

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

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## **Abstract**

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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  $\alpha$ -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.

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## List of Abbreviations

4EBP1	Eukaryotic translation initiation factor 4E binding protein 1
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AS	160 Akt substrate 160kD
ATP	Adenosine triphosphate
BCAA	Branched-chain amino acid
BCAT2	Branched-chain aminotransferase 2
BCKDH	Branched-chain $\alpha$ -keto acid dehydrogenase complex
DAG	Diacylglycerol
Deptor	DEP-domain-containing mTOR-interacting protein
eEF2K	Eukaryotic elongation factor 2 kinase
eIF4A	Eukaryotic translation initiation factor 4A
eIF4B	Eukaryotic translation initiation factor 4B
eIF4E	Eukaryotic translation initiation factor 4E
eIF4F	Eukaryotic translation initiation factor 4F
eIF4G	Eukaryotic translation initiation factor 4G
FKBP12	FK506-binding protein 12 kDa
GAP	GTPase activating protein
Gab-1	Grb2-associated-binding protein 1
GDP	Guanosine diphosphate
GLUT4	Glucose transporter type-4
Grb2	Growth factor receptor-bound protein 2

hVps34	Human vacuolar protein sorting 34
IGF1	Insulin-like growth factor 1
IKK	Inhibitor kappa B (IkB) kinase
IKK- $\beta$	IkB kinase beta
IL-6	Interleukin-6
IRS	Insulin receptor substrate
JNK	c-jun N-terminal kinase
KIC	$\alpha$ -ketoisocaproic acid
KIV	$\alpha$ -ketoisovaleric acid
KMV	$\alpha$ -keto- $\beta$ -methylvaleric acid
mLST8	Mammalian lethal with Sec 13 protein 8
MODY	Maturity-onset diabetes of the young
mSin1	Mammalian stress-activated protein kinase (SAPK)-interacting protein-1
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
Nck1	Non-catalytic region of tyrosine kinase adaptor protein 1
NF- $\kappa$ B	Inhibitor kappa B (IkB) kinase (IKK)-nuclear factor $\kappa$ B
PDCD4	Programmed cell death 4
PDK1	3-phosphoinositide dependent protein kinase-I
PH	Pleckstrin-homology
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol-4,5-biphosphate



PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PPAR- $\gamma$	Peroxisome proliferator-activated receptor gamma
PRAS40	Proline-rich Akt substrate of 40 kDa
Protor-1	Protein observed with rictor-1
Raptor	Rapamycin-sensitive adaptor protein of mTOR
Rheb	Ras homologue in the brain
Rictor	Rapamycin-insensitive companion of mTOR
ROS	Reactive oxygen species
S6K1	Ribosomal protein S6 Kinase 1
Ser/S	Serine
SH2	SRc homology-2
SOCS1	Suppressor of cytokine signaling 1
SOCS3	Suppressor of cytokine signaling 3
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
Thr/T	Threonine
TNF- $\alpha$	Tumor necrosis factor-alpha
TSC1/2	Tuberous sclerosis complex 1/2

## 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<sup>1</sup>. An estimated 439 million people will have T2DM by 2030<sup>2</sup>. 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  $\beta$ -cells in the pancreas<sup>3</sup>. 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<sup>2</sup>. This is because high fat diets display pro-inflammatory effects both in vitro and in vivo<sup>8</sup>. Inflammation, a characteristic of obesity and insulin resistance has been directly linked to T2DM. Literature shows that pro-inflammatory cytokines like tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), could have implications in the development of insulin resistance. TNF- $\alpha$  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<sup>9</sup>.

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<sup>6</sup>.

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<sup>5</sup>. 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<sup>174</sup>. Also, incubation of KIC (200 $\mu$ M), a metabolite of leucine, in L6 myotubes resulted in a 45% suppression in insulin-stimulated glucose uptake<sup>174</sup>.

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

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### 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<sup>158</sup>. Besides locomotion and storage, skeletal muscle is important in the maintenance of systemic glucose metabolism and is the most abundant insulin-sensitive tissue<sup>148</sup>. 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<sup>148</sup>.

#### 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<sup>10</sup>. The IR is a heterotetrameric membrane glycoprotein comprising of two  $\alpha$  subunits as well as two  $\beta$  subunits that are joined by disulfide bonds<sup>11</sup>. Insulin binds to the extracellular  $\alpha$  subunit of the receptor, inducing conformational changes bringing both  $\alpha$  subunits together. This leads to the auto-phosphorylation of the IR's Tyr<sup>960</sup> residue, allowing for its binding with the phospho-tyrosine binding domain of the insulin receptor substrates (IRSs)<sup>12</sup>.

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<sup>dok</sup> and adaptor protein with a PH and SH2 domain (APS)<sup>13,14</sup>. IRS-1 and IRS-2 are widely distributed 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<sup>307</sup>, Ser<sup>612</sup> and Ser<sup>1101</sup> 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<sup>178</sup>. 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<sup>207-212</sup>. 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<sup>109</sup>. On the other hand, in a mice study, where they mutated the Ser<sup>302</sup> site to alanine, there was no change in insulin action<sup>234</sup>.

The first IRS discovered was IRS-1, which has 21-22 tyrosine phosphorylation sites<sup>15,16</sup> and 50 serine/threonine phosphorylation sites<sup>245</sup>. 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<sup>238</sup>. Ablation of this phosphatase in muscle<sup>239</sup> and liver<sup>240</sup> 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<sup>17</sup>. 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<sup>18</sup>. 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<sup>250</sup>. 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<sup>19</sup>.

### 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<sup>20</sup>. 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<sup>27,28</sup>. 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-bisphosphate (PIP<sub>2</sub>). PI3K phosphorylates PIP<sub>2</sub> at position 3 of the inositol ring to generate a lipid second messenger known as phosphatidylinositol 3,4,5-trisphosphate (PIP<sub>3</sub>)<sup>21</sup>. 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<sup>22</sup>.

Lipid phosphatases can have negative effects on insulin signaling. Phosphatase and tensin homolog (PTEN) dephosphorylates PIP<sub>3</sub>, thus antagonizing PI3K signaling<sup>37,38</sup>. PTEN is a tumour suppressor that is disrupted in human cancers as well<sup>236</sup>. PTEN inhibition has increased Akt activation, thus not only affecting insulin action<sup>237</sup>, but also cell growth and survival pathways<sup>236</sup>.

Studies have also demonstrated that PTEN deletion in skeletal muscle resulted in increased insulin sensitivity, highlighting its role in the pathogenesis of insulin resistance<sup>39,40</sup>.

Many of the effects of PI3K-derived PIP<sub>3</sub> 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<sup>30</sup>. For example, PDK-1 phosphorylates and activates the Thr<sup>308</sup> residue of Akt<sup>31</sup>. Simultaneous binding of PDK-1 and Akt to phosphoinositides like PIP<sub>3</sub> facilitates conformational changes, allowing for interaction between PDK-1 and Akt<sup>251</sup>. This makes Akt more prone to be phosphorylated by PDK-1<sup>251</sup>. For full Akt activation, the Ser<sup>473</sup> residue also needs to be phosphorylated, which is accomplished by the mammalian target of rapamycin complex 2 (mTORC2)<sup>23,24</sup>. Mutations in either Akt or PI3K cause severe insulin resistance highlighting the importance of these two proteins in the insulin signaling pathway<sup>246</sup>.

