The role of leucine and its metabolite (KIC) in insulin signalling and glucose transport in L6 myotubes

Mahshid Moghei

A thesis submitted to the faculty of graduate studies in partial fulfillments of the requirements for the degree of

Master of Science

Graduate Program in Kinesiology and Health Science
York University
Toronto, Ontario
December 2015

© Mahshid Moghei, 2015

Abstract

Branched-chain Amino Acids (BCAAs) are known to have positive effects in metabolic health through weight management and muscle protein synthesis. However, elevated levels of BCAAs (particularly leucine) and their metabolites have also been implicated in the development of insulin resistance and type 2 diabetes mellitus (T2DM). This study examines the dose-dependent effect of leucine in the presence or absence of other amino acids on glucose transport in L6 rat myotubes. Here we report that leucine significantly suppresses insulin-stimulated glucose uptake in skeletal muscle cells and particularly at 150 μM, there is a 75% reduction in insulin-mediated glucose transport ($p<0.01$). This occurs in parallel with increased activation of proteins involved in the mammalian/mechanistic target of rapamycin complex 1 (mTORC1) pathway ($p<0.05$), which suggests a link between increased mTORC1 activity and insulin resistance. Interestingly, the suppressive effect of leucine on glucose transport disappears in the presence of other amino acids. We also illustrate that leucine's metabolite, α-ketoisocaproic acid (KIC) inhibits insulin-stimulated glucose uptake at 200 μM by 45% concurrent with increased activation of the mTORC1 pathway ($p<0.05$). Finally, siRNA knockdown of the branched-chain aminotransferase 2 mitochondrial (BCAT2) enzyme which catalyzes the reversible conversion of leucine to KIC, ameliorated the inhibitory effect of KIC on glucose transport ($p<0.05$), suggesting that the impairing effects of KIC on glucose transport occur through its conversion back to leucine. Taken together, our results show that in L6 myotubes, leucine and its metabolite significantly suppress insulin-mediated glucose transport. Moreover, modulating the activity of the BCAT2 enzyme could be a new therapeutic approach in patients with high BCAA levels in conditions such obesity and T2DM.

Acknowledgements

I would like to express my gratitude to my wonderful teacher and supervisor, Dr. Ola Adegoke. Without your continuous support and guidance, I would not be where I am today. Thank you for believing in me and supporting me throughout this amazing journey. Thank you for challenging me to work harder and strive for independence and excellence. And finally, thank you for being a great mentor!

Another thanks to my amazing lab members for their help and all the fun times and laughs we had together.

Lastly, I want to thank my loving and supportive family without whom I could not achieve this. I love you endlessly!

Table of Contents

Abstract....ii

Acknowledgments....iii

Table of Contents....iv

List of Figures....vii

Abbreviations....viii

1.0 Introduction....1

2.0 Literature Overview....3

2.1 Insulin Signalling Within Skeletal Muscle....3

2.1.1 Insulin Receptor and Insulin Receptor Substrates....3

2.1.2 IRS-1/PI3K/Akt Pathway....4

2.1.3 PI3K/Akt/mTOR Pathway....6

2.2 Insulin Resistance and T2DM....7

2.3 Causes of Insulin Resistance....7

2.3.1 Genetics....7

2.3.2 Lack of Physical Activity and Obesity....9

2.3.3 Nutrition....11

2.3.3.1 High Lipid Intake....11

2.3.3.2 High Protein Intake....13

2.4 Molecular Mechanisms of Insulin Resistance....15

2.4.1 Accumulation of Lipid Intermediate....15

2.4.2 Chronic Inflammation....16

2.4.3 Oxidative Stress....18

2.4.4 mTORC1/S6K1 Pathway....19

2.5 Mammalian Target of Rapamycin (mTOR)....................20

2.5.1 Mammalian Target of Rapamycin Complex 1 (mTORC1)....................20

2.5.2 Mammalian Target of Rapamycin Complex 2 (mTORC2)....................21

2.5.3 mTORC1 and Downstream Substrates....................21

2.5.3.1 Ribosomal Protein S6 Kinase-1 (S6K1)....................22

2.5.3.2 Eukaryotic translation initiation factor 4E-binding protein 1(4E-BP1)......22

2.6 Upstream Regulators of mTORC1....................23

2.6.1 Growth Factors....................23

2.6.2 Stress....................24

2.6.3 Amino Acids....................24

2.7 Branched-chain Amino Acids (BCAAs) and Metabolic Health....................26

2.7.1 BCAA Metabolic Pathway....................27

2.7.2 The link between BCAAs, mTORC1 and Insulin Resistance- BCAA Paradox....................31

2.8 Rationale....................33

2.9 Objective....................34

3.0 Hypothesis....................34

4.0 Manuscript....................37

5.0 Materials and Methods....................40

6.0 Results....................46

The effect of leucine on glucose transport in the presence of other amino acids **(Fig 4)** 46

The effect of medium composition (presence of other amino acids) on leucine-mediated stimulation of mTORC1 **(Fig 5)** 46

The effect of leucine alone on glucose uptake in L6 myotubes **(Fig 6)**....................47

Dose-dependent effect of leucine on mTORC1 activation in L6 myotubes **(Fig 7)** 48

Basal Glucose Uptake is Higher in the Presence of Other Amino Acids **(Fig 8)** 48

The effect of KIC on glucose transport and insulin signalling in L6 myotubes **(Fig 9)** 49

Treatment with KIC results in activation of mTORC1/S6K1 pathway **(Fig 10)**....................50

KIC-mediated regulation of glucose transport appears to involve mTORC1 **(Fig 11)** 50

The effect of rapamycin on KIC-mediated regulation of mTORC1 **(Fig 12)** 51

KIC-mediated suppression of glucose transport is due to its intracellular conversion to leucine **(Fig 13 A)** 52

BCAT2 knockdown efficiency **(Fig 13 B)** 53

The effect of BCAT2 knockdown on KIC-mediated regulation of mTORC1 Pathway **(Fig 14)** 53

7.0 Figures....................54

8.0 Discussion....................65

9.0 Future Directions....................72

10.0 References....................73

11.0 Appendix....................85

List of Figures

Figure 1. General overview of insulin signalling pathway and proteins involved in glucose transport....................35

Figure 2. Defective insulin signalling as a result of excess amino acid levels................36

Figure 3. BCAA metabolic pathway 30

Results

Figure 4. The effect of leucine on glucose transport in the presence of other amino acids....................54

Figure 5. The effect of medium composition (presence of other amino acids) on leucine-mediated stimulation of mTORC1....................55

Figure 6. The effect of leucine alone on glucose uptake in L6 myotubes....................56

Figure 7. Dose-dependent effect of leucine on mTORC1 activation in L6 myotubes....................57

Figure 8. Glucose Uptake is Higher in the Presence of Other Amino Acids....................58

Figure 9. The effect of KIC on glucose transport and insulin signalling in L6 myotubes....................59

Figure 10. Treatment with KIC results in activation of mTORC1/S6K1 pathway.... 60

Figure 11. KIC-mediated regulation of glucose transport appears to involve mTORC1... 61

Figure 12. The effect of rapamycin on KIC-mediated regulation of mTORC1...............62

Figure 13 A. KIC-mediated suppression of glucose transport is due to its intracellular conversion to leucine....................63

Figure 13 B. The efficiency of BCAT2 knockdown....................63

Figure 14. The effect of BCAT2 knockdown on KIC-mediated regulation of mTORC1 pathway....................64

Abbreviations

4EBP1	eukaryotic translation initiation factor 4E binding protein 1
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AS 160	Akt substrate 160kD
ATP	adenosine triphosphate
BCAA	branched chain amino acid
BCAT2	branched-chain aminotransferase 2 mitochondrial
BCKDH	branched chain a-keto acid dehydrogenase complex
Crk	chicken tumor virus number 10 regulator of kinase
DAG	diacylglycerol
Deptor	DEP-domain-containing mTOR-interacting protein
eEF2K	eukaryotic elongation factor 2 kinase
eIF4A	eukaryotic translation initiation factor 4A
eIF4B	eukaryotic translation initiation factor 4B
eIF4E	eukaryotic translation initiation factor 4E
eIF4F	eukaryotic translation initiation factor 4F
eIF4G	eukaryotic translation initiation factor 4G
FKBP12	FK506-binding protein 12 kDa
GAP	GTPase activating protein
GDP	guanosine diphosphate
GLUT4	glucose transporter type-4
Grb2	growth factor receptor-bound protein 2
GTP	guanosine-5'-triphosphate

hVps34	human vacuolar protein sorting 34
IGF1	insulin-like growth factor 1
IKK	inhibitor kappa B (IkB) kinase
NF-kB	inhibitor kappa B (IkB) kinase (IKK)-nuclear factor kB
IKK-β	IkB kinase beta
IL-6	interleukin-6
IRS	1 insulin receptor substrate 1
JNK	c - Jun N-terminal kinase
KIC	α-ketoisocaproic acid
KIV	α-ketoisovaleric acid
KMV	α-keto-β-methylvaleric acid
mLST8	mammalian lethal with Sec 13 protein 8
MODY	maturity-onset diabetes o f the young
mSinl	mammalian stress-activated protein kinase (SAPK)- interacting protein-1
mTOR	mammalian target of rapamycin
mTORC 1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
Nck1	non-catalytic region of tyrosine kinase adaptor protein 1
PDCD4	programmed cell death 4
PDK1	3-phosphoinositide dependent protein kinase-1
PH	pleckstrin-homology
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol-4,5-biphosphate
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PKB	protein kinase B

PKC	protein kinase C
PPAR-y	peroxisome proliferator-activated receptor gamma
PRAS40	proline-rich Akt substrate of 40kDa
Proctor	protein observed with rictor
Raptor	rapamycin-sensitive adaptor protein o f mTOR
Rheb	ras homologue in the brain
Rictor	rapamycin-insensitive companion o f mTOR
ROS	reactive oxygen species
S6K1	ribosomal protein S6 kinase 1
Ser/S	serine
SH2	src homology-2
T2DM	type 2 diabetes mellitus
Thr/T	threonine
TNF-α	tumor necrosis factor -alpha
TSC 1/2	tuberous sclerosis complex 1/2

1.0 Introduction

Diabetes is a major health concern in today's world and its global burden is staggering. According to the International Diabetes Federation, approximately 1 in every 12 people is diabetic with an estimated 387 million cases worldwide. Intriguingly, for every diabetic person, another person suffers from impaired glucose metabolism which increases their risk for other conditions such as heart disease and stroke [1].

T2DM accounts for about 90% of all cases of diabetes worldwide and its precursor is a condition known as insulin resistance which affects millions of people, particularly in Western societies[2]. It is one of the main causes of morbidity and mortality and a hallmark of chronic diseases such as obesity, T2DM, cardiovascular disease and metabolic syndrome.[3,4] Nutrient overload is one of the main causes for the development of insulin resistance. Although high fat diets are generally considered to be the major cause of metabolic abnormalities, more recent evidence suggests that consumption of high protein diets is also associated with insulin resistance. [5–7]

High protein diets are consistently used for weight management in obese individuals. They have been shown to have positive metabolic effects such as weight loss, increased basal metabolic rate, increased muscle mass and loss of fat mass.[8–10] The underlying mechanisms for some of these positive effects are increased thermogenesis and prolonged feelings of satiety [10,11]. It has been shown that BCAAs are the main components in high protein diets, which exert these beneficial effects. [6,8] Of the three BCAAs (leucine, isoleucine and valine), leucine has gained considerable attention due to its strong ability to activate mTORC1, the master regulator of cell growth, proliferation and protein synthesis. [12–15]

Despite their benefits, diets consisting of high levels of proteins have been shown to be implicated in the development of insulin resistance and T2DM.[5,6,16] Increased circulating levels of BCAAs, specifically leucine, have been shown to be associated with hyperactivation of mTORC1, leading to the impairment of insulin signalling pathway and consequently the development of insulin resistance. In addition, elevated BCAA levels are frequently observed in human and rodent models of obesity [6,17]. Therefore, high protein/BCAA diets can act as a double-edged sword by demonstrating beneficial effects in the management of obesity while having the ability to negatively affect insulin sensitivity and ultimately cause insulin resistance and T2DM. [6]

Since leucine is the most potent BCAA in activating the mTORC1 pathway [18,19], many studies have examined its effect on insulin signalling, however, current evidence is still inconclusive and many questions remain to be addressed. Moreover, the dose-dependent effect of leucine (within a range that is physiologically-relevant) on glucose transport and mTORC1 signalling has not yet been determined. In addition, since dietary protein contains amino acids other than BCAAs, it is essential to study the effect of a combination of amino acids on insulin signalling and mTORC1 pathway. It is also important to examine the mTORC1 pathway in response to specific metabolites of leucine, since the amino acid undergoes metabolic changes intracellularly that can affect the regulation of glucose transport and insulin signalling. Understanding the effect of leucine-mediated mTORC1 activation and glucose transport within skeletal muscle can be helpful in explaining the underlying mechanisms of BCAAs' action in the development and progression of insulin resistance. This can ultimately help in the prevention and management of T2DM and reduce its burden.

2.0 Literature Review

2.1 Insulin Signalling Within Skeletal Muscle

Skeletal muscle comprises about 40-50% of the adult human body weight [20,21]. Thus, it is the most abundant insulin-sensitive tissue mediating about 85% of all insulin-stimulated glucose disposal under normal physiological conditions. [21,22] In humans, insulin-stimulated glucose transport into skeletal muscle is the key mechanism through which disposal of ingested glucose occurs. Insulin-mediated glucose transport requires tightly regulated, multistep processes involving crosstalk with other signalling pathways.

2.1.1 Insulin Receptor and Insulin Receptor Substrates

Upon binding to the insulin receptor (IR) on the sarcolemma of skeletal muscle, insulin increases the tyrosine kinase activity of insulin receptor. The insulin receptor is a member of the large tyrosine kinase family of transmembrane signaling receptors and plays an important role in metabolic regulation and glucose homeostasis. The receptor is composed of two extracellular α subunits, each connected to a β subunit and linked to each other by disulfide bonds forming a heterotetramer. [23] When endogenous ligands such as insulin and insulin-like growth factor 1 (IGF-1) bind the α subunits, they cause autophosphorylation of the β subunits exclusively on tyrosine residues.[24] This tyrosine phosphorylation mediates the interaction of insulin with its receptors. Upon its activation, the insulin receptor phosphorylates other proteins such as the insulin receptor substrates 1/2 (IRS-1, 2,3,4). The four members of the IRS family differ in their subcellular localization, tissue distribution and their ability to bind to insulin receptors. In fact, IRS-1

plays a major role in insulin signaling within skeletal muscle, while IRS-2 appears to regulate hepatic insulin action. [25]

As a main substrate of the IR, IRS-1 plays a major role in transmitting insulin-dependent signals that propagate many biological processes such as cell growth and cellular uptake of glucose. [26] IRS proteins are composed of an N-terminal region containing a pleckstrin homology (PH) domain and a phosphotyrosine-binding (PTP) domain that work together to ensure proper substrate phosphorylation by the activated IR. The C-terminal part of the IRS proteins contains various tyrosine phosphorylation motifs that act as docking sites for multiple signalling molecules that express Src homology-2 (SH-2) binding domains such as phosphatidylinositol-3-kinase (PI3K), phosphotyrosine phosphatase (SHP-2), non-catalytic region of tyrosine kinase adaptor protein 1 (Nck1), chicken tumor virus number 10 regulator of kinase (Crk) and growth factor receptor-bound protein 2 (Grb-2). [25,26] The PH domain of IRS-1 plays a critical role in promoting the interaction of the protein with IR, thereby regulating the tyrosine phosphorylation of IRS-1.[26] It has been shown that in addition to regulating IR-IRS1 interactions, the PH domain of IRS-1 also regulates the ability of the protein to signal to downstream targets such as PI3K. Therefore, impairing the function of the PH domain (ie. in insulin resistance states) could both inhibit tyrosine phosphorylation of IRS-1 by the IR and alter efficient signal transduction to downstream targets. [27]

2.1.2 IRS-1/PI3K/Akt Pathway

The PI3K-Akt pathway is a key regulator of cell proliferation, growth, survival and glucose metabolism. Extensive evidence suggests that this pathway is frequently deregulated in various cancer types, making its components molecular targets in cancer.[28] The family of lipid kinases, PI3Ks, is known to regulate essential cellular processes such as cell survival, proliferation and differentiation. PI3Ks are divided into three classes based on their structure and substrate specificity. The most commonly studied are the class I enzymes that are activated directly by cell surface receptors. In the insulin signalling pathway, PI3Ks are the major downstream substrates of IRS-1 and play pivotal roles in insulin-mediated glucose transport and translocation of the glucose transporter type 4 (GLUT4) from intracellular vesicles to the plasma membrane. Class IA PI3Ks which are a subset of Class I PI3Ks, are heterodimers comprised of a p110 catalytic subunit and a p85 regulatory subunit. [29] The p85 regulatory subunit binds to IRS-1 and the p110 catalytic subunit interacts with and phosphorylates phosphatidylinositol in the cell membrane.

Upon interaction of IRS-1 with the p85 regulatory subunit of PI3K, the IRS-1/PI3K complex is recruited to the cell membrane where PI3K catalyzes the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). Then, PIP3 allows the recruitment and activation of other kinases such as phosphatidylinositol-dependent protein kinase 1 (PDK1) and Akt. Akt then becomes activated upon phosphorylation on Thr308 and Ser473 residues. [(Figure 1)] When Akt is active, it phosphorylates Akt Substrate of 160 kD (AS160) and facilitates the translocation of Glucose Transporter Type 4 (GLUT4), the protein responsible for insulin-stimulated glucose transport in skeletal muscle and adipose tissue, to the sarcolemma to allow glucose entry into the cell. [14,22,30]

Akt, also known as protein kinase B, is a serine/threonine kinase which exists as three structurally similar isoforms: Akt1, Akt2 and Akt3 that are expressed in most tissues [29,31] All three isoforms contain an N-terminal PH domain, a C-terminal regulatory domain and a central serine/threonine catalytic domain. Activation of Akt begins with its translocations to the plasma membrane facilitated by docking of PIP3 to the PH domain in the N-terminal region of Akt.[29] This interaction results in a conformational change in Akt and exposes two important phosphorylation residues:Th308 and Ser473. In order to fully activate Akt, both amino acid residues must be phosphorylated. [29,31]

2.1.3 PI3K/Akt/mTOR Pathway

Once activated, Akt signalling can be propagated to many substrates including mTOR, a key regulator of protein translation. One of the well-studied substrates of Akt is the mammalian/mechanistic target of rapamycin complex 1 (mTORC1). Akt can activate mTORC1 by phosphorylating PRAS40 on T246 and TSC2 on multiple sites such as Ser 939, Ser 1086 and T1422 and mitigating their inhibitory effect on mTORC1[29,32] Although mTORC1 is a downstream substrate of Akt, Akt itself can be activated by another mTOR complex, mTORC2 on S473 residues.[31] The PI3K/Akt/mTOR pathway is constitutively activated in many cancer cell types and one of the involved mechanisms is the loss of tumor suppressor PTEN (phosphatase and tensin homolog). [28,29,31] As a lipid phosphatase, PTEN normally suppresses the activation of PI3K/Akt/mTOR pathway by removing a phosphate group from PIP3 and converting it to PIP2 , therefore, PTEN mutations or deletions are constantly observed in a variety of human cancers [33]. Amplifications or mutations of PI3K and Akt are other mechanisms that lead to constitutive activation of this pathway.[28,29,31]

2.2 Insulin Resistance and T2DM

Type 2 diabetes mellitus (T2DM) is a growing health concern accounting for about 90% of all cases of diabetes. In 2014, the WHO estimated the global prevalence of T2DM to be 9% among adults aged 18 years and older [2]. T2DM is characterized by hyperglycemia as a result of either insufficient secretion of the hormone insulin, defects in insulin action or both. [4,34,35] Therefore, unlike in Type 1 diabetes mellitus (T1DM) where there is autoimmune destruction of β cells of the pancreas, in T2DM the body may produce insulin but it cannot effectively use it. T2DM is often preceded by a condition known as insulin resistance where peripheral target tissues such as liver, skeletal muscle and adipose tissue become unresponsive to the function of insulin, resulting in suppression of glucose uptake and metabolism. [3,4,35] Insulin is an essential hormone that regulates carbohydrate, fat and protein metabolism in the body and deregulation of its signalling is the hallmark of many complex metabolic and physiological disorders including obesity, cardiovascular disease and cancers. [5,6,16]. Hence, it is crucial to understand the molecular mechanisms that regulate the development and pathogenesis of insulin resistance. In addition, since skeletal muscle is responsible for about 85% of postprandial glucose disposal and is the predominant site for insulin-stimulated glucose uptake, the study of insulin resistance in skeletal muscle is essential. [5,6]

2.3 Causes of Insulin Resistance

2.3.1 Genetics

Genetic predisposition plays a major role in the development of insulin resistance and T2DM. Some of the early evidence came from classical experimental paradigms with twin, family and population studies. It was clearly demonstrated that the more closely two individuals were related, the more similar their glucose tolerance status. [35] Therefore, first

degree families of individuals with T2DM have a 3 times higher chance of developing the disease than individuals with no family history of the disease. Furthermore, the concordance rate of T2DM is significantly higher in monozygotic twins (70%) compared to dizygotic twins (20-30%).[35–37] Individuals who have one parent with T2DM have a 40% lifetime risk of developing the disease and those with both parents affected have a 70% chance of developing the disease in during their lifetime [36].

Thus, it is clear that T2DM has a strong genetic component with 30-70% of the risk attributed to genetics and a large variety of genes involved. [38,39]

To date, multiple candidate genes for T2DM have been identified in various populations worldwide. However, it is still not clear exactly how many genes are involved and how they affect the development of the condition. Candidate genes were selected and studied because of their involvement in insulin action, glucose metabolism, β cell function and other metabolic conditions affecting T2DM. So far, some of the most promising candidate genes are Peroxisome Proliferator-Activated Receptor Gamma (*PPARγ*), Transcription Factor 7-Like 2 (T-Cell Specific, HMG-Box) (*TCF7L2*), Potassium Channel, Inwardly Rectifying Subfamily J, Member 11 (*KCNJ11*), and Calcium-Activated Neutral Proteinase 10 (*CAPN10*).

