Effect of Inflammation on Ketoisocaproic Acid Induced Insulin Resistance in Skeletal Muscle Cells

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Abstract

Branched-chain amino acids (BCAAs) have displayed metabolic benefits, and play a role in muscle protein synthesis. However, elevated levels of BCAAs and their metabolites have been linked to the pathogenesis of insulin resistance and type 2 diabetes mellitus. It has been demonstrated in my lab that α-ketoisocaproic acid (KIC), a metabolite of leucine, inhibited insulin-stimulated glucose uptake, but is converted back to leucine in order to do so. Inflammation, a feature of insulin resistance may modulate the effects of amino acids and their metabolites on insulin action. Thus, I analyzed whether or not there was an additive effect of KIC and inflammation on insulin-stimulated glucose transport in L6 myotubes. Results emphasize previous findings, that even in the presence of inflammation, KIC is converted back to leucine to inhibit insulin-stimulated glucose uptake, suggesting that interventions altering BCAA pathway flux may help in the management/prevention of insulin resistance.
Acknowledgements

Thank you first and foremost to Dr. Adegoke, without your guidance and help throughout my journey, this would not have been possible. Thanks for taking a chance on someone with minimal lab experience.

Next, thank you to my lab mates for all the help. Thank you to Brendan for teaching me everything and helping me whenever needed, and thank you Stephen, Jasmine, Abdurahman, and Glory for your help as well.

Finally thank you to my parents, as without you this wouldn’t be possible.
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4EBP1</td>
<td>Eukaryotic translation initiation factor 4E binding protein 1</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>AS</td>
<td>160 Akt substrate 160kD</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCAA</td>
<td>Branched-chain amino acid</td>
</tr>
<tr>
<td>BCAT2</td>
<td>Branched-chain aminotransferase 2</td>
</tr>
<tr>
<td>BCKDH</td>
<td>Branched-chain α-keto acid dehydrogenase complex</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>Deptor</td>
<td>DEP-domain-containing mTOR-interacting protein</td>
</tr>
<tr>
<td>eEF2K</td>
<td>Eukaryotic elongation factor 2 kinase</td>
</tr>
<tr>
<td>eIF4A</td>
<td>Eukaryotic translation initiation factor 4A</td>
</tr>
<tr>
<td>eIF4B</td>
<td>Eukaryotic translation initiation factor 4B</td>
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<td>eIF4E</td>
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</tr>
<tr>
<td>eIF4F</td>
<td>Eukaryotic translation initiation factor 4F</td>
</tr>
<tr>
<td>eIF4G</td>
<td>Eukaryotic translation initiation factor 4G</td>
</tr>
<tr>
<td>FKBP12</td>
<td>FK506-binding protein 12 kDa</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>Gab-1</td>
<td>Grb2-associated-binding protein 1</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter type-4</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>hVps34</td>
<td>Human vacuolar protein sorting 34</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor kappa B (IkB) kinase</td>
</tr>
<tr>
<td>IKK-β</td>
<td>IkB kinase beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun N-terminal kinase</td>
</tr>
<tr>
<td>KIC</td>
<td>α-ketoisocaproic acid</td>
</tr>
<tr>
<td>KIV</td>
<td>α-ketoisovaleric acid</td>
</tr>
<tr>
<td>KMV</td>
<td>α-keto-β-methylvaleric acid</td>
</tr>
<tr>
<td>mLST8</td>
<td>Mammalian lethal with Sec 13 protein 8</td>
</tr>
<tr>
<td>MODY</td>
<td>Maturity-onset diabetes of the young</td>
</tr>
<tr>
<td>mSin1</td>
<td>Mammalian stress-activated protein kinase (SAPK)-interacting protein-1</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>mTORC2</td>
<td>Mammalian target of rapamycin complex 2</td>
</tr>
<tr>
<td>Nck1</td>
<td>Non-catalytic region of tyrosine kinase adaptor protein 1</td>
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<td>NF-κB</td>
<td>Inhibitor kappa B (IkB) kinase (IKK)-nuclear factor κB</td>
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<td>Programmed cell death 4</td>
</tr>
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<td>PDK1</td>
<td>3-phosphoinositol dependent protein kinase-1</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin-homology</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol-4,5-biphosphate</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol-3,4,5-triphosphate</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>PRAS40</td>
<td>Proline-rich Akt substrate of 40 kDa</td>
</tr>
<tr>
<td>Protor-1</td>
<td>Protein observed with rictor-1</td>
</tr>
<tr>
<td>Raptor</td>
<td>Rapamycin-sensitive adaptor protein of mTOR</td>
</tr>
<tr>
<td>Rheb</td>
<td>Ras homologue in the brain</td>
</tr>
<tr>
<td>Rictor</td>
<td>Rapamycin-insensitive companion of mTOR</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S6K1</td>
<td>Ribosomal protein S6 Kinase 1</td>
</tr>
<tr>
<td>Ser/S</td>
<td>Serine</td>
</tr>
<tr>
<td>SH2</td>
<td>SRC homology-2</td>
</tr>
<tr>
<td>SOCS1</td>
<td>Suppressor of cytokine signaling 1</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Suppressor of cytokine signaling 3</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>Thr/T</td>
<td>Threonine</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TSC1/2</td>
<td>Tuberous sclerosis complex 1/2</td>
</tr>
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</table>
1.0 Introduction

Type 2 Diabetes Mellitus (T2DM) is a chronic metabolic disorder and its prevalence is increasing drastically where it is beginning to be regarded as an epidemic in some countries\(^1\). An estimated 439 million people will have T2DM by 2030\(^2\). T2DM is characterized by high blood glucose levels (hyperglycemia) and insulin resistance. Insulin resistance refers to a suppressed response to insulin. T2DM differs from Type 1 Diabetes Mellitus (T1DM), as T2DM refers to an issue with the tissue responding to insulin instead of a lack of insulin production due to the destruction of β-cells in the pancreas\(^3\). Two factors in the onset of T2DM include genetic factors and lifestyle choices. Genetics plays a significant role, as having relatives with T2DM significantly increases the risk of developing T2DM. Medical conditions like obesity can intensify or give rise to T2DM.

Lifestyle choices can increase the likelihood of developing T2DM. High fat diets are extensively studied in their role in the development of T2DM\(^2\). This is because high fat diets display pro-inflammatory effects both in vitro and in vivo\(^6\). Inflammation, a characteristic of obesity and insulin resistance has been directly linked to T2DM. Literature shows that pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α), could have implications in the development of insulin resistance. TNF-α activates c-Jun N-terminal kinases (JNK), which ultimately leads to phosphorylation of the serine residues of insulin receptor substrate (IRS-1), which inhibits insulin signaling\(^9\).

Calorie restriction is often suggested for obese individuals for weight loss, but this can lead to a loss of muscle mass. High branched-chain amino acid (BCAA; leucine, valine, isoleucine) diets have been recommended to counter this loss in muscle mass. These high BCAA diets exhibit positive effects such as stimulating muscle protein synthesis, and regulating body weight and
glucose homeostasis. BCAAs, like insulin, behave as anabolic signals for growth of tissues through the activation of mammalian target of rapamycin complex 1 (mTORC1). This is especially true for leucine, which activates mammalian target of rapamycin (mTOR) signalling.

Elevated circulating levels of BCAAs have also been associated with an increase risk in developing T2DM. BCAA supplementation results in sustained activation of mTORC1 (hyper-activation) and its substrates. This hyper-activation results in a feedback loop, promoting phosphorylation of serine residues of IRS-1. Ultimately, BCAAs have a paradoxical role for obese individuals, as although they may increase protein synthesis and maintain muscle mass, they might also have implications in the pathogenesis of T2DM by reducing insulin sensitivity. Since leucine activates mTORC1, there have been numerous studies trying to elucidate its potential effects on insulin signaling and its role in the pathogenesis of insulin resistance. In previous studies in my lab, leucine significantly suppressed insulin-stimulated glucose uptake. Also, incubation of KIC (200µM), a metabolite of leucine, in L6 myotubes resulted in a 45% suppression in insulin-stimulated glucose uptake.

As stated earlier, inflammation is a characteristic of both insulin resistance and obesity, which can modulate the effect of KIC on insulin sensitivity. Thus, I analyzed the potential additive or synergistic effects of KIC in the context of inflammation on insulin-stimulated glucose uptake. This was important in order to elucidate potential mechanisms involved in the pathogenesis of insulin resistance. This will help in uncovering potential interventions that can help manage or treat dysregulations in BCAA metabolism that may have implications on the management of insulin resistance in type 2 diabetes and cardiovascular diseases.
2.0 Literature Review

2.1 Insulin Signaling Within Skeletal Muscle

Skeletal muscle makes up to 30-40% of our body mass, and is important for amino acid and glucose metabolism and thermogenesis. Besides locomotion and storage, skeletal muscle is important in the maintenance of systemic glucose metabolism and is the most abundant insulin-sensitive tissue. Insulin is one of the main hormones in regulating glucose metabolism. Insulin controls a wide variety of biological processes and many aspects of metabolism and growth such as glucose transport, glycogen synthesis and protein synthesis.

2.1.1 Insulin Receptor and Insulin Receptor Substrates

Insulin and IGF-1 elicits their effects by binding to the insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF-IR) respectively. Despite the preferential binding to their own respective receptors, both insulin and IGF-1 can bind to the others’ receptor with reduced affinity. The IR is a heterotetrameric membrane glycoprotein comprising of two α subunits as well as two β subunits that are joined by disulfide bonds. Insulin binds to the extracellular α subunit of the receptor, inducing conformational changes bringing both α subunits together. This leads to the auto-phosphorylation of the IR’s Tyr residue, allowing for its binding with the phospho-tyrosine binding domain of the insulin receptor substrates (IRSs).

Thus far there are 12 substrates of the IR elucidated. These include: IRS-1, IRS-2, IRS-3, IRS-4, IRS-5, IRS-6, growth factor receptor-bound protein 2 (Grb2)-associated-binding protein 1 (Gab-1), three isoforms of SH2 (Src homology 2), p62 dok and adaptor protein with a PH and SH2 domain (APS). IRS-1 and IRS-2 are widely disturbed within the body, while IRS-3 is only
present in adipocytes and the brain, IRS-4 in embryonic tissues or cell lines, IRS-5 in kidney and liver, and IRS-6 in skeletal muscle.

In muscle, serine phosphorylation of IRS residues, such as Ser\textsuperscript{307}, Ser\textsuperscript{612} and Ser\textsuperscript{1101} can reduce the ability of Phosphatidylinositol 3-kinase (PI3K) to be activated by IRS itself. Serine phosphorylation of IRS-1 results in a negative feed-back control mechanism to uncouple IR-IRS complexes\textsuperscript{178}. This impairs tyrosine phosphorylation and thus affecting how the signal is transmitted downstream. Serine phosphorylation reduces the ability of IRS-1 to recruit PI3K, minimizing PI3K activation\textsuperscript{207-212}. Recent studies have emphasized the importance of these serine residues in insulin resistance, as replacing the serine residues of IRS-1 with alanine in vivo has prevented high fat diet induced insulin resistance\textsuperscript{109}. On the other hand, in a mice study, where they mutated the Ser\textsuperscript{302} site to alanine, there was no change in insulin action\textsuperscript{234}.

The first IRS discovered was IRS-1, which has 21-22 tyrosine phosphorylation sites\textsuperscript{15,16} and 50 serine/threonine phosphorylation sites\textsuperscript{245}. IRS-1 is expressed in insulin sensitive tissues such as skeletal muscle, adipose tissue and liver. Phosphorylation of IRS-1 tyrosine residues is crucial in transmitting the signal from the IR to downstream substrates. The insulin receptor and IRS tyrosine phosphorylation is transient and is dephosphorylated by protein tyrosine phosphatase 1B\textsuperscript{238}. Ablation of this phosphatase in muscle\textsuperscript{239} and liver\textsuperscript{240} resulted in improved insulin sensitivity.

IRS-1’s importance in insulin signaling has been emphasized with knockout IRS-1 mice exhibiting peripheral insulin resistance and decreased growth\textsuperscript{17}. However, IRS-2 compensates for this loss and allows for the transduction of the insulin signal. IRS-2, like IRS-1 can engage with PI3K, and thus relay the signal downstream\textsuperscript{18}. Tang et al. emphasize the importance of IRS-2, as in an IRS-1 knockout mouse model, IRS-2 was upregulated in the liver and skeletal muscle, and
this upregulation of IRS-2 prevented an increase in blood glucose associated with insulin resistance\textsuperscript{250}. IRS-2’s importance is highlighted with its knockout, resulting in the development of T2DM, increased adiposity, and insulin resistance in liver and skeletal muscle\textsuperscript{19}.