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<sup>25</sup>. Akt2 plays an important role in mediating insulin action on metabolism, while Akt1 is more associated with growth<sup>241</sup>. 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<sup>159,160</sup>. 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<sup>233</sup>. TCB1D4 activates Rab proteins<sup>242</sup> that are responsible for about half

of the effect of insulin on GLUT4<sup>243</sup>. In fat cells Rab10 specifically is known for its role in the biogenesis of GLUT4-containing transport vesicles<sup>244</sup>.

### **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)<sup>97</sup>. 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<sup>147</sup> (figure 1). Akt can also activate mTORC1 through phosphorylation of proline rich AKT substrate 40 kDa (PRAS40), alleviating PRAS40's inhibition of mTORC1<sup>33</sup>.

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<sup>36</sup>. 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.<sup>233</sup>

## **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<sup>89</sup>. mTOR with various other protein interactions



forms two distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2)<sup>90-91</sup>. 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<sup>92</sup>.

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)<sup>176,7</sup>. Raptor is an important protein in this complex, as it functions to regulate the assembly of the complex and recruit substrates for mTOR<sup>161</sup>. On the other hand, the role of mLST8 seems to be unclear, as knockdown in vivo exhibited no difference in mTORC1 activity<sup>161</sup>. PRAS40, FKBP12 and Deptor function as negative regulators of mTORC1, and inhibit mTORC1 when recruited to the complex<sup>175</sup>. When mTORC1 is active, the opposite occurs, mTORC1 directly phosphorylates PRAS40 and Deptor, reducing the physical interaction and further activating mTORC1 signaling<sup>176-177</sup>.

### 2.2.2 Mammalian Target of Rapamycin Complex 2 (mTORC2)

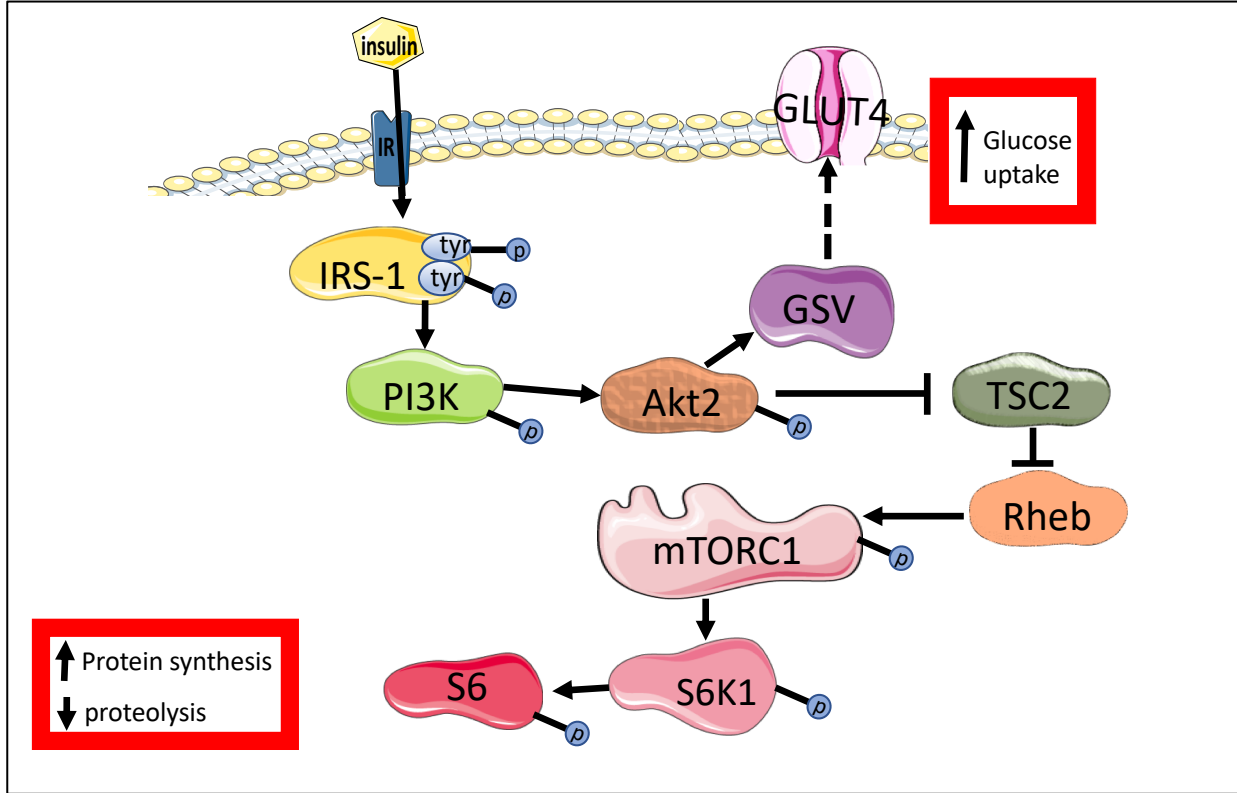
Unlike mTORC1, the regulation of mTORC2 is poorly understood. Only growth factors can activate mTORC2. It plays a major role in Akt, serum and glucocorticoid-regulated kinase (SGK) and protein kinase C (PKC) activation.

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. Protor-1 interacts with Rictor, although its physiological function is unclear<sup>147</sup>.

mTORC2 is responsible for phosphorylating Ser473 of Akt2. mTORC2 indirectly through Akt signaling plays an important role in the pathogenesis of diabetes and cancers<sup>89</sup>.

### 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)<sup>93</sup>.



**Figure 1: Simiplified 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)<sup>94,95</sup>. 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<sup>163</sup>. It also phosphorylates programmed cell death 4 (PDCD4), which is a tumor suppressor that negatively regulates eukaryotic initiation factor 4A<sup>163</sup>. 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)<sup>164</sup>. S6K1 is evidently very important in the regulation of translation, allowing for protein synthesis<sup>96</sup>.

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<sup>ser307</sup> phosphorylation reduces Akt activation, attenuating insulin responses, such as glucose uptake and glycogen synthesis (Figure 2)<sup>118</sup>.

## **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<sup>422</sup> residue of IRS-1. Phosphorylation at this site then recruits SCF<sup>β-TRCP</sup> E3 ligase complex to degrade IRS-1, which can have major implications on insulin sensitivity<sup>248</sup>. However, Hoehn et al. suggested that IRS-1 degradation is a consequence of insulin resistance instead of it causing insulin resistance<sup>249</sup>.

### 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<sup>117</sup>.

### 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<sup>97</sup>.

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<sup>98-100</sup>.

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<sup>101</sup>.

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<sup>102</sup>.

### **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<sup>5</sup>. 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<sup>5</sup>.