What makes the *PPARγ* a strong candidate gene is that it encodes the nuclear receptor PPARγ, a molecular target for thiazolidinedione which is a class of insulin-sensitizing drugs used to treat T2DM. [36] *TCF7L2* shows one of the strongest associations with T2DM. It is involved in the Wnt/ β-canenin signalling pathway which works to increase insulin sensitivity and pancreatic islet development. High risk *TCF7L2* genotype reduces insulin secretion, thereby, confirming the importance of *TCF7L2* variants in β cell function. [38,39] *KCNJ11* is part of the ATP-sensitive potassium channel with a major role in regulating the release of hormones insulin and glucagon, therefore, mutations in this

gene can affect insulin secretion.[35–38] *CAPN10 gene* encodes ubiquitous, calcium-dependent cysteine proteases. Calpain-10 is a protein encoded by the *CAPN10* gene. It is an important protein in β cell function and emerging evidence suggests that it plays a role in facilitating GLUT4 translocation, therefore, variations in calpain-10 activity can modulate insulin secretion and enhance susceptibility to T2DM. [36–39]

Maturity-onset diabetes of the young (MODY) is an uncommon type of diabetes that usually occurs before the age of 25 in less than 5% of all cases of T2DM. The pathophysiology of MODY involves mutations in transcription factor genes that are critical in β cell function. Molecular genetics studies have shown that there are at least six forms of MODY caused by various gene mutations. MODY1 is caused by mutations in hepatocyte nuclear factor-4α (*HNF4A*) while MODY2 results from glucokinase (*GCK*) gene mutations. Mutations in the hepatocyte nuclear factor-1α (*HNF1A*) gene result in MODY3 which is the most frequent cause of the disease. Finally, MODY5 and 6 are caused by mutations of the hepatocyte nuclear factor-1β (*HNF1B*) and neurogenic differentiation 1(*NEUROD1*) genes respectively.

Even though genetic factors are a significant component of T2DM development, it is clear that environmental factors also play a major role in the progression of the disease. Two key environmental risk factors contributing to T2DM are obesity and a sedentary lifestyle (physical inactivity).

2.3.2 Lack of Physical Activity and Obesity

The increasing cases of T2DM in recent years can be primarily explained by the dramatic growth in obesity rates worldwide. It has been estimated that up to about 80% of all new T2DM cases are attributed to obesity [40]. Body fat distribution is another important factor in T2DM risk. The waist-to-hip ratio (WHR) which is a measure of abdominal

obesity, has a stronger association with T2DM than the standard measure of obesity, BMI.[41] Physical inactivity is another major risk factor for T2DM. Regular physical activity not only maintains a healthy weight, but also improves glucose and fat metabolism which reduce T2DM risk.

There are two well-defined mechanisms of glucose uptake by muscle: one is insulin-dependent which occurs at rest and post and postprandially and the other through exercise. [42] Epidemiological studies strongly support the effect of exercise in preventing obesity and T2DM. Moderate exercise such as brisk walking or cycling for more than 30 minutes per day has been shown to significantly lower the risk of T2DM [42–44]. Studies have shown that even low intensity aerobic exercise is beneficial in enhancing skeletal muscle insulin-stimulated glucose disposal and GLUT4 protein content.[43,45] Other studies have found that the effect of aerobic exercise on improving insulin signalling is dose and intensity-dependent. [41] A combination of aerobic and resistance training may be more effective in regulating glucose uptake than any of them alone. This is because resistance training results in increased muscle mass and can contribute to glucose uptake without modifying the muscle's intrinsic ability to respond to insulin, whereas aerobic exercise enhances insulin's action without altering muscle mass. [42]

The key mechanisms responsible for the beneficial effects of physical activity on insulin sensitivity include enhancement of glucose transport mediated by increased expression of GLUT4 proteins and translocation of GLUT4 from intracellular vesicles to sarcolemma. [45–48] Other benefits of physical activity on insulin sensitivity include promotion of muscle vascularization and mitochondrial biogenesis. [41] Kennedy et. al. [49] demonstrated that even a single bout of moderate intensity exercise is effective in promoting the translocation of GLUT4 to the plasma membrane in skeletal muscle of T2DM patients. Other studies have also found that contraction-mediated glucose

transport via increased GLUT4 translocation, involves AMPK activation and is insulin-independent. [42,47,50] Despite the importance of physical activity in the prevention and treatment of insulin resistance and T2DM, nutrition has a more profound impact on regulation of insulin resistance since insulin is only secreted in response to nutrient availability.

2.3.3 Nutrition

Food is an important regulator of insulin sensitivity and nutrient overload can trigger the progression of metabolic disorders such as T2DM and cardiovascular disease [4,15,51]. Two key nutrients that have been the focus of research in studies of skeletal muscle glucose metabolism are lipid and protein.

2.3.3.1 High Lipid Intake

High fat diets combined with a sedentary lifestyle are major contributing factors for the high prevalence of obesity and T2DM in Western lifestyle. Epidemiological evidence supports the effect of high fat diets in the development of obesity and other physiological complications. Such studies indicate a direct relationship between the amount of dietary fat consumed and the level of obesity. Overconsumption and weight gain associated with high fat diets have consistently been shown to be caused by the high caloric density and low satiety properties of such diets.[52]

The role of dietary fat in the development of T2DM has received clinical interest for many years. Many previous studies have established that fat-enriched diets significantly alter the composition of cell membrane phospholipids. [53] These cell membrane alterations have been shown to affect insulin binding and action, GLUT4 translocation and other cellular functions which are all membrane-mediated events. [53,54] Because of their close

contact with the lipid bilayer, membrane-associated events such as the coupling between transporters and receptors, are influenced by dietary fat. Several studies have examined the effect of increasing plasma fatty acid levels by infusion of lipid in humans and rodents and found an inhibitory effect on insulin-mediated activation of the IRS-1/PI3K/Akt pathway in skeletal muscle.[55,56] Other studies have found similar effects of high plasma free fatty acid levels on impairment of the IRS-1/PI3K/Akt activity and have attributed these effects mainly to defects in IRS-1 tyrosine phosphorylation and ultimately inhibition of insulin-induced glucose transport. [57]

Therefore, rises in plasma free fatty acid concentrations result in the development of insulin resistance through inhibition of insulin-stimulated glucose transport. However, it is important to note that different types of fatty acids exert different effects on insulin signalling. For instance, Harding et al. found diets with a higher polyunsaturated saturated fat (PUFA) to saturated fat ratio, reduced the risk of T2DM independent of age, sex, family history of T2DM, physical activity, protein intake and smoking status [58]. The beneficial effects of n-3 PUFA have been shown to include reduction of serum lipids and lipoproteins, lowering blood pressure and impairment of platelet aggregation which are factors that help reduce the risk of T2DM. In animal studies, diets enriched with PUFA enhanced peripheral glucose utilization [59]. Conversely, saturated fat and animal fat intake has been shown to be strongly associated with increased risk of T2DM. [53,58] This is because a greater saturated fatty acid content of membrane phospholipids has been reported to increase insulin resistance [53,54,60].

Trans fatty acids (TFAs) which are created through the transformation of PUFAs from their natural *cis* form to the *trans* form are abundant in processed foods in Western diets and have been shown to have implications in insulin resistance and T2DM [61]. Salmerón et al. examined the effect of TFAs on the risk of T2DM in a large sample of women and

found a positive association [62]. Their results were consistent with previous human and animal studies indicating adverse metabolic effects associated with TFAs intake on lipoprotein metabolism and insulin sensitivity. [61,63] It has been suggested that TFAs may reduce insulin sensitivity by promoting systemic inflammation as reflected by increased levels of inflammatory markers TNFα, IL-6, and C-reactive protein [61].

2.3.3.2 High Protein Intake

High protein diets have frequently been used in the management of obesity. The Recommended Daily Allowance for protein is 0.8 g/kg of body weight, however, some studies suggest that consuming higher amounts of proteins produce positive metabolic effects. High protein diets have been shown to increase thermogenesis, energy expenditure, satiety and assist weight loss. [10] In particular, BCAAs have received more attention because of their anabolic effects on muscle and their anti-obesity effects. Studies suggest that diets high in BCAAs often have positive effects in weight management, glucose homeostasis and protein synthesis [6,64–66]. In addition, amino acids, particularly leucine, stimulate protein synthesis by promoting mTORC1 activity, leading to skeletal muscle anabolism and growth [12,14,67–69]. The anabolic effect of amino acid intake on muscle is due to increased muscle protein synthesis [30,48,64,67,70]. The major signalling factors involved in synthesis of proteins in skeletal muscle are mTORC1 and its two downstream substrates, ribosomal protein S6 kinase 1 (S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP-1) [12,70,71]. Of all three BCAAs, leucine has been shown to be the most potent nutrient signal in activating mTORC1[13,14,68].

Consumption of dietary protein increases the secretion of insulin into the bloodstream which enhances the clearance of glucose from the blood. However, in the long term, high

intake of dietary protein is associated with a higher risk of T2DM in healthy individuals. [72]In obese individuals however, consumption of high protein diets might be helpful for improving insulin sensitivity in the presence of weight loss which has a significant beneficial effect on enhancing insulin sensitivity. Furthermore, in studies where obese or T2DM subjects consumed high protein diets without weight loss, the results are inconclusive, indicating the importance of weight loss in improving insulin sensitivity and glucose metabolism.[72]

In a study done by Linn et al. plasma insulin concentrations were found to be elevated not only by a single protein-rich meal but also by long term high protein intake in healthy non-obese individuals. [73] They found prolonged protein intake (for 6 months) was associated with increased fasting glucose production, increased gluconeogenesis and reduced insulin sensitivity. Other observational studies have also shown associations between long-term high protein intake and the risk for developing T2DM and metabolic syndrome [74].

Despite the beneficial anti-obesity effects associated with BCAA consumption, emerging evidence suggests elevated levels of circulating BCAAs may also be implicated in the development of insulin resistance and T2DM [5,6]. Normal BCAA metabolism appears to be altered in insulin resistance, leading to high blood concentrations of BCAAs. One of the proposed mechanisms relating high levels of BCAAs to insulin resistance is the persistent activation of mTORC1/S6K1 pathway as a result of excess leucine levels, which subsequently causes an impairment of insulin signalling [6,75]. Hyper-phosphorylation of $S6K1^{T389}$ due to amino acid overload results in a negative feedback loop leading to phosphorylation of IRS-1 on serine residues ($Ser^{612,307,1101}$) rather than the normal tyrosine residues ($Tyr^{608,628}$). [23,71] Serine phosphorylation of IRS-1 impairs its function and inhibits further signalling to P13K/AKT pathway. [23, (Figure 2)] As a

result, GLUT4 cannot be recruited to the plasma membrane, leading to impairment of glucose transport and the development of insulin resistance and T2DM. [14,71] The importance of persistent activation of the mTORC1/S6K1 pathway has been shown in animal studies with disruptions in S6K1 activity. For instance, Um et al. reported that *S6K1*-deficient mice are protected against obesity and insulin resistance, thereby, signifying the importance of S6K1 in negative regulation of insulin signalling. [76]

2.4 Molecular Mechanisms of Insulin Resistance

2.4.1 Accumulation of Lipid Intermediates

Studies have consistently demonstrated that lipid-induced insulin resistance may result from accumulation of intracellular lipid metabolites which have deleterious effects on insulin-mediated IRS-1 tyrosine phosphorylation. [56] Specifically, intracellular lipid intermediates diacylglycerols (DAGs) and ceramides have been shown to be implicated in the pathogenesis of insulin resistance and T2DM. Ceramides are mainly membrane lipids and are the precursors for the formation of sphingomyelin, one of the primary lipids in the lipid bilayer. In rodent models, rises in hepatic and muscle ceramide content has been found to be associated with insulin resistance and can be toxic in certain cell types such as the pancreatic β cells and cardiomyocytes [77]. The role of ceramides in insulin resistance came from observations that they inhibit insulin-stimulated glucose transport and they do so by inhibiting insulin-mediated activation of Akt [78].

The lipid second messenger DAG is produced upon activation of the phosphoinositide signalling pathway which plays important roles in cell signalling, lipid signalling and membrane trafficking [79]. DAG is produced from PIP2 and is a physiological activator of protein kinase C (PKC) in addition to interacting with other signalling molecules such as small G proteins. Persistent activation of PKC has been shown to correlate with increased

serine phosphorylation of the insulin receptors, an effect that mediates insulin resistance.[80,81] The effect of DAG on insulin resistance can be explained by genetic manipulations of the enzyme that acylates DAGs into triglycerides. Liu et al. examined the effect of overexpression of diacylglycerol acyltransferase (DGAT) in skeletal muscle of mice and found that these mice exhibit increased levels of triglycerides, reduced levels of ceramides and DAGs and improved muscle and whole-body insulin sensitivity [80]. This finding recapitulates “the athlete’s paradox” phenomenon which refers to endurance athletes being very insulin-sensitive despite having high triglyceride contents [77]. Liu el al. also found DGAT overexpression to attenuate DAG-mediated PKC activation and downstream c-Jun N-terminal kinase 1(JNK1) response. JNK1 is a a stress mediator protein kinase activated by various environmental stresses, inflammatory cytokines and growth factors. JNK1 phosphorylates IRS-1 on Ser307 residues and suppresses the insulin-induced IRS-1/PI3K pathway activation.[80]

Thus, accumulation of intracellular lipid intermediates such as DAG and ceramide in skeletal muscle and liver may be a common mechanism leading to impairment of insulin of signalling and insulin resistance.

2.4.2 Chronic Inflammation

Chronic and low grade inflammation is associated with the pathogenesis of chronic diseases such as T2DM, atherosclerosis and cancer. Inflammation is a physiological process characterized by increased circulating levels of proinflammatory cytokines or elevated count of white blood cells. In general, inflammation is a protective mechanism required for initiating tissue repairing, healing wounds and battling infections in the body. However, overactivation of the inflammatory response has deleterious effects. In states of

obesity and T2DM, proinflammatory cytokines enter the bloodstream from adipose tissue and liver and cause systemic inflammation.

Early studies in T2DM patients observed lowering of blood glucose levels in response to the anti-inflammatory drug aspirin [82]. In rodent models, an increase in the proinflammatory cytokine TNF-α, was also observed in the adipose tissue of obese/insulin-resistant mice, indicating an association with insulin resistance [83]. More recent animal and human studies found elevated levels of inflammatory markers such as C reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1), interleukin 6 (IL-6) and other inflammation mediators in the plasma of obese patients and animals [83,84]. TNF-α has been shown to inhibit normal IRS-1 signalling [83]. Other signalling molecules such as JNK1 (c-Jun N-terminal kinase) and IκB kinase β (IKKβ) have also been found to be elevated in the adipose tissue and liver of obese patients and their activation appears to involve TNF-α-mediated IRS-1 inhibition. [84]

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex involved in survival and cytokine production, therefore, it regulates many genes involved in inflammation. IKKβ is a central regulator of inflammatory responses and is the primary kinase mediating NF-κB activation.[85] The IKKβ/NF-κB pathway is a key inflammation signaling pathway and is extensively studied in cancer and obesity. IKKβ inhibits insulin signaling by phosphorylating IRS-1 at multiple serine residues including Ser^{307} in adipocytes.[84] The inhibitory effects of IKKβ on insulin signalling is supported by evidence from animal studies. Arkan et al. found that IKKβ knockout mice demonstrated improved insulin sensitivity and greater protection from high fat diet-induced insulin resistance in both the liver and muscle. [86]

Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear receptor responsible for lipid synthesis and storage as well as regulation of cellular differentiation,

proliferation and metabolism.[56,84] It regulates a number of genes involved in insulin signalling, including the ones that control the expression of TNF-α and other proinflammatory cytokines.[87] Reduction of PPARγ has been shown to contribute to insulin resistance. It has been suggested that TNF-α inhibits the function of PPARγ which induces insulin resistance [84,87]. Importantly, PPARγ agonists such as thiazolidinediones, can antagonize the synthesis of TNF-α or its action in adipocytes. [88]

2.4.3 Oxidative Stress

Oxidative stress results from the imbalance between the production of reactive oxygen species (ROS) in the body and their disposal. ROS is mainly produced in the mitochondria when fatty acids or glucose are oxidized to generate ATP or heat. [84] Even though ROS production is necessary for normal signal transduction in cells, its overproduction will cause oxidative stress. In conditions of obesity, excess levels of fatty acids and glucose in the mitochondria produce oxidative stress, since obesity is normally associated with hyperglycemia and hyperlipidemia. [84] ROS can also be induced by other factors such as hypoxia.

Oxidative stress has been reported to be closely associated with insulin resistance and T2DM. Under diabetic conditions, glucose toxicity occurs as a result of chronic hyperglycemia which impairs insulin biosynthesis and secretion.[89] Production of ROS as a consequence of hyperglycemia has been shown to decrease insulin gene expression and secretion and ultimately β-cell dysfunction. Lipotoxicity is also involved in inhibition of β-cell function found in T2DM. Exposure of islets to free fatty acids has been shown to induce ROS and reduce insulin secretion and β-cell dysfunction.[89]

Furthermore, activation of the JNK pathway is also involved in pancreatic β-cell dysfunction observed in T2DM. This pathway is induced by many factors including

increased levels of free fatty acids, inflammatory cytokines, endoplasmic reticulum (ER) stress and ROS which are all involved in the development of T2DM. [89]The JNK pathway has been reported to be involved in ROS-mediated reduction of insulin gene expression and its suppression can protect β-cell from ROS damage. [90] Previous studies also suggest that ROS can inhibit the IRS-1/PI3K pathway and thereby, disrupt insulin-mediated GLUT4 translocation in 3T3-L1 adipocytes. [91] The importance of ROS implication in T2DM is highlighted by animal studies in which ROS suppression in obese/ type-2 diabetic mice was shown to rescue β-cell function and insulin sensitivity and lead to improved glucose tolerance.[92]

2.4.4 mTORC1/S6K1 Pathway

Chronic activation of the mTORC1/S6K1 pathway is one of the mechanisms involved in the development of T2DM and other pathophysiological conditions such as obesity and cancer. Persistent activation of mTORC1 as a result of nutrient overload, results in phosphorylation and activation of S6K1 and subsequent serine phosphorylation of IRS-1 which disrupts its normal activity and degrades the protein. This negative feedback loop has been shown to have profound implications in insulin resistance and T2DM. In-vivo studies further support the implication of S6K1/IRS-1 negative feedback loop in the pathogenesis of insulin resistance and T2DM, whereby S6K1-deficient mice show improved insulin sensitivity despite being on a chronic high fat diet.[76] Therefore, the mTORC1/S6K1 pathway has major implications in the development of insulin resistance and T2DM.

2.5 Mammalian Target of Rapamycin (mTOR)

The mammalian/mechanistic target of rapamycin (mTOR), a 289-kD protein, is a serine/threonine protein kinase belonging to the family of PI3K-related kinases. [93] The mTOR signalling pathway integrates intracellular and extracellular signals and is a central regulator of cell growth, metabolism, proliferation, survival, protein synthesis and ribosome biogenesis. mTOR interacts with several other proteins to form two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) with different subunit compositions, cellular functions and type of regulation. [93–96] One distinguishing feature of the two complexes is that although the immunosuppressant drug, rapamycin, perturbs mTORC1 signalling, short-term treatment with rapamycin does not inhibit mTORC2 signalling. [94] Since mTOR is generally deregulated in states of obesity, T2DM and cancer, it is important to understand the processes involved in the mTOR signaling network.

2.5.1 Mammalian Target of Rapamycin Complex 1 (mTORC1)

mTORC1 is comprised of five proteins: mTOR, the catalytic subunit of the complex, regulatory-associated protein of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8, also known as GbL), DEP-domain-containing mTOR-interacting protein (Deptor) and proline-rich AKT substrate 40 kDa (PRAS40). [14,94,97] It has been suggested that Raptor affects mTORC1 activity by regulating the complex assembly and recruiting mTOR substrates. Even though mLST8 is a positive regulators of mTORC1, its function in mTORC1 activity is still unclear as in-vivo deletion of this protein does not affect mTORC1 function [93,97]. PRAS40 and Deptor are characterized as a negative regulator of mTORC1. When mTORC1 activity is reduced, PRAS40 and Deptor are recruited to the complex where they inhibit mTORC1. PRAS40 has been suggested to

regulate mTORC1 activity by directly inhibiting substrate biding. When mTORC1 becomes activated, it directly phosphorylates PRAS40 and Deptor, thereby reducing their physical interaction with mTORC1.[93,95]

2.5.2 Mammalian Target of Rapamycin Complex 2 (mTORC2)

Some of the proteins are common to mTORC1 and mTORC2. In addition to mTOR, mLST8 and Deptor, mTORC2 has three other components: rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein (mSIN1) and protein observed with Rictor-1 (Protor-1). [93,94] Although mTORC2 is not as well characterized as mTORC1, its components have been identified and studied. Rictor and mSIN1 have been shown to interact with and stabilize each other, forming the structural foundation of mTORC2. [98,99] Rictor also interacts with Proctor-1 but the exact physiological function of this interaction is not yet clear. Similar to its effect in mTORC1, Deptor is a negative regulator of mTORC2 activity and it is the only endogenous inhibitor of mTORC2 so far. In contrast to what is observed with mTORC1, mLST8 is essential for mTORC2 function. This is supported by studies in which mLST8 knockout significantly reduced the stability and activity of mTORC2. [97]

2.5.3 mTORC1 and Downstream Substrates

The focus of my thesis will be on mTORC1 since it is the prominent regulator of cell growth and metabolism and integrates anabolic signals from amino acids and growth factors. Protein synthesis, an anabolic process required for cell growth is by far the most well-known process controlled by mTORC1. mTORC1 positively regulates protein synthesis by phosphorylating its two downstream substrates: the eukaryotic translation initiation factor (eIF) 4E-binding protein 1 (4E-BP1) and p70 ribosomal S6 kinase 1

(S6K1). Phosphorylation of these proteins allows mRNA biogenesis and translation and promotes protein synthesis. [96,100]

2.5.3.1 Ribosomal Protein S6 Kinase-1 (S6K1)

S6K1 particularly regulates protein synthesis, ribosome biogenesis and insulin-mediated anabolic responses. S6K1 belongs to the serine/threonine protein kinases family. Phosphorylation of S6K1 by mTORC1 occurs on the T389 residues. Activation of S6K1 by mTORC1 stimulates mRNA biogenesis, cap-dependent translation and elongation, and translation of ribosomal proteins through regulation of proteins such as the tumor suppressor programmed cell death 4 (PDCD4), ribosomal protein S6 and eukaryotic elongation factor 2 kinase (eEF2K). As a result, S6K1 promotes protein synthesis by enhancing the translational capacity of the cell. [100]

2.5.3.2 Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1)

4E-BP1 is involved in protein translation. Regulation of protein translation is controlled with ribosome recruitment at the 5’ end of an mRNA at the position of a start codon. A number of translation initiation factors facilitate ribosome binding to the 5’ end of an mRNA. There is a cap structure specific to the 5’ end of all nuclear-transcribed mRNAs which is specifically recognized by eukaryotic translation initiation factor 4E (eIF4E). eIF4E is one of the protein components of the trimeric complex known as eIF4F. The other two proteins are eIF4A, which is an RNA helicase and eukaryotic translation initiation factor 4G (eIF4G) and is a scaffolding molecule. eIF4E provides the essential interface between mRNA and the recruitment of eIF4A and eIF4G necessary for translation initiation. 4E-BP1 belongs to a family of translation repressor proteins and it

negatively regulates mRNA translation through its effect on eIF4E and eIF4G. 4E-BP1 competes with eIF4G to bind to dorsal surface of eIF4E. When 4E-BP1 is in a hypophosphorylated state, it prevents the formation of the eIF4F complex by preventing the recruitment of eIF4G to the 5' cap of mRNA. However, upon phosphorylation by mTORC1, a conformational alteration releases eIF4E, allowing it to recruit eIF4G to the 5' cap of mRNAs to allow cap-dependent translation. Thus, phosphorylation of 4E-BP1 by mTORC1 inhibits the suppression of mRNA translation.