2.1.2 IRS-1/PI3K/Akt Pathway

The IRS-1/PI3K/Akt signaling pathway is central to insulin signaling. Once IRS-1 is activated through its phosphorylation by the IR, it relays this signal to PI3K (figure 1). PI3K is a heterodimeric lipid kinase with a wide array of functions, such as growth and differentiation, synthesis and degradation of lipids, proteins and carbohydrates, and membrane trafficking\textsuperscript{20}. PI3K consists of a regulatory and a catalytic subunit. Recruitment and activation of PI3K depends on the binding of two SH2 domains in the regulatory subunits of PI3K to the tyrosine-phosphorylated IRS-1\textsuperscript{27,28}. In skeletal muscle of humans, the regulatory subunit binds to IRS-1 via IRS-1’s SH2 domains, allowing for PI3K activation. This activation allows for the catalytic subunit to phosphorylate phosphatidylinositol 4,5-biphosphate (PIP\textsubscript{2}). PI3K phosphorylates PIP\textsubscript{2} at position 3 of the inositol ring to generate a lipid second messenger known as phosphatidylinositol 3,4,5-trisphosphate (PIP\textsubscript{3})\textsuperscript{21}. The importance of PI3K in insulin signaling is emphasized in the inhibition of PI3K. Martin et al. demonstrated that PI3K inhibition with wortmannin resulted in a reduced translocation of glucose transporter 4 (GLUT4), which is a protein necessary for glucose uptake\textsuperscript{22}.

Lipid phosphatases can have negative effects on insulin signaling. Phosphatase and tensin homolog (PTEN) dephosphorylates PIP\textsubscript{3}, thus antagonizing PI3K signaling\textsuperscript{37,38}. PTEN is a tumour suppressor that is disrupted in human cancers as well\textsuperscript{236}. PTEN inhibition has increased Akt activation, thus not only affecting insulin action\textsuperscript{237}, but also cell growth and survival pathways\textsuperscript{236}.
Studies have also demonstrated that PTEN deletion in skeletal muscle resulted in increased insulin sensitivity, highlighting its role in the pathogenesis of insulin resistance\(^{39,40}\).

Many of the effects of PI3K-derived PIP\(_3\) are mediated by a subset of AGC protein kinase family members including Akt, p70 ribosomal S6 kinase (S6K), and serum and glucocorticoid-induced protein kinase (SGK). 3-phosphoinositide-dependent protein kinase 1 (PDK-1) is the major upstream kinase responsible for phosphorylating substrates regulated by PI3K\(^{30}\). For example, PDK-1 phosphorylates and activates the Thr\(^{308}\) residue of Akt\(^{31}\). Simultaneous binding of PDK-1 and Akt to phosphoinosotides like PIP\(_3\) facilitates conformational changes, allowing for interaction between PDK-1 and Akt\(^{251}\). This makes Akt more prone to be phosphorylated by PDK-1\(^{251}\). For full Akt activation, the Ser\(^{473}\) residue also needs to be phosphorylated, which is accomplished by the mammalian target of rapamycin complex 2 (mTORC2)\(^{23,24}\). Mutations in either Akt or PI3K cause severe insulin resistance highlighting the importance of these two proteins in the insulin signaling pathway\(^{246}\).

There are three different isoforms of Akt: Akt1, Akt2, and Akt3. Akt1 and Akt2 are involved in the insulin signaling pathway in both skeletal muscle and adipose tissue. On the contrary, Akt3 is not activated by insulin in adipose tissue, skeletal muscle or the liver\(^{25}\). Akt2 plays an important role in mediating insulin action on metabolism, while Akt1 is more associated with growth\(^{241}\). Akt2 has major implications in insulin-stimulated glucose uptake, as it is important for the translocation of GLUT4 through the phosphorylation of Akt substrate of 160 kDa (AS160) by Akt2\(^{159,160}\). AS160, otherwise known as TCB1D4 and its homolog TCB1D1, are phosphorylated by Akt to elicit its effects on contraction-mediated glucose uptake, and insulin-stimulated glucose uptake\(^{233}\). TBC1D4 activates Rab proteins\(^{242}\) that are responsible for about half
of the effect of insulin on GLUT4\textsuperscript{243}. In fat cells Rab10 specifically is known for its role in the biogenesis of GLUT4-containing transport vesicles\textsuperscript{244}.

2.1.3 PI3K/Akt/mTOR Pathway

In skeletal muscle once Akt is activated by PDK-1 and mTORC2, it results in the activation/phosphorylation of downstream substrates. Akt phosphorylates tuberous sclerosis complex protein 2 (TSC-2), which is an inhibitor of mTORC1 through Ras homologue enriched in brain (Rheb)\textsuperscript{97}. Akt phosphorylation of TSC-2 induces the degradation of the tumour suppressor complex consisting of TSC-2 and tuberous sclerosis complex protein 1 (TSC-1). This allows for the activation of the mTORC1 complex\textsuperscript{147} (figure 1). Akt can also activate mTORC1 through phosphorylation of proline rich AKT substrate 40 kDa (PRAS40), alleviating PRAS40’s inhibition of mTORC1\textsuperscript{33}.

Another role of Akt includes phosphorylating glycogen synthase kinase 3 (GSK-3), resulting in its inactivation, which in turn allows for activation of glycogen synthase for glycogen accumulation in the liver and skeletal muscle\textsuperscript{36}. Akt also mediates insulin’s effect on inhibiting lipolysis. Akt phosphorylates phosphodiesterase 3B, resulting in a decrease in cyclic AMP levels, which then can inhibit lipolysis in adipocytes\textsuperscript{233}.

2.2 Mammalian Target of Rapamycin (mTOR)

The mammalian/mechanistic target of rapamycin (mTOR) protein is a serine/threonine kinase that belongs to the PI3K-related kinases family. mTOR functions as a growth regulator and nutrient sensor and controls a wide array of cellular processes such as cell growth, differentiation, proliferation and overall metabolic homeostasis\textsuperscript{89}. mTOR with various other protein interactions
forms two distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). It is imperative to understand the complexity and features of mTOR signaling as its dysregulation has had severe implications on cancer, obesity and T2DM.

2.2.1 Mammalian Target of Rapamycin Complex 1 (mTORC1)

mTORC1 regulates cell growth through its phosphorylation of downstream substrates. This allows for the stimulation of anabolic processes like mRNA translation and the inhibition of catabolic processes such as autophagy.

mTORC1 consists of six components: mTOR, the catalytic subunit of the complex; regulatory-associated protein of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8, also known as GbL), PRAS40, FK506 binding protein (FKBP12) and DEP-domain-containing mTOR-interacting protein (Deptor). Raptor is an important protein in this complex, as it functions to regulate the assembly of the complex and recruit substrates for mTOR. On the other hand, the role of mLST8 seems to be unclear, as knockdown in vivo exhibited no difference in mTORC1 activity. PRAS40, FKBP12 and Deptor function as negative regulators of mTORC1, and inhibit mTORC1 when recruited to the complex. When mTORC1 is active, the opposite occurs, mTORC1 directly phosphorylates PRAS40 and Deptor, reducing the physical interaction and further activating mTORC1 signaling.
2.2.2 Mammalian Target of Rapamycin Complex 2 (mTORC2)

Unlike mTORC1, the regulation of mTORC2 is poorly understood. Only growth factors can activate mTORC2. It plays a major role in Akt, serum and glucocorticoid-regulated kinase (SGK) and protein kinase C (PKC) activation.

mTORC2 consists of six different proteins, some that are also in mTORC1. These are: mTOR, rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein (mSIN1), protein observed with Rictor-1 (Protor-1), mLST8 and Deptor. Rictor and mSIN1 are responsible for stabilizing each other in establishing a structural foundation for the complex. Deptor, like in mTORC1, is a negative regulator of mTORC2 activity. mLST8 is vital for mTORC2 activity, as knockout of this protein results in reduced stability and activity of the complex. Poctor-1 interacts with Rictor, although its physiological function is unclear.

mTORC2 is responsible for phosphorylating Ser473 of Akt2. mTORC2 indirectly through Akt signaling plays an important role in the pathogenesis of diabetes and cancers.

2.2.3 mTORC1 and Downstream Substrates

As stated earlier, mTORC1 is a regulator of growth and is responsible for numerous cellular processes. mTORC1 mediates these effects by phosphorylating its downstream substrates. The two best characterized downstream effectors of mTORC1 are p70 ribosomal S6 kinase 1 (S6K1, Figure 1) and eukaryotic translation initiation factor (eIF) 4E-binding protein 1 (4E-BP1).
Figure 1: Simplified overview of insulin signaling pathway and proteins involved in glucose transport.

Insulin binds to the insulin receptor, resulting in the activation of the insulin receptor and the tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1). Then IRS-1 interacts with PI3K, activating it. Subsequently, PI3K activation allows for the activation of Akt. Akt activation allows for the translocation of GLUT4 to the plasma membrane, resulting in the uptake of glucose by the cell. Akt also phosphorylates TSC2, removing TSC2’s inhibition on Rheb allowing for the subsequent activation of mTORC1. Further downstream substrates of mTORC1 are activated allowing for anabolic processes like protein synthesis to take place.
2.2.3.1 Ribosomal Protein S6 Kinase-1 (S6K1)

p70 ribosomal S6 kinase 1 (S6K1), as the name suggests, phosphorylates ribosomal protein S6 (rpS6), which is a component of the small ribosomal subunit (40S, figure 1)\(^{94,95}\). It is directly phosphorylated by mTORC1 at the Thr389 residue. It is a serine/threonine kinase a part of the AGC kinase family. It plays an integral role in translation, as it regulates translation initiation by phosphorylating the cap binding complex of eukaryotic initiation factor 4B\(^{165}\). It also phosphorylates programmed cell death 4 (PDCD4), which is a tumor suppressor that negatively regulates eukaryotic initiation factor 4A\(^{165}\). S6K1 is also important in the elongation step of translation, as it inactivates eukaryotic elongation factor-2 kinase (eEF2K), a negative regulator of eukaryotic elongation factor 2 (eEF2)\(^{164}\). S6K1 is evidently very important in the regulation of translation, allowing for protein synthesis\(^{96}\).

The mTORC1/S6K1 pathway has a major role in anabolic processes like protein synthesis, but has recently been linked to insulin resistance. High levels of BCAAs can maintain mTORC1 activity, and thus this mTORC1/S6K1 hyper-activation results in a feedback loop phosphorylating Ser307 of IRS-1. IRS-1\(^{\text{Ser307}}\) phosphorylation reduces Akt activation, attenuating insulin responses, such as glucose uptake and glycogen synthesis (Figure 2)\(^{118}\).

2.3 Upstream Regulators of mTORC1

2.3.1 Growth Factors

As stated earlier, mTORC1 regulates cell growth in response nutrients, mitogens, cellular energy status and various stressors. It senses and then transmits these signals onto downstream substrates. Yoneyama et al. demonstrated that prolonged mTORC1 activation by insulin-like
growth factor in L6 muscle cells resulted in a negative feedback loop phosphorylating the Ser\(^{422}\) residue of IRS-1. Phosphorylation at this site then recruits SCF\(^{β-TRCP}\) E3 ligase complex to degrade IRS-1, which can have major implications on insulin sensitivity\(^{248}\). However, Hoehn et al. suggested that IRS-1 degradation is a consequence of insulin resistance instead of it causing insulin resistance\(^{249}\).

### 2.3.2 Stress

mTORC1 respond to numerous stresses related to energy, nutrient and oxygen levels. In regards to energy levels, when ATP is low due to glucose deprivation, it results in the inhibition of mTORC1. The decrease in ATP levels results in greater AMP/ATP ratio, and thus activation of 5'AMP-activated protein kinase (AMPK). AMPK phosphorylates TSC2, which ultimately results in mTORC1’s suppression. Under low oxygen levels or hypoxia, mTORC1 may be inhibited. Hypoxia reduces ATP levels, and thus inhibits mTORC1, as stated above through AMPK. Hypoxia can also suppress mTORC1 through regulated in development and DNA damage response 1 (REDD1). REDD1 inhibits mTORC1 by controlling the release of TSC2 from 14-3-3, and stabilizing the interaction of TSC1 and TSC2. DNA damage, glucocorticoids and oxidizing agents may also inhibit mTORC1 with the induction of REDD1\(^{117}\).

