & Goodyear, L. J. 2004. Cloning and characterization of mouse 5'-AMP-activated protein kinase gamma3 subunit. *Am J Physiol Cell Physiol,* **286,** C283-292.

Zerial, M. & McBride, H. 2001. Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol,* **2,** 107-117.

Zierath, J. R. 1995. In vitro studies of human skeletal muscle. Hormonal and metabolic regulation of glucose transport. *Acta Physiol Scand,* **155,** 1-96.

Zierath, J. R. 2002. Invited review: Exercise training-induced changes in insulin signaling in skeletal muscle. *J Appl Physiol,* **93,** 773-781.