2.6 Upstream Regulators of mTORC1

2.6.1 Growth Factors

As mentioned earlier, mTORC1 is the better characterized form of the two mTOR complexes and it senses and integrates inputs from intracellular and extracellular cues such as growth factors, energy status, stress, oxygen and amino acids. [94] Activation of mTORC1 by insulin or insulin-like growth factor 1 (IGF-1) occurs via Akt. Activated Akt stimulates mTORC1 signalling by phosphorylating and inhibiting the tuberous sclerosis complex (TSC1/2).[93,101] TSC1/2 inhibits the activity of mTORC1 by functioning as a GTPase-activating protein (GAP) towards the Ras homolog enriched in brain (Rheb) GTPase, which acts as an activator of mTORC1. [93,102,103] When Rheb is in its GTP-bound form, it directly interacts with mTORC1 resulting in strong stimulation of the kinase. Hence, TSC1/2 negatively regulates mTORC1 by converting Rheb into its inactive GDP-bound form. [93] Akt also regulates mTORC1 in a TSC1/2-independent manner by phosphorylating PRAS40 (an inhibitor of mTORC11) and dissociating it from raptor.

2.6.2 Stress

In addition to growth factors, many stress signals also act on mTORC1 through the TSC1/2 complex. The best characterized stress signals to date are DNA damage, low oxygen and low energy levels. The mTORC1 pathway directly senses low ATP levels through a mechanism involving AMPK. In response to hypoxia or low energy status, AMPK phosphorylates TSC1/2 and stimulates its GAP activity towards Rheb. Furthermore, AMPK phosphorylates raptor, leading to inhibition of mTORC1 through allosteric mechanisms. [94,96] Hypoxia also leads to low energy levels as a result of impaired mitochondrial respiration. Hypoxia stimulates the expression of regulated in development and DNA damage responses 1 (REDD1) gene, which suppresses mTORC1 through a TSC1/2-dependent mechanism.[95] It has been shown that in response to hypoxia, REDD1 inhibits the TSC2/14–3–3 interaction and mTORC1 activity [104]. DNA damage can also negatively regulate mTORC1 activity through multiple mechanisms that involve p53-dependent upregulation of AMPK. [94]

2.6.3 Amino Acids

In addition to being the building blocks of proteins, amino acids, particularly leucine, are able to stimulate mTORC1 activity leading to increased muscle protein synthesis and decreased proteolysis. [30,64,70,105] The importance of amino acids in organismal growth and homeostasis was appreciated decades ago when early studies showed that rats deprived of leucine displayed significant weight loss, muscle waste and eventually death [106]. It was also observed that amino acid deprivation results in rat liver autophagy (the process of cellular self-eating via lysosomal degradation).[107] Leucine and arginine have been shown to be the most potent stimulators of mTORC1 activity among all the amino acids [94]. In mammalian and yeast cell lines, withdrawal of amino acids from the culture media

significantly suppresses mTORC1 activity [108]. When there are sufficient levels of amino acids in the cell, mTORC1 becomes active due to its lysosomal localization [109]. Amino acid-dependent activation of mTORC1and its localization to the lysosome require the Rag GTPases.[13,94,109,110] Rag proteins are the central regulators of amino acid signalling to mTORC1 because they regulate the subcellular localization of mTORC1 and its activity. In mammals there are four Rag proteins and they exist as heterodimers where RagA and RagB bind to either RagC or RagD [110]. Amino acids control the nucleotide loading of the Rag proteins causing them to switch to an active conformation, which then bind and activate mTORC1. When the Rag proteins are in the RagA/B ^{GTP}, Rag C/D ^{GDP} form, they display maximum activity and binding to mTORC1 [110]. When active, the Rag GTPases recruit mTORC1 to the lysosomal surface where it directly interacts with Rheb to become activated. [93] On the lysosomal surface, the Rag GTPases dock on a complex called Ragulator. This complex is essential for amino acid-mediated activation of mTORC1 similar to Rag GTPases because it recruits mTORC1 to the lysosome in response to amino acids. The localization of the Ragulator and the Rag GTPases on the lysosomal surface suggests an important role for this organelle in amino acid sensing by the mTORC1 pathway. Recently, a model of amino acid sensing has been proposed in which amino acids accumulate in the lysosomal lumen and initiate signaling through a mechanism requiring the vacuolar H^+-adenoside triphosphate ATPase (v-ATPase). [13] When v-ATPase subunits are depleted, amino acid-induced translocation of mTORC1 to the lysosomal surface and downstream signaling is blocked. The v-ATPase directly interacts with the Ragulator and provides a link between Rag GTPase and the v-ATPase on the lysosomal surface. Moreover, the mTORC1 pathway regulates the expression of v-ATPase, suggesting that a feedback loop exists between mTORC1and lysosome function.[13,111]

Another protein implicated in amino acid sensing by mTORC1 is the class 3 PI3K or the mammalian vacuolar protein sorting 34 homologue (hVPS34). Amino acids cause an increase in intracellular calcium levels which stimulate mTORC1 and hVps34 activation. This rise in intracellular calcium levels promotes the binding of calmodulin to hVps34.[112]

Interestingly, a recent study by Demetriades et al. found the TSC1/2 complex is also involved in the amino acid-mediated regulation of mTORC1, thereby placing this complex at the intersection of all signalling inputs to mTORC1[109]. They proposed a model in which TSC1/2 complex is part of the molecular machinery required for mTORC1 to respond to amino acid starvation. They found that TSC1/2 complex responds to amino acid starvation by changing its subcellular localization. They also showed that upon amino acid withdrawal, TSC2 is required for mTORC1 to be fully released from the lysosome. [109]

Therefore, elevated levels of amino acids can result in hyperactivation of the mTORC1 pathway which can create an abnormal metabolic response and lead to the development of insulin resistance.

2.7 Branched-chain Amino Acids (BCAAs) and Metabolic Health

BCAAs refer to the three essential amino acids: leucine, isoleucine and valine. These amino acids cannot be synthesized in the body, thus, they must be obtained through diet. BCAAs comprise a large part of dietary amino acids. The percentage of BCAAs in food sources varies between 20 and 25% of total protein content. [19] There is abundant evidence for the critical role of BCAAs in regulating the maintenance and growth of skeletal muscle by enhancing protein synthesis [64,67,113]and inhibiting protein breakdown[114]. BCAAs also play important roles in attenuating exercise-induced muscle damage and delayed-onset muscle soreness[114,115], lowering the loss of lean body mass during weight

loss[116] and preserving and restoring muscle mass in patients with liver disease such as advanced cirrhosis [19]. BCAAs and insulin are anabolic signals that affect the growth of energy-consuming tissues, mediated in part through their ability to activate the mTORC1 pathway. Even though BCAAs are believed to improve many aspects of health, several lines of evidence suggest their implication in the development of insulin resistance and T2DM. Therefore, the idea that dietary BCAAs or their supplementation might be beneficial in preventing metabolic disorders is controversial.

2.7.1 BCAA Metabolic Pathway

The metabolism of BCAAs involves two common steps. The first step occurs in most peripheral tissues except the liver. This step involves the reversible transamination of BCAAs and is catalyzed by the mitochondrial isoform of the enzyme branched-chain amino acid transferase (BCATm) encoded by the *BCAT2* gene. In the transamination step catalyzed by BCATm, an α-amino group of BCAAs is transferred to α-ketoglutarate to form the three branched-chain ketoacids: α-ketoisocaproic acid (KIC), α-ketoisovaleric acid (KIV), and α-keto-β-methylvaleric acid (KMV). While BCATm is expressed in most non-neuronal tissues except the liver, the cytosolic isoform of the enzyme (BCATc) is expressed in the central nervous system and peripheral nerves.[66]

The second step in BCAA catabolic pathway is the irreversible oxidative decarboxylation of the ketoacids catalyzed by the mitochondrial branched-chain α-ketoacid dehydrogenase complex (BCKDH) which yields the three CoA derivatives of BCAAs: propionyl-CoA, acetoacetate and acetyl-CoA.[117,118] The metabolic pathway further continues and produces intermediates used in the TCA cycle. The BCKDH complex contains three enzymes: E1(branched-chain α-ketoacid decarboxylase), E2 (dihydrolipoyl

transacylase) and E3 (dihydrolipoyl dehydrogenase). Phosphorylation and dephosphorylation of the E1 α subunit regulates the activity of BCKDH complex. [5] The first product of leucine catabolism, α-ketoisocaproicacid (KIC) has been the focus of research as there is some evidence for its involvement in mTORC1 activation and protein synthesis. Studies in primary adipocytes have shown KIC to be as effective as leucine in stimulating mTORC1signalling [70]. However, this could be due to the conversion of KIC back to leucine, since the first reaction in leucine catabolism is reversible and therefore, increased leucine levels may account for increased stimulation of mTORC1 signalling in response to KIC treatment. [119] In fact, She et al. examined mTORC1 signalling in BCAT2 knockout and wild-type mice to investigate the role of leucine metabolism in mTORC1 signalling pathway [66]. They observed increased phosphorylation of S6K1 and 4E-BP1 only in the skeletal muscle of wild-type mice suggesting that conversion of leucine to KIC may not be essential for stimulating mTORC1 activity by leucine. Other studies have also reported that leucine is a direct-acting nutrient signal and its metabolism may not be required for mTORC1 activation in adipose tissue.[18] Because current data is inconclusive, further research is needed to understand whether KIC can promote mTORC1 activation independently of leucine or if conversion of KIC back to leucine is required to promote mTORC1 activity.

Another metabolite of leucine β-hydroxy-β-methylbutyrate (HMB) has also been studied in the context of muscle protein turnover. HMB has been shown to stimulate protein synthesis and increase skeletal muscle hypertrophy via the mTORC1 pathway in rats [120]. Furthermore, an in-vitro study by Girón et al., found that conversion of leucine to HMB is required for potent stimulation of mTORC1 and enhancing protein synthesis in L6 myotubes and that HMB is more effective than leucine in stimulating protein synthesis in skeletal muscle.[121] HMB has also been reported to have anti-catabolic

effects, attenuate muscle proteolysis during exercise and increase muscle strength and performance.[120,122] Even though a combination of HMB, resveratrol and metformin has been recently shown to improve insulin sensitivity in C2C12 muscle cells, the independent effect of HMB on insulin signalling has not yet been studied.[123] Since, this particular metabolite of leucine is able to stimulate mTORC1 activity, it would be interesting to determine if and how it is implicated in the development of insulin resistance.

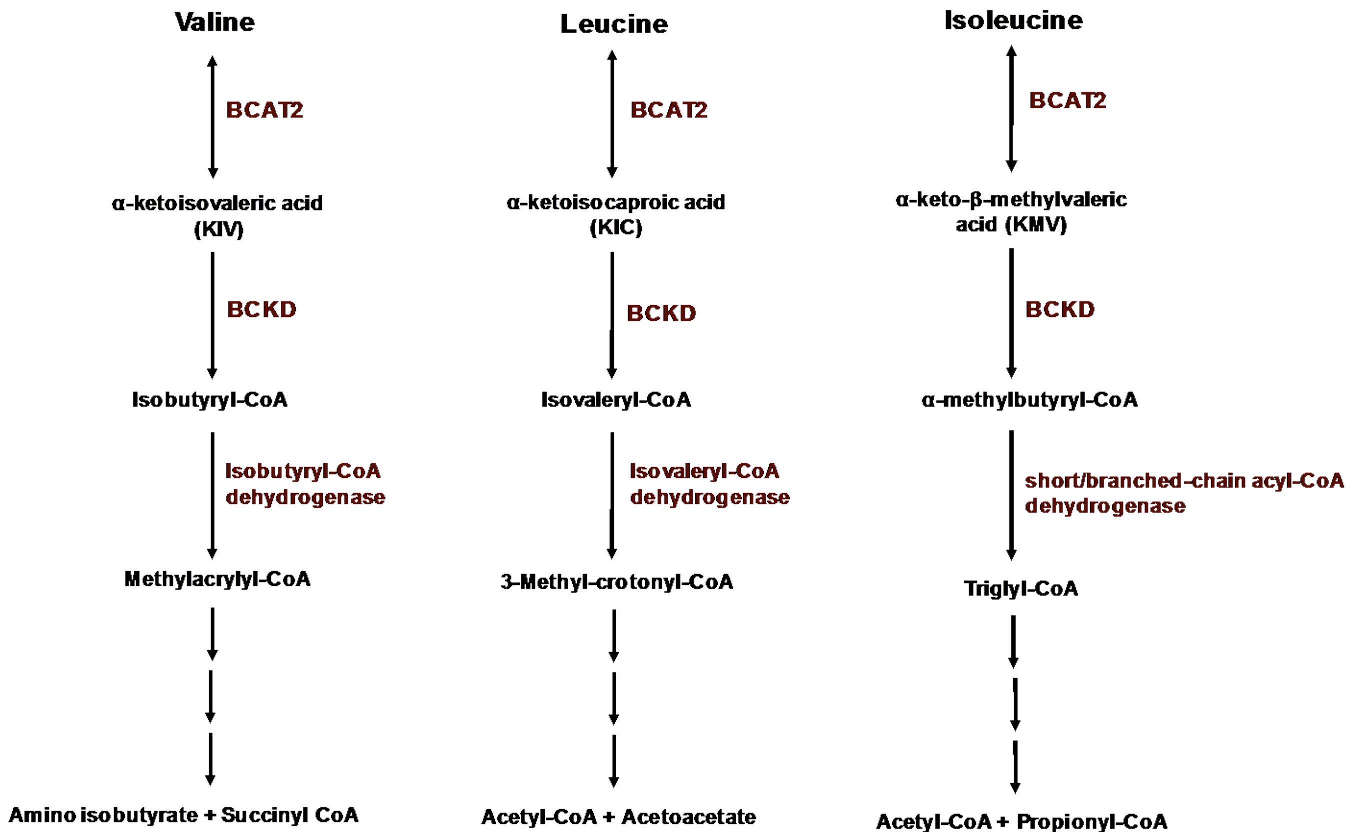


Fig 3. BCAA metabolic pathway. The three branched-chain amino acids, leucine, isoleucine, and valine are deaminated via the action of the branched-chain aminotransferase (BCAT2) enzyme. This step is reversible. The subsequent α-ketoacids then undergo oxidative decarboxylation via the action of the enzyme complex, branched-chain ketoacid dehydrogenase (BCKD). This reaction yields the CoA derivatives of the decarboxylated ketoacids. The third step in BCAA catabolism is a dehydrogenation step that involve three individual enzymes, one for each of the CoA derivatives generated via the BCKD reaction. The remainder of the catabolic pathways for the three BCAAs diverges and generates substrates for the Krebs cycle.
Figure adapted from "Branched-chain amino acids in metabolic signalling and insulin resistance", by C.J. Lynch and S.H. Adams, 2014, *Nature Reviews Endocrinology*, 10, 723–736.

2.7.2 The link between BCAAs, mTORC1 and Insulin Resistance-BCAA Paradox

As mentioned earlier, BCAAs have positive anabolic effects, however, paradoxically, they have been shown to be associated with obesity, insulin resistance and T2DM.[6] A lot of the evidence comes from metabolomics studies. Metabolomics is the comprehensive measurement of processes involving the identification and quantification of cellular metabolites and is a widely used technique to study metabolic diseases. Metabolic profiling has provided new insight into the mechanisms that underlie the development of insulin resistance and T2DM.

Huffman et al. measured various metabolic intermediates (including acylcarnitines, amino acids and fatty acids) using mass spectrometry in obese individuals who are at risk of T2DM. They found elevated concentrations of these intermediates to be closely related to insulin resistance and impaired pancreatic response. [124] Other metabolomics studies observed similar findings. In a study done by Newgard et al., mass spectrometry techniques were used to analyze and compare plasma samples of lean and obese insulin-resistant individuals. BCAAs and their metabolites C3 and C5 acylcarnitines, along with other amino acids such as glutamine and alanine were strongly correlated with the development of insulin resistance. [75] In addition, Suhre et al. found elevated levels of metabolic biomarkers of diabetes including sugar metabolites, ketone bodies and BCAAs using a multiplatform metabolomics approach in an epidemiological cohort. [125]

Clinical studies also show positive correlations between increased BCAA levels and insulin resistance, HOMA index and HbA_1 levels [5,126,127] Shah et al. examined plasma samples from 500 overweight/obese subjects at baseline and 6 months after weight loss. They used metabolic profiling of 60 metabolites and biochemical assays of ketones, insulin, glucose and non-essential fatty acids and found that a metabolite signature that

represents BCAA catabolism is associated with insulin resistance and can be improved with weight loss [128]. Interestingly, Laferrère et al. compared the metabolic effects of two methods of weight loss: dietary intervention and gastric bypass surgery in two groups of obese type-2 diabetic individuals with identical weight loss. Using mass spectrometry metabolomics, they showed that the level of BCAAs and their related metabolites were reduced much more significantly after gastric bypass surgery than a dietary intervention program resulting in the same amount of weight loss. This indicates that the reduction in circulating BCAA levels, improvements in glucose tolerance and BCAA oxidation observed in these patients involve mechanisms other than weight loss. [129] Furthermore, longitudinal studies in different groups have indicated that increased circulating levels of BCAAs predict future insulin resistance and T2DM.[7,130] Although such associations are repeatedly observed in human models, the mechanisms underlying the relationship between high blood BCAA levels and insulin resistance remain to be fully elucidated.

So far, two potential mechanisms have emerged that explain how BCAAs may contribute to the development of insulin resistance and T2DM. The first mechanism suggests that increasing levels of dietary BCAAs results in hyperactivation of the mTORC1 pathway which promotes insulin resistance through serine phosphorylation of IRS-1. The second mechanism proposes that excess BCAA levels are a biomarker of impaired BCAA metabolism, while these impairments could also result in accumulation of toxic BCAA metabolites that stimulate β-cell mitochondrial dysfunction and stress signalling associated with insulin resistance and T2DM. [6] Therefore, BCAAs can have positive and negative consequences. They are beneficial in increasing muscle mass due to their highly anabolic effects, but can also exert negative effects on insulin signalling and cause insulin resistance. Thus, further studies are needed to fully understand their mechanism of action and implication in insulin resistance and T2DM.

2.8 Rationale

Despite the growing research on the effect of high protein/leucine diets on mTORC1 activation and implications in insulin resistance, current evidence on the exact molecular mechanism that mediates these effects still remains inconclusive. Although BCAAs and their metabolites have been shown to be implicated in the development of insulin resistance and T2DM [7,8,17], some studies suggest beneficial roles of high protein/BCAA levels in improving insulin sensitivity and glucose uptake in skeletal muscle [65,68,119,131–133]. Thus, it is imperative to understand how leucine and its metabolites regulate glucose transport and mTORC1/S6K1 signalling in skeletal muscle since it is the predominant tissue for insulin-induced glucose disposal. Most studies that have examined glucose transport and mTORC1 signalling in response to leucine treatment in skeletal muscle have tested supraphysiological concentrations of leucine, which do not correlate with normal physiological levels. Therefore, it is important to determine the effect of leucine in a concentration range that is more physiologically relevant. Furthermore, under normal circumstances, leucine is consumed along with other amino acids; therefore, it is also essential to understand the effect of leucine on glucose transport and mTORC1 signalling in the presence of other amino acids.

Moreover, it is not exactly known whether the effects observed with leucine on glucose transport in-vitro are due to its intracellular catabolism. More specifically it is not known whether leucine metabolite KIC can independently regulate insulin sensitivity in L6 myotubes.

2.9 Objective

1) Examine the molecular mechanisms that mediate the effect of leucine supplementation on insulin-stimulated glucose uptake and mTORC1 pathway activation in L6 rat skeletal muscle cells. More specifically, determine whether leucine's effect is dose-dependent, using physiologically relevant concentrations.

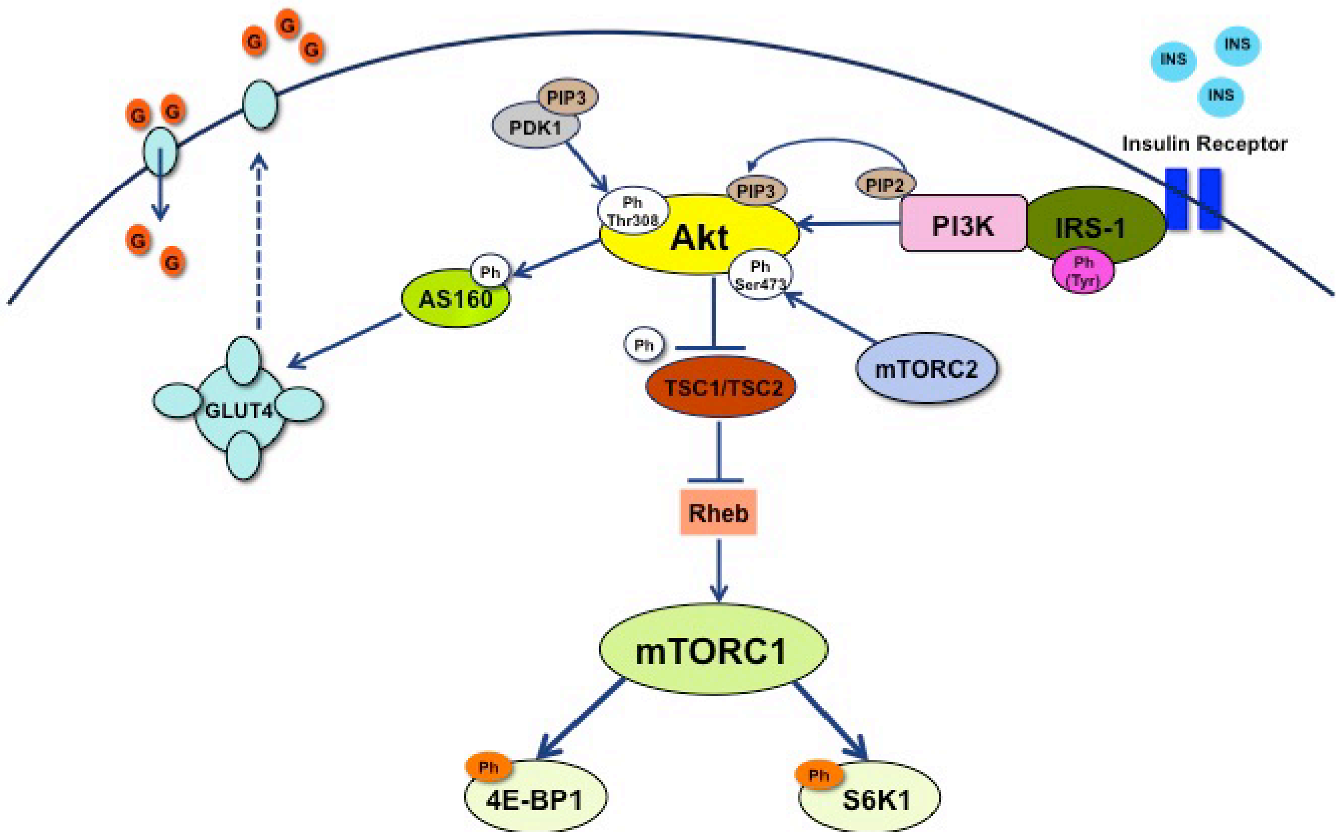
2) Examine the dose-dependent effect of leucine on insulin-induced glucose uptake and mTORC1 signalling in the presence of other amino acids.