### 2.3.3 Amino Acids

Amino acids, in addition to being building blocks for proteins, also act as nutrient signals to induce cell growth. Cells can initiate anabolic processes like protein synthesis based on the availability of amino acids. Thus, amino acid sensing is critical for controlling cellular metabolism\(^{97}\).
In response to amino acid levels the Rag GTPases control the localization of mTORC1 to the lysosome. In the presence of nutrients like amino acids, RagA/B is GTP loaded while RagC/D is GDP loaded. These are the active conformations of the Rag GTPases and allows for mTORC1 translocation to the lysosomal surface, where it interacts with its kinase activator, Rheb.

Two important amino acid sensors for mTORC1 include Sestrin1 and Sestrin2, which are both leucine sensors. Sestrins negatively regulate mTORC1 upstream of GATOR2, a positive regulator of mTORC1. When deprived of leucine, Sestrin1 and Sestrin2 binds to GATOR2, resulting in its inhibition. When leucine is available, Sestrin2 is activated and thus disassociates from GATOR2, relieving this inhibitory effect and allowing for the stimulation of mTORC1.

Similar to Sestrin1 and Sestrin2, CASTOR1 and CASTOR2, arginine sensors for mTORC1 behave similarly. Under arginine deprived conditions, the arginine sensors either as a homodimer (CASTOR1) or a heterodimer (CASTOR1 and CASTOR2), bind to GATOR2 to inhibit GATOR2. Thus, similarly to leucine, when arginine is present, this inhibition is lifted and GATOR2 can positively regulate mTORC1.

2.3.3.1 Branched-chain Amino Acids (BCAAs)

There are three branched-chain amino acids (BCAAs) and they are vital in stimulating protein synthesis. These are leucine, isoleucine and valine. Leucine seems to be the most important in stimulating protein synthesis in the skeletal muscle. BCAA-rich diets or BCAA supplementation has numerous positive benefits such as muscle protein synthesis, regulating body weight, reducing protein breakdown and glucose homeostasis. BCAAs, like insulin, act as anabolic signals that affects the growth of energy consuming tissues. Similar to insulin, they regulate growth through the activation of mTORC1. BCAA-rich diets have been proposed for better metabolic health,
although with recent advances in this field, this claim might be controversial as BCAA’s might have implications in the onset of insulin resistance\textsuperscript{5}.

2.3.3.2 BCAA Catabolic Pathway

BCAA metabolism happens in a two-step process. In the first step, the mitochondrial isoform of branched-chain amino acid transaminase, (BCATm) encoded by the BCAT2 gene catalyzes the transamination of the BCAAs into their respective α-keto acids. The α-amino group is transferred onto α-ketoglutarate, and thus leucine is converted to α-ketoisocaproic acid (KIC), isoleucine to 2-keto-3-methylvaleric acid (KMV), and valine to α-ketoisovaleric acid (KIV). These BCAAs are the only amino acids that share a common first step enzyme (BCAT2) in their metabolism. BCAT2 has a preferential binding to the BCAAs, as it prefers isoleucine the most, then leucine and valine.

The second step of BCAA catabolism is catalyzed by the enzyme branched-chain α-keto acid dehydrogenase (BCKDH) complex. This complex consists of three subunits: a branched-chain α-keto acid decarboxylase (E1), a dihydrolipoyl transacylase (E2), and a dihydrolipoyl dehydrogenase (E3). BCKDH catalyzes the oxidative decarboxylation of the branched-chain α-keto acid products formed in the BCAT transamination reaction yielding three CoA derivatives. Isovaleryl-CoA is produced from KIC, isobutyryl-CoA from KIV, and alpha-methylbutyryl-CoA from KMV, with NADH also being produced. After further breakdown acetyl-CoA, succinyl-CoA and propionyl-CoA from leucine, valine and isoleucine respectively can be shuttled and used in the tricarboxylic acid (TCA) cycle. The activity of BCKDH is regulated by BCKDH Kinase (BDK). BDK phosphorylates BCKD at the E1 α subunit at the Ser239 residue to inactivate BCKDH\textsuperscript{5,113,114}. In the presence of BCKDH substrates, the mitochondrial phosphatase 2C
(PP2Cm) binds to the BCKDH complex and induces dephosphorylation, activating the complex. The BCKDH complex activity is downregulated in obese and diabetic animals resulting in increased levels of BCAAs, which is often associated with insulin resistance. This downregulation of BCKDH activity is consistent with Lian et al demonstrating a downregulation in PP2Cm in liver and adipose tissue of diabetic mice.

Leucine is an important BCAA as it can activate mTORC1. There have also been various reports of amino acid metabolites activating mTORC1. Moghei et al. demonstrated that KIC in leucine starvation conditions can activate mTORC1, but this effect was attenuated once BCAT2 was knocked down. In another study with the presence of an amino transaminase inhibitor, KIC could not activate mTORC1, implying that leucine was necessary for mTORC1 activation. This suggested KIC is converted back to leucine to elicit its effects on mTORC1 activation, as the transamination reaction by BCAT2 is reversible.

Another metabolite of leucine that has been extensively studied is beta-hydroxy-beta-methylbutyrate (HMB). HMB is used as a nutritional supplement to aid in sport performance. HMB is known to have an anti-catabolic effect on skeletal muscle. Eley et al. demonstrated how HMB supplementation prevented phosphorylation of kinases that inhibit the elongation step of mRNA translation. HMB also increased mRNA translation. In a study conducted by Pinheiro et al., they demonstrated that HMB supplementation increased maximum strength production in rat skeletal muscle. HMB supplementation also increased ATP content and glycogen content in red and white portions of gastrocnemius muscle of rats. Pimental et al. demonstrated that HMB induced skeletal muscle hypertrophy and that this was through significant increases in mTORC1 activation, with the increased phosphorylation of S6K1 in the extensor digitorum longus muscle. In fact, Giron et al. demonstrated that HMB was more
effective in activating mTORC1 and stimulating protein synthesis than leucine\(^{192}\). In regards to insulin sensitivity, one study demonstrated that HMB supplemented to rats with a high fat diet improved insulin sensitivity\(^{193}\). Sharawy et al. claim that HMB decreases GLUT-2 expression, which leads to less fructose transported into the liver, and thus the potential mechanism in which HMB improves insulin sensitivity\(^{193}\). In another study, metformin, resveratrol and HMB together improved insulin sensitivity. These reports are contradictory considering that HMB increases mTORC1 and increased mTORC1 activity has been correlated with insulin resistance, while Sharawy et al. demonstrated increased insulin sensitivity with HMB\(^{193}\). Since leucine has implications in insulin resistance and it is evident HMB plays a major role in activating mTORC1, further research is required to understand HMB’s effect on insulin sensitivity and how mTORC1 plays a role in this.

Leucine often is the culprit in eliciting insulin resistance in regards to BCAAs, but recently there has been a study looking at the catabolic intermediate of valine, 3-hydroxyisobutyrate (3-HIB) that may play a role. This mechanism differs from the mTORC1 hyperactivation concept. In the context of 3-HIB, it behaves as a peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1\(\alpha\)) paracrine regulator of trans-endothelial FA transport. Jang et al. demonstrated in endothelial cells that 3-HIB increased trans-endothelial FA uptake by the cells from the media\(^{183}\). Knockdown of upstream enzymes preventing 3-HIB formation abrogated this trans-endothelial FA uptake, while knockdown of downstream enzymes resulting in 3-HIB accumulation demonstrated an increase in triglyceride levels\(^{183}\). Treatment of mice for two weeks with 3-HIB resulted in insulin resistance via hyperinsulinemic euglycemic clamp experiments. 3-HIB treatment resulted in an increased lipid signature in the skeletal muscle, activating PKC-\(\theta\), and in turn the phosphorylation of Akt was blunted resulting in
reduced glucose tolerance⁷⁸⁴. Consistent with this study, diabetic subjects have increased serum 3-HIB levels⁷⁸⁴-⁷⁸⁵. Diabetic mice have also displayed a decrease in the downstream enzyme that catabolizes 3-HIB⁷⁸⁶. All of this data emphasizes 3-HIB’s potential role in eliciting insulin resistance and requires further investigating.

**Figure 2: General overview of how inflammation and BCAAs induces insulin resistance.**

TNF-α and homocysteine are pro-inflammatory factors that can activate JNK, which in turn inhibits IRS-1 and its ability to relay the insulin signal downstream. BCAAs activate mTORC1 and S6K1 and sustain this activation, creating a feedback loop to inhibit IRS-1 signaling. Thus, inflammation and BCAAs may have an additive/synergistic effect toward phosphorylating IRS-1 serine residues to prevent tyrosine phosphorylation. This results in a reduced activation of Akt, and reduced glucose uptake by the cell.
2.3.3.3 The link between BCAAs, mTORC1 and Insulin Resistance

Amino acids are important as building blocks for proteins and as stated earlier can be important nutrient signals for cell growth and activating other metabolic processes. BCAAs regulate protein synthesis, and leucine in particular, can activate mTORC1. Despite these benefits, BCAAs have a paradoxical role, as increased levels of BCAAs are present in insulin resistant states like obesity and T2DM\textsuperscript{103-105}. In clinical studies, BCAA levels in the blood positively correlate with insulin resistance\textsuperscript{106,151-153}. BCAA levels have also been predictive of future insulin resistance or T2DM in several studies\textsuperscript{104,107}. These observations pose the question of whether or not the increased levels of BCAAs are correlated with insulin resistance or cause insulin resistance.

Despite reduced insulin sensitivity, mTORC1 is oddly still activated due to excess nutrients. Specifically, the three BCAAs, leucine, isoleucine and valine are potent activators of mTORC1. A rat model demonstrated that high levels of BCAAs maintained mTORC1 activity, resulting in the phosphorylation of IRS-1 serine residues, leading to insulin resistance\textsuperscript{103}.

Similar to BCAAs, their metabolites also have been implicated in the development of insulin resistance. Metabolomic studies show a positive correlation between BCAA metabolite levels in the blood with T2DM and insulin resistance\textsuperscript{103,151,153}. In particular high concentrations of KIC has been associated with insulin resistance in humans\textsuperscript{172} and animals\textsuperscript{173}. It is still unknown if these metabolites cause insulin resistance or if their increased levels just happen to be a symptom of insulin resistance.

Many studies have shown a correlation between both BCAA metabolites and insulin resistance, but very few have looked at the cause and effect of BCAA metabolites. Moghei et al. analyzed the effect of KIC in eliciting insulin resistance. They demonstrated that KIC inhibited insulin-stimulated glucose transport with increased mTORC1 activity\textsuperscript{174}. This inhibition was
abrogated once BCAT2 was knocked down, suggesting that KIC was converted back to leucine to elicit the inhibition in insulin-stimulated glucose transport\textsuperscript{174}.

2.4 Insulin Resistance and T2DM

Type 2 diabetes mellitus (T2DM) is the most common endocrine disorder affecting over 170 million individuals, and this number could rise to 365 million by the year 2030\textsuperscript{108}. T2DM can also lead to more health complications such as stroke, neuropathy, ischemic heart disease, retinopathy and nephropathy\textsuperscript{108}. Type 1 diabetes mellitus (T1DM) differs from T2DM, as T1DM solely involves the autoimmune destruction of beta cells of the pancreas, resulting in a deficiency in insulin production\textsuperscript{110}. On the other hand, the major pathophysiological event that contributes to the pathogenesis of T2DM is the resistance of target tissues, specifically the liver, skeletal muscle and adipose tissue to normal circulating levels of insulin.

Insulin is an important hormone in our body responsible for allowing skeletal muscle and adipose tissue to absorb glucose from our blood, lowering blood glucose levels and preventing hyperglycemia. It also acts to store excess glucose and controls glucose production in the liver\textsuperscript{111}. Impairment in insulin action results in reduced glucose uptake and metabolism by the targeted tissues, thus it is imperative to investigate ways to prevent and manage insulin resistance.

2.5 Causes of Insulin Resistance

2.5.1 Genetics

Genetics play a key role in the pathogenesis of insulin resistance. Mutations in the insulin receptor gene are implicated in several rare forms of insulin resistance. Leprechaunism Rabson-Mendenhall syndrome or the Type-A syndrome of insulin resistance patients usually require at
least hundred-fold more insulin than a typical diabetic patient\textsuperscript{41,42}. Most of these patients have nonsense or missense mutations in either the ligand binding domain or tyrosine kinase domain of the IR\textsuperscript{43,44}. Mutations in IRS-1 also can result in insulin resistance. Studies display an association between a single-nucleotide polymorphism (SNP) in IRS-1(Gly972Arg) and T2DM\textsuperscript{45,46}. A T608R missense mutation in IRS-1 resulted in decreased insulin signaling, but this appears to be very rare\textsuperscript{47}.