### 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  $\alpha$ -keto acids. The  $\alpha$ -amino group is transferred onto  $\alpha$ -ketoglutarate, and thus leucine is converted to  $\alpha$ -ketoisocaproic acid (KIC), isoleucine to 2-keto-3-methylvaleric acid (KMV), and valine to  $\alpha$ -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  $\alpha$ -keto acid dehydrogenase (BCKDH) complex. This complex consists of three subunits: a branched-chain  $\alpha$ -keto acid decarboxylase (E1), a dihydrolipoyl transacylase (E2), and a dihydrolipoyl dehydrogenase (E3). BCKDH catalyzes the oxidative decarboxylation of the branched-chain  $\alpha$ -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  $\alpha$  subunit at the Ser239 residue to inactivate BCKDH<sup>5,113,114</sup>. In the presence of BCKDH substrates, the mitochondrial phosphatase 2C

(PP2Cm) binds to the BCKDH complex and induces dephosphorylation, activating the complex<sup>179</sup>. The BCKDH complex activity is downregulated in obese and diabetic animals resulting in increased levels of BCAAs<sup>180-182</sup>, 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<sup>179</sup>.

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<sup>174</sup>. In another study with the presence of an amino transaminase inhibitor, KIC could not activate mTORC1, implying that leucine was necessary for mTORC1 activation<sup>116</sup>. This suggested KIC is converted back to leucine to elicit its effects on mTORC1 activation, as the transamination reaction by BCAT2 is reversible<sup>116</sup>.

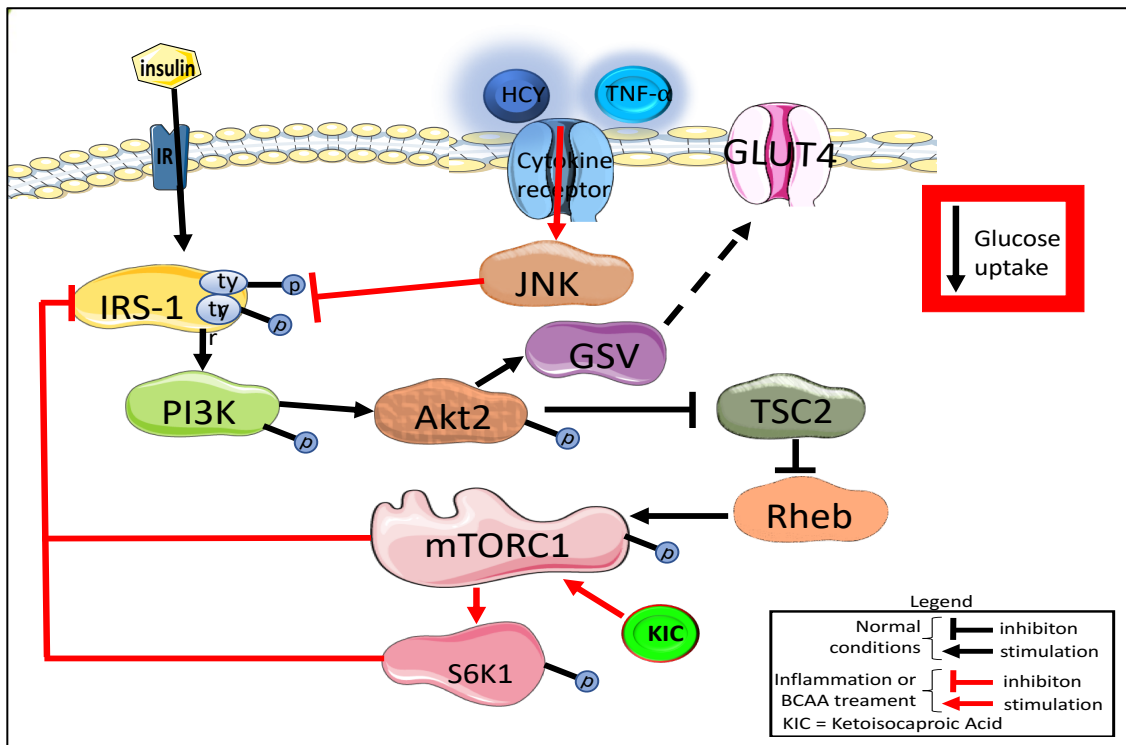
Another metabolite of leucine that has been extensively studied is beta-hydroxy-beta-methylbutyrate (HMB). HMB is used as a nutritional supplement to aid in sport performance. HMB is known to have an anti-catabolic effect on skeletal muscle<sup>187-188</sup>. Eley et al. demonstrated how HMB supplementation prevented phosphorylation of kinases that inhibit the elongation step of mRNA translation<sup>189</sup>. HMB also increased mRNA translation<sup>189</sup>. In a study conducted by Pinheiro et al., they demonstrated that HMB supplementation increased maximum strength production in rat skeletal muscle<sup>189</sup>. HMB supplementation also increased ATP content and glycogen content in red and white portions of gastrocnemius muscle of rats<sup>190</sup>. 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<sup>191</sup>. In fact, Giron et al. demonstrated that HMB was more



effective in activating mTORC1 and stimulating protein synthesis than leucine<sup>192</sup>. In regards to insulin sensitivity, one study demonstrated that HMB supplemented to rats with a high fat diet improved insulin sensitivity<sup>193</sup>. 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<sup>193</sup>. 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<sup>193</sup>. 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<sup>183</sup>. 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<sup>183</sup>. 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 tolerance<sup>184</sup>. Consistent with this study, diabetic subjects have increased serum 3-HIB levels<sup>184-185</sup>. Diabetic mice have also displayed a decrease in the downstream enzyme that catabolizes 3-HIB<sup>186</sup>. 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- $\alpha$  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<sup>103-105</sup>. In clinical studies, BCAA levels in the blood positively correlate with insulin resistance<sup>106,151-153</sup>. BCAA levels have also been predictive of future insulin resistance or T2DM in several studies<sup>104,107</sup>. 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<sup>103</sup>.

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<sup>103,151,153</sup>. In particular high concentrations of KIC has been associated with insulin resistance in humans<sup>172</sup> and animals<sup>173</sup>. 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<sup>174</sup>. 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<sup>174</sup>.

## **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<sup>108</sup>. T2DM can also lead to more health complications such as stroke, neuropathy, ischemic heart disease, retinopathy and nephropathy<sup>108</sup>. 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<sup>110</sup>. 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<sup>111</sup>. 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<sup>41,42</sup>. Most of these patients have nonsense or missense mutations in either the ligand binding domain or tyrosine kinase domain of the IR<sup>43,44</sup>. 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<sup>45,46</sup>. A T608R missense mutation in IRS-1 resulted in decreased insulin signaling, but this appears to be very rare<sup>47</sup>.

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<sup>48</sup>. 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<sup>49,50</sup>. 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<sup>51</sup>. 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<sup>138</sup>.

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<sup>165-168</sup>. Thus, exercise and insulin can have an additive effect on GLUT4 translocation, resulting in greater glucose uptake<sup>139,169-171</sup>.

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<sup>140-142</sup>. 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<sup>143-145</sup>.

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<sup>252</sup>.

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<sup>145</sup>. 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<sup>146</sup>. 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*<sup>221-224</sup>. Most cross-sectional studies display a positive correlation between dietary fat intake and obesity<sup>225-227</sup>. This is further emphasized when formerly obese individuals prevent relapse with a lower fat intake and maintain their weight more effectively<sup>120</sup>.

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<sup>121</sup>. Studies have demonstrated that the ability for GLUT4 to shuttle glucose into the cell is sensitive to changes in the membrane lipid bilayer<sup>122-123</sup>. High fat feeding and obesity can affect the composition and structure of this membrane, resulting in a decrease in GLUT4 activity<sup>124-125</sup>. Rosholt et al. demonstrated reduced GLUT4 activity in skeletal muscle of rat as a result of a high fat diet<sup>126</sup>.