3) Determine the effect of KIC treatment on insulin-stimulated glucose transport and mTORC1 signalling in L6 myotubes.

4) Examine whether KIC-mediated regulation of glucose transport and mTORC1 activity is independent of its intracellular conversion to leucine

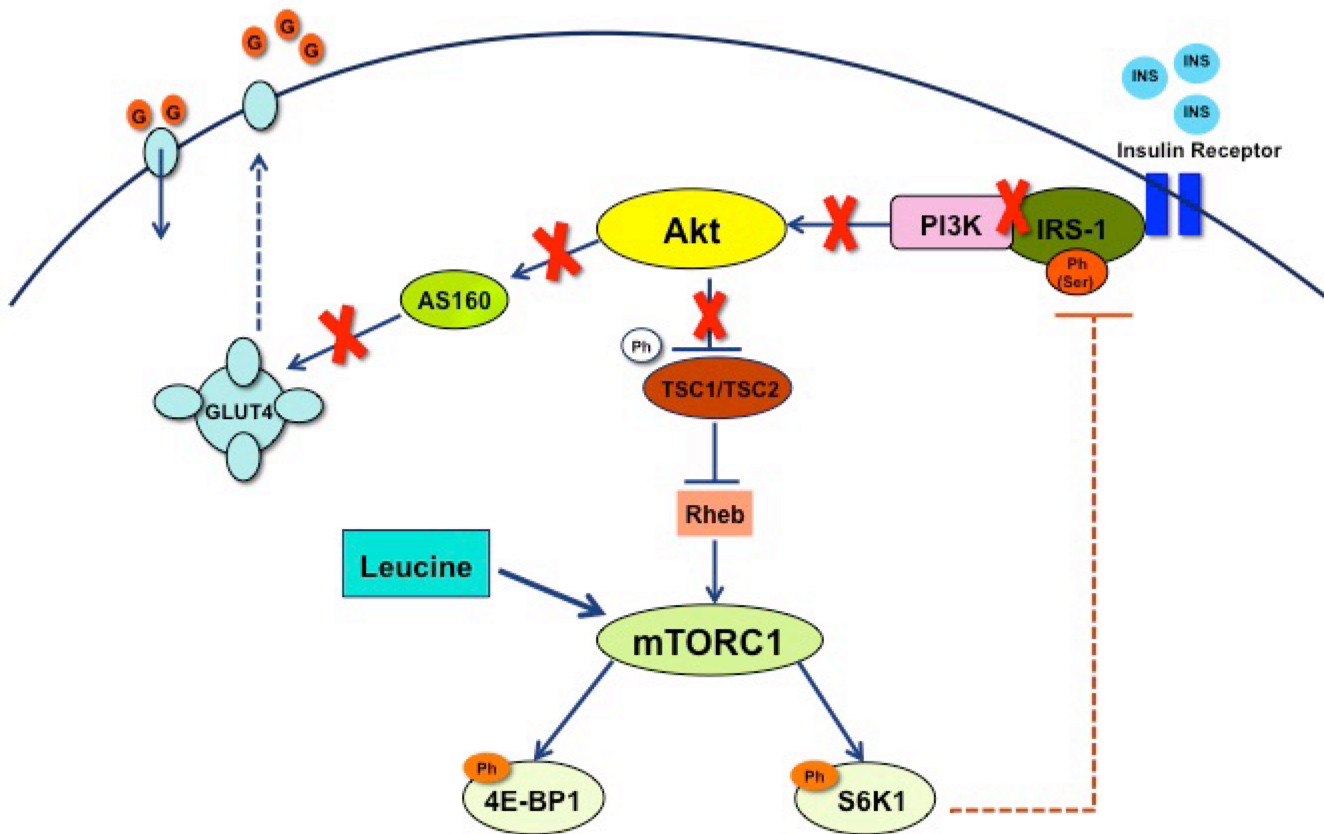
3.0 Hypothesis

I hypothesize that leucine treatment will suppress insulin-stimulated glucose transport in skeletal muscle through increased activation of mTORC1/S6K1 signalling. I also hypothesize that KIC can exert similar effects on insulin-stimulated glucose uptake and mTORC1 signalling in L6 myotubes. Finally, I hypothesize that KIC-mediated regulation of insulin signalling and mTORC1 pathway is at least in part due to its intracellular conversion back to leucine.



<u>Figure 1.</u> General overview of insulin signalling pathway and proteins involved in glucose transport

Insulin binds to the insulin receptor leading to activation of the receptor and thus tyrosine phosphorylation of the insulin receptor substrate 1 (IRS-1). This causes IRS-1 to interact with phosphatidylinositol-3-kinase (PI3K), facilitating the conversion of phosphatidylinositol-4, 5-bisphosphate (PIP2) to phosphatidylinositol-3, 4, 5-triphosphate (PIP3). PIP3 then allows the recruitment of other kinases such as PDK1, which phosphorylates Akt. Upon its activation, Akt phosphorylates and inhibits Akt substrate of 160 kD (AS160), thereby allowing the translocation of GLUT4 to the plasma membrane and leading to glucose uptake into the cell. In addition, Akt activates mTORC1 by phosphorylating TSC1/2 and removing its inhibitory restraint on Rheb. Amino acids can directly activate mTORCl through the Ragulator complex on the lysosomal surface which acts as a docking site for Rag GTPases. When mTORC1 is recruited to the lysosomal membrane, it interacts with Rheb in its GTP-bound form and it becomes activated. Figure adapted from “Insulin resistance in the nervous system”, by B. Kim and E. L. Feldman, 2012, *Trends in Endocrinology and Metabolism*, 23, 133-141.



<u>Figure 2.</u> Defective insulin signalling as a result of excess amino acid levels

Amino acids (particularly leucine) are potent stimulators of mTORC1 activity. Thus, amino acid overload can result in hyperactivation of mTORC1, leading to the formation of a negative feedback loop from S6K1 to IRS-1. Phosphorylation of IRS-1 on serine residues instead of the normal tyrosine residues leads to inhibition of its function and its degradation. This defect in insulin signalling pathway can ultimately result in insulin resistance and inhibition of glucose uptake.
Figure adapted from "Upstream of the mammalian target of rapamycin: do all roads pass through mTOR?", by M. N. Corradetti and K. L. Guan, 2006, *Oncogene*, 25, 6347–6360.

4.0 Manuscript

Leucine and its metabolite, α-ketoisocaproic acid, suppress insulin-stimulated glucose transport in L6 myotubes

Mahshid Moghei [1], Olasunkanmi A.J. Adegoke [1]

Muscle Health Research Centre, School of Kinesiology and Health Science, York University, Toronto, ON, M3J 1P3

Corresponding Author: Dr. Olasunkanmi A.J. Adegoke
Muscle Health Research Centre, School of Kinesiology and Health Science, York University, Toronto, ON, Canada. Tel: 416-7362100 Ext 20887. Fax: 416-736-5774. Email: oadegoke@yorku.ca

Key words: Insulin resistance, skeletal muscle, glucose transport, leucine, KIC, mTORC1,

Introduction

Type 2 diabetes mellitus (T2DM) is a global health concern and a major risk factor for morbidity and mortality. It is preceded by a condition known as insulin resistance which is characterized by a reduction in the body's sensitivity to the hormone insulin. [4] Many factors can contribute to the development of insulin resistance and T2DM with nutrition being one of the major ones. In addition to high fat diets, high protein diets have recently been shown to be closely associated with the development of insulin resistance and T2DM.[8]

Branched-chain amino acid (BCCAs) are often used for their positive effects in metabolic health They have many positive effects such as regulation of body weight, muscle protein synthesis and glucose homeostasis.[6] Therefore, BCAA supplementations are often used for weight loss in people with obesity. Among all three BCAAs, leucine has the strongest effect on muscle protein synthesis through the activation of the mammalian/mechanistic target of rapamycin complex 1 (mTORC1). Recently, studies have suggested that despite their beneficial effects, high concentrations of plasma BCAAs are associated with a higher risk of T2DM. This has been supported by studies in human and rodent models which found elevated level of circulating BCAAs and their metabolites in the plasma of insulin resistant/Type-2 diabetic subjects. Hyperactivation of the mTORC1 pathway and the subsequent impairment of normal insulin receptor substrate-1 (IRS-1) function has been shown as a mechanism contributing to the development of insulin resistance and T2DM.[5–7,130] In contrast, other studies have shown benefits of BCAAs, particularly leucine, in stimulating glucose transport and improving insulin sensitivity in skeletal muscle. [68,119,132,133] Therefore, it is not exactly known how BCAAs may be involved in the development of insulin resistance and T2DM. The objectives of this study were to examine the dose-dependent effect of leucine on insulin-

stimulated glucose uptake in the presence or absence of other amino acids in L6 skeletal muscle cells. We also examined how leucine metabolite, α-ketoisocaproic acid (KIC), affects glucose transport and mTORC1 signalling in control and in cells where BCAT2 (branched-chain aminotransferase 2 mitochondrial) enzyme has been depleted. This enzyme catalyzes the reversible transamination of leucine to KIC. We found that leucine significantly impairs insulin-stimulated glucose transport, concurrent with increased mTORC1 activation, suggesting a link between impaired insulin signalling and mTORC1 activity. KIC was also found to inhibit insulin-stimulated glucose transport through upregulation of mTORC1 activity. Interestingly, depleting the BCAT2 enzyme attenuates the inhibitory effect of KIC on insulin-induced glucose transport, suggesting that in fact it is the intracellular conversion of KIC to leucine which causes the impairment of glucose transport. Our results suggest that leucine and KIC impair insulin-stimulated glucose transport in L6 myotubes and therefore, may be implicated in the development of insulin resistance and T2DM.

5.0 Materials and Methods

5.1 Reagents

The growth medium (GM) used for cell growth was α- Modification of Eagle's Medium (AMEM) purchased from Wisent (#310-010-CL), supplemented with 10% fetal bovine serum (FBS) (Gibco #26050-088) and 1% Antibiotic-Antimycotic (Wisent #450-115-EL). Phosphate Buffered Saline (PBS) and Trypsin were also purchased from Wisent. The medium used for differentiation of cells (DM) consisted of AMEM, 1% antibiotic-antimycotic and 2% horse serum (HS) (Gibco #26050088). RPMI 1640 (a medium free of amino acids and serum) was used as the starvation medium and was purchased from United States Biologicals (#R8999-04A). L-Leucine (#61-90-5) and Sodium 4-methyl-2-oxovalerate (KIC) (#K0629) were purchased from Sigma Aldrich. RPMI 1640 without leucine (a medium containing all amino acids except leucine) was used for leucine dose-dependent experiment in the presence of other amino acids and was purchased from United States Biologicals (#R8999-12). BCAT2 (#111-125) and scramble (negative control) siRNA (#S1452) oligosachharides were purchased from Sigma-Aldrich. Lipofectamine RNAiMAX was purchased from Life technologies (#13778-150). Opti-MEM 1X Reduced Serum Medium was purchased from Life Technologies (#31985-070). Immobilon Western HRP chemiluminescence substrate was obtained from Fischer-Scientific (#WBKLS0500). [^{3}H]-2-deoxyglucose was purchased from Perkin Elmer (#NET549).

5.2 Cell Culture

L6 rat skeletal muscle myoblasts were purchased from American Type Culture Collection. Cells cells were cultured in 10 cm plates with growth medium composed of AMEM supplemented with 10% FBS and 1% Antibiotic-Antimycotic and allowed to propagate at 37°C and 5% CO_2 in a cell culture incubator until they reached ~ 80% confluency. Next, they were counted and seeded at a density of $2x10^5$/well in either 6-well plates for western blot experiments (described in section 3.5) or 10^5 cells/well for 12-well plates for glucose transport (described in section 3.3). Cells were allowed to proliferate for 48 hours or until they became confluent. Following the incubation period, the medium was switched to DM every 48 hours and cells were allowed to differentiate into myotubes until D5 when the experiments were performed (Description below).

5.3 Glucose Transport Assay

Glucose uptake assay, a measure of insulin action, was performed using radiolabeled 2- deoxyglucose (2-DG). When 2-DG is taken up by glucose transporters, it is phosphorylated to 2-DG-6-phosphate (2-DG6P); however, it cannot be further metabolized and therefore accumulates in the cell. The addition of radiolabeled glucose ([^{3}H]-2-deoxyglucose) as the tracer to 2-DG in the transport solution allows for the entry of the radioactively tagged glucose into the cells along with normal 2-DG glucose. [134] Therefore, we can measure the level of glucose uptake by determining the amount of radioactivity present in the cell.

On Day 5 of differentiation, myotubes were starved for 4 hours in RPMI (complete starvation medium, free of amino acids and serum). Following the starvation period, myotubes were treated with the first treatment, either RPMI (without amino acids and serum) or RPMI (containing all other amino acids except leucine) in which leucine was

added at various concentrations (150, 350, 600, 800 and 1800 μM) for 30 minutes. For the KIC experiment, following the 4 hr starvation, myotubes were treated with RPMI (free of amino acids and serum) containing two different concentrations of KIC (200 and 400 μM) for 30 minutes. Subsequently, the incubation with leucine or KIC continued in the presence or absence of 100 nM of insulin for 20 minutes. The cells were then rinsed twice with HEPES (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid) buffered saline, which is an organic chemical buffering agent. They were then incubated in 300 μL of transport solution (HEPES buffer, 10μM 2-deoxyglucose, 0.5 μCi/mL [^{3}H]-2-deoxyglucose) for 5 minutes at 37°. Following the 5-minute incubation period, the transport solution was removed and the cells were immediately rinsed with ice-cold stop solution (0.9% Saline) three times to stop the reaction and prevent any further glucose uptake. Subsequently, 1mL of 0.05M NaOH was added to each well and the cells were scraped and collected on ice. Samples were then stored at -20°C for further analysis. During analysis, 200 μL of each sample was used to conduct protein assay and the remaining 800 μL was added to 4-5 mL of Scintillation fluid (Ecolite+, MP Biomedicals #01882475) in liquid scintillation vials. The amount of radioactivity in each sample was counted using a Liquid Scintillation Counter (Tri-Carb Liquid Scintillation Counter). Rate of glucose transport was expressed per μg of protein. (See Appendix A and B)

5.4 siRNA Gene Silencing

L6 myoblasts were plated in 6-well plates at a density of $2x10^5$ cells/ well. After 48 hours, medium was shifted to DM. On day 3 of differentiation, myotubes
were transfected with 50 nM of BCAT2 siRNA or 50 nM of scramble siRNA (negative control) using Lipofectamine RNAiMAX reagent according to the manufacturer's instructions (Life technologies). Lipofectamine RNAiMAX reagent was diluted in Opti-

MEM medium. BCAT2 siRNA and the scramble siRNA were diluted in Opti-MEM medium. Next diluted siRNAs were added to diluted Lipofectamine RNAiMAX reagent in 1:1 ratio and were allowed to incubate for 5 minutes at room temperature. Finally, 250 μL of the siRNA-lipid complex was added to respective wells containing 1mL of antibiotics-free a-MEM with 2% HS. Twenty four hours following transfection, 1mL of a-MEM containing 2% HS and 1% Ab-Am was added to each well. On day 5 of differentiation (48 hours following transfection) some cells were harvested to test the efficiency of the BCAT2 knockdown using immunoblot analysis. The remaining wells were either used for glucose transport assay (refer to section 5.3) or western blot analysis (refer to section 5.5). (See Appendix A and C)

5.5 Cell Harvesting for Western Blot Analysis

Following the treatments, cells were rinsed with PBS. Then 100 μL of lysis buffer [1mM EDTA, 2% sodium dodecyl sulphate (SDS), 25 mM Tris-HCL pH 7.5, 10μL/mL protease inhibitor (Sigma Aldrich #P8340), 10μL/mL phosphatase inhibitor (Sigma Aldrich # P5726) and 1mM DTT (Research Organics #2190D-A101X) was added to each well of the 6-well plate. The cells were then scraped and transferred into 1.5 mL Eppendorf tubes using a 1mL Syringe. Repeated aspiration and expulsion was used to ensure breakdown of the cell lysate. Cells were stored at -20°C for further analysis.

5.6 Protein Assay and Western Blot Analysis

The Pierce BCA Protein Assay Kit (Thermo Scientific #23225) was used to determine protein concentration. The KC4 plate reader software (Bio-Tek Instruments Inc.) was used to obtain an absorbance reading of each well at a wavelength of 550 nanometers. A standard curve was used to estimate the volume required to load 25 μg of protein into one

well of a polyacrylamide gel. Equal amounts of protein were loaded into each well of the gel. The proteins were separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Following gel electrophoresis, they were transferred onto polyvinylidene difluoride (PVDF) membranes. Next, membranes were incubated for one hour in 5% non-fat milk in Tris Buffered Saline with Tween (TBST) at room temperature to block non-specific antigen binding. Subsequently, they were quickly rinsed 3 times with TBST for 5 minutes and then incubated overnight at 4°C with the desired primary antibody.

Primary antibodies*:* ph-S6K1^{Th389} 1:1000 dilution (Cell Signalling #9205), ph-IRS-1^{Ser612} 1: 1000 dilution (Cell Signalling #3203), ph-S6 $^{Ser\ 235/236}$ 1:1000 dilution (Cell Signalling # 4858), Gamma-Tubulin 1:10000 dilution (Sigma Aldrich #T6557). BCAT2 1:1000 dilution (Sigma Aldrich #111-125).

Following the overnight incubation in primary antibody, membranes were quickly rinsed 2 times and 3 times for 5 minutes each with TBST and then incubated in secondary antibody for three hours at room temperature.

Secondary Antibodies*:* Anti-rabbit (CST # 7074) and Anti-mouse (CST #7076) antibodies were used with the dilution of 1:10000. Subsequently, membranes were rinsed again 3 times for 5minutes each with TBST before HRP chemical luminescent substrate was applied to them. Kodak molecular imaging system was used for signal visualization and the images were quantified with Carestream molecular imaging software (version 5 .0.3.33)

4.7 Graphical Representations of Glucose Transport and Western Blots

Glucose transport data is normalized to the control group with no amino acid, KIC or insulin (CTL-insulin). This was done by dividing the amount of [^{3}H]-2-deoxyglucose (pmol) transported into cells in each well by the concentration of protein found in each well (μg). The glucose transport value (pmol/μg) was then expressed as percentage of glucose transport in myotubes that were not treated with amino acid, KIC or insulin. Western blots for ph-S6K1, ph-IRS-1 and ph-Akt were normalized to the control group which was not treated with amino acid or KIC, but treated with insulin (CTL+ins)

4.8 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 software. Data presented here are means ± SEM. One-way analysis of variance (ANOVA) was used and Tukey's post-hoc tests were done to measure statistically significant differences among means. Two-tailed T-tests were also performed to measure difference between two groups (ie. +ins vs –ins). Significance was determined as $p < 0.05$. Bars with different letters are significantly different. Letters shared among groups indicate they are not significantly different.