Mutations in Akt2 has also been exhibited in patients with diabetes. A rare missense mutation (R274H) in Akt2 leads to a loss of kinase activity\textsuperscript{48}. Tribbles homolog 3 (TRIB3) gene located on chromosome 20p13 has been implicated with insulin resistance. Specifically, a Q84R polymorphism in TRIB3 gene has been associated with insulin resistance and decreased insulin-stimulated Akt phosphorylation\textsuperscript{49,50}. A mutation in AS160 at position 363 results in a premature stop codon, which was identified in a patient with severe postprandial hyperinsulinemia, thus reducing glucose transport\textsuperscript{51}. Thus, mutations in relevant genes is one of the many ways in which insulin resistance can arise.

### 2.5.2 Lack of Physical Activity and Obesity

Obesity has been linked to T2DM for decades, as a common feature of both is insulin resistance. One proposed mechanism observed in obesity is the upregulation of protein tyrosine phosphatases, dephosphorylating tyrosine residues on IRS-1 and terminating insulin signaling. Also, in both obesity and T2DM there is a reduction in GLUT4 translocation\textsuperscript{138}.

Physical activity is a preventative strategy employed for both obesity and T2DM. There has also been a link between physical inactivity and the onset of diabetes. Physical activity can decrease the risk of T2DM by increasing insulin sensitivity. Evidence suggests that exercise allows
for greater glucose uptake by the skeletal muscles by increased GLUT4 translocation to the membrane. In this case exercise acts similarly to insulin to induce GLUT4 translocation\textsuperscript{165-168}. Thus, exercise and insulin can have an additive effect on GLUT4 translocation, resulting in greater glucose uptake\textsuperscript{139,169-171}.

Intracellular signaling mechanisms differ between insulin and exercise. As stated before, insulin through a series of steps stimulates PI3K and then Akt for GLUT4 translocation. In exercise it differs, as using a PI3K inhibitor, wortmannin, did not inhibit exercise-induced glucose uptake\textsuperscript{140-142}. A single bout of exercise can increase whole body glucose disposal. Not only does exercise increase translocation of this GLUT4, but consistent exercise results in greater GLUT4 expression\textsuperscript{143-145}.

In exercise, increased contraction-stimulated glucose uptake is linked to increases in AMPK phosphorylation. AMPK phosphorylates TBC1D1 resulting in its deactivation, which allows for GTP to react with Rab proteins on the GLUT4 vesicles, thus allowing for greater GLUT4 translocation and glucose uptake into the cell\textsuperscript{252}.

Epidemiological studies have emphasized these findings, as physical inactivity may be a risk factor for T2DM. In a study, exercise training significantly increased skeletal muscle GLUT4 protein by 23\% in men with T2DM and by 39\% in nondiabetic men\textsuperscript{145}. This emphasizes the importance of physical activity in managing insulin resistant states like obesity and T2DM.

2.5.3 Nutrition

Nutrition can play a major role in the onset of insulin resistance. Chronic overconsumption of energy, especially in the absence of adequate physical activity, leads to weight gain and excess abdominal fat, which leads to insulin resistance and development of T2DM\textsuperscript{146}. An appropriate diet
can be a preventative strategy or treatment for insulin resistance or T2DM. High lipid, protein and carbohydrate diets have been studied for their implications in insulin resistance.

2.5.3.1 High Lipid Intake

High fat diets have been one of the major contributors to obesity and insulin resistance. There is a link between high fat diets and obesity in vivo. Epidemiological studies also mirror what is demonstrated in vivo\textsuperscript{221-224}. Most cross-sectional studies display a positive correlation between dietary fat intake and obesity\textsuperscript{225-227}. This is further emphasized when formerly obese individuals prevent relapse with a lower fat intake and maintain their weight more effectively\textsuperscript{120}.

Insulin-stimulated glucose uptake by the skeletal muscle depends on the translocation of the GLUT4 transporter to the membrane. Zierath et al. found that insulin resistance, induced by high fat intake, resulted in a decrease in the translocation of the GLUT4 transporter in muscle of mice\textsuperscript{121}. Studies have demonstrated that the ability for GLUT4 to shuttle glucose into the cell is sensitive to changes in the membrane lipid bilayer\textsuperscript{122-123}. High fat feeding and obesity can affect the composition and structure of this membrane, resulting in a decrease in GLUT4 activity\textsuperscript{124-125}. Rosholt et al. demonstrated reduced GLUT4 activity in skeletal muscle of rat as a result of a high fat diet\textsuperscript{126}.

Increased fatty acid concentrations in the blood are often associated with insulin resistant states such as obesity and T2DM\textsuperscript{127-130}, even in children insulin resistance correlated with higher free fatty acid concentrations in the blood\textsuperscript{253}. A cross-sectional study also showed the inverse relationship between fatty acid plasma levels and insulin sensitivity\textsuperscript{131}. Randle et al. demonstrated how free fatty acids compete with glucose for substrate oxidation, thus they speculated that this increase in fatty acid oxidation was linked to insulin resistance. The proposed mechanism was that
increased levels of fatty acids resulted in increased levels of intra-mitochondrial acetyl CoA/CoA and NADH/NAD+ ratios, and thus inactivation of pyruvate dehydrogenase. This leads to increases in citrate levels, leading to phosphofructokinase inhibition, which is an important rate limiting step in glycolysis. This leads to an accumulation in glucose-6-phosphate, which inhibits hexokinase II activity, resulting in an increase in glucose concentrations in the cell, leading to reduced glucose uptake\textsuperscript{132-134}.

High fatty acid levels in the blood also resulted in a decrease in insulin-stimulated IRS-1 tyrosine phosphorylation in skeletal muscle\textsuperscript{136}. Elevations in fatty acids resulted in a decrease in IRS-1 associated PI3K activation\textsuperscript{135}. Fatty acid metabolites also affect insulin signaling. Metabolites like diacylglycerol (DAG), fatty acyl CoA, and ceramides activate serine/threonine kinase cascades, which leads to phosphorylation of serine/threonine residues of IRSs. This leads to impaired insulin signaling, as PI3K is not activated, and downstream events like glucose transport cannot take place\textsuperscript{137}. DAG can also activate protein kinase Ce, which impairs auto-phosphorylation of Thr\textsuperscript{960} of the insulin receptor and thus IRS-1 tyrosine phosphorylation, by phosphorylating the Thr\textsuperscript{1160} site in the insulin receptor activation loop, specifically in the liver\textsuperscript{235}.

2.5.3.2 High Protein Intake

High protein diets have often been recommended to combat obesity\textsuperscript{150}. High protein diets have been associated with increased insulin sensitivity. Dietary proteins have an insulinotropic effect, thus promoting insulin release, and glucose uptake by the tissues\textsuperscript{119}.

Specifically, BCAAs have been often recommended for weight management. BCAAs act as anabolic signals to activate protein synthesis, preventing loss of muscle mass in weight management programs. BCAAs, particularly leucine, activates mTORC1. mTORC1 then activates
its downstream substrates Ribosomal Protein S6 Kinase-1 (S6K1) and Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) allowing for protein synthesis to occur\textsuperscript{93}.

Despite these positive benefits, high protein intake for longer periods of time have been associated with the development of insulin resistance. High levels of BCAAs are present in insulin resistance and T2DM\textsuperscript{103}. Despite positive anti-obesity effects and the stimulation of protein synthesis, high levels of BCAAs can result in insulin resistance through the sustained activation of the mTORC1-S6K1 pathway, as stated previously. Persistent activation of this pathway results in a feedback loop, which phosphorylates IRS-1 at its serine residues such as Ser307, Ser612, Ser1101 (figure 2). This results in reduced activation of the tyrosine residues of IRS-1, and consequently the signal cannot be transmitted downstream, which would prevent Akt activation. With the lack of Akt activation, important processes like glucose uptake and glycogen synthesis cannot take place\textsuperscript{118}.

\textbf{2.5.3.3 High Carbohydrate Intake}

With high fat diets being the main focus of diet induced insulin resistance, studies about carbohydrate diets and implications on diseases and mortality have gone under the radar. In a recent study, high carbohydrate diet had a positive correlation with total mortality, and non-cardiovascular disease mortality\textsuperscript{154}. Another study in rats demonstrated that high fructose and sucrose (simple carbohydrates) intake produced a decline in insulin sensitivity in liver and peripheral tissues\textsuperscript{155}. Although when this is translated to human studies, there is no consistent data suggesting these simple sugars reduce insulin sensitivity. When looking at the role of complex carbohydrates and their roles on insulin sensitivity, again there is some inconsistencies. High amylose diets increased insulin sensitivity, while high amylopectin diets display the opposite\textsuperscript{155}.
In another study Pérez-Jiménez et al. looked at the effect of a high carbohydrate diet in comparison to high saturated fat diet, which is known for eliciting insulin resistance. They demonstrated significantly reduced plasma insulin levels and steady state plasma glucose levels with the high carbohydrate group compared to the high saturated fat diet^{157}.

2.6 Molecular Mechanisms of Insulin Resistance

2.6.1 Chronic Inflammation

Inflammation is a key factor in the pathogenesis of obesity-associated insulin resistance^{52}. Both adipocytes and macrophages secrete pro-inflammatory cytokines and induce insulin resistance. Increased secretion of the chemokine monocyte chemoattractant protein-1 (MCP-1) by adipocytes, results in macrophage accumulation into adipose tissues and induce insulin resistance^{53}. This is evident, as the deletion of MCP-1 or its receptor CCR2 improves insulin sensitivity and actually alleviates inflammation in mice^{54,55}.

Homocysteine is a pro-inflammatory factor that promotes inflammation in both in vitro and in vivo^{213-214}. Li et al. demonstrated that homocysteine impaired glucose uptake in rat adipocytes and rats^{215}. Other cytokines released in obesity that induce insulin resistance include tumor necrosis factor α (TNF-α), interleukin-1β (IL-1b) and interleukin-6 (IL-6). Upregulation of TNF-α for example results in c-Jun N-terminal kinases (JNK) to be activated, which in turn can interact with the Ser^{307} residue on IRS-1 and thus down regulate IRS-1 tyrosine phosphorylation to hinder downstream signaling^{56}. JNK’s importance in mediating insulin resistance is evident, as knockdown of JNK protects against obesity-induced insulin resistance^{247}.

TNF-α role in insulin sensitivity was emphasized when Li et al. demonstrated that TNF-α inhibition in 3T3-L1 adipocytes partially improved insulin-stimulated glucose uptake that was
originally suppressed with TNF-α. The inhibitions were mediated by inhibiting proteins involved in the inflammatory pathways such as JNK and Inhibitor kappa B (IκB) kinase (IKK)-nuclear factor κB (Nf-κB)\textsuperscript{254}. This emphasizes the link between these inflammatory pathways and insulin sensitivity.

Another factor in activating inflammation in obesity include the activation of Toll-like receptors (TLRs) especially activation of TLR-2 and TLR-4. TLRs, especially TLR-4 are activated by fatty acids and endotoxinemia, which are both features of obesity\textsuperscript{216}. They both induce inflammation by activating the NF-κB pathway\textsuperscript{57}. Mice with decreased TLR-2 and TLR-4 signaling proteins are protected from diet-induced obesity and insulin resistance\textsuperscript{58-60}.

### 2.6.2 Accumulation of Lipid Intermediate

Accumulation of lipids especially fatty acids are believed to cause insulin resistance through multiple mechanisms. This accumulation of lipids in non-adipose tissues like skeletal muscle can result in lipotoxicity. Increased hydrolysis of circulating triglycerides can lead to skeletal muscle insulin resistance\textsuperscript{61}. Elevated circulating levels of free fatty acids are observed in obesity and induce activation of JNK, IκB kinase (IKK) and protein kinase C (PKC), which may lead to the phosphorylation of IRS-1 at the ser\textsuperscript{307} residue\textsuperscript{62}. Palmitate specifically promotes insulin resistance by stimulating cytokine production, JNK activation and endoplasmic reticulum (ER) stress\textsuperscript{58,63}. Palmitate also activates NF-κB, and studies inhibiting this pathway reverse lipid-induced insulin resistance\textsuperscript{64,65}.