Increased fatty acid concentrations in the blood are often associated with insulin resistant states such as obesity and T2DM<sup>127-130</sup>, even in children insulin resistance correlated with higher free fatty acid concentrations in the blood<sup>253</sup>. A cross sectional study also showed the inverse relationship between fatty acid plasma levels and insulin sensitivity<sup>131</sup>. 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<sup>+</sup> 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<sup>132-134</sup>.

High fatty acid levels in the blood also resulted in a decrease in insulin-stimulated IRS-1 tyrosine phosphorylation in skeletal muscle<sup>136</sup>. Elevations in fatty acids resulted in a decrease in IRS-1 associated PI3K activation<sup>135</sup>. 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<sup>137</sup>. DAG can also activate protein kinase C $\epsilon$ , which impairs auto-phosphorylation of Thr<sup>960</sup> of the insulin receptor and thus IRS-1 tyrosine phosphorylation, by phosphorylating the Thr<sup>1160</sup> site in the insulin receptor activation loop, specifically in the liver<sup>235</sup>.

### **2.5.3.2 High Protein Intake**

High protein diets have often been recommended to combat obesity<sup>150</sup>. High protein diets have been associated with increased insulin sensitivity. Dietary proteins have an insulintropic effect, thus promoting insulin release, and glucose uptake by the tissues<sup>119</sup>.

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<sup>93</sup>.

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<sup>103</sup>. 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<sup>118</sup>.

### **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<sup>154</sup>. Another study in rats demonstrated that high fructose and sucrose (simple carbohydrates) intake produced a decline in insulin sensitivity in liver and peripheral tissues<sup>155</sup>. 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<sup>155</sup>.

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<sup>157</sup>.

## **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<sup>52</sup>. 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<sup>53</sup>. This is evident, as the deletion of MCP-1 or its receptor CCR2 improves insulin sensitivity and actually alleviates inflammation in mice<sup>54,55</sup>.

Homocysteine is a pro-inflammatory factor that promotes inflammation in both in vitro and in vivo<sup>213-214</sup>. Li et al. demonstrated that homocysteine impaired glucose uptake in rat adipocytes and rats<sup>215</sup>. Other cytokines released in obesity that 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) to be activated, which in turn can interact with the Ser<sup>307</sup> residue on IRS-1 and thus down regulate IRS-1 tyrosine phosphorylation to hinder downstream signaling<sup>56</sup>. JNK's importance in mediating insulin resistance is evident, as knockdown of JNK protects against obesity-induced insulin resistance<sup>247</sup>.

TNF- $\alpha$  role in insulin sensitivity was emphasized when Li et al. demonstrated that TNF- $\alpha$  inhibition in 3T3-L1 adipocytes partially improved insulin-stimulated glucose uptake that was

originally suppressed with TNF- $\alpha$ . The inhibitions were mediated by inhibiting proteins involved in the inflammatory pathways such as JNK and Inhibitor kappa B (IkB) kinase (IKK)-nuclear factor  $\kappa$ B (Nf- $\kappa$ B)<sup>254</sup>. 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 endotoxemia, which are both features of obesity<sup>216</sup>. They both induce inflammation by activating the NF- $\kappa$ B pathway<sup>57</sup>. Mice with decreased TLR-2 and TLR-4 signaling proteins are protected from diet-induced obesity and insulin resistance<sup>58-60</sup>.

### **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<sup>61</sup>. Elevated circulating levels of free fatty acids are observed in obesity and induce activation of JNK, IkB kinase (IKK) and protein kinase C (PKC), which may lead to the phosphorylation of IRS-1 at the ser<sup>307</sup> residue<sup>62</sup>. Palmitate specifically promotes insulin resistance by stimulating cytokine production, JNK activation and endoplasmic reticulum (ER) stress<sup>58,63</sup>. Palmitate also activates NF- $\kappa$ B, and studies inhibiting this pathway reverse lipid-induced insulin resistance<sup>64,65</sup>.

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- $\theta$  and inducing IRS-1<sup>Ser307</sup>

phosphorylation<sup>66</sup>. 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<sup>67-69</sup>.

Ceramides, a type of lipid molecule, also have been shown to induce insulin resistance via PKC and JNK activation<sup>62,70</sup>. This happens with the activation PKC- $\zeta$ , which phosphorylates the thr<sup>34</sup> residue of the Pleckstrin Homology (PH) domain of Akt. This leads to a loss of PIP<sub>3</sub> binding to the PH domain, and thus the inhibition of Akt<sup>71-73</sup>. 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<sup>208</sup>.

### **2.6.3 Oxidative Stress**

Low levels of reactive oxygen species (ROS) can enhance insulin action<sup>74,75</sup>, 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<sup>76</sup>. Increased ROS levels have been observed in obese and diabetic states in adipose tissue<sup>77</sup> and in muscle<sup>78</sup>. This increased oxidative stress leads to the activation of stress kinases, which phosphorylate serine residues of IRS proteins, ultimately resulting in insulin resistance<sup>77-79</sup>.

#### 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<sup>80,81</sup>. 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<sup>82-85</sup>. Hyperglycemia also activates the PKC pathway by inducing synthesis of DAG<sup>86</sup> and causes insulin resistance by increasing IRS-1<sup>ser307</sup> phosphorylation<sup>87,88</sup>.

## 2.7 Rationale

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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<sup>103-107</sup>. 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**

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- 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**

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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.

### 3.0 Manuscript

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## **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- $\alpha$  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.<sup>1</sup> The main underlying cause of T2DM is insulin resistance. Insulin resistance is when the tissues response to insulin is reduced<sup>3</sup>. 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<sup>6</sup>. Despite this anabolic benefit,

circulating BCAA levels have been positively correlated with insulin resistance<sup>106,151-153</sup>. 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<sup>5</sup>.

Hernandez et al. demonstrated that BCAA catabolic enzyme mRNA are downregulated in individuals with T2DM compared to healthy individuals<sup>232</sup>. He also showed that BCAAs and their metabolites were upregulated in individuals with T2DM<sup>232</sup>. 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<sup>174</sup>. They also demonstrated that ketoisocaproic acid (KIC), the metabolite of leucine, suppressed insulin-stimulated glucose uptake as well<sup>174</sup>. 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<sup>174</sup>. 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<sup>52</sup> 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