6.0 Results

The effect of leucine on glucose transport in the presence of other amino acids

Since dietary proteins contain amino acids other than leucine, we sought to determine how the presence of other amino acids (medium composition) affects insulin-stimulated glucose uptake in L6 cells. We tested the dose-dependent effect of leucine in a medium that contains all the other amino acids but leucine. Since most studies in the literature have worked with supraphysiological concentrations of leucine, we chose to work with concentrations that are within a physiologically relevant range (0-1600 μM). In the presence of other amino acids, leucine does not significantly alter insulin-stimulated or basal glucose transport levels relative to control. The insulin effect within the control group was 100%, while for other concentrations of leucine it ranged from 24-48%, suggesting that in the presence of other amino acids, the effect of insulin becomes weaker since basal glucose uptake is higher compared to control **(Fig 4)**

The effect of medium composition (presence of other amino acids) on leucine-mediated stimulation of mTORC1

To determine how the presence of other amino acids affects leucine-mediated stimulation of mTORC1 signalling and examine if the effects observed with glucose transport are mediated through mTORC1 activation, we examined the phosphorylation levels of proteins involved in the mTORC1 pathway. Leucine treatment in a medium containing all other amino acids significantly promotes basal and insulin-stimulated phosphorylation of $S6K1^{T389}$ at all concentrations tested relative to control ($p < 0.05$). Insulin-stimulated levels of ph-$S6K1^{T389}$ are increased by about 40-65% relative to control while basal

levels are higher by about 2.5-4 fold compared to control. Interestingly, within the control group, insulin caused a 9X increase in ph-S6K1^{T389} levels, while in the treatment groups the insulin effect ranged from 1.5-3 folds. Insulin-stimulated and basal phosphorylation of IRS-1^{Ser612} also show a trend for a dose-dependent increase relative to control, although non-significantly. Insulin caused a 3X increase in ph-IRS-1^{Ser612} in the control group, whereas within the other treatment groups, the effect of insulin ranged from 80-140%. Therefore, leucine promotes basal and insulin-stimulated phosphorylation of mTORC1 pathway proteins and more importantly, in the presence of other amino acids, basal levels of ph-S6K1^{T389} and IRS-1^{Ser612} are higher relative to control **(Fig 5)**

The effect of leucine alone on glucose uptake in L6 myotubes

To determine whether leucine, independent of other amino acids, can regulate glucose transport, we examined the dose-response effect of leucine at 150, 350, 600, 800 and 1600 μM. There is an overall inhibitory effect of leucine treatment (at all concentrations) on insulin-stimulated glucose transport relative to control ($p<0.05$). However, the effect is stronger at 150 μM, where there is a 75% reduction in insulin-stimulated glucose transport relative to control ($p <0.01$). We did not observe a significant difference in basal levels of glucose uptake across all treatments. The insulin effect within the control group was 110% while in the treatment groups it ranged from 40-70%, indicating that in the presence of leucine, the effect of insulin on glucose transport is less evident. It appears that the inhibitory effect of leucine on insulin-induced glucose uptake becomes less pronounced at higher concentrations, suggesting that there might be a positive shift in glucose uptake levels in response to leucine and insulin at concentrations much higher than physiological levels. **(Fig 6)**

Dose-dependent effect of leucine on mTORC1 activation in L6 myotubes

To understand whether leucine-mediated suppression of glucose transport occurs via increased mTORC1 pathway activation and whether its effects are dose-dependent, we treated myotubes with various concentrations of leucine (150, 350, 600, 800 or 1600 μM) and then examined mTORC1 signalling. Leucine in the presence of insulin, stimulates mTORC1 activation in a dose-dependent manner. This is observed by the significant upregulation of $S6K1^{T389}$ phosphorylation with increasing leucine concentrations ($p<0.05$). Interestingly, the basal levels of ph- $S6K1^{T389}$ are barely detectable, suggesting that the combined effect of leucine and insulin on mTORC1 activation is much more potent than leucine alone. This is expected since both leucine and insulin are powerful signaling inputs that activate mTORC1. Furthermore, there is a trend for a non-significant upregulation in insulin-stimulated ph-$IRS\text{-}1^{Ser612}$ levels, while basal levels are barely detectable. Overall, our results demonstrate that leucine impairs insulin signalling through increasing mTORC1 activation. **(Fig 7)**

Basal Glucose Uptake is Higher in the Presence of Other Amino Acids

Since basal levels of glucose uptake appeared to be higher in our first experiment carried out in the presence of other amino acids (Fig 3), compared to our second experiment where the role of leucine alone on glucose transport was examined (Fig 5), we used these data to compare basal glucose uptake levels in a medium without amino acids versus a medium that contains all amino acids. When a combination of amino acids is present in the medium, basal glucose uptake rate is significantly higher compared to when there are no amino acids in the medium. Insulin-stimulated glucose transport is also higher in the presence of all amino acids, although non-significantly. Interestingly, in the presence of a

combination of amino acids, insulin increases glucose uptake by about 90%, while in the absence of amino acids, the effect of insulin is 120%. Therefore, the magnitude of insulin effect is smaller in the presence of amino acids, meaning that the basal levels are already so high, such that the availability of insulin will not significantly alter glucose uptake levels. These results demonstrate that an amino acid mixture enhances basal glucose uptake levels in skeletal muscle cells, particularly in the absence of insulin, indicating there might be pathways other than the PI3K-Akt signalling pathway involved in amino acid-mediated glucose transport. **(Fig 8)**

The effect of KIC on glucose transport and insulin signalling in L6 myotubes

Since KIC is a metabolite of leucine and it has been shown to be involved in mTORC1 pathway activation, we were interested in examining whether it has a similar effect to leucine on glucose transport. Furthermore, the role of KIC in insulin-stimulated glucose transport has not been examined in a physiologically-relevant concentration range. We tested the effect of KIC treatment on insulin-stimulated glucose transport at 200 and 400 μM in L6 cells to mimic upper-range physiological levels and to see if there is a dose-dependent effect. Supplementation with 200 μM of KIC suppresses insulin-mediated glucose transport by 45% ($p<0.05$). It also causes a non-significant suppression of basal glucose transport at this concentration. The magnitude of insulin effect within the control group is similar to the treatment groups (55-60%). **(Fig 9)**

Treatment with KIC results in activation of mTORC1/S6K1 pathway

To determine whether the effect of KIC on glucose transport is associated with changes in the mTORC1 pathway activity, phosphorylation of three major proteins involved in the mTORC1 pathway were examined: $S6K1^{T389}$, $IRS\text{-}1^{Ser612}$ and Akt^{Ser473}. We chose to examine Akt activity because growth factor-mediated activation of mTORC1 occurs via Akt [14,94]. Moreover, we tested the effect of KIC on mTORC1 signalling at two different concentrations, 200 and 400 μM to try to mimic upper-range physiological levels and to see if there is a dose-dependent effect. KIC treatment at 200 μM significantly stimulates mTORC1 activity as shown by a 125% increase in ph-$S6K1^{T389}$ and a 55% increase in ph-$IRS\text{-}1^{Ser612}$ levels. Supplementation with KIC at 400μM also caused a non-significant upregulation in ph-$S6K1^{T389}$ and ph-$IRS\text{-}1^{Ser612}$ levels. However, KIC treatment did not significantly modulate phosphorylation level of Akt^{Ser473} possibly because Akt activation is dependent on the insulin-mediated PI3K pathway rather than KIC-mediated mTORC1 activation, therefore, the presence of KIC in the medium may mask the full effect of insulin on Akt activation. Our results suggest that leucine metabolite KIC, can also stimulate mTORC1 activity similar to leucine. **(Fig 10)**

KIC-mediated regulation of glucose transport involves mTORC1

Since suppression of glucose transport by KIC occurred in parallel with increased mTORC1 activity, I examined whether rapamycin, a specific inhibitor of mTORC1, could ameliorate the KIC-mediated suppression of glucose transport. As shown before, 200 μM of KIC suppressed insulin-stimulated glucose transport by 40%. ($p < 0.05$) However, co-incubation of 50 nM of rapamycin and 200μM of KIC reversed the inhibitory effect of KIC on glucose transport ($p < 0.05$). The magnitude of insulin effect is

higher in the control group where insulin increased glucose uptake by 75% while in the treatment groups the effect of insulin ranged from 30-45%. These results demonstrate the involvement of mTORC1 in KIC-mediated regulation of glucose transport and that inhibition of mTORC1 reverses the negative effects of KIC on glucose transport. **(Fig 11)**

The effect of rapamycin on KIC-mediated regulation of mTORC1

To link the effect of rapamycin on KIC-induced suppression of insulin-stimulated glucose transport to mTORC1, we measured ph-S6K1^{T389}, ph-S6$^{Ser235/236}$ and ph-IRS-1^{Ser612} levels. As expected, rapamycin significantly inhibited phosphorylation of ph-S6K1^{T389}, ph-IRS-1 ^{Ser612}, and ph-S6$^{Ser235/236}$ ($p<0.05$). KIC-mediated stimulation of mTORC1 activity is inhibited in the presence of rapamycin, as shown by the reduced phosphorylation levels of IRS- ^{Ser612} and S6$^{Ser235/236}$ in the presence of rapamycin. We did not observe ph-S6K1^{T389} levels in in the co-incubation of rapamycin and KIC. This could be due to the particularly strong inhibition of rapamycin on phosphorylation of S6K1 such that even the presence of insulin and KIC was not sufficient to activate S6K1. **(Fig 12)**

KIC-mediated suppression of glucose transport is due to its intracellular conversion to leucine

Since the first step in the catabolism of leucine to its metabolite KIC, is reversible, one could speculate that the KIC-mediated inhibition of insulin-stimulated glucose transport is because KIC is being converted to leucine and that these negative effects are caused by leucine and not KIC. To our knowledge, the effect of KIC itself on insulin-stimulated glucose transport in L6 myotubes has not been examined. Therefore, we used an siRNA gene silencing technique to knockdown the BCAT2 enzyme that catalyzes the reversible conversion of leucine to KIC. By doing this, we aimed to "block" the intracellular conversion of KIC to leucine and then examine whether KIC can independently regulate glucose transport and mTORC1 activation in skeletal muscle cells. We knocked down the BCAT2 enzyme in our L6 cells and then supplemented them with KIC in the presence or absence of insulin. Depletion of the BCAT2 enzyme, attenuates the KIC-mediated suppression of insulin-induced glucose transport ($p<0.05$), indicating the observed effects are due to the conversion of KIC back to leucine. The magnitude of insulin effect in all treatment groups was similar to control, ranging from 45% to 60%. Therefore, it appears that KIC cannot independently regulate glucose transport in L6 myotubes and that its effects are mainly caused by its reversible conversion to leucine. **(Fig 13 A)**

The efficiency of BCAT2 knockdown is demonstrated in **Fig 13 B.**

The effect of BCAT2 knockdown on KIC-mediated regulation of mTORC1 pathway

We observed that the inhibitory effect of KIC on glucose transport is due to its intracellular conversion to leucine. Now, we wanted to determine whether KIC-mediated regulation of the mTORC1 pathway also depends on its conversion to leucine. We knocked down the BCAT2 enzyme and then examined the effect of KIC supplementation on mTORC1 pathway activation. Interestingly, we observed that depleting the BCAT2 enzyme significantly suppresses the KIC-mediated activation of mTORC1 ($p<0.05$) as shown by the phosphorylation levels of $S6K1^{T389}$, $IRS\text{-}1^{S612}$ and Akt^{S473}. This further supports the notion that conversion of KIC to leucine is required for its regulation of insulin signalling and mTORC1 activation. **(Fig 14)**

7.0 Figures

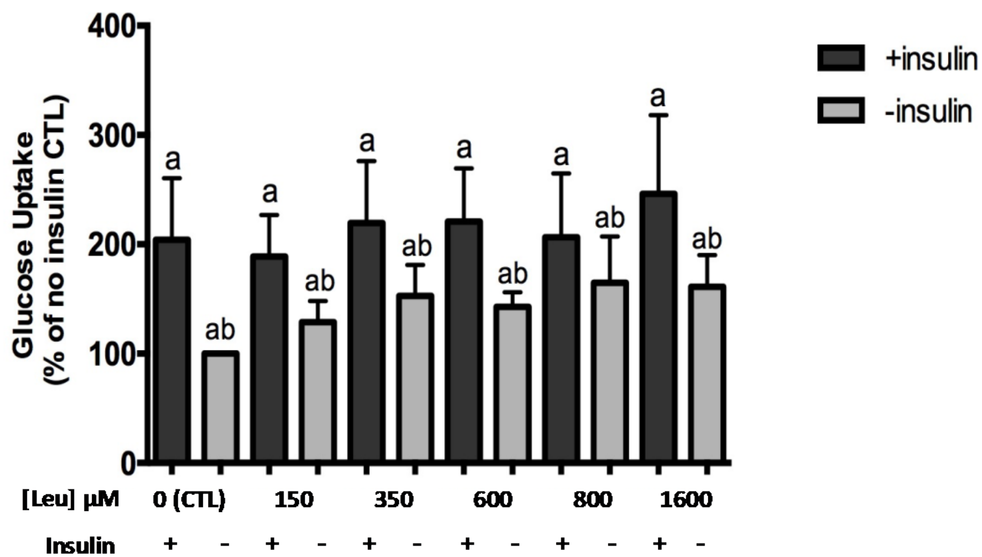


Fig 4. Glucose uptake rate in response to different leucine concentrations in the presence of other amino acids

L6 myoblasts were differentiated until day 5. On D5, they were starved for 4 hours in RPMI (without amino acids and serum). They were then incubated in a medium that contained all amino acids except leucine and then supplemented with different [leucine] for 30 minutes. Finally, the myotubes were incubated for another 20 minutes in the presence or absence of 100 nM of insulin. Glucose uptake assay was then performed. Rate of glucose transport is expressed as % CTL (no amino acids or insulin). Mean ± SEM; n=3 independent experiments with 3-6 replicates per treatment within each experiment.

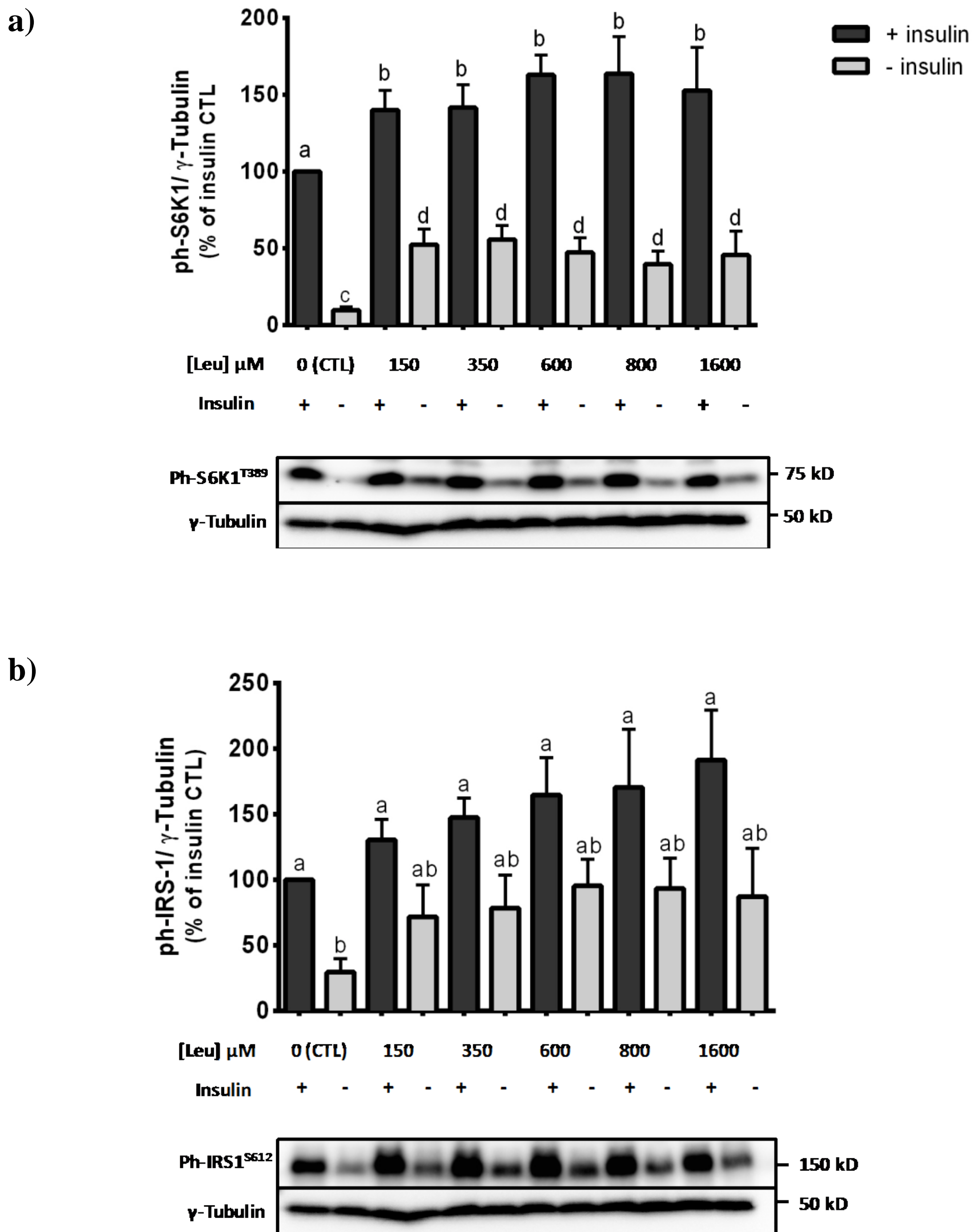


Fig 5. Leucine-mediated activation of mTORC1 in the presence of other amino acids

Graphical representation and western blot analysis of **a)** ph-S6K1^{T389}, **b)** ph-IRS1 ^{Ser612} and gamma tubulin. L6 myoblasts were differentiated until D5. On D5, they were starved for 4 hours in RPMI (without amino acids and serum). They were then incubated in a medium that contained all amino acids except leucine and then supplemented with different [leucine] for 30 minutes, followed by a 20-minute incubation in the presence or absence of 100 nM of insulin. Lastly, samples were harvested for immunoblot analysis. Data is presented as % CTL (no amino acids +ins). Mean ± SEM; n=3 independent experiments with 3-6 replicates per treatment within each experiment ($p < 0.05$).

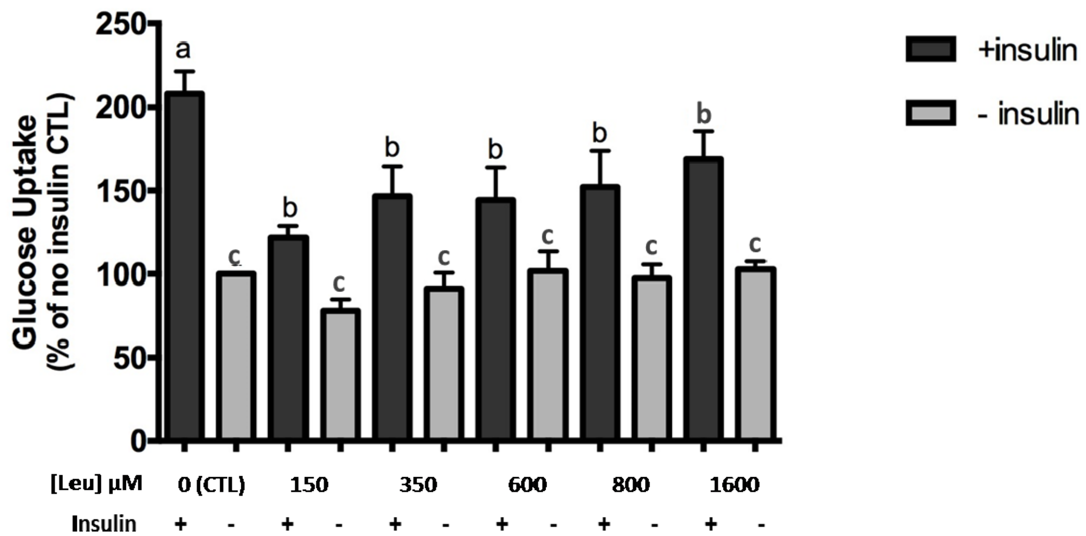


Fig 6. Leucine impairs insulin-stimulated glucose transport in L6 myotubes

L6 myoblasts were differentiated for 5 days. On D5, they were starved for 4 hours in RPMI (without amino acids and serum) and then treated with different leucine concentrations for 30 minutes. They were then incubated in the presence or absence of 100 nM of insulin for 20 minutes. Glucose uptake assay was then performed. Rate of transport is expressed per µg of protein. Rate of glucose transport is expressed as % CTL (no amino acids or insulin). Mean ± SEM; n=4 independent experiments with 3-6 replicates per treatment within each experiment. ($p < 0.05$). "a" significantly different from "b" ($p<0.01$).

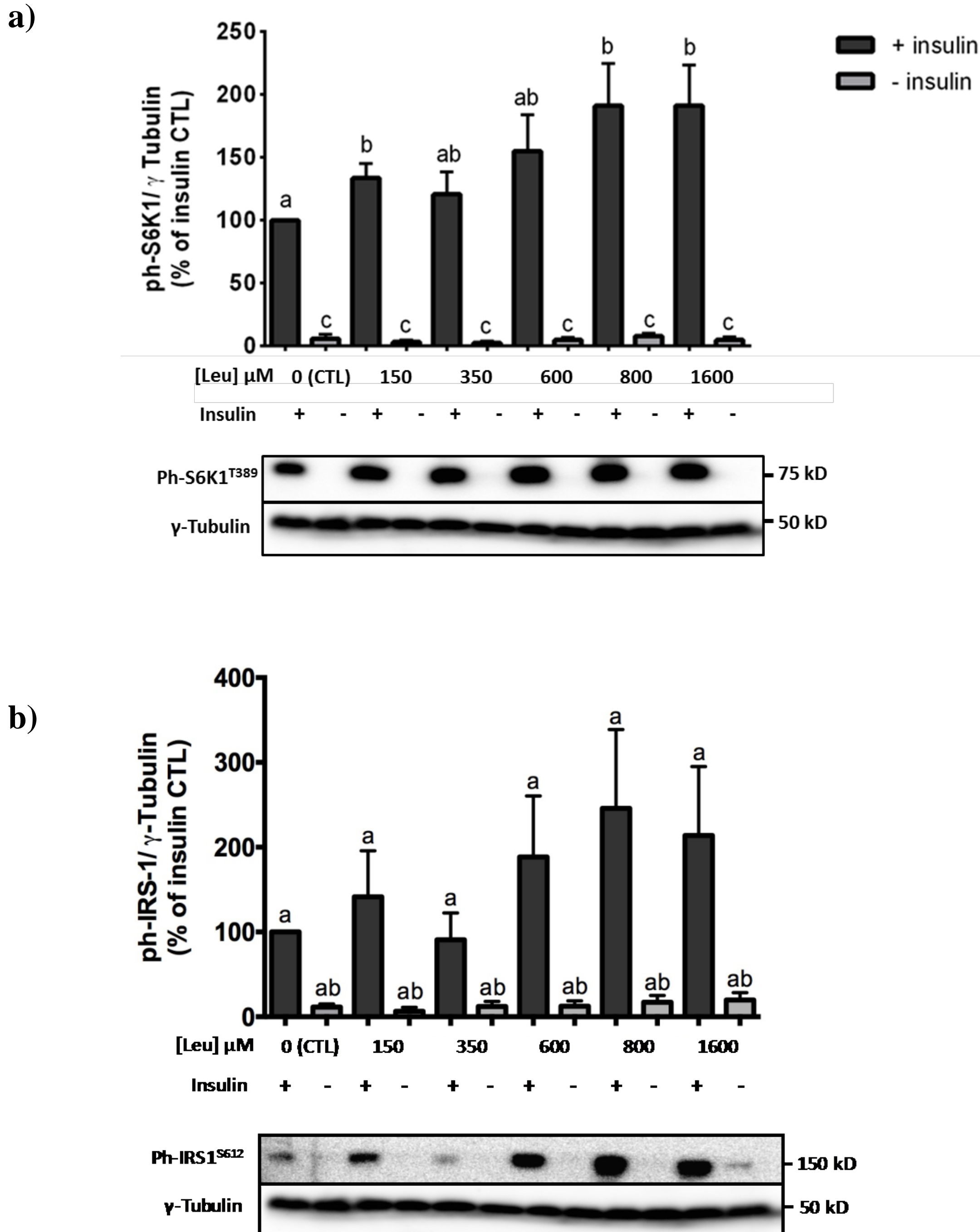


Fig 7. Leucine impairs glucose transport via upregulation of mTORC1 activity

Graphical representation and western blot analysis of **a)** ph-S6K1 ^{T389}, **b)** ph-IRS1 ^{S612} and gamma tubulin. L6 myoblasts were differentiated until day 5. On D5, following 4 hours of starvation in RPMI (without amino acids and serum), they were incubated with various leucine concentrations for 30 minutes. They were then incubated in the presence or absence of 100 nM of insulin for 20 minutes. Lastly, samples were harvested for immunoblot analysis. Data is expressed as % CTL (no amino acids +insulin). Mean ± SEM; n=3 independent experiments with 3-6 replicates per treatment within each experiment. Bars with different letters are significantly different ($p<0.05$).