Lipid metabolite DAG has shown a key role in the induction of insulin resistance, as increased levels results in muscle insulin resistance by activating PKC-θ and inducing IRS-1\textsuperscript{Ser307}.
phosphorylation. DAG’s role is evident as studies have demonstrated that reducing DAG levels in skeletal muscle protects mice from high fat diet induced insulin resistance.

Ceramides, a type of lipid molecule, also have been shown to induce insulin resistance via PKC and JNK activation. This happens with the activation PKC-ζ, which phosphorylates the thr residue of the Pleckstrin Homology (PH) domain of Akt. This leads to a loss of PIP binding to the PH domain, and thus the inhibition of Akt. Thus, inhibiting ceramide synthesis ameliorates insulin resistance. Huang et al. analyzed the correlation between ceramides and insulin sensitivity. In C2C12 myotubes they demonstrated that dysregulations in lipid metabolism that caused an increase in ceramide production, reduced Akt activation and glucose uptake. When inhibiting ceramide production by inhibiting ceramide synthase, these reductions in Akt and glucose uptake were attenuated, emphasizing the link between ceramide accumulation and insulin resistance.

2.6.3 Oxidative Stress

Low levels of reactive oxygen species (ROS) can enhance insulin action, conversely a high concentration of ROS results in oxidative stress. ROS is often produced due to mitochondrial dysfunction and is a by-product of the electron transport chain. Increased ROS levels have been observed in obese and diabetic states in adipose tissue and in muscle. This increased oxidative stress leads to the activation of stress kinases, which phosphorylate serine residues of IRS proteins, ultimately resulting in insulin resistance.
2.6.4 Hyperglycemia

Hyperglycemia is when glucose concentrations in the blood are abnormally high. This can alter insulin sensitivity in muscle and increase insulin secretion from beta cells\textsuperscript{80,81}. Hyperglycemia can activate JNK and cause the addition of an N-acetylglucosamine (O-GlcNAcylation) to IRS-1. This O-GlcNAcylation to IRS-1 downregulates tyrosine phosphorylation\textsuperscript{82-85}. Hyperglycemia also activates the PKC pathway by inducing synthesis of DAG\textsuperscript{86} and causes insulin resistance by increasing IRS-1\textsuperscript{ser307} phosphorylation\textsuperscript{87,88}. 

2.7 Rationale

Recent advances in research demonstrate the potential paradoxical effects of BCAAs. The effects of high protein/BCAA diets remain inconclusive in regard to insulin resistance and mTORC1 activation. Despite the beneficial roles of BCAAs, including increased protein synthesis, and regulating body weight, BCAAs and their metabolites have been implicated in the development of insulin resistance and T2DM\textsuperscript{103-107}. From previous work in my lab, KIC, the metabolite for leucine inhibited insulin-stimulated glucose uptake under normal conditions. When BCAT2 was knocked down, this suppression was attenuated.

Thus, I analyzed KIC in the context of inflammation, as inflammatory factors released in conditions like insulin resistance and obesity may modulate KIC-induced insulin resistance. Furthermore, I confirmed whether or not KIC elicits these effects on insulin-stimulated glucose uptake and insulin signaling or if it is converted back to leucine through BCAT2 to elicit these effects. This helps to determine the role of BCAA metabolism in the pathogenesis of insulin resistance and if interventions affecting BCAA metabolism can help in the management of insulin resistance in type 2 diabetes and cardiovascular diseases.
2.8 Objectives

1) Examine the effect of inflammation on insulin-stimulated glucose uptake and its effects on insulin signaling in L6 rat skeletal muscle cells.

2) Examine whether inflammation modifies the effect of KIC on insulin-stimulated glucose uptake and how it affects insulin signaling.

3) Examine whether the effect of KIC and inflammation on insulin-stimulated glucose uptake and insulin signaling are independent of BCAT2.

2.9 Hypothesis

I hypothesized that KIC treatment in an inflammatory environment results in an additive suppression in insulin-stimulated glucose uptake and insulin signaling in skeletal muscle. I also hypothesized that KIC elicits its effects on insulin-stimulated glucose uptake and insulin signaling due to its intracellular conversion back to leucine, even in the presence of inflammation.
Effects of Inflammation and Ketoisocaproic Acid on Glucose Metabolism in Muscle Cells

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Key words: Insulin resistance, skeletal muscle, glucose transport, leucine, KIC, mTORC1, inflammation, glycogen synthesis
Abstract

Despite the anabolic benefits of branched-chain amino acids (BCAAs) and their metabolites, they have also been implicated in the suppression of insulin sensitivity in skeletal muscle. In our lab we have shown that leucine and its metabolite ketoisocaproic acid (KIC) inhibit insulin-stimulated glucose uptake. The knockdown of branched-chain aminotransferase 2 (BCAT2) attenuated this suppression. In this current study I analyzed the effect of inflammation, a key feature of insulin resistance and how it may influence KIC’s effect on insulin-stimulated glucose uptake. Incubating cells in KIC did not significantly suppress insulin-stimulated glucose uptake. Co-incubation of L6 myotubes with pro-inflammatory factors homocysteine, interleukin-6 and tumor necrosis factor-α with KIC showed a significant suppression of insulin-stimulated glucose uptake (p<0.05). Once again, BCAT2 knockdown attenuated the effect of KIC, even in the presence of inflammation. These results suggest that KIC is converted back to leucine to elicit its effects on insulin-stimulated glucose uptake.

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder and its prevalence is growing. The main underlying cause of T2DM is insulin resistance. Insulin resistance is when the tissues response to insulin is reduced. Many factors such as nutrition and lifestyle choices can give rise to or worsen insulin resistance.

This study focuses on the role of nutrition and insulin resistance, specifically, the main constituents of high protein diets that have been extensively researched, branched chain amino acids (BCAAs). BCAAs, especially leucine, is known for activating protein synthesis with the activation of mammalian target of rapamycin (mTORC1) signaling. Despite this anabolic benefit,
circulating BCAA levels have been positively correlated with insulin resistance\textsuperscript{106,151-153}. It has been suggested that leucine maintains mTORC1 activation, creating a feedback loop, which phosphorylates the serine residues of insulin substrate receptor-1 (IRS-1), and thus inhibiting insulin signaling downstream\textsuperscript{5}.

Hernandez et al. demonstrated that BCAA catabolic enzyme mRNA are downregulated in individuals with T2DM compared to healthy individuals\textsuperscript{232}. He also showed that BCAAs and their metabolites were upregulated in individuals with T2DM\textsuperscript{232}. Thus, the question remains, do BCAAs cause insulin resistance or are increased BCAA levels a consequence of insulin resistance and T2DM.

Previously, Moghei et al. demonstrated that leucine inhibited insulin-stimulated glucose uptake\textsuperscript{174}. They also demonstrated that ketoisocaproic acid (KIC), the metabolite of leucine, suppressed insulin-stimulated glucose uptake as well\textsuperscript{174}. When branched-chain aminotransferase 2 (BCAT2), the enzyme responsible for the reversible catabolism of leucine to KIC was knocked down, KIC’s suppressive effect was attenuated. This suggests KIC is converted back to leucine to elicit these effects\textsuperscript{174}. Similar to previous reports, Moghei et al. showed that mTORC1 is upregulated with leucine and this could be the mechanism in which glucose uptake is suppressed.

Thus, we built off the previous work of Moghei et al. and examined the potential additive effect of KIC and inflammation on insulin-stimulated glucose uptake. This was necessary as inflammation is a feature of both obesity and insulin resistance\textsuperscript{52} that may modulate KIC’s effect on insulin signaling. Ultimately, confirming the role of BCAA metabolites on insulin sensitivity in the context of inflammation, can help elucidate potential therapeutic strategies in treating/managing insulin resistance.
Methods and Materials

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 agents (Wisent #450-115-EL). Phosphate Buffered Saline (PBS, #311-010-CL) and Trypsin (#325-043-CL) 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 (#R8999-12). Tumor Necrosis Factor α was purchased from Shendoah Biotechnology (#300-18) and Sodium 4-methyl-2-oxovalerate (KIC) (#K0629) was purchased from Sigma Aldrich. Homocysteine was purchased from Sigma Aldrich (#69453). BCAT2 (B7312) and scramble (negative control) siRNA (#SIC001) oligosaccharides were purchased from Sigma-Aldrich. Glycogen carrier was purchased from Sigma Aldrich (#G8751-5G). 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). [3H]-2-deoxyglucose (NET549) and [U-14C]-D-glucose (NEC042X) was purchased from Perkin Elmer, Massachusetts.
Cell Culture

L6 rat skeletal muscle myoblasts were purchased from American Type Culture Collection. Cells were cultured in 10 cm plates with growth medium comprised of α-MEM supplemented with 10% FBS and 1% Antibiotic-Antimycotic agents and grown at 37°C and 5% CO² in a cell culture incubator until they were approximately 70-80% confluent. Next, cells were counted and seeded with 2x10⁵ cells/well in 6-well plates for western blot experiments or 10⁵ cells/well for 12-well plates for glucose transport experiments. Cells were allowed to proliferate for 48 hours or until they became 90-100% confluent to be switched into DM. Cells were replenished with fresh DM every 24-48 hours and cells were allowed to differentiate into myotubes until D5 or D6 when the experiments were performed (Description below).

siRNA Gene Silencing

L6 myoblasts were plated in 6-well plates (for western blotting) and 12 well plates (for glucose uptake assay) at a density of 2x10⁵ cells/well and 1x10⁵ cells/well respectively. After 48 hours, medium was shifted to DM. On day 3 of differentiation, myotubes were transfected with 10 µM of scramble siRNA (negative control) or 10 µM BCAT2 siRNA using Lipofectamine RNAiMAX reagent according to the manufacturer’s instructions (Life technologies). Lipofectamine RNAiMAX reagent was diluted in Opti-MEM medium. Scramble siRNA and the BCAT2 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, for 6 well plates, 250 µL of the siRNA-lipid complex was added to wells containing 1mL of antibiotic-free α-MEM containing 2% HS. For 12 well plates, 125 µL of the siRNA-lipid
complex was added to wells containing 0.5 mL of antibiotic-free α-MEM with 2% HS. Twenty-four hours following transfection, 1mL of α-MEM containing 2% HS, 1% Ab-Am, and the pro-inflammatory factors (homocysteine, TNF-α and IL-6) were added to each well of the 6 well plates, and 0.5mL of α-MEM containing 2% HS, 1% Ab-Am, and the pro-inflammatory factors was added to the wells of the 12 well plates. On day 5 of differentiation (48 hours following transfection) the media was replenished with differentiation media containing pro-inflammatory factors. On day 6 of differentiation 6 well plates were harvested for insulin signaling and to test the efficiency of the BCAT2 knockdown using immunoblot analysis (refer to section 4.6). The 12 well plates were used for glucose transport assay (refer to section 4.5)

**Inflammation and KIC Supplementation**

**Homocysteine Experiments**

Homocysteine experiments were done to test the effect of homocysteine a pro-inflammatory factor on insulin-stimulated glucose uptake. On D5 of differentiation, fully differentiated myotubes were incubated in various concentrations (10, 15, 20, 30, 40, 50, 100, 200, 500, 1000 μM) of homocysteine in DM for 24 hours. Normal physiological levels of homocysteine are usually around 5-15 μM, but intermediately elevated homocysteine levels are between 31-100 μM, while excess of 100 μM is severely elevated levels of homocysteine. Homocysteine levels elevated above 15 μM is characterized as hyperhomocysteinemia. Following this incubation period, on D6 myotubes cells were starved for four hours with RPMI (complete starvation medium, free of amino acids and serum). After starvation cells were incubated with the various concentrations of homocysteine and 100nM of insulin for 20 minutes. Cells were then subjected to a glucose uptake assay (refer to section 4.5) and harvested for western blot analysis (refer to section 4.6).
**KIC and Homocysteine Experiment**

KIC and homocysteine were incubated together to analyze the effect of inflammation on KIC-induced insulin resistance and how inflammation may modulate amino acid signaling. On D5 of differentiation fully differentiated myotubes were incubated in homocysteine in DM at various concentrations (10, 15, 50, and 500 μM) of homocysteine for 24 hours. Following this incubation period, on D6, myotubes cells were starved for four hours with RPMI (complete starvation medium, free of amino acids and serum). After starvation, cells were incubated with various concentrations of homocysteine stated previously with or without 200 μM KIC for 30 minutes. Then following these 30 minutes, myotubes were incubated with homocysteine with or without 200 μM of KIC and with or without 100 nM of insulin for 20 minutes (Appendix A). Cells were then subjected to a glucose uptake assay (refer to section 4.5) and harvested for western blot analysis (refer to section 4.6).