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### Reagents

The growth medium (GM) used for cell growth was  $\alpha$ -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  $\alpha$  was purchased from Shendoah Biotechnology (#300-18) and Sodium 4-methyl-2- oxoalate (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). [ $^3\text{H}$ ]-2-deoxyglucose (NET549) and [ $\text{U-}^{14}\text{C}$ ]-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  $\alpha$ -MEM supplemented with 10% FBS and 1% Antibiotic-Antimycotic agents and grown at 37°C and 5% CO<sub>2</sub> in a cell culture incubator until they were approximately 70-80% confluent. Next, cells were counted and seeded with  $2 \times 10^5$  cells/well in 6-well plates for western blot experiments or  $10^5$  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  $2 \times 10^5$  cells/well and  $1 \times 10^5$  cells/well respectively. After 48 hours, medium was shifted to DM. On day 3 of differentiation, myotubes were transfected with 10  $\mu$ M of scramble siRNA (negative control) or 10  $\mu$ M 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  $\mu$ L of the siRNA-lipid complex was added to wells containing 1mL of antibiotic-free  $\alpha$ -MEM containing 2% HS. For 12 well plates, 125  $\mu$ L of the siRNA-lipid

complex was added to wells containing 0.5 mL of antibiotic-free  $\alpha$ -MEM with 2% HS. Twenty-four hours following transfection, 1mL of  $\alpha$ -MEM containing 2% HS, 1% Ab-Am, and the pro-inflammatory factors (homocysteine, TNF- $\alpha$  and IL-6) were added to each well of the 6 well plates, and 0.5mL of  $\alpha$ -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  $\mu$ M) of homocysteine in DM for 24 hours. Normal physiological levels of homocysteine are usually around 5-15  $\mu$ M, but intermediately elevated homocysteine levels are between 31-100  $\mu$ M, while excess of 100  $\mu$ M is severely elevated levels of homocysteine<sup>217</sup>. Homocysteine levels elevated above 15  $\mu$ M is characterized as hyperhomocysteinemia<sup>217</sup>. 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  $\mu\text{M}$ ) 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  $\mu\text{M}$  KIC for 30 minutes. Then following these 30 minutes, myotubes were incubated with homocysteine with or without 200  $\mu\text{M}$  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- $\alpha$ Test Experiments**

The effect of TNF- $\alpha$  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- $\alpha$  in DM at various concentrations (2, 5, 10ng/ml) for 24 hours. For TNF- $\alpha$ , I used values consistent in the literature indicative of inflammatory states<sup>218-220</sup>. Following this incubation period on D6 myotubes cells were starved for four hours with TNF- $\alpha$  in RPMI (complete starvation medium, free of amino acids and serum). After starvation, cells were incubated with various concentrations of TNF- $\alpha$  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- $\alpha$  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- $\alpha$ , 10ng/ml of IL-6 and 50 $\mu$ M 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  $\mu$ M of KIC for 30 minutes. Then they were incubated with or without pro-inflammatory factors, with or without 200  $\mu$ M 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- $\alpha$ , 10ng/ml of IL-6 and 50 $\mu$ M 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  $\mu$ M 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<sup>255</sup>. 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 ( $[^3\text{H}]$ -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  $\mu\text{L}$  of transport solution (HEPES buffer, 10 $\mu\text{M}$  2-deoxyglucose, 0.5  $\mu\text{Ci/mL}$   $[^3\text{H}]$ -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  $\mu\text{L}$  was collected for a protein assay. These samples were stored at -20°C for protein assays. The remaining 800  $\mu\text{L}$  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  $\mu\text{g}$  of protein per 5 minutes.

### **Cell Harvesting for Western Blot Analysis**

Following the treatments, cells were rinsed with 1mL of PBS. Then 100  $\mu\text{L}$  of lysis buffer [1mM EDTA, 2% sodium dodecyl sulphate (SDS), 25 mM Tris-HCL pH 7.5, 10 $\mu\text{L}/\text{mL}$  protease inhibitor (Sigma Aldrich #P8340), 10 $\mu\text{L}/\text{mL}$  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  $-20^{\circ}\text{C}$  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+  $\mu\text{g}$  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) 0.2 $\mu\text{m}$  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.

Primary Antibody	Dilution	Company Purchased From	Secondary Antibody Used
ph-S6K1 <sup>Thr389</sup>	1:1000	Cell Signaling #9205	Anti-rabbit (CST #7074)
ph-S6 <sup>Ser235/236</sup>	1:1000	Cell Signaling #4858	Anti-rabbit (CST #7074)
ph-Akt <sup>Ser473</sup>	1:1000	Cell Signaling #9271	Anti-rabbit (CST #7074)
Gamma-Tubulin	1:1000	Sigma Aldrich #T6557	Anti-mouse (CST #7076)
ph-SAPK/JNK <sup>Thr183/Tyr185</sup>	1:1000	Cell Signaling #9255	Anti-mouse (CST #7076)
BCAT2	1:1000	Sigma Aldrich #B7312	Anti-rabbit (CST #7074)
ph-IRS-1 <sup>Ser612</sup>	1:1000	Cell Signaling #3203	Anti-rabbit (CST #7074)
ph-Glycogen Synthase	1:1000	Cell Signaling #47043	Anti-rabbit (CST#7074)

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 [<sup>3</sup>H]-2-deoxyglucose (pmol) transported into cells each well by the concentration of protein found in each well (μg). This value is expressed as the rate of glucose uptake. The glucose transport value (pmol/μg), was the rate of glucose uptake per 5 minutes. Rate of glycogen synthesis is measured by CPM per μg 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 γ-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 μCi/ml of [U-<sup>14</sup>C]-D-glucose, with or with insulin (100nM), with or without KIC (200μM), and with or without the pro-inflammatory factors as explained in 4.4.5 for one hour. Then 200 μl 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l of glycogen carrier, 80  $\mu$ l of saturated sodium sulfate, and 1.2 ml of ice cold ethanol was added to each sample. The tubes were then vortexed and 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  $\mu$ l of double distilled water. 450  $\mu$ l 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  $\pm$  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  $\mu$ g 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,  $\gamma$ -tubulin. Significance was determined as  $p < 0.05$ . Bars with \* denote significant differences.

## Results

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### **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  $\mu\text{M}$  and 50  $\mu\text{M}$  of homocysteine ( $p < 0.05$ , Fig 5c, Supplementary Fig S3B).

### **Effect of TNF- $\alpha$ on Insulin-Stimulated Glucose Uptake and JNK Phosphorylation**

Exposure of L6 myotubes to TNF- $\alpha$  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- $\alpha$ 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 (50 $\mu$ M), IL-6 (10ng/ml), and TNF- $\alpha$  (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- $\alpha$ 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- $\alpha$  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- $\alpha$  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- $\alpha$  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- $\alpha$  on JNK phosphorylation (Fig 11, Supplementary Fig S8A).

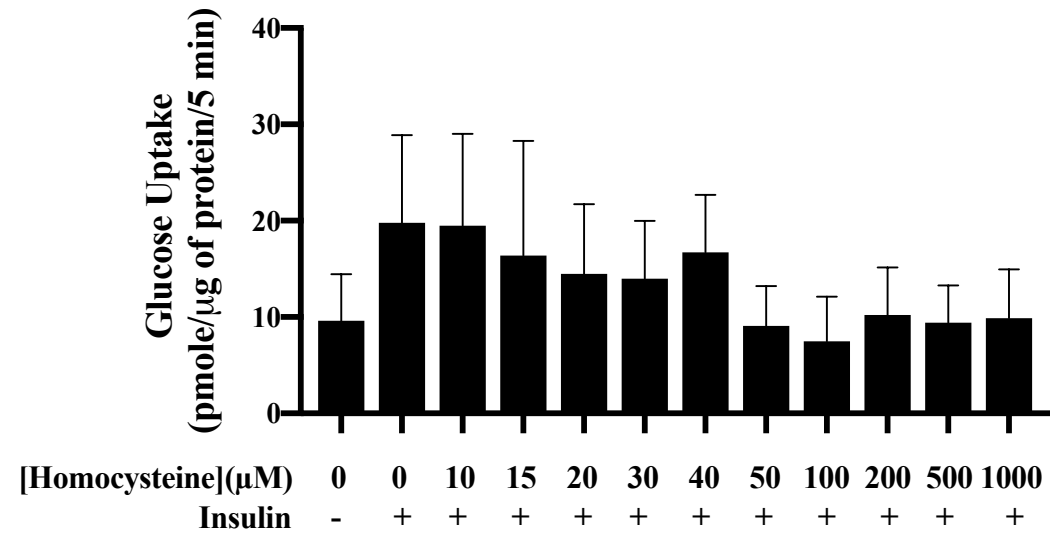
### **Effect of BCAT2 Knockdown on KIC, Homocysteine, IL-6 and TNF- $\alpha$ Induced Glycogen Synthesis**