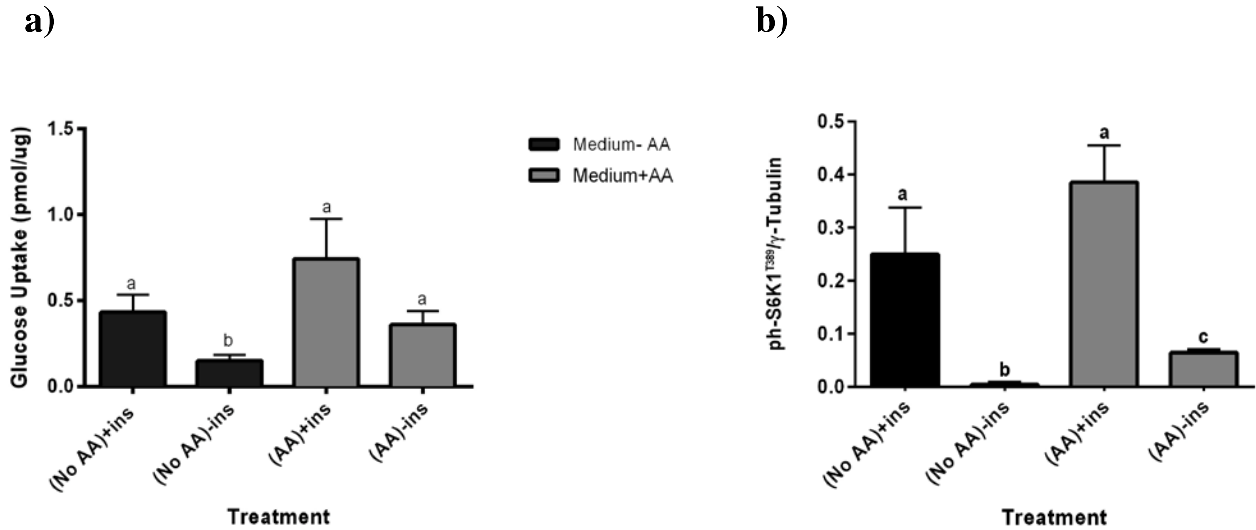


Fig 8. An amino acid mixture enhances basal glucose uptake rate and ph-S6K1^{T389} levels in L6 myotubes

Derived from data in Figure 3 and Figure 5.
L6 myoblasts were differentiated for 5 days. On D5, they were starved for 4 hours in RPMI (without amino acids and serum) and then incubated in a medium that contained either all amino acids, or no amino acids at all for 30 minutes. They were then incubated in the presence or absence of 100 nM of insulin for 20 minutes. Glucose uptake assay was then performed. Rate of transport is expressed per µg of protein. Mean ± SEM; n=3 independent experiments with 3-6 replicates per treatment within each experiment. Bars with different letters are significantly different ($p < 0.05$).

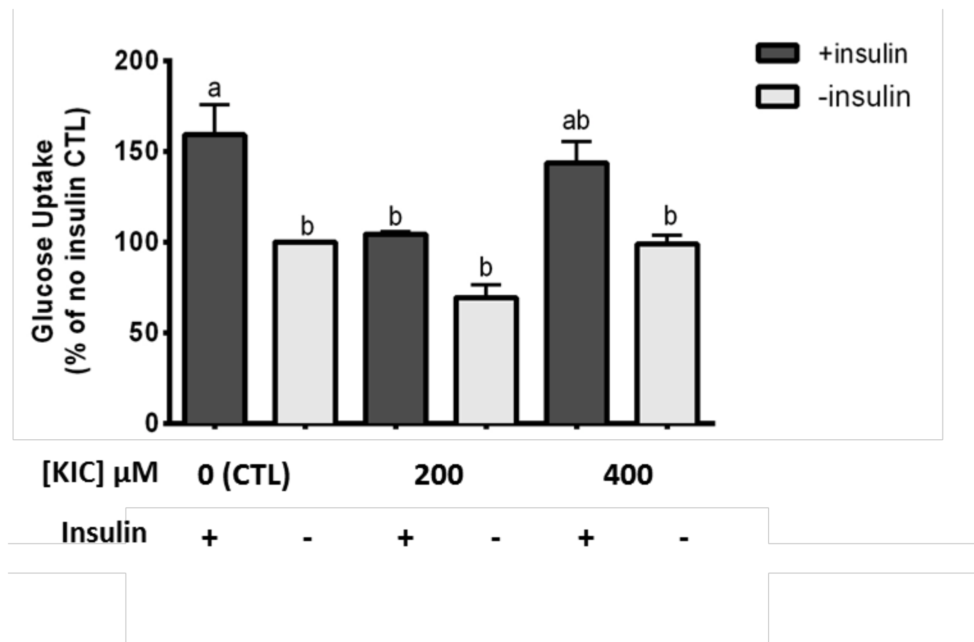


Fig 9. Supplementation with 200µM of KIC significantly suppresses insulin-stimulated glucose uptake in L6 cells

L6 myoblasts were differentiated until day 5. On D5, following 4 hours of starvation in RPMI (without amino acids and serum), they were treated with 200 or 400 µM of KIC for 30 minutes. They were then further incubated in the presence or absence of 100 nM of insulin for 20 minutes. Glucose uptake assay was then performed. Rate of transport is expressed per µg of protein. Rate of glucose transport is expressed as % CTL (no amino acids or insulin). Mean ± SEM; n=4 independent experiments with 3-6 replicates per treatment within each experiment. Bars with different letters are significantly different ($p < 0.05$).

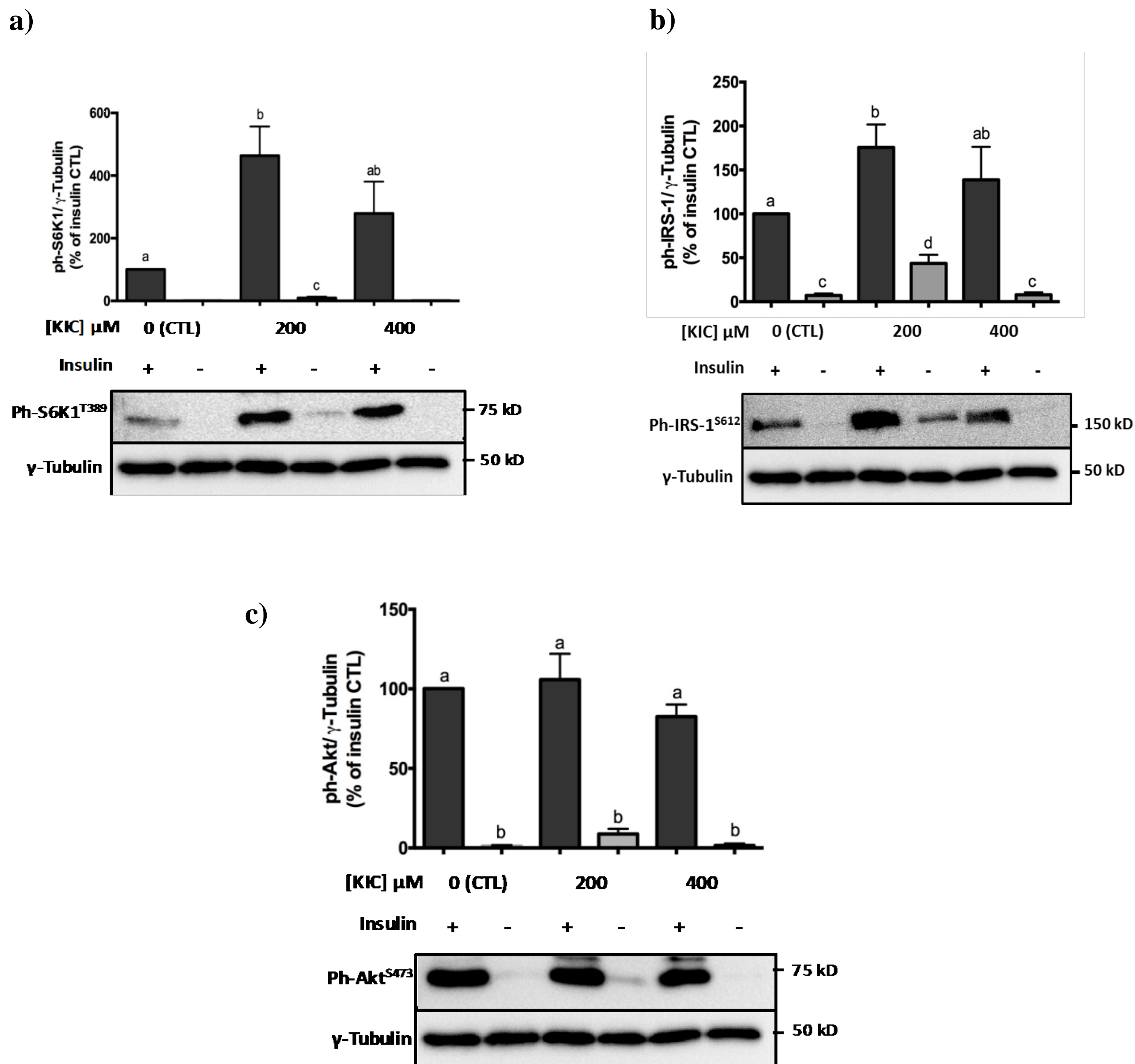


Fig 10. The role of KIC in mTORC1/S6K1 pathway activation

Graphical representation and western blot analysis of **a**) ph-S6K1^{T389}, **b**) ph-IRS-1 ^{Ser612}, **c**) ph-Akt ^{Ser473} and gamma tubulin. L6 myoblasts were differentiated until D5. On D5, following 4 hours of starvation in RPMI (free of amino acids and serum), myotubes were incubated with different [KIC] for 30 minutes. They were then incubated in the presence or absence of 100 nM of insulin for 20 minutes. Samples were then harvested for immunoblot analysis. Data is expressed as % CTL (no KIC +insulin). Mean ± SEM; n=3 independent experiments with 3-6 replicates per treatment within each experiment. Bars with different letters are significantly different ($p < 0.05$).

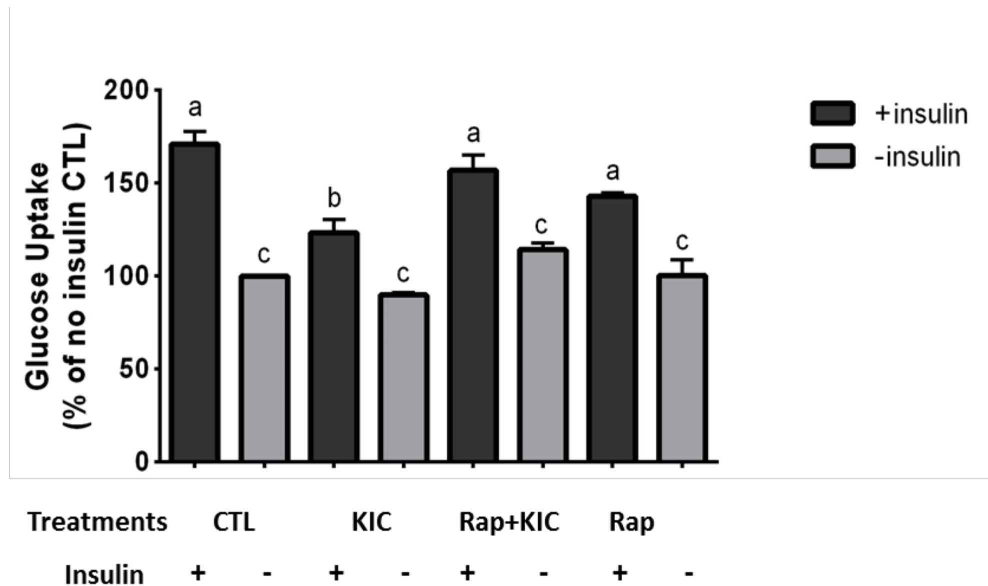


Fig 11. KIC-mediated suppression of glucose transport involves mTORC1

L6 myoblasts were differentiated until day 5. On D5, following 4 hours of starvation in RPMI (free of amino acids and serum), myotubes were supplement with either KIC, KIC plus 50 nM of rapamycin, or 50 nM of rapamycin for 30 minutes. They were then incubated in the presence or absence of 100 nM of insulin for another 20 minutes. Glucose uptake assay was then performed to determine the level of glucose uptake. Rate of glucose transport is expressed as % CTL (no KIC or insulin). Mean ± SEM; n=3 independent experiments with 3-6 replicates per treatment within each experiment. Bars with different letters are significantly different ($p<0.05$).

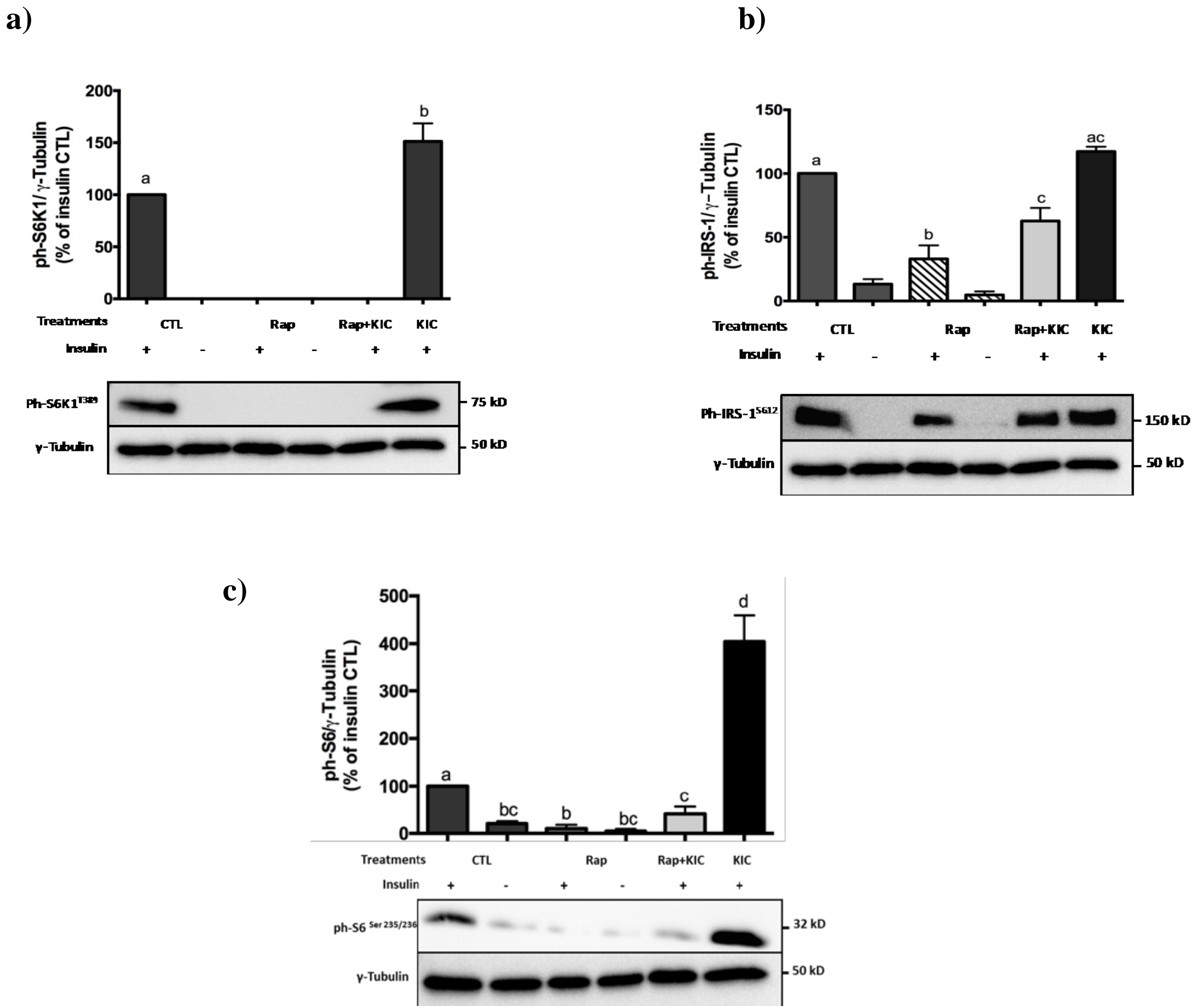
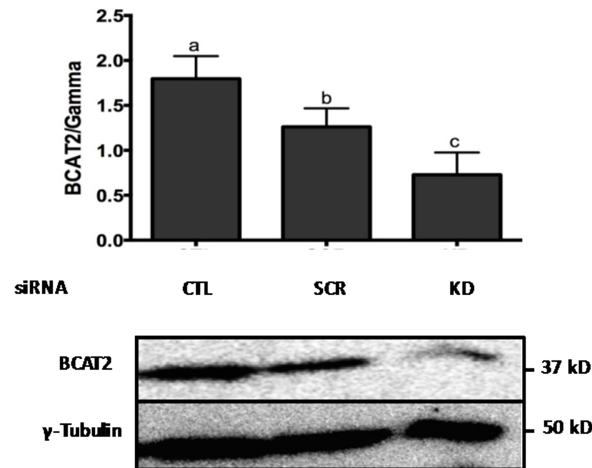


Fig 12. The effect of rapamycin on KIC-mediated activation of mTORC1

Graphical representation and western blot analysis of **a)** ph-S6K1^{T389} and **b)** ph-IRS-1 ^{Ser612}, **c)** ph-S6$^{Ser235/236}$ and gamma tubulin. L6 myoblasts were differentiated until day 5. On D5, following 4 hours of starvation in RPMI (free of amino acids and serum), myotubes were supplement with either 200µM of KIC alone, 200µM KIC plus 50 nM of rapamycin, or 50 nM of rapamycin for 30 minutes. They were then incubated in the presence or absence of 100 nM of insulin for 20 minutes. Samples were then harvested for immunoblot analysis. Data is expressed as % CTL (no KIC +insulin). Mean ± SEM; n=3 independent experiments with 3-6 replicates per treatment within each experiment. Bars with different letters are significantly different ($p < 0.05$).

a)



b)

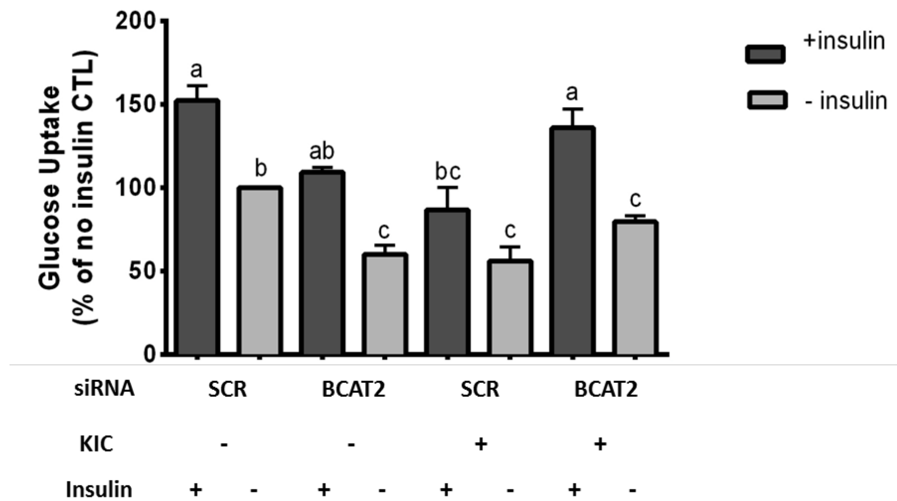


Fig 13. BCAT2 knockdown attenuates the inhibitory effect of KIC on glucose transport

L6 myoblasts were differentiated until day 3. On D3, they were transfected with siRNA targeting the BCAT2 enzyme or scramble siRNA. They were then allowed to differentiate further until day 5. On D5, samples were either harvested for immunoblot analysis **a)** or **b)** following 4 hours of starvation in RPMI (free of amino acids and serum), myotubes were supplement with or without 200 μM KIC for 30 minutes. They were then incubated in the presence or absence of 100 nM of insulin for another 20 minutes. Glucose uptake assay was then performed. Rate of glucose transport is expressed as % CTL (SCR siRNA, no KIC, no insulin). Mean ± SEM; n=3 independent experiments with 3-6 replicates per treatment within each experiment. Bars with different letters are significantly different ($p<0.05$).

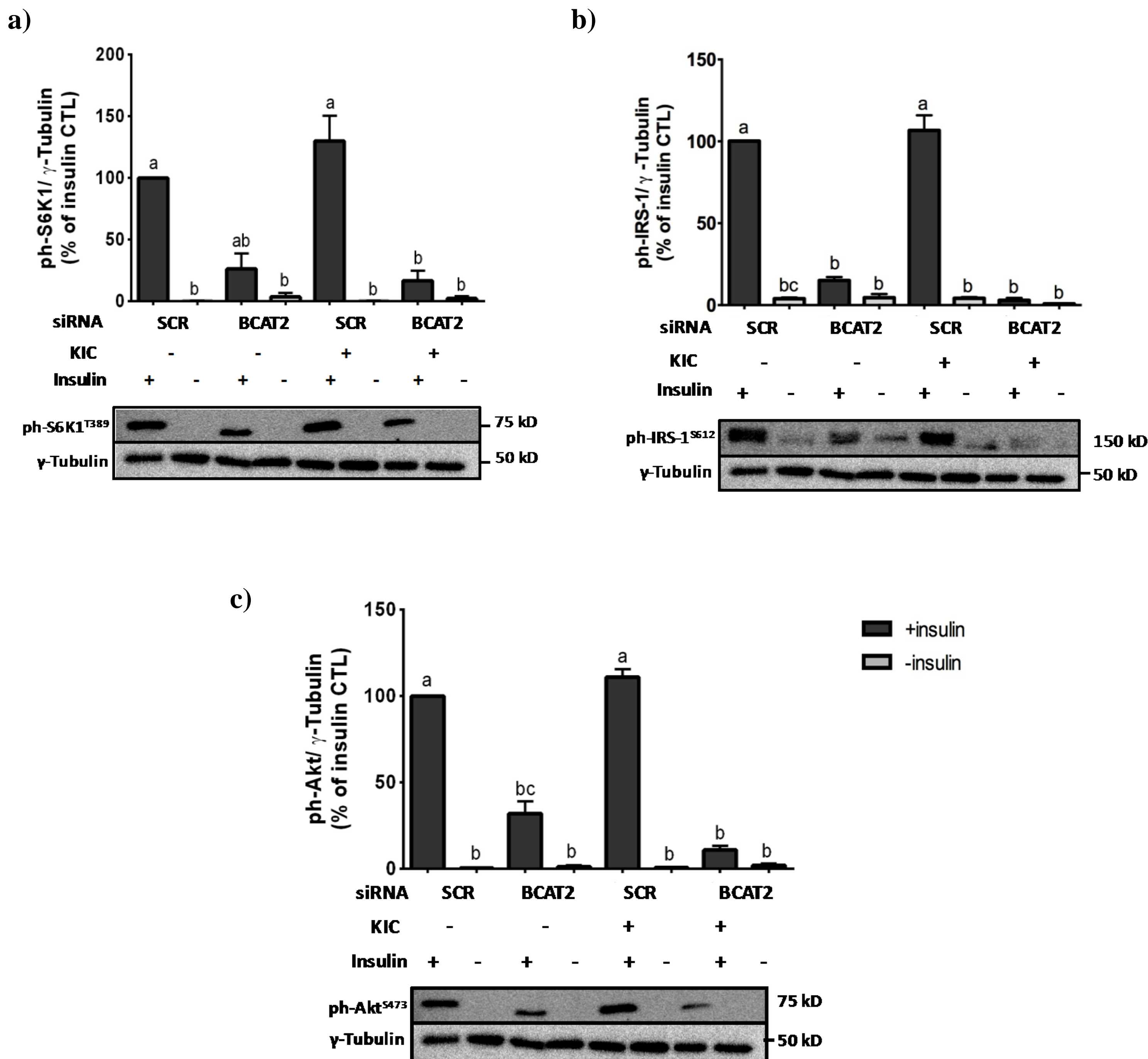


Fig 14. The effect of BCAT2 knockdown on KIC-mediated regulation of mTORC1

Graphical representation and western blot analysis of **a)** ph-S6K1^{T389}, **b)** ph-IRS-1 ^{Ser612}, **c)** ph-Akt ^{Ser473} and gamma tubulin. L6 myoblasts were differentiated until D3. On D3, they were transfected with siRNA targeting the BCAT2 enzyme or scramble siRNA. They were then allowed to differentiate further until day 5. On D5, following 4 hours of starvation in RPMI (free of amino acids and serum), myotubes were supplement with or without 200 μM of KIC for 30 minutes, followed by another 20-min incubation in the presence or absence of 100 nM of insulin. Samples were then harvested for immunoblot analysis. Data is expressed as % CTL (no KIC +insulin). Mean ± SEM; n=3 independent experiments with 3-6 replicates per treatment within each experiment. Bars with different letters are significantly different ($p<0.05$).