**TNF-α Test Experiments**

The effect of TNF-α on insulin-stimulated glucose uptake and JNK activation was tested to produce a more robust inflammatory response. On D5 of differentiation, fully differentiated myotubes were incubated in TNF-α in DM at various concentrations (2, 5, 10ng/ml) for 24 hours. For TNF-α, I used values consistent in the literature indicative of inflammatory states218-220. Following this incubation period on D6 myotubes cells were starved for four hours with TNF-α in RPMI (complete starvation medium, free of amino acids and serum). After starvation, cells were incubated with various concentrations of TNF-α stated previously with or without 100 nM of insulin for 20 minutes. Cells were then subjected to a glucose uptake assay (refer to section 4.5) and harvested for western blot analysis (refer to section 4.6).
**Pro-Inflammatory Factors and KIC Experiments**

To ensure a robust inflammation response was produced, KIC was incubated with homocysteine, TNF-α and IL-6 (pro-inflammatory factors) to analyze the effect of KIC and inflammation on insulin-stimulated glucose uptake and insulin signaling. On D4 of differentiation, fully differentiated myotubes were incubated in DM with 5 or 10ng/ml of TNF-α, 10ng/ml of IL-6 and 50μM of homocysteine for 48 hours. This media with the pro-inflammatory factors was replenished 24 hours later. Following this incubation period, on D6, myotubes were starved for four hours with pro-inflammatory factors in RPMI (complete starvation medium, free of amino acids and serum). After starvation, cells were incubated with the pro-inflammatory factors stated previously with or without 200 μM of KIC for 30 minutes. Then they were incubated with or without pro-inflammatory factors, with or without 200 μM KIC and with or without 100 nM of insulin for 20 minutes (Appendix B). Cells were then subjected to a glucose uptake assay (refer to section 4.5) and harvested for western blot analysis (refer to section 4.6).

**BCAT2 Knockdown with Pro-Inflammatory Factors and KIC Experiments**

On D3 of differentiation BCAT2 was knocked down as explained in section 4.3. On D4 of differentiation, fully differentiated myotubes were incubated in DM with 5 or 10ng/ml of TNF-α, 10ng/ml of IL-6 and 50μM for 48 hours. This media with the pro-inflammatory factors was replenished 24 hours later. Following this incubation period, on D6, myotubes were starved for four hours with RPMI (complete starvation medium, free of amino acids and serum). After starvation, cells were incubated with the pro-inflammatory factors stated previously with or without 200 μM of KIC and with or without 100 nM of insulin for 20 minutes. Cells were then
subjected to a glucose uptake assay (refer to section 4.5) and harvested for western blot analysis (refer to section 4.6).

**Glucose Transport Assay**

Glucose uptake assay was performed using radiolabeled 2-deoxyglucose (2-DG). 2-DG was used instead of glucose, as 2-DG does not undergo glycolysis in the cell, so it can accumulate and be measured accurately\(^{255}\). 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. Radiolabeled 2-DG ([\(^3\)H]-2-deoxyglucose) is employed as the tracer to 2-DG in the transport solution, which allows for the entry of the radiolabeled 2-DG into the cells along with unlabelled 2-DG glucose. Therefore, level of glucose uptake is measured by determining the amount of radioactivity present in the cell. Following treatments, cells were then rinsed twice with 37.1°C HEPES (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid) buffered saline. 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 37.1°C. Following the 5-minute incubation period, cells were placed on ice where 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 stop any glucose uptake. Next, 1mL of ice-cold 0.05M NaOH was added to each well and the cells were scraped and collected. For analysis, 200 μL was collected for a protein assay. These samples were stored at -20°C for protein assays. The remaining 800 μL was added to 3.5 mL of Scintillation fluid (Ecolite+, MP Biomedicals #01882475) in liquid scintillation vials. The amount of radioactivity in each vial was counted using a Liquid Scintillation Counter (Tri-Carb Liquid
Scintillation Counter). Rate of glucose uptake in figures 3a, 4, 7, 8, and 9a was expressed as picomole per μg of protein per 5 minutes.

**Cell Harvesting for Western Blot Analysis**

Following the treatments, cells were rinsed with 1mL of 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 cocktail (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 with the use of a 1mL syringe the lysate was transferred from the 6 wells into 1.5 mL Eppendorf tubes. Repeated collection and expulsion of the lysate was used to ensure breakdown of the cell lysate. Lysates were stored at -20°C for further analysis.

**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 acquire an absorbance reading of each well at a wavelength of 550 nanometers. A standard curve was used to estimate the volume needed 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 for figures 3b, 5a-c, and 6. For figures 9b, 10a-c, 11, and 13 protein assays were not completed, so equal amounts of protein were not loaded into the wells of the gel. The proteins were separated on 10% or 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Following gel electrophoresis, they were transferred onto polyvinylidene difluoride (PVDF) 0.2um pore sized membranes. Transfer efficiency was checked with a ponceau dye treatment to the membranes ensuring the protein was transferred. This dye was
then washed off with 3 5-minute washes of Tris Buffered Saline with Tween (TBST). Next, membranes were incubated for one hour in 5% non-fat milk in TBST at room temperature to block non-specific antigen binding. Subsequently, they were quickly rinsed 3 times, 5 minutes each with TBST at room temperature and then incubated overnight at 4°C with the primary antibody of interest.

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Following the overnight incubation in primary antibody, membranes were quickly rinsed, and then rinsed 3 times for 5 minutes each with TBST and were incubated in a secondary antibody for three hours at room temperature. Secondary antibodies were diluted into a 5% milk with TBST solution before incubation with the membranes.

**Secondary Antibodies:** Anti-rabbit (CST # 7074) or Anti-mouse (CST #7076) antibodies were used with the dilution of 1:10000. Subsequently, membranes were rinsed 3 times for 5 minutes each with TBST before HRP chemical luminescent substrate (Millipore Sigma #WBKLS0500) was applied to them. BioRAD ChemiDoc XRS+ was used for signal visualization and the images were quantified with Image Lab software (version 7).
Graphical Representations of Glucose Transport, Glycogen Synthesis and Western Blots

Glucose transport data was expressed by dividing the amount of $[^3]H$-2-deoxyglucose (pmol) transported into cells each well by the concentration of protein found in each well ($\mu$g). This value is expressed as the rate of glucose uptake. The glucose transport value (pmol/$\mu$g), was the rate of glucose uptake per 5 minutes. Rate of glycogen synthesis is measured by CPM per $\mu$g of protein per one hour. Western blots for ph-S6, ph-S6K1, BCAT2, ph-glycogen synthase, ph-JNK and ph-Akt were adjusted by the $\gamma$-tubulin values, and these values were used in the western blot expression figures. These values were not normalized. Total proteins were not used, as I did not get a satisfactory level of stripping. It is important to note that total protein levels may not change under acute supplementation with KIC, but may with inflammation for 24 or 48 hours, which may affect the results of the western blotting.

Glycogen Synthesis Assay

Cells were seeded in a 6 well and treated like in section 4.4.5. Due to excessive cell death, and to ensure enough myotubes to carry out glycogen synthesis, 5.5mM of D-glucose was added to RPMI for starvation media, and cells were starved two hours at 37.1°C. This starvation media was aspirated, and respective wells were supplemented with non-labelled D-glucose (5.5mM) and 0.2 $\mu$Ci/ml of [U-$^{14}$C]-D-glucose, with or with insulin (100nM), with or without KIC (200$\mu$M), and with or without the pro-inflammatory factors as explained in 4.4.5 for one hour. Then 200 $\mu$l of the media with respective conditions in the well was collected and put into scintillation vials containing 3.5ml of scintillation fluid to count total radioactivity. The rest of
the media in the well was aspirated, and glycogen synthesis reaction was stopped by putting plates onto ice and washing wells twice with ice cold PBS. Wells were then treated with 450 μl of KOH (1M) and put on a shaker for 15 minutes to lyse the cells. Lysates were then transferred from wells into Eppendorf tubes and heated at approximately 65°C for 5 minutes. 50 μl of each sample was transferred into a different Eppendorf tube for protein measurement via BCA protein assay as explained in section 4.7. Next 100 μl of glycogen carrier, 80 μl of saturated sodium sulfate, and 1.2 ml of of ice cold ethanol was added to each sample. The tubes were then vortexed and was stored at -20°C overnight for precipitation. The next day samples were centrifuged at 10000 rpm for 20 minutes at room temperature forming a pellet. The supernatant was discarded, and the pellet was dissolved in 500 μl of double distilled water. 450 μl of this was transferred to scintillation vials filled with 3.5ml of scintillation fluid for radioactivity counting.

**Statistical Analysis**

Statistical analyses were performed using GraphPad Prism 7 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. Conditions were not normalized; absolute values were used. For glucose uptake assays, rate of glucose uptake is expressed as pmole of glucose per μg of protein per 5 minutes. For western blot data, each condition is represented as the respective protein or its phosphorylation state over the loading control, γ-tubulin. Significance was determined as p <0.05. Bars with * denote significant differences.
Results

Effect of Homocysteine and KIC on Insulin-Stimulated Glucose Uptake and Insulin Signaling

Homocysteine did not have a significant effect on insulin-stimulated glucose uptake (Fig 3a). Homocysteine did not have an effect on insulin action, as there was no significant effect on Akt phosphorylation (Fig 3b, Supplementary Fig S1A, S1C, S1E). KIC significantly suppressed insulin-stimulated glucose uptake, but co-incubation of homocysteine and KIC did not further suppress insulin-stimulated glucose uptake (Fig 4). Also, KIC with or without homocysteine did not have a significant effect on Akt phosphorylation (Fig 5a, Supplementary Fig S2A). KIC and homocysteine did not exert any significant effect on mTORC1 activation as determined by S6K1 phosphorylation (Fig 5b, Supplementary Fig S3A). KIC had a significant suppression on the phosphorylation of S6 (p <0.05, Fig 5c, Supplementary Fig S3B), which suggests a suppression in protein synthesis. There was also a significant suppression of S6 phosphorylation when KIC was co-incubated with 15 µM and 50 µM of homocysteine (p <0.05, Fig 5c, Supplementary Fig S3B).

Effect of TNF-α on Insulin-Stimulated Glucose Uptake and JNK Phosphorylation

Exposure of L6 myotubes to TNF-α did not affect either insulin-stimulated phosphorylation of the isoform p46 of JNK (Fig 6, Supplementary Fig S4A) or insulin-stimulated glucose uptake (Fig 7).
Effect of Homocysteine, IL-6, TNF-α and KIC on Insulin-Stimulated Glucose Uptake and Insulin Signaling

KIC did not have a significant effect on insulin-stimulated glucose uptake (Fig 8); however, when KIC was co-incubated with homocysteine (50µM), IL-6 (10ng/ml), and TNF-α (10ng/ml), a significant effect on insulin-stimulated glucose uptake was observed (Fig 8). This suggests that inflammation may worsen the suppressive effect of KIC on insulin-stimulated glucose uptake.

The Effect of Homocysteine, IL-6, TNF-α and KIC on Insulin-Stimulated Glucose Uptake, Insulin Signaling and JNK phosphorylation in the Presence and Absence of BCAT2

There was a 64% reduction in insulin-stimulated glucose uptake when myotubes were co-incubated with KIC, homocysteine, IL-6 and TNF-α in the scrambled (SCR) group (p < 0.05, Fig 9a). When BCAT2 was knocked down, no significant effect of KIC and the pro-inflammatory factors was detected (Fig 9a). BCAT2 knockdown resulted in a 39% decrease in BCAT2 expression comparing the average SCR and BCAT2 conditions, although this was not statistically significant (Fig 9b, Supplementary Fig S5A). This suggests the possibility that KIC is converted back to leucine, even in the context of inflammation to elicit its effects in suppressing insulin-stimulated glucose uptake.

In regards to insulin signaling, there was no significant effect of KIC, homocysteine, IL-6 and TNF-α on Akt phosphorylation in the SCR or BCAT2 knockdown conditions (Fig 10a, Supplementary Fig S6A). Similarly, there was no significant effect of KIC, homocysteine, IL-6 and TNF-α on both S6K1 (Fig 10b, Supplementary Fig S7A) and S6 phosphorylation (Fig 10c, Supplementary Fig S6B) in the SCR or BCAT2 knockdown conditions.
There was no significant effect of KIC, homocysteine, IL-6 and TNF-α on JNK phosphorylation (Fig 11, Supplementary Fig S8A).