I analyzed the effect of KIC, homocysteine, IL-6 and TNF- $\alpha$  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- $\alpha$  co-incubation on glycogen synthase phosphorylation either (Fig 13, Supplementary Fig S9A).

## Figures

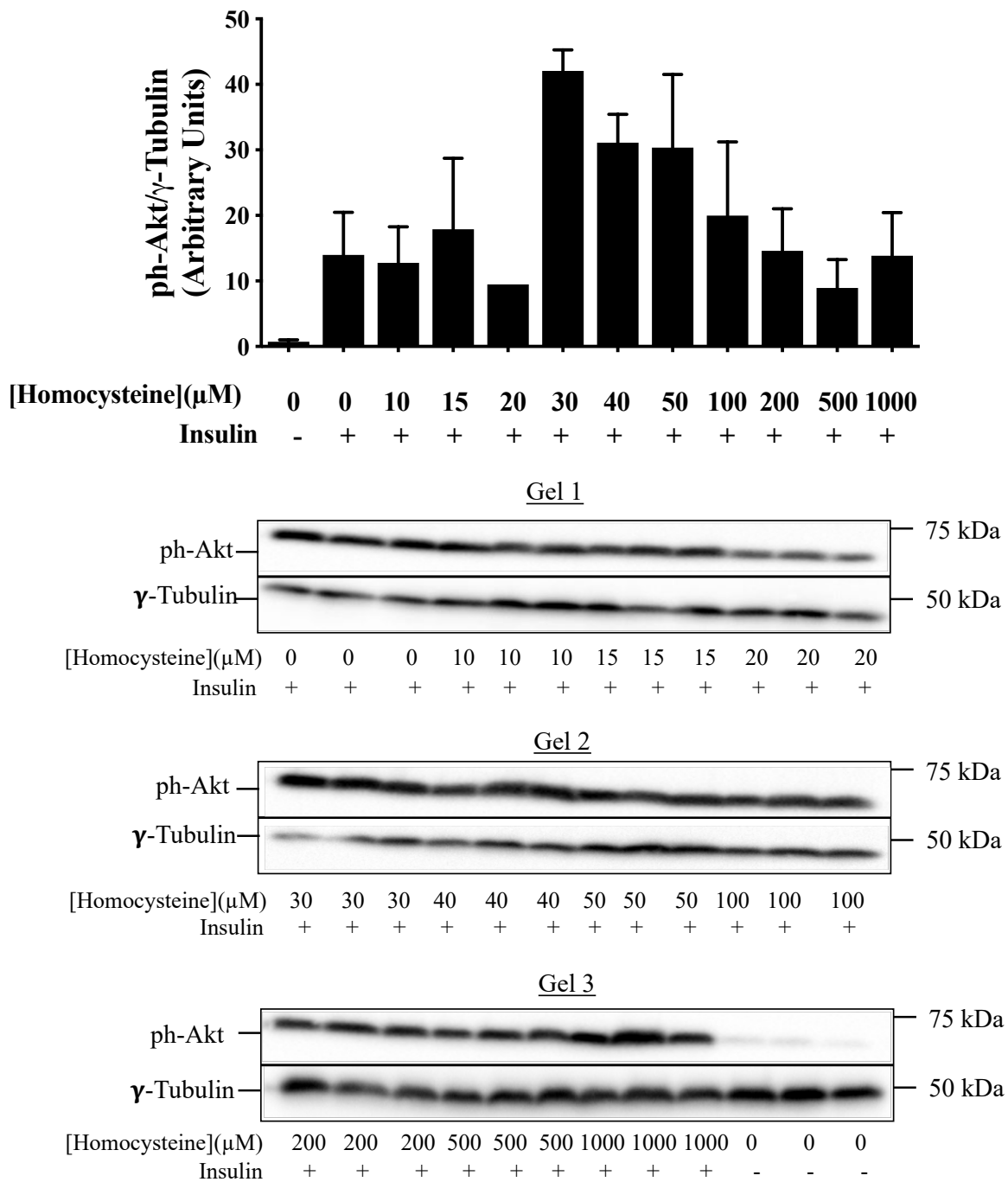
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**Figure 3a: Effect of homocysteine on insulin-stimulated glucose uptake**

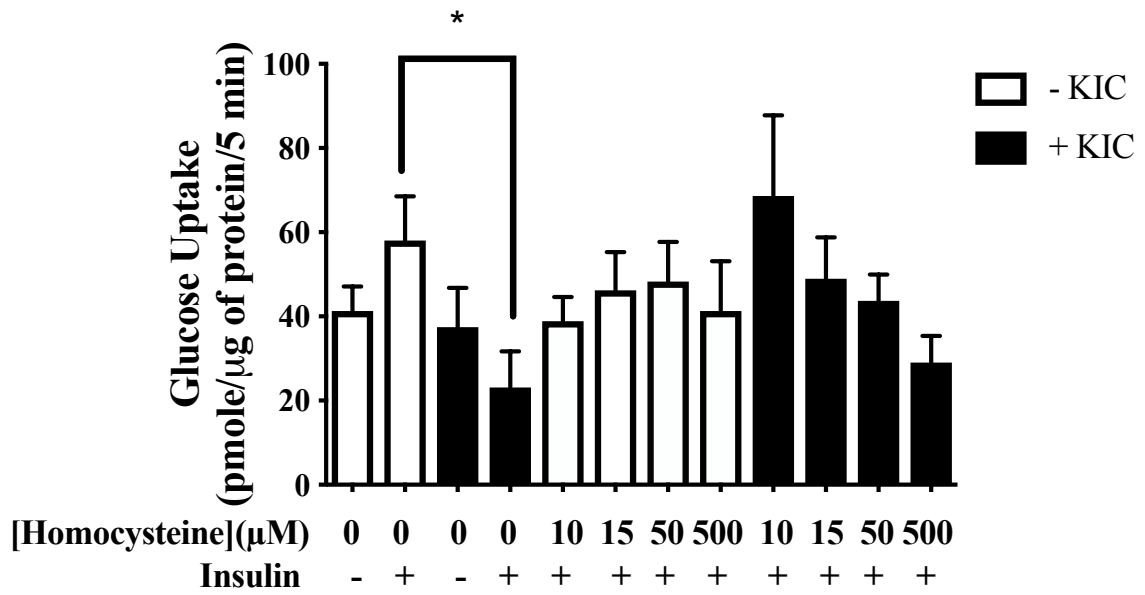
n=3 independent experiments with 3 replicates per experiment except for [Homocysteine] of 15, 20, 30, 40, n=2 independent experiments with 3 replicates. Data presented as Means  $\pm$  SEM.