8.0 Discussion

We used an in-vitro model to examine the effect of leucine and its metabolite KIC on insulin-stimulated glucose transport. My first experiment was examining the effect of different leucine concentrations on glucose uptake and mTORC1 activation in the presence of other amino acids. This is because most dietary proteins that people consume for weight loss or building muscle mass contain other amino acids in addition to leucine. Thus, we sought to determine how the availability of other amino acids affects leucine's regulation of glucose uptake and mTORC1 signalling. In a medium that contains all other amino acids except leucine, supplementation with various leucine concentrations does not alter glucose transport rates in L6 myotubes. This may be due to a possible stimulatory response in glucose uptake associated with the presence of a combination of amino acids rather than just one (ie. leucine).

Although the exact mechanisms are unknown, some in-vivo studies suggest that a combination of all amino acids may result in greater endogenous insulin secretion and greater GLUT4 translocation to the plasma membrane as a result of increased phosphorylation of AS160.[132,133] AS160 is an inhibitor of GLUT4 translocation, however, once phosphorylated by Akt, its inhibitory effect on GLUT4 translocation is removed, resulting in the recruitment of GLUT4 from intracellular storage pools to the plasma membrane. In addition, a mixture of amino acids that contains isoleucine and arginine has been shown to increase glucose uptake levels. [133]

Furthermore, studies that show amino acid mixtures improve glucose transport have worked with concentrations from 2.5 to10 mM which are way beyond physiological levels [135]. When looking at mTORC1 activation, the availability of other amino acids caused a significant stimulation in phosphorylation of S6K1^{T389} in response to various

leucine concentrations. We also observed a dose-dependent but non-significant increase in ph-IRS-1^{Ser612} levels. Moreover, in the presence of other amino acids, basal glucose uptake levels were higher compared to control, which is why the magnitude of insulin effect was not as strong as the control group.

A number of studies have observed elevated levels of BCAAs in obese/insulin-resistant individuals[75,124,126], therefore, it is important to investigate their in-vitro effect in skeletal muscle. Since leucine is the strongest BCAA in activating mTORC1 and the effects of high protein diets on insulin resistance occur, at least in part through the mTORC1/S6K1 pathway, I examined the effect of various concentrations of leucine on glucose transport and mTORC1 signalling in skeletal muscle cells. This was to determine whether leucine in the absence of other amino acids can regulate glucose transport dose-dependently. We found that leucine significantly impairs insulin-stimulated glucose transport in L6 cells ($p<0.05$) and this inhibition is particularly more significant at 150 μM ($p<0.01$). Interestingly, we observed a more significant reduction in glucose uptake in response to 150 μM leucine compared to higher levels. This could possibly be due to a dual mechanism associated with leucine's action such that at lower concentrations (closer to physiological levels) it can impair insulin-stimulated glucose uptake, however, as you increase the concentration, its effect gradually shifts to a more positive side and stimulates glucose uptake due to unknown mechanism. This could be explained by the stronger synergistic effect of leucine and insulin at higher leucine concentrations. It may also be due to either increased expression of GLUT4 proteins or redistribution of the GLUT4 vesicles to the plasma membrane in response to high leucine levels.

Moreover, there is some discrepancy between our results and some previous findings in the literature. For instance, a recent study by Liu et al. examined the dose-dependent effect of leucine on glucose uptake in C2C12 skeletal muscle cells [68]. They tested various

leucine concentrations (0 to 8 mM) and found that leucine facilitated insulin-stimulated glucose uptake, particularly at 2 mM. Nishitani et al. also suggested a stimulatory effect of leucine on glucose uptake, particularly at high concentrations (2-3 mM) under insulin-free conditions [119]. However, these studies examined the effect of leucine at supraphysiological concentrations which many not be relevant to normal physiological conditions. The physiological concentration of leucine in rodents is around 140-150 μM [17] and in humans it is in the range of 100-150 μM [136–138], therefore, it would not be physiologically appropriate to test such high concentrations of leucine (2-8 mM) as these studies did. Therefore, results from different studies may vary depending on many factors such as experimental procedures and concentration of leucine tested. Thus, further studies need to be done to fully elucidate the mechanism of leucine's action in insulin signalling and glucose uptake in skeletal muscle.

Interestingly we found that the rate of basal glucose uptake is significantly higher in a medium containing all amino acids, compared to one that does not. As mentioned earlier, there are certain amino acids in the mixture that can stimulate glucose uptake even in the absence of insulin, such as isoleucine and arginine[133], therefore, even in the absence of insulin, a mixture of amino acids can stimulate glucose transport.

Next we determined whether suppression of glucose uptake by leucine occurs via mTORC1. We observed a trend for a dose-dependent upregulation in mTORC1 as shown by increased phosphorylation of S6K1^{T389}. This is consistent with previous findings in the literature showing increased activation of mTORC1 with leucine treatment [22,] although no studies have examined the dose-dependent effect of leucine on phosphorylation of mTORC1 proteins in a physiologically-relevant range. This is an important question because the effect of leucine on mTORC1 signalling may be dose-dependent. Serine phosphorylation of IRS-1 was also increased in response to leucine treatment, although

non-significantly. Thus, our results suggest that leucine promotes mTORC1/S6K1 activation and increases abnormal IRS-1 function as a result of S6K1-mediated negative feedback loop on IRS-1.

Next we sought to determine how the first product of leucine's catabolism (KIC) affects glucose uptake and mTORC1 activation. As mentioned before, previous studies have shown KIC to be involved in mTORC1 signalling [139], however, its effects on glucose transport have not been elucidated. Therefore, we were interested to examine the role of KIC in insulin –stimulated glucose transport in L6 cells. Since it is possible that leucine exerts its effects through metabolic changes such as its conversion to KIC, we sought to examine whether KIC by itself can regulate glucose transport. Our results suggest that KIC functions in a similar manner to leucine in glucose transport and mTORC1 signalling. We observed a significant reduction in insulin-induced glucose uptake with 200 uM of KIC treatment. Nishitani et. al [119] compared the effect of 2 mM of KIC and leucine on glucose uptake in the soleus muscle of rats. They found KIC was able to promote glucose uptake at 2 mM, although its effect was weaker than leucine. However, once again, they used supraphysiological levels of KIC (1-8 mM), which makes their findings questionable and it may not be applicable to normal physiological conditions. This is because the physiological concentration of KIC is around 30-35 uM[136,137,140], therefore, it would not be logical to test such high concentrations of KIC as Nishitani et al. did. We also observed an upregulation in ph-S6K1^{T389} , ph-IRS-1S^{612} and p-Akt^{S473} levels which suggest increased mTORC1 activity. Therefore, KIC suppresses insulin-stimulated glucose transport in L6 myotubes concurrent with increased mTORC1 activation and therefore, can be implicated in the development of insulin resistance.

Since we observed a reduction in insulin-stimulated glucose uptake in response to 200 uM of KIC and this effect was associated with increased mTORC1 activity, we were

interested to determine if the effects were specifically mTORC1-dependent. Because in our previous experiment, 200 uM of KIC treatment caused a more significant reduction in insulin-induced glucose uptake, we chose 200 uM as our testing concentration in this experiment. As observed before, KIC significantly suppressed insulin-mediated glucose uptake ($p<0.05$). Interestingly, the addition of rapamycin to KIC ameliorated the inhibitory effect of KIC on glucose transport ($p<0.05$), indicting the specific involvement of mTORC1 in KIC-mediated regulation of glucose transport. We next examined the effect of KIC and rapamycin on KIC-mediated regulation of mTORC1 activation. Co-incubation of KIC with rapamycin, suppressed the KIC-induced phosphorylation of IRS-1^{Ser612}, $S6K1^{T389}$ and $S6^{Ser235/236}$, indicating that the effect of KIC on insulin signalling is mTORC1-dependent. In the presence of KIC and rapamycin, ph-$S6k1^{T389}$ levels were not detectable at all. This could possibly be due to the specifically strong inhibition of rapamycin on phosphorylation of S6K1 such that even the presence of insulin and KIC was not sufficient to activate S6K1.

Our next question was to determine whether KIC can by itself regulate glucose transport and mTORC1 signalling in L6 myotubes, or if the effects we observed are due to the intracellular conversion of KIC to leucine. This is because the first step in leucine metabolism, catalyzed by the BCAT2 enzyme, is reversible and KIC can be converted back to leucine intracellularly. When we knocked down the BCAT2 enzyme and supplemented the cells with KIC, we found that in the scramble condition (control), KIC suppressed insulin-stimulated glucose transport as expected; however, in the knockdown condition, KIC-mediated suppression of insulin-induced glucose transport was abolished. To our knowledge, the effect of the BCAT2 enzyme in KIC-mediated regulation of glucose transport in L6 skeletal muscle cells has not been studied. She et al. examined the effect of whole body BCAT2 knockout in mice and found that disruption of BCAT2

leads to improved insulin sensitivity in mice despite elevated BCAA levels, indicating an important role for BCAT2 in the development of insulin resistance and T2DM [66]. Our results indicate that negative effects associated with KIC treatment on insulin-stimulated glucose transport are due to the intracellular conversion of KIC to leucine and that in fact, it is leucine which impairs insulin signalling.

Finally, we probed the effect of BCAT2 knockdown on KIC-mediated regulation of mTORC1 signalling. Unfortunately, we did not observe a strong stimulation of ph-$S6K1^{T389}$ and ph-$IRS\text{-}1^{S612}$ levels in response to KIC treatment in our scramble condition. This could be due to the high level of cell death and cell toxicity associated with siRNA gene silencing which could diminish or mask the effect of KIC on activation of mTORC1. However, interestingly, we found that BCAT2 knockdown significantly suppresses KIC-mediated mTORC1 activation as shown by ph-$S6K1T^{389}$ and ph-IRS-$1S^{612}$ levels in the knockdown condition. These results further support our findings that KIC-mediated regulation of glucose transport and mTORC1/S6K1 activation occur through the reversible conversion of KIC to leucine. Therefore, targeting this enzyme could be a new therapeutic approach in the treatment of insulin resistance and T2DM.

In conclusion, my results demonstrate that leucine can impair insulin-stimulated glucose transport in skeletal muscle through a negative feedback mechanism involving increased mTORC1/S6K1 activation which leads to insulin resistance. The inhibitory effect of leucine on glucose transport disappears in a mixture of other amino acids, possibly due to the presence of amino acids such as isoleucine and arginine which stimulate glucose uptake. Similar to leucine, its metabolite, KIC can also impair insulin-induced glucose transport concurrent with increased mTORC1 activation in rat skeletal muscle cells. Our novel findings indicate that diminishing the activity of the BCAT2 enzyme reverses the KIC-mediated suppression of glucose transport, supporting the

notion that KIC exerts its effects through its intracellular conversion to leucine. This indicates that altering BCAA metabolism may be a new strategy in certain pathological conditions such as T2DM. Even though BCAA supplementation may be beneficial in overweight/obese individuals for weight management, excess levels could lead to the development of insulin resistance via persistent mTORC1 activation and loss of normal IRS-1 function.

Some of the limitations of my study include the lack of examining total protein levels of S6K1, IRS-1 and Akt. However, since the measures we were interested in examining were not likely to modulate total protein levels, we used gamma tubulin as our loading control to normalize our protein levels. Furthermore, to determine whether leucine-mediated regulation of glucose transport is completely mTORC1 dependent, I could have used the mTORC1 inhibitor, rapamycin in my treatments. This would allow us to find out if leucine-mediated regulation of glucose uptake involves other mechanisms. Also, to further support our findings in the BCAT2 experiments, we could have used other methods to abolish the activity of the enzyme, such as specific inhibitor of BCAT2.

9.0 Future Directions

Even though my results have shown some novel findings with regards to the effect of KIC on glucose transport and mTORC1 activation, further studies are needed to fully examine KIC-mediated regulation of insulin signalling. Some future work could include:

1. Knocking down the BCAT2 enzyme and supplementing the cells with leucine to confirm our findings that leucine and not its metabolite, mediates the negative effects observed with glucose transport and mTORC1 activation
2. Examining the role of another downstream metabolite of leucine (HMB) on glucose transport and mTORC1 signalling and comparing its effect to KIC. This is because HMB has been shown to stimulate protein synthesis via mTORC1 and therefore, could possibly be implicated in insulin resistance and T2DM.

2. Examining the effect of markers of inflammation on leucine/KIC regulation of glucose transport since inflammation can lead to the development of insulin resistance

10.0 References

1. International Diabetes Federation. IDF Diabetes Atlas. *Key Find*. 2014. http://www.idf.org/diabetesatlas/update-2014.

2. WHO. World Health Organization. *Diabetes Fact sheet N°312*. 2014.

3. National Institute of Health. Insulin Resistance and Pre-diabetes. *Diabetes*. 2009;09.

4. Singh B, Saxena A. Surrogate markers of insulin resistance: A review. *World J Diabetes*. 2010;1(2):36-47.

5. Lu J, Xie G, Jia W, Jia W. Insulin resistance and the metabolism of branched-chain amino acids. *Front Med*. 2013;7(1):53-59.

6. Lynch CJ, Adams SH. Branched-chain amino acids in metabolic signalling and insulin resistance. *Nat Rev Endocrinol*. 2014;10(12):723-736.

7. McCormack SE, Shaham O, McCarthy M a, et al. Circulating branched-chain amino acid concentrations are associated with obesity and future insulin resistance in children and adolescents. *Pediatr Obes*. 2012:52-61.

8. Macotela Y, Emanuelli B, Bång AM, et al. Dietary leucine - an environmental modifier of insulin resistance acting on multiple levels of metabolism. *PLoS One*. 2011;6(6).

9. Westerterp-Plantenga MS, Lemmens SG, Westerterp KR. Dietary protein – its role in satiety, energetics, weight loss and health. *Br J Nutr*. 2012;108:S105-S112.

10. Halton TL, Hu FB. The effects of high protein diets on thermogenesis, satiety and weight loss: a critical review. *J Am Coll Nutr*. 2004;23(5):373-385.

11. Paddon-jones D, Westman E, Mattes RD, Wolfe RR, Astrup A. Protein, weight management, and satiety 1– 4. 2008;87:1558-1561.

12. Schriever SC, Deutsch MJ, Adamski J, Roscher A a., Ensenauer R. Cellular signaling of amino acids towards mTORC1 activation in impaired human leucine catabolism. *J Nutr Biochem*. 2013;24(5):824-831.

13. Efeyan A, Zoncu R, Sabatini DM. Amino acids and mTORC1: From lysosomes to disease. *Trends Mol Med*. 2012;18:524-533.

14. Tato I, Bartrons R, Ventura F, Rosa JL. Amino acids activate mammalian target of rapamycin complex 2 (mTORC2) via PI3K/Akt signaling. *J Biol Chem*. 2011;286:6128-6142.

15. Yoshizawa F. New Therapeutic Strategy for Amino Acid Medicine: Notable Functions of Branched Chain Amino Acids as Biological Regulators. *J Pharmacol Sci*. 2012;118:149-155.

16. Krebs M, Krssak M, Bernroider E, et al. Mechanism of Amino Acid – Induced Skeletal Muscle Insulin Resistance in Humans. *Diabetes*. 2002;51(March):599-605.

17. She P, Van Horn C, Reid T, Hutson SM, Cooney RN, Lynch CJ. Obesity-related elevations in plasma leucine are associated with alterations in enzymes involved in branched-chain amino acid metabolism. *Am J Physiol Endocrinol Metab*. 2007;293(6):E1552-E1563.

18. Lynch CJ, Halle B, Fujii H, et al. Potential role of leucine metabolism in the leucine-signaling pathway involving mTOR. *Am J Physiol Endocrinol Metab*. 2003;285:E854-E863.

19. Marchesini G, Marzocchi R, Noia M, Bianchi G. Branched-chain amino acid supplementation in patients with liver diseases. *J Nutr*. 2005;135(6 SUPPL.):1596S - 601S.

20. Eng D, Ma H-Y, Gross MK, Kioussi C. Gene Networks during Skeletal Myogenesis. *ISRN Dev Biol*. 2013;2013:1-8.

21. Stump CS, Henriksen EJ, Wei Y, Sowers JR. The metabolic syndrome: role of skeletal muscle metabolism. *Ann Med*. 2006;38:389-402.

22. Wei Y, Chen K, Whaley-Connell AT, Stump CS, Ibdah J a, Sowers JR. Skeletal muscle insulin resistance: role of inflammatory cytokines and reactive oxygen species. *Am J Physiol Regul Integr Comp Physiol*. 2008;294(December 2007):R673-R680.

23. Gual P, Le Marchand-Brustel Y, Tanti J-F. Positive and negative regulation of insulin signaling through IRS-1 phosphorylation. *Biochimie*. 2005;87:99-109.

24. Lee J, Pilch PF. The insulin receptor: structure, function, and signaling. *Am J Physiol*. 1994;266(2 Pt 1):C319-C334.

25. Sesti G, Federici M, Hribal ML, Lauro D, Sbraccia P, Lauro R. Defects of the insulin receptor substrate (IRS) system in human metabolic disorders. *FASEB J*. 2001;15(12):2099-2111.

26. Farhang-fallah J, Randhawa VK, Klip A, Bar-sagi D, Nimnual A, Rozakis-adcock M. The Pleckstrin Homology (PH) Domain-Interacting Protein Couples the Insulin Receptor Substrate 1 PH Domain to Insulin Signaling Pathways Leading to The Pleckstrin Homology (PH) Domain-Interacting Protein Couples the Insulin Receptor Substrate 1 PH Dom. *Mol Cell Biol*. 2002;22(20):7325-7336.

27. Vainshtein I, Kovacina KS, Roth RA. The insulin receptor substrate (IRS)-1 pleckstrin homology domain functions in downstream signaling. *J Biol Chem*. 2001;276(11):8073-8078.

28. Yap TA, Garrett MD, Walton MI, Raynaud F, de Bono JS, Workman P. Targeting the PI3K–AKT–mTOR pathway: progress, pitfalls, and promises. *Curr Opin Pharmacol*. 2008;8(4):393-412.

29. Liu Pixu, Cheng Hailing, Roberts Thomas M ZJJ. Targeting the phosphoinositide 3-kinase (PI3K) pathway in cancer. 2011;8(8):627-644.

30. Bolster DR, Jefferson LS, Kimball SR. Regulation of protein synthesis associated with skeletal muscle hypertrophy by insulin-, amino acid- and exercise-induced signalling. *Proc Nutr Soc*. 2004;63(2):351-356.

31. Lopiccolo J, Blumenthal GM, Bernstein WB, Dennis PA. Targeting the PI3K/Akt/mTOR pathway: effective combinations. 2009;11(301):32-50.

32. Inoki K, Li Y, Zhu T, Wu J, Guan K-L. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol*. 2002;4(9):648-657.

33. Dahia PLM. PTEN, a unique tumor suppressor gene. *Endocr Relat Cancer*. 2000;7(2):115-129.

34. American Diabetes Association. Diabetes Mellitus and Other Categories of Description of Diabetes. *World Health*. 2005;28(Suppl 1):224102.

35. McCarthy M, Menzel S. The genetics of type 2 diabetes. *Br J Clin Pharmacol*. 2001;51:195-199.

36. Ahlqvist E, Singh Ahluwalia T, Groop L. Genetics of Type 2 diabetes. *Curr Sci*. 2002;83(12):1477-1482.

37. Freeman H. Type-2 diabetes: a cocktail of genetic discovery. *Hum Mol Genet*. 2006;15(Review Issue 2):R202-R209.

38. Grant RW, Moore AF, Florez JC. Genetic Architecture of Type 2 Diabetes: Recent Progress and Clinical Implications. *Diabetes Care*. 2009;32(6):1107-1114.

39. Doria A, Patti M-E, Kahn CR. The emerging genetic architecture of T2DM. 2013;18(9):1199-1216.

40. Lean M. Obesity: burdens of illness and strategies for prevention or management. *Drugs of Today*. 2000;36(11):773-784.

41. Balkau B, Mhamdi L, Oppert J, et al. Physical Activity and Insulin Sensitivity. 2008;57(October):2613-2618.

42. Colberg SR, Sigal RJ, Fernhall B, et al. Exercise and Type 2 Diabetes: The American College of Sports Medicine and the American Diabetes Association: joint position statement. *Diabetes Care*. 2010;33(12):e147-e167.

43. Hu FB. Sedentary lifestyle and risk of obesity and type 2 diabetes. *Lipids*. 2003;38:103-108.

44. Boule NG, Haddad E, Kenny GP, Wells GA, Sigal RJ. Effects of Exercise on Glycemic Control and Body Mass in Type 2 Diabetes Mellitus A Meta-analysis of Controlled Clinical Trials. 2001;286(10):11-15.

45. Hawley J a, Lessard SJ. Exercise training-induced improvements in insulin action. *Acta Physiol (Oxf)*. 2008;192(1):127-135.

46. Kraniou GN, Cameron-smith D, Hargreaves M, Giorgos N. Acute exercise and GLUT4 expression in human skeletal muscle : influence of exercise intensity. 2006:934-937.

47. Richter EA, Hargreaves M. Exercise, GLUT4, and Skeletal Muscle Glucose Uptake. *Physiol Rev*. 2013;93(3):993-1017.

48. Miller SL, Tipton KD, Chinkes DL, Wolf SE, Wolfe RR. Independent and combined effects of amino acids and glucose after resistance exercise. *Med Sci Sports Exerc*. 2003;35(3):449-455.

49. Kennedy JW, Hirshman MF, Gervino E V, et al. Acute exercise induces GLUT4 translocation. 1999;(35):1-6.

50. Winder W., Hardie D. AMPK a metabolic master switch: possible roles in T2DM. *Rev Lit Arts Am*. 1998;11(6):5-8.

51. Fonseca V a. Defining and characterising the progression of type 2 diabetes. *Br J Diabetes Vasc Dis*. 2008;8(2 Suppl):S3-S9.