**Effect of BCAT2 Knockdown on KIC, Homocysteine, IL-6 and TNF-α Induced Glycogen Synthesis**

I analyzed the effect of KIC, homocysteine, IL-6 and TNF-α co-incubation on glycogen synthesis in the presence and absence of BCAT2 by measuring glycogen synthesis and phosphorylated glycogen synthase.

Since there was only one glycogen synthesis experiment completed, statistics could not be completed, and therefore significance could not be determined (Fig 12). There was no significant effect of KIC on insulin-stimulated glycogen synthase phosphorylation. There was no significant effect of KIC, homocysteine, IL-6 and TNF-α co-incubation on glycogen synthase phosphorylation either (Fig 13, Supplementary Fig S9A).
Figure 3a: Effect of homocysteine on insulin-stimulated glucose uptake

n=3 independent experiments with 3 replicates per experiment except for [Homocysteine] of 15, 20, 30, 40, n=2 independent experiments with 3 replicates. Data presented as Means ± SEM.
Figure 3b: Effect of homocysteine on insulin-stimulated Akt phosphorylation

n= 3 independent experiments with 3 replicates except for [Homocysteine] of 15, 30, 40, n=2 independent experiments with 3 replicates and for [Homocysteine] of 20, n=1 independent experiment with 3 replicates. Data presented as Means ± SEM.
Figure 4: The effect of KIC and homocysteine on insulin-stimulated glucose uptake

n= 4 independent experiments with 3 replicates per experiment. Data presented as Means ± SEM. * (p<0.05).
a) Graph showing the effect of homocysteine and insulin on ph-Akt/γ-Tubulin expression. The x-axis represents the concentration of homocysteine (µM) and the y-axis represents the arbitrary units of ph-Akt/γ-Tubulin. The graph includes a control (KIC -) and treatment (KIC +) condition. The data points are represented by bars with error bars, and a * indicates statistical significance.

Ph-Akt: 75 kDa and 50 kDa bands are visible.

b) Graph showing the effect of homocysteine and insulin on ph-S6K1/γ-Tubulin expression. The x-axis represents the concentration of homocysteine (µM) and the y-axis represents the arbitrary units of ph-S6K1/γ-Tubulin. The graph includes a control (KIC -) and treatment (KIC +) condition. The data points are represented by bars with error bars.

Ph-S6K1: 75 kDa and 50 kDa bands are visible.
Figure 5: Effect of KIC and homocysteine on insulin signaling

n=5 independent experiments with 3 replicates per experiment. Data presented as Means ± SEM. * (p<0.05).
Figure 6: Effect of TNF-α on JNK phosphorylation

n=4 independent experiments with 3 replicates per experiment. Data presented as Means ± SEM.
Figure 7: Effect of TNF-α on insulin-stimulated glucose uptake

n=3 independent experiments with 3 replicates per experiment. Data presented as Means ± SEM. * (p<0.05)
Figure 8: Effect of pro-inflammatory factors and KIC on insulin-stimulated glucose uptake

n=3 independent experiments with 3 replicates per experiment. Data presented as Means ± SEM. * (p<0.05)
Figure 9: BCAT2 attenuates reduction of insulin-stimulated glucose uptake in the presence of KIC and inflammation

n=3 independent experiments with 3 replicates per experiment. Data presented as Means ± SEM. * (p<0.05)
a)  

![Graph showing ph-Akt/γ-Tubulin levels](image)

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b)  

![Graph showing ph-S6K1/γ-Tubulin levels](image)

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<tr>
<td>ph-S6K1/γ-Tubulin (Arbitrary Units)</td>
<td>+</td>
<td>SCR siRNA</td>
<td>+</td>
<td>BCAT2 siRNA</td>
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Figure 10: The effect of a BCAT2 knockdown with KIC and inflammation on insulin signaling

n=3 independent experiments with 3 replicates per experiment. Data presented as Means ± SEM.
Figure 11: The effect of KIC and inflammation with and without the presence of BCAT2 on JNK phosphorylation

n=3 independent experiments with 3 replicates per experiment. Data presented as Means ± SEM.
Figure 12: The effect of KIC and inflammation with and without the presence of BCAT2 on glycogen synthesis.

n=1 independent experiment with 3 replicates per experiment. Data presented as Means ± SEM.
Figure 13: The effect of KIC and inflammation with and without the presence of BCAT2 on glycogen synthase phosphorylation.

n=3 independent experiments with 3 replicates per experiment. Data presented as Means ± SEM.
Discussion

Despite the role of BCAAs in stimulating protein synthesis, there has been a positive correlation between circulating BCAA levels and their metabolites, and insulin resistance\textsuperscript{103-105}. This poses the questions of whether high levels of BCAAs and their metabolites are a symptom of insulin resistance, or if BCAAs and BCAA metabolites play a causative role in eliciting insulin resistance.

In previous studies, my lab has demonstrated the effect of leucine supplementation and KIC supplementation on insulin-stimulated glucose uptake. Not only has leucine, in the absence of other amino acids, reduced insulin-stimulated glucose uptake, its metabolite KIC also suppressed insulin-stimulated glucose uptake by 45\%\textsuperscript{174}. The literature supports this, as high circulating concentrations of KIC has been associated with insulin resistance in humans\textsuperscript{172} and animals\textsuperscript{173}.

Building off this previous work, it was important to examine the effect of KIC in the presence of inflammation, as inflammation is a feature of insulin resistance\textsuperscript{204-206}. Cytokines released in conditions like obesity that may induce insulin resistance include tumor necrosis factor $\alpha$ (TNF-$\alpha$), interleukin-1$\beta$ (IL-1$\beta$) and interleukin-6 (IL-6). Upregulation of TNF-$\alpha$ for example, results in c-Jun N-terminal kinases (JNK) activation, which in turn can phosphorylate the Ser$^{307}$ residue on IRS-1 and thus down regulate IRS-1 tyrosine phosphorylation to hinder downstream signaling\textsuperscript{56}.

To establish inflammation, I examined the effect of homocysteine a metabolite of cysteine, on insulin-stimulated glucose uptake. Hyperhomocysteinemia has been implicated as an independent risk factor of coronary heart disease\textsuperscript{194} and insulin resistance\textsuperscript{215}. Hyperhomocysteinemia ranges $\sim$15-50$\mu$M in humans\textsuperscript{195}. In epidemiological studies
hyperhomocysteinemia has been associated with insulin resistance\textsuperscript{196-197}. Homocysteine is a pro-inflammatory factor and has also been displayed to promote inflammation both in vitro and in vivo\textsuperscript{198-199}. In my studies, there was not a significant suppression of insulin-stimulated glucose uptake with the incubation of homocysteine.

It was also important to analyze the effect of homocysteine on the phosphorylation of Akt(s473), which is a strong marker for insulin signaling, and is important for the translocation of GLUT4 in order for insulin-stimulated glucose uptake to take place. There was an increase in pAkt at 30µM of Hcy, although this was not significant. Despite not reaching significance, an increase in Akt phosphorylation was still surprising. This was not expected as inflammation is often linked with insulin resistance and thus reduces insulin signaling, but there has been some literature to suggest Akt is activated in the presence of inflammation\textsuperscript{228-229}. Op den Kamp et al. demonstrated that increases in Akt in a cachexic state could imply impaired Akt activity, as downstream targets of Akt were reduced\textsuperscript{229}, which was consistent with our data displaying a further reduction in S6K1 and S6 phosphorylation at 15 and 50 µM of homocysteine (Fig 5b, 5c, Supplementary Fig S3A, S3B).

Homocysteine, as stated earlier, did not have a significant effect on insulin-stimulated glucose uptake, but at a physiological level of 50 µM, there was a slight decrease. This indicated that inflammation may have been established and thus lead me into my subsequent studies. Next, I examined the effects of KIC on insulin-stimulated glucose uptake in the presence of homocysteine (50µM). There was no significant effect on insulin-stimulated glucose uptake with the co-incubation of KIC and homocysteine.

Previous studies have implicated a role of mTORC1 in the pathogenesis of insulin resistance from BCAAs. The hyper-activation of mTORC1/S6K1 is said to create a feedback
loop phosphorylating the serine residue of IRS-1 and thus hindering insulin signaling (figure 2) 5,103,118. I analyzed the effect of KIC and homocysteine on pS6K1 (Thr389). With solely KIC there was not an increase in pS6K1. There was also a decrease in pS6K1 with the addition of inflammation, although not statistically significant. As displayed in figure 1, when JNK is activated in an inflammatory state it can inhibit IRS-1 and insulin signalling, which can allude to the reduced pS6K1 signalling. In terms of protein synthesis, a hallmark benefit of BCAAs and their metabolites, there was a decrease in insulin-stimulated S6 phosphorylation. There was a further decrease with inflammation, but as explained above, inflammation does hinder insulin signaling through IRS-1. Failing to reproduce KIC’s effect on S6 phosphorylation is a shortcoming of this thesis, as the effect of KIC and BCAAs on S6 phosphorylation has already been established174,256.

I established a more robust inflammatory signal with TNF-α, IL-6 and homocysteine co-incubation. Both IL-6 and TNF-α are pro-inflammatory cytokines. I incubated the L6 myotubes in these pro-inflammatory factors for 48 hours opposed to the 24-hour incubation period I implemented previously. During exercise pro-inflammatory cytokines like IL-6 are released establishing an acute inflammatory response and thus increasing glucose uptake201-203. My study induced a more chronic inflammatory signal not associated with exercise stimulated glucose uptake, as longer IL-6 incubation times show reduced insulin-stimulated glucose uptake with increased JNK activation in muscle203.

With a 48-hour incubation period, KIC with multiple pro-inflammatory signals suppressed insulin-stimulated glucose uptake (Fig 8). Previous work in my lab showed when BCAT2 is knocked down the effect of KIC is ameliorated. Even in the context of inflammation that may modulate KIC’s effect on insulin signaling, BCAT2 knockdown attenuated this
suppression (Fig 9a). This emphasizes the notion that KIC’s conversion back to leucine is suppressing glucose uptake not KIC. This also highlights the importance of BCAT2 in insulin signaling and how increased catabolism of BCAAs may help in the management/prevention of insulin resistance.

Next, I analyzed how mTORC1 is implicated in the attenuation of suppressed insulin-stimulated glucose uptake by KIC and the pro-inflammatory factors. Unlike in figure 5, although not significant, both S6 and S6K1 phosphorylation was increased with the addition of KIC consistent with the notion that mTORC1 is hyper-activated. However, this effect was reduced in BCAT2 conditions, suggesting that KIC is converted back to leucine to elicit its effects on insulin-stimulated glucose uptake and mTORC1 activation.

I analyzed the effect of KIC and inflammation in the absence and presence of BCAT2 with another pathway of glucose metabolism further validating the development of insulin resistance under these conditions. In the SCR condition, inflammation resulted in a 39% greater glycogen synthase phosphorylation, and KIC and inflammation together resulted in a 70% greater glycogen synthase phosphorylation, although neither of these were statistically significant. In the BCAT2 knockdown conditions, inflammation increased glycogen synthase phosphorylation by 49%, but it was not statistically significant. KIC did not further this phosphorylation like in the SCR condition further emphasizing KIC’s intracellular conversion back to leucine.

To better assess the effects of KIC and inflammation together on insulin-stimulated glucose uptake in the presence and absence of BCAT2 it would be necessary to do these experiments in vivo. A shortcoming of this thesis is not examining total protein levels of Akt, S6K1, S6, IRS-1 and JNK and using γ-tubulin as a loading control to normalize protein levels. I
attempted to use stripping buffer, but this procedure could not be optimized as it resulted in no protein levels on the membrane, therefore γ-tubulin was used. An acute supplementation of KIC may not change total protein levels, but inflammation for 24 or 48 hours may. Thus, for higher accuracy in the future, it would be necessary to look at total protein levels. Another shortcoming of this study was not getting a good signal for IRS-1. Showing increased levels of Ser\textsuperscript{612} phosphorylated IRS-1 would help emphasize the paradox of BCAAs and the mechanism in which they suppress insulin-stimulated glucose uptake. Difficulty in obtaining a signal for the phosphorylation of IRS-1 could be due to its degradation by activating inflammatory pathways. Both proteins suppressor of cytokine signaling 1 (SOCS1) and suppressor of cytokine signaling 3 (SOCS3) are induced in inflammation and can cause ubiquitylation and subsequent degradation of IRS-1\textsuperscript{257}. Not obtaining significance for many of the figures could be attributed to a lack of sample size and is a major shortcoming of this thesis. Lastly, struggling to finish enough glycogen synthesis experiments and producing an effect of insulin is also a shortcoming, as an alternate pathway of glucose metabolism could further emphasize KIC’s role in inflammation on insulin sensitivity.