**Figure 3b: Effect of homocysteine on insulin-stimulated Akt phosphorylation**

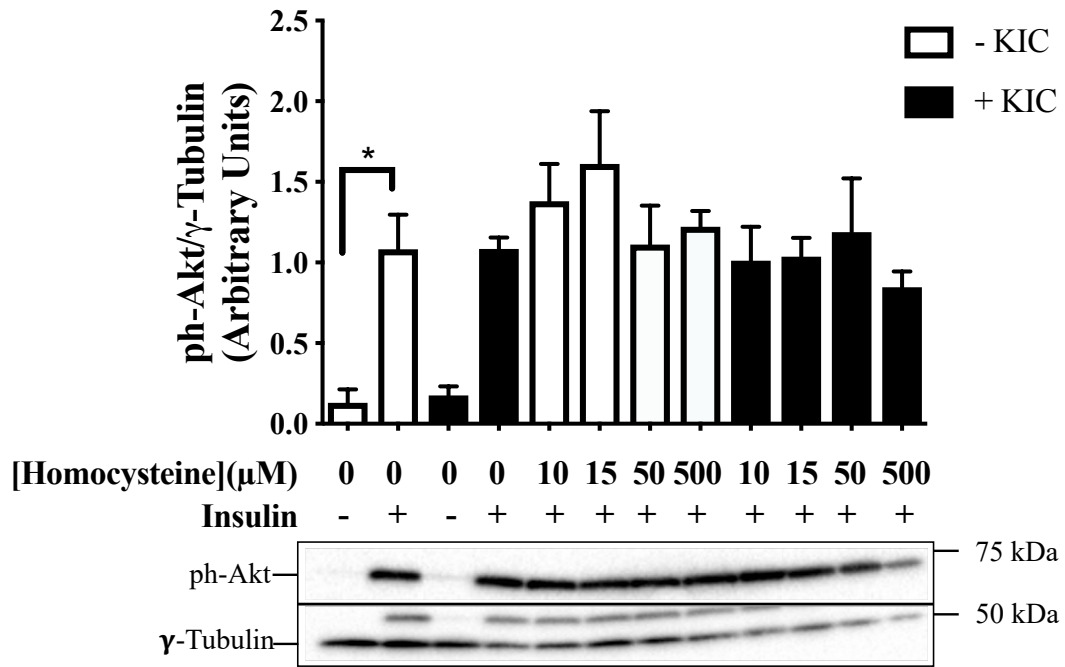
n= 3 independent experiments with 3 replicates except for [Homocysteine] of 15, 30, 40, n=2 independent experiments with 3 replicates and for [Homocysteine] of 20, n=1 independent experiment with 3 replicates. Data presented as Means  $\pm$  SEM.



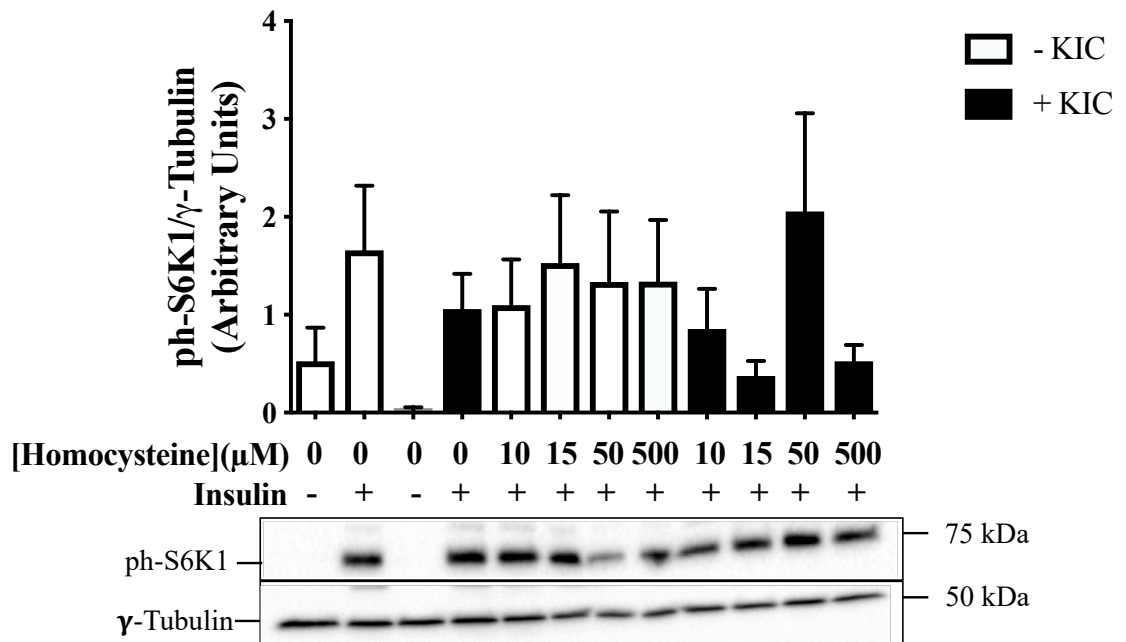
**Figure 4: The effect of KIC and homocysteine on insulin-stimulated glucose uptake**

n= 4 independent experiments with 3 replicates per experiment. Data presented as Means  $\pm$  SEM. \* (p<0.05).

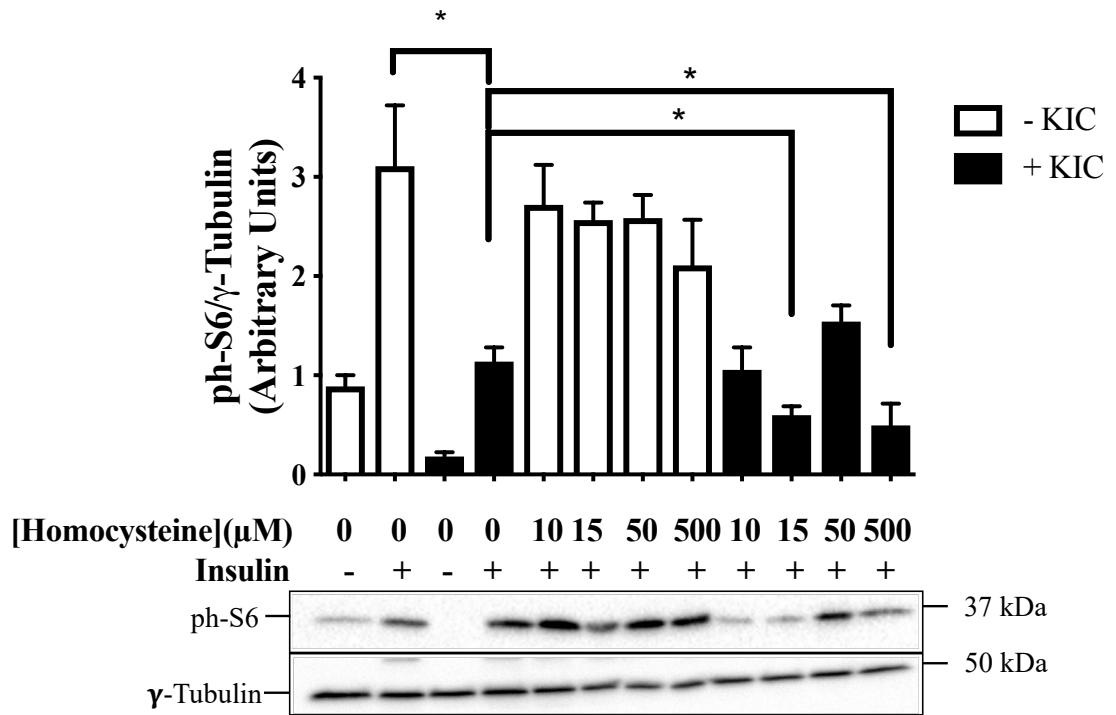
a)



b)

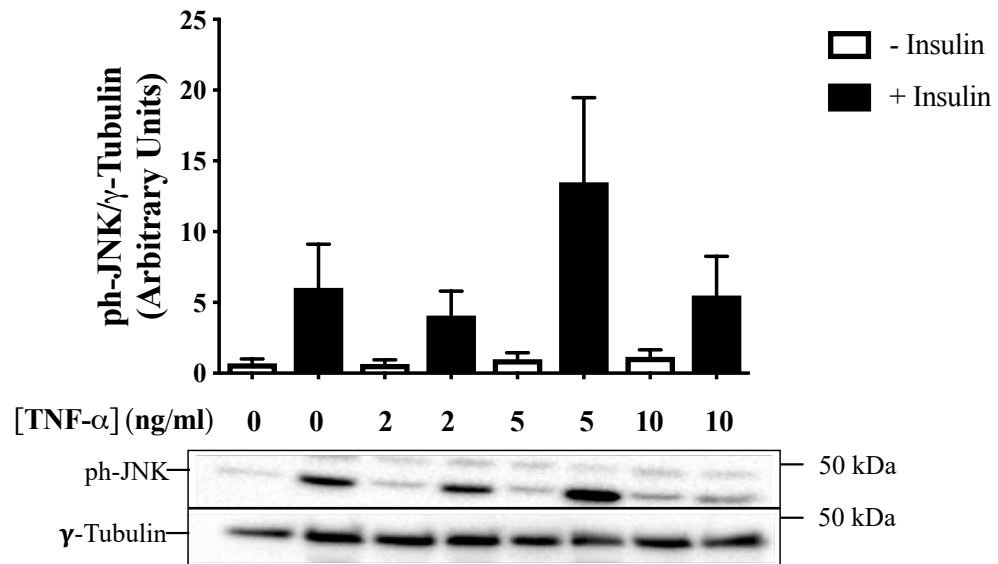


c)



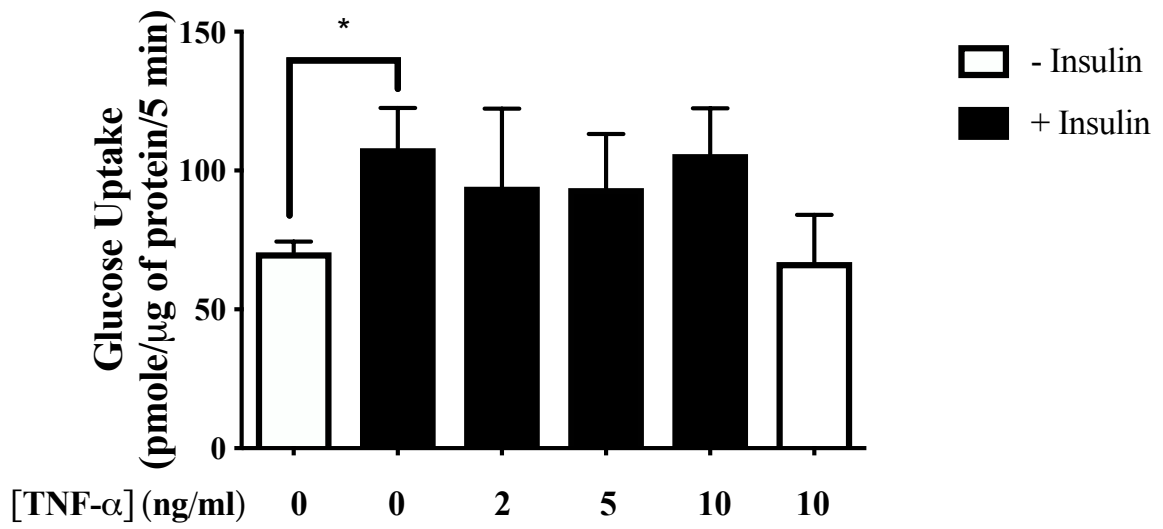
**Figure 5: Effect of KIC and homocysteine on insulin signaling**

n=5 independent experiments with 3 replicates per experiment. Data presented as Means ± SEM. \* (p<0.05).



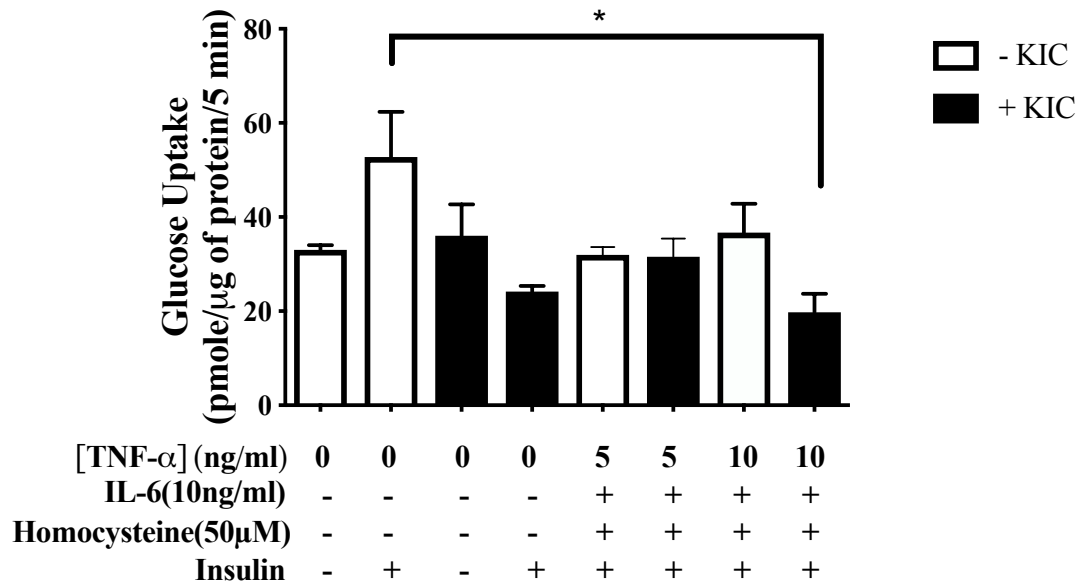
**Figure 6: Effect of TNF- $\alpha$  on JNK phosphorylation**

n=4 independent experiments with 3 replicates per experiment. Data presented as Means  $\pm$  SEM.



**Figure 7: Effect of TNF- $\alpha$  on insulin-stimulated glucose uptake**