52. Golay A, Bobbioni E. The role of dietary fat in obesity. *Int J Obes Relat Metab Disord*. 1997;21.

53. Field C., Ryan E., Thomson AB., Clandinin M. Diet fat composition alters membrane phospholipid composition , insulin binding , and glucose metabolism in adipocytes from control and diabetic animals . C J Field , E A Ryan , A B Thomson and M T Clandinin Diet Fat Composition Alters Membrane Phospholip. *J Biol Chem*. 1990;265(19):11143-11150.

54. Abbott SK, Else PL, Atkins TA, Hulbert AJ. Fatty acid composition of membrane bilayers: Importance of diet polyunsaturated fat balance. *Biochim Biophys Acta - Biomembr*. 2012;1818(5):1309-1317.

55. Dresner A, Laurent D, Marcucci M, et al. Effects of free fatty acids on glucose transport and IRS-1–associated phosphatidylinositol 3-kinase activity. *J Clin Invest*. 1999;103(2):253-259.

56. Morino K, Petersen KF, Shulman GI. Molecular Mechanisms of Insulin Resistance in Humans and Their Potential Links With Mitochondrial Dysfunction. *Diabetes*. 2006;55(Supplement 2):S9-S15.

57. Yu C, Chen Y, Cline GW, et al. Mechanism by Which Fatty Acids Inhibit Insulin Activation of Insulin Receptor Substrate-1 (IRS-1) associated Phosphatidylinositol 3 Kinase Activity in Muscle. *J Biol Chem*. 2002;277(52):50230-50236.

58. Harding a.-H. Dietary Fat and the Risk of Clinical Type 2 Diabetes: The European Prospective Investigation of Cancer-Norfolk Study. *Am J Epidemiol*. 2004;159(1):73-82.

59. Opara E, Garfinkel M, Hubbard V, Burch W, Akwari O. Effect of fatty acids on insulin release: role of chain length and degree of unsaturation. *Am J Physiol*. 1994;266(4):E635-E639.

60. Storlien, Pan, Kriketos. Skeletal muscle membrane lipids and insulin resistance. *Lipids*. 1996;31(1):S261-S265.

61. Odegaard AO, Pereira M a. Trans fatty acids, insulin resistance, and type 2 diabetes. *Nutr Rev*. 2006;64(8):364-372.

62. Salmeron J, Hu FB, Manson JE, et al. Dietary fat intake and risk of type 2 diabetes in women. *Am J Clin Nutr*. 2001;73(6):1019-1026.

63. Christiansen E, Schnider S, Palmvig B, Tauber-Lassen E, Pedersen O. Intake of a diet high in trans monounsaturated fatty acids or saturated fatty acids. Effects on postprandial insulinemia and glycemia in obese patients with NIDDM. *Diabetes Care*. 1997;20(5):881-887.

64. Norton LE, Layman DK. Branched-Chain Amino Acids in Exercise Leucine Regulates Translation Initiation of Protein Synthesis in Skeletal. 2006:533-537.

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

66. She P, Reid TM, Bronson SK, et al. Disruption of BCATm in mice leads to increased energy expenditure associated with the activation of a futile protein turnover cycle. *Cell*. 2009;6(3):181-194.

67. Greiwe JS, Kwon G, McDaniel ML, Semenkovich CF. Leucine and insulin activate p70 S6 kinase through different pathways in human skeletal muscle. *Am J Physiol Endocrinol Metab*. 2001;281:E466-E471.

68. Liu H, Liu R, Xiong Y, et al. Leucine facilitates the insulin-stimulated glucose uptake and insulin signaling in skeletal muscle cells: Involving mTORC1 and mTORC2. *Amino Acids*. 2014;46:1971-1979.

69. Kimball SR, Jefferson LS. Control of translation initiation through integration of signals generated by hormones, nutrients, and exercise. *J Biol Chem*. 2010;285:29027-29032.

70. Lynch CJ, Hutson SM, Patson BJ, Vaval A, Vary TC. Tissue-specific effects of chronic dietary leucine and norleucine supplementation on protein synthesis in rats. *Am J Physiol Endocrinol Metab*. 2002;283:E824-E835.

71. Tremblay F, Marette A. 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*. 2001;276:38052-38060.

72. Rietman a, Schwarz J, Tomé D, Kok FJ, Mensink M. High dietary protein intake, reducing or eliciting insulin resistance? *Eur J Clin Nutr*. 2014;(November 2013):1-7.

73. Linn T, Santosa B, Grönemeyer D, et al. Effect of long-term dietary protein intake on glucose metabolism in humans. *Diabetologia*. 2000;43:1257-1265.

74. Ricci G, Canducci E, Pasini V, et al. Nutrient intake in Italian obese patients: Relationships with insulin resistance and markers of non-alcoholic fatty liver disease. *Nutrition*. 2011;27(6):672-676.

75. Newgard CB, An J, Bain JR, et al. A branched-chain amino acid related metabolic signature. 2009;9(4):311-326.

76. Um SH, Frigerio F, Watanabe M, et al. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature*. 2004;431(7005):200-205.

77. Samuel VT and SGI. Integrating mechanisms for insulin resistance. *Cell*. 2013;148(5):852-871.

78. Chaurasia B, Summers SA. Ceramides – Lipotoxic Inducers of Metabolic Disorders. *Trends Endocrinol Metab*. 2015;26(10):538-550.

79. Ohanian J, Ohanian V. Lipid second messenger regulation: the role of diacylglycerol kinases and their relevance to hypertension. *J Hum Hypertens*. 2001;15(2):93-98.

80. Liu L, Zhang Y, Chen N, Shi X, Tsang B, Yu Y. Upregulation of myocellular DGAT1 augments triglyceride synthesis in skeletal muscle and protects against fat-induced insulin resistance. *J Clin Invest*. 2007;117(6):1679-1689.

81. Itani SI, Zhou Q, Pories WJ, MacDonald KG, Dohm GL. Involvement of protein kinase C in human skeletal muscle insulin resistance and obesity. *Diabetes*. 2000;49(8):1353-1358.

82. Shoelson SE, Lee J, Goldfine AB. Review series Inflammation and insulin resistance. *J Clin Invest*. 2006;116(7):1793-1801.

83. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science*. 1993;259(5091):87-91.

84. Ye J. Mechanisms of insulin resistance in obesity Jianping. *Front Med*. 2013;7(1):14-24.

85. Ashida N, Senbanerjee S, Kodama S, et al. IKKβ regulates essential functions of the vascular endothelium through kinase-dependent and -independent pathways. *Nat Commun*. 2011;2(May):318.

86. Arkan MC, Hevener AL, Greten FR, et al. IKK-beta links inflammation to obesity-induced insulin resistance. *Nat Med*. 2005;11(2):191-198.

87. Soldo-Jureša D, Metelko Ž. PPARγ - A new concept of treatment? *Diabetol Croat*. 2009;38(2):23-29.

88. Moller DE. Potential role of TNF-alpha in the pathogenesis of insulin resistance and type 2 diabetes. *Trends Endocrinol Metab*. 2000;11(6):212-217.

89. Kaneto H, Katakami N, Matsuhisa M, Matsuoka T. Role of Reactive Oxygen Species in the Progression of Type 2 Diabetes and Atherosclerosis. *Mediators Inflamm*. 2010;2010:1-11.

90. Kaneto H, Xu G, Fujii N, Kim S, Bonner-Weir S, Weir GC. Involvement of c-Jun N-terminal kinase in oxidative stress-mediated suppression of insulin gene expression. *J Biol Chem*. 2002;277(33):30010-30018.

91. Rudich A, Tlrosh A, Potashnik R, Hemi R, Kanety H, Bashan N. Prolonged oxidative stress impairs insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. *Diabetes*. 1998;47(10):1562-1569.

92. Kaneto H, Kajimoto Y, Miyagawa J, et al. Beneficial effects of antioxidants in diabetes : Possible protection ... 1999.

93. Laplante M, Sabatini DM. mTOR signaling at a glance. *J Cell Sci*. 2010;123:4001-4006.

94. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell*. 2013;149(2):274-293.

95. Zoncu R, Sabatini DM, Efeyan A. mTOR: from growth signal to diabetes and cancer. *Nat Rev Mol Cell Biol*. 2012;12(1):21-35. doi:].

96. Hay N, Sonenberg N. Upstream and downstream of mTOR. 2004:1926-1945.

97. Guertin DA, Stevens DM, Thoreen CC, et al. Ablation in Mice of the mTORC Components raptor, rictor, or mLST8 Reveals that mTORC2 Is Required for Signaling to Akt-FOXO and PKCα, but Not S6K1. *Dev Cell*. 2006;11(6):859-871.

98. Frias MA, Thoreen CC, Jaffe JD, et al. mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. *Curr Biol*. 2006;16(18):1865-1870.

99. Jacinto E, Loewith R, Schmidt A, et al. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol*. 2004;6(11):1122-1128.

100. Um SH, D'Alessio D, Thomas G. Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. *Cell Metab*. 2006;3(6):393-402.

101. Potter CJ, Pedraza LG, Xu T. Akt regulates growth by directly phosphorylating Tsc2. *Nat Cell Biol*. 2002;4(9):658-665.

102. Inoki K, Li Y, Xu T, Guan K. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling service Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. 2003;(734):1829-1834.

103. Manning. ARTBD, Cantley. PPRLC, John B and. Tuberous Sclerosis Complex Gene Products, Tuberin and Hamartin, Control mTOR Signaling by Acting as a GTPase-Activating Protein Complex toward Rheb. *Curr Biol*. 2003;13:1317-1323.

104. Deyoung MP, Horak P, Sofer A, Sgroi D, Ellisen LW. Hypoxia regulates TSC1 / 2 – mTOR signaling and tumor suppression through REDD1-mediated 14 – 3 – 3 shuttling. *Genes (Basel)*. 2008:239-251.

105. Williams BD, Stuart CA, Lane W. Oral Branched-Chain Amino Acids Decrease Whole-Body Proteolysis. :47-54.

106. Said a K, Hegsted DM. Response of adult rats to low dietary levels of essential amino acids. *J Nutr*. 1970;100:1363-1375.

107. Mortimore GE, Schworer CM. Induction of autophagy by amino-acid deprivation in perfused rat liver. *Nature*. 1977;270(5633):174-176.

108. Kroemer, Guido. Marino, Guillermo. Levine B. Autophagy and the integrated stress response. *Mol Cell*. 2010;40(2):280-293.

109. Demetriades C, Doumpas N, Teleman A a. Regulation of TORC1 in response to amino acid starvation via lysosomal recruitment of TSC2. *Cell*. 2014;156(4):786-799.

110. Sekiguchi T, Hirose E, Nakashima N, Ii M, Nishimoto T. Novel G Proteins, Rag C and Rag D, Interact with GTP-binding Proteins, Rag A and Rag B. *J Biol Chem*. 2001;276(10):7246-7257.

111. Peña-Llopis S, Vega-Rubin-de-Celis S, Schwartz JC, et al. Regulation of TFEB and V-ATPases by mTORC1. *EMBO J*. 2011;30(16):3242-3258.

112. Gulati P, Gaspers LD, Dann SG, et al. Amino acid mediated activation of mTORC1 by hVps34. 2009;7(5):456-465.

113. Escobar J, Frank JW, Suryawan A, et al. Leucine and alpha-ketoisocaproic acid, but not norleucine, stimulate skeletal muscle protein synthesis in neonatal pigs. *J Nutr*. 2010;140(8):1418-1424.

114. Matsumoto K, Koba T, Hamada K, Sakurai M, Higuchi T, Miyata H. Branched-chain amino acid supplementation attenuates muscle soreness, muscle damage and inflammation during an intensive training program. *J Sport Med Phys Fit*. 2009;49(4):424-431.

115. Ra S-G, Miyazaki T, Ishikura K, et al. Combined effect of branched-chain amino acids and taurine supplementation on delayed onset muscle soreness and muscle damage in high-intensity eccentric exercise. *J Int Soc Sports Nutr*. 2013;10(1):51.

116. Layman DK. The role of leucine in weightloss. *J Nutr*. 2003:249-251.

117. Brosnan JT, Brosnan ME. Branched-Chain Amino Acids : Metabolism , Physiological Function , and Application. 2006;(3):207-211.

118. Shimomura Y, Murakami T, Nagasaki M, et al. Regulation of branched-chain amino acid metabolism and pharmacological effects of branched-chain amino acids. *Hepatol Res*. 2004;30:3-8.

119. Nishitani S, Matsumura T, Fujitani S, Sonaka I, Miura Y, Yagasaki K. Leucine promotes glucose uptake in skeletal muscles of rats. *Biochem Biophys Res Commun*. 2002;299:693-696.

120. Pimentel GD, Rosa JC, Lira FS, et al. β-Hydroxy-β-methylbutyrate (HMβ) supplementation stimulates skeletal muscle hypertrophy in rats via the mTOR pathway. *Nutr Metab (Lond)*. 2011;8(1):11.

121. Girón MD, Vílchez JD, Salto R, et al. Conversion of leucine to β-hydroxy-β-methylbutyrate by α-keto isocaproate dioxygenase is required for a potent stimulation of protein synthesis in L6 rat myotubes. *J Cachexia Sarcopenia Muscle*. 2015;1:n/a - n/a.

122. Pinheiro CHDJ, Gerlinger-Romero F, Guimarães-Ferreira L, et al. Metabolic and functional effects of beta-hydroxy-beta-methylbutyrate (HMB) supplementation in skeletal muscle. *Eur J Appl Physiol*. 2012;112(7):2531-2537.

123. Bruckbauer A, Zemel MB. Synergistic effects of metformin, resveratrol, and hydroxymethylbutyrate on insulin sensitivity. *Diabetes, Metab Syndr Obes Targets Ther*. 2013;6:93-102.

124. Huffman K, Shah S, Stevens R, et al. Metabolic Intermediates and Insulin Action in Overweight to Obese , Inactive Men and. *Diabetes Care*. 2009;32(9).

125. Suhre K, Meisinger C, Döring A, et al. Metabolic Footprint of Diabetes: A Multiplatform Metabolomics Study in an Epidemiological Setting. *PLoS One*. 2010;5(11):e13953.

126. Badoud F, Lam KP, Dibattista A, et al. Serum and adipose tissue amino acid homeostasis in the metabolically healthy obese. *J Proteome Res*. 2014;13(7):3455-3466.

127. Lackey DE, Lynch CJ, Olson KC, et al. Regulation of adipose branched-chain amino acid catabolism enzyme expression and cross-adipose amino acid flux in human obesity. *Am J Physiol Endocrinol Metab*. 2013;304(11):E1175-E1187.

128. Shah SH, Crosslin DR, Haynes CS, et al. Branched-chain amino acid levels are associated with improvement in insulin resistance with weight loss. *Diabetologia*. 2012;55(2):321-330.

129. Laferrère B, Reilly D, Arias S, et al. Differential metabolic impact of gastric bypass surg. 2013;3(80).

130. Wang TJ, Larson MG, Vasan RS, et al. Metabolite profiles and the risk of developing diabetes. *Nat Med*. 2011;17(4):448-453.

131. Nishitani S, Takehana K, Fujitani S, Sonaka I. Branched-chain amino acids improve glucose metabolism in rats with liver cirrhosis. 2005:1292-1300.

132. Kleinert M, Liao Y-H, Nelson JL, Bernard JR, Wang W, Ivy JL. An amino acid mixture enhances insulin-stimulated glucose uptake in isolated rat epitrochlearis muscle. *J Appl Physiol*. 2011;111(April 2011):163-169.

133. Bernard JR, Liao Y-H, Doerner PG, et al. An amino acid mixture is essential to optimize insulin-stimulated glucose uptake and GLUT4 translocation in perfused rodent hindlimb muscle. *J Appl Physiol*. 2012;113(5):97-104.

134. Yamamoto N, Ueda M, Sato T, et al. Measurement of glucose uptake in cultured cells. *Curr Protoc Pharmacol*. 2011;(December):1-22.

135. Selvi R, Angayarkanni N, Asma B, Seethalakshmi T, Vidhya S. Amino acids influence the glucose uptake through GLUT4 in CHO-K1 cells under high glucose conditions. *Mol Cell Biochem*. 2010;344(1-2):43-53.

136. Luzi L, Giordano M, Caloni, Castellino P. Effects of insulin and amino acids on leucine metabolism in young and middle-aged humans. *Eur J Nutr*. 2001;40(3):106-112. http://www.ncbi.nlm.nih.gov/pubmed/11697442.

137. Castellino P, Luzi L, Simonson DC, Haymond M, DeFronzo RA. Effect of insulin and plasma amino acid concentrations on leucine metabolism in man. Role of substrate availability on estimates of whole body protein synthesis. *J Clin …*. 1987;80(December):1784-1793.

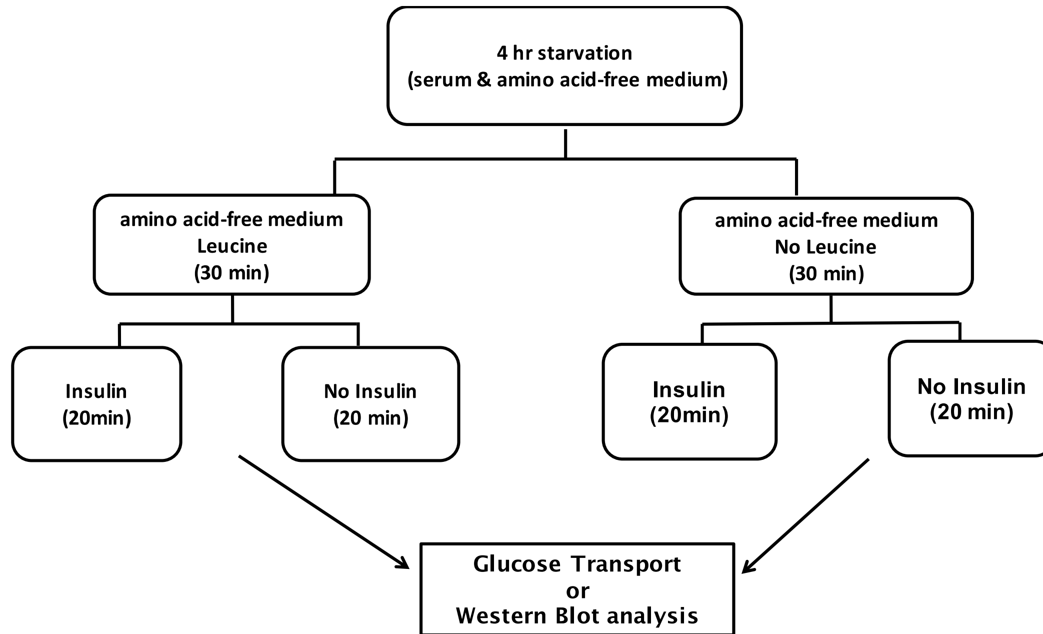
138. Alvestrand A, Hagenfeldt L, Merli M, Oureshi A, Eriksson LS. Influence of leucine infusion on intracellular amino acids in humans. *Eur J Clin Invest*. 2008;20(3):293-298.

139. Xu G, Kwon G, Cruz WS, Marshall C a., McDaniel ML. Metabolic regulation by leucine of translation initiation through the mTOR-signaling pathway by pancreatic β-cells. *Diabetes*. 2001;50(25):353-360.

140. She P, Van Horn C, Reid T, Hutson SM, Cooney RN, Lynch CJ. Obesity-related elevations in plasma leucine are associated with alterations in enzymes involved in branched-chain amino acid metabolism. *Am J Physiol Endocrinol Metab*. 2007;293(6):E1552-E1563.

11.0 Appendix

A) Experiment Outline:



Myotubes were starved for 4h in serum and amino acid-free medium. They were then incubated in an amino acid-free medium to which leucine was added at various concentrations for 30 minutes. Following this, the cells were incubated with or without insulin for 20 minutes and harvested for either western blot analysis or glucose transport assay.

B) *Glucose Transport Assay*

Solutions

Hepes Buffer Saline:

- 140 mM NaCl
- 20 mM Hepes-Na, pH 7.4
- 5 mM KCl
- 2.5 mM $MgSO_4$
- 1.0 mM $CaCl_2$

Stop Solution:

- 0.9% NaCl (Saline)

2-DG Stock Solution:

- 10 mM 2-Deoxy-D-Glucose in Hepes buffer

Transport Solution (TS):

- Prepare in Hepes buffer
- 10 µM 2-Dexocy-Glucose
- 0.5 µCi/mL ^{3}H 2-Deoxy-Glucose

Procedures

1. On the designated radioactive bench in the lab, wash cells two times with Hepes Buffered Saline (HBS) at room temperature and aspirate any remaining buffer
2. Add 300 µl of of Transport Solution (TS) per well for a 12-well plate.
3. Incubate the plates for 5 minutes at 37°. Be sure to not exceed this time.
4. Aspirate away the Transport Solution (TS) quickly and wash the wells thoroughly three times with ice-cold Stop Solution (0.9% Saline). Aspirate to dryness.
5. While on ice, add 1.0 mL of 0.05N NaOH to each well in the plate.
6. Scrape the cells and transfer 0.8 mL of the contents into plastic Scintillation vials already filled with 4-5 mL of Scintillation fluid.
7. Transfer the remaining contents into 1.5 mL Eppendorf tubes (to be used for protein assay).

8. Count the amount of radioactivity in each vial using the Scintillation counter and measure the amount of radioactivity in each sample.

C) *RNAi Gene Silencing*

Materials

- Opti-MEM (Life technologies: cat # 31985-070)
- Lipofectamine RNAiMAX reagent (Life technologies: cat #13778-150)
- siRNA scramble and BCAT2 (Sigma Aldrich)
- Growth Medium (GM) without antibiotics (AMEM (Wisent Inc. Cat # 310-010-CL) supplemented with 10% FBS (Cat # 12484-028))
- Growth Medium (GM) with antibiotics (AMEM (Wisent Inc. Cat # 310-010-CL) supplemented with 10% FBS (Cat # 12484-028) and 1% Ab-Am (Wisent Inc. Cat # 450-115-EL))
- 15 ml polypropylene conical tubes (BD Falcon Ref #372096)
- 6 well plates (Cellstar: cat # 657160)

Procedures:

1. In the cell culture hood, prepare three 15 ml tubes and label as Optimum, "A", "B" and "C"

   A→ Lipofectamine+ Opti-MEM

   B→ BCAT2 siRNA+Opti-MEM

   C→ Scramble siRNA+Opti-MEM

2. Pour Optimum solution into the designated 15 ml-Optimum tube (without touching the mouth of the bottle).
3. Add 120 μl of Opti-MEM +5 μl of Lipofectamine to tube "A".
4. Add 2-3 μl of BCAT2 siRNA and 122-123 μl of Opti-MEM to tube "B".
5. Add 2-3 μl of scramble siRNA and 122-123 μl of Opti-MEM to tube "C".
6. Add mix "A" to mix "B" in 1:1 ratio. (For example, 125 μl of "A" + 125 μl of "B".
7. Wait at least 5 minutes.
8. While waiting, add 1 mL/well of GM without antibiotics, and add cells to each well.
9. Add 250 μl/well of diluted mixture to each well.
10. Following 24 hours, add 1 mL/well of GM (with antibiotics).