One of the future steps in this study includes the ensuring that KIC is converted back to leucine in the L6 myotubes, and this could be done with the use of HPLC. Comparing KIC supplementation in both SCR and BCAT2 knockdown condition gives insight to whether or not KIC is converted back to leucine in these cells, as I would expect a higher leucine level in control muscle cells compared to BCAT2 depleted muscle cells.

Once KIC’s conversion back to leucine is confirmed, as described above, it is imperative to study whether increasing the catabolic flux of the BCAA catabolic pathway can help attenuate the suppression in insulin-stimulated glucose uptake.
Hernandez-Alvarez et al. have demonstrated a decrease in BCAT2 and BCKD mRNA levels in T2DM\(^{232}\). As a result, BCAA metabolism is hindered, and there are higher levels of BCAAs and their metabolites as well. Thus, increasing BCAA metabolism may help in reducing the increased levels of BCAAs that are prevalent in insulin resistance. One potential method of stimulating greater BCAA metabolism is the use of B-vitamins. Vitamin B6’s active form pyridoxal 5’-phosphate (PLP) is a co-factor of transaminases like BCAT2\(^{230}\). Other B-vitamins like thiamine and riboflavin increase the activity of BCKDH\(^{231}\). Thus B-vitamin supplementation with amino acids like leucine may help prevent the dysregulations in the catabolic enzymes in this BCAA metabolic pathway, that are often characteristic of T2DM.

In conclusion, I provide further emphasis that even in the presence of inflammation, KIC’s effect on insulin-stimulated glucose uptake is attenuated with the knockdown of BCAT2. This emphasizes the importance of leucine and how upregulating the catabolism of leucine can be a therapeutic strategy in managing insulin resistance.
4.0 Future Directions

1. Examine the effect of both KIC and leucine in inflammation in vivo as well.

2. Examine if an anti-inflammatory intervention such as omega-3s can alleviate the significant reduction in KIC mediated insulin-stimulated glucose uptake.

3. Examining the effect of overexpressing BCAT2 to assess whether or not leucine’s suppression of insulin-stimulated glucose uptake is attenuated with enhanced BCAA flux.

4. Examine the effect of branched-chain keto dehydrogenase (BCKD) and how its knockdown or overexpression through branched-chain keto dehydrogenase kinase (BDK) knockdown can affect insulin sensitivity.
5.0 References

50. Prudente S, Scarpelli D, Chandalia M, Zhang YY, Morini E, Del Guerra S, Perticone F, Li


Regulation by Leucine of Translation Initiation Through the mTOR-Signaling Pathway by Pancreatic β-Cells. *Diabetes*, 50(2), 353-360.


phosphorylation mediates lipid-induced hepatic insulin resistance. *J. Clin. Invest.*, 126, 4361–4371


A) Experimental Outline for Homocysteine Experiments

On Day 5 of differentiation myotubes were incubated with or without homocysteine for 24 hours. Myotubes were starved for 4h in serum and amino acid-free medium. They were then incubated in an amino acid-free medium with KIC for 30 minutes. Following this, the cells were incubated with or without insulin for 20 minutes and then a glucose uptake assay was conducted and cells were harvested for western blot analysis.
On Day 5 of differentiation, myotubes were incubated with or without pro-inflammatory factors for 48 hours. Media with pro-inflammatory factors was replenished at the 24-hour mark. Myotubes were starved for 4h in serum and amino acid-free medium. They were then incubated in an amino acid-free medium with KIC for 30 minutes. Following this, the cells were incubated with or without insulin for 20 minutes and then a glucose uptake assay was conducted and cells were harvested for western blot analysis.
C) Glucose Transport Assay Solutions

Hepes Buffer Saline:
- 140 mM NaCl (Wisent #600-082-LG)
- 20 mM Hepes-Na, pH 7.4 (Research Organics #6003H)
- 5mM KCl (Fisher Scientific #M-12445)
- 2.5 mM MgSO₄ (Bioshop #10034-99-8)
- 1.0 mM CaCl₂ (Bioshop #10035-04-8)

Stop Solution:
- 0.9% NaCl (Saline) (Wisent #600-082-LG)

2-DG Stock Solution:
- 10 mM 2-Deoxy-D-Glucose in Hepes buffer

Transport Solution (TS):
Prepare in Hepes buffer
10 uM 2-Deoxy-Glucose
0.5 uCi/mL H³ 2-Deoxy-Glucose (Perkin Elmer #NET549250UCI)

Glucose Uptake Procedures

1. On the designated radioactive bench in the lab, wash cells two times with 400ul of 37°C Hepes Buffered Saline (HBS) at room temperature and aspirate any remaining buffer
2. Add 300ul of room temperature Transport Solution per well for a 12-well plate.
3. Incubate the plates for 5 minutes at 37°C. Be sure to not exceed this time.
4. Aspirate away the Transport Solution quickly and wash the wells thoroughly three times with 1ml of ice-cold Stop Solution (0.9% Saline) while on ice. Aspirate to dryness.
5. While on ice, add 1.0 mL of ice-cold 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 3.5 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.
**D) 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 (Cat#08-772-1B)
- 12 well plates (Cat#665180)

**Procedures:**

1. In the cell culture hood, prepare three 15 ml tubes and label “A”, “B” and “C” and one 50 ml tube labelled as “Optimum”

A→Lipofectamine+ Opti-MEM B→BCAT2 siRNA+Opti-MEM C→Scramble siRNA+Opti-MEM

2. Pour 18ml Optimum solution into the designated 50 ml-Optimum tube (without touching the mouth of the bottle).
3. Add 120 µl of Opti-MEM/well + 5 µl of Lipofectamine/well to tube “A”.
4. Add 3 µl of BCAT2 siRNA/well and 122 µl of Opti-MEM/well to tube “B”.
5. Add 3 µl of scramble siRNA/well and 122 µl of Opti-MEM/well to tube “C”.
6. Add tube “A” to tube “B” and tube “C” in 1:1 ratio. (For example, 125µl of “A” to tube “B” and 125µl of tube “A” into tube “C”).
7. Wait at least 5 minutes.
8. While waiting, add 1 mL/well and 0.5ml/well of GM without antibiotics into the 6 well and 12 well plates respectively, and add 125000 or 250000 cells to each 12 well or 6 well respectively.
9. Add 250 µl or 125µl/well of diluted tube “B” and “C” to 6 well plate wells or 12 well plate wells respectively.
10. Following 24 hours, add 1 mL/well of GM (with antibiotics and pro-inflammatory factors) into the 6 well and 0.5mL/well of GM (with antibiotics and pro-inflammatory factors) into the 12 wells.
E) Glycogen Synthesis Assay

1) Cells were starved for three hours (2ml/well for 6 well plate)
   a. Prepare test media containing [U^{14}C]-D-Glucose (0.2µCi/ml) – 2 µl of
      [U^{14}C]-D-Glucose (50µCi/0.5ml)/ml of media
2) Incubate cells with test media (1ml/well) containing respective conditions, 5.5mM unlabelled glucose and [U^{14}C]-D-Glucose to wells for one hour.
3) After 1 hour, collect 200 µl from each well and transferred into scintillation tubes for total counting.
4) Remaining media was aspirated, and wells were washed with ice cold PBS twice.
5) Subsequently cells were lysed with the addition of 450 µl of KOH (1M) to each well and were placed on the rocker for 15 minutes.
6) Cells were transferred from wells into Eppendorf tubes and put onto heat (65°C) for 5 minutes.
7) For protein determination 50 µl was removed and put into different Eppendorf tubes.
8) Glycogen carrier was added (100 µl) [stock solution is 25mg/ml]
9) 80 µl of saturated sodium sulfate was added to each tube
10) 1.2 ml of ice cold 100% ethanol was added to each tube.
11) Tubes were vortexed and placed in freezer (-20°C) overnight for precipitation.
12) After overnight precipitation tubes were centrifuged (20min/10000rpm) at room temperature.
13) Supernatant was discarded, and the pellet was dissolved in 500 µl of double distilled water.
14) Finally, 450 µl of this solution was transferred to scintillation vials containing 3.5 ml of scintillation fluid for counting.
7.0 Supplementary Data

a)  

![Image of ph-Akt with molecular weight markers: 60 kDa.]

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<th>Homocysteine (µM)</th>
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<th>0</th>
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b)  

![Image of γ-Tubulin with molecular weight markers: 48 kDa.]

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c)  

![Image showing a gel with bands labeled as ph-Akt and 60 kDa marker.]

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Ph-Akt  
50 kDa  

γ-Tubulin  
50 kDa  

Homocysteine (µM)  
30 30 30 40 40 40 50 50 50 100 100 100

Insulin  
+ + + + + + + + + + + +
**Figure S1) Effect of homocysteine on insulin-stimulated Akt phosphorylation**

Original blots for ph-Akt (Supplementary Figure S1A, S1C, S1E) and γ-tubulin (Supplementary Figure S1B, S1D, S1F) for Figure 3b.
Figure S2: Effect of homocysteine and KIC on insulin-stimulated Akt phosphorylation

Original blots for ph-Akt (Supplementary Figure S2A) and γ-tubulin (Supplementary Figure S2B) for Figure 5a.
a)

![Image of a gel with bands labeled ph-S6k1 and ph-S6, with molecular weight markers at 75 kDa and 37 kDa.]

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b)

![Image of a gel with bands labeled ph-S6k1 and ph-S6, with molecular weight marker at 37 kDa.]

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<td>KIC (200 µM)</td>
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**Figure S3) Effect of homocysteine and KIC on insulin signaling**

Original blots for ph-S6K1 (Supplementary Figure S3A) and ph-S6 (Supplementary Figure S3B) for Figure 5b and 5c. Original blots for γ-tubulin (Supplementary Figure S3C) was used for both Figure 5b and 5c.

| Homocysteine(µM) | 0   | 0   | 0   | 10  | 15  | 50  | 500 | 10  | 15  | 50  | 500 |
|------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Insulin          | -   | +   | -   | +   | +   | +   | -   | -   | -   | -   | -   | -   |
| KIC(200µM)       | -   | -   | +   | -   | -   | -   | -   | +   | +   | +   | +   | +   |

**Figure S3) Effect of homocysteine and KIC on insulin signaling**

Original blots for ph-S6K1 (Supplementary Figure S3A) and ph-S6 (Supplementary Figure S3B) for Figure 5b and 5c. Original blots for γ-tubulin (Supplementary Figure S3C) was used for both Figure 5b and 5c.
Figure S4) Effect of TNF-α on JNK phosphorylation

Original blots for ph-JNK (Supplementary Figure S4A) and γ-tubulin (Supplementary Figure S4B) for Figure 6. JNK has two isoforms, 46p and 48p, I chose to quantify 46p throughout, as both have similar functions, but just different structure.
Figure S5) BCAT2 attenuates reduction of insulin-stimulated glucose uptake in the presence of inflammation and KIC

Original blots for BCAT2 (Supplementary Figure S5A) and γ-tubulin (Supplementary Figure S5B) for Figure 9b.
### a)

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### b)

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Figure S6) The effect of a BCAT2 knockdown with KIC and inflammation on insulin signaling

Original blots for ph-Akt (Supplementary Figure S6A) and ph-S6 (Supplementary Figure S6B) for Figure 10a and 10c respectively. Original blots for γ-tubulin (Supplementary Figure S6C) was used in Figure 10a and 10c.
Figure S7) The effect of a BCAT2 knockdown with KIC and inflammation on insulin signaling

Original blots for ph-S6K1 (Supplementary Figure S7A), and γ-tubulin (Supplementary Figure S7B) for Figure 10b.
**Figure S8** The effect of KIC and inflammation with and without the presence of BCAT2 on JNK phosphorylation

Original blots for ph-JNK (Supplementary Figure S8A), and γ-tubulin (Supplementary Figure S8B) for Figure 11. JNK has two isoforms, 46p and 48p, I chose to quantify 46p throughout, due to the fact they have similar functions, just different structures.
b) Figure S9) The effect of KIC and inflammation with and without the presence of BCAT2 on Glycogen Synthase phosphorylation

Original blots for ph-glycogen synthase (Supplementary Figure S9A), and γ-tubulin (Supplementary Figure S9B) for Figure 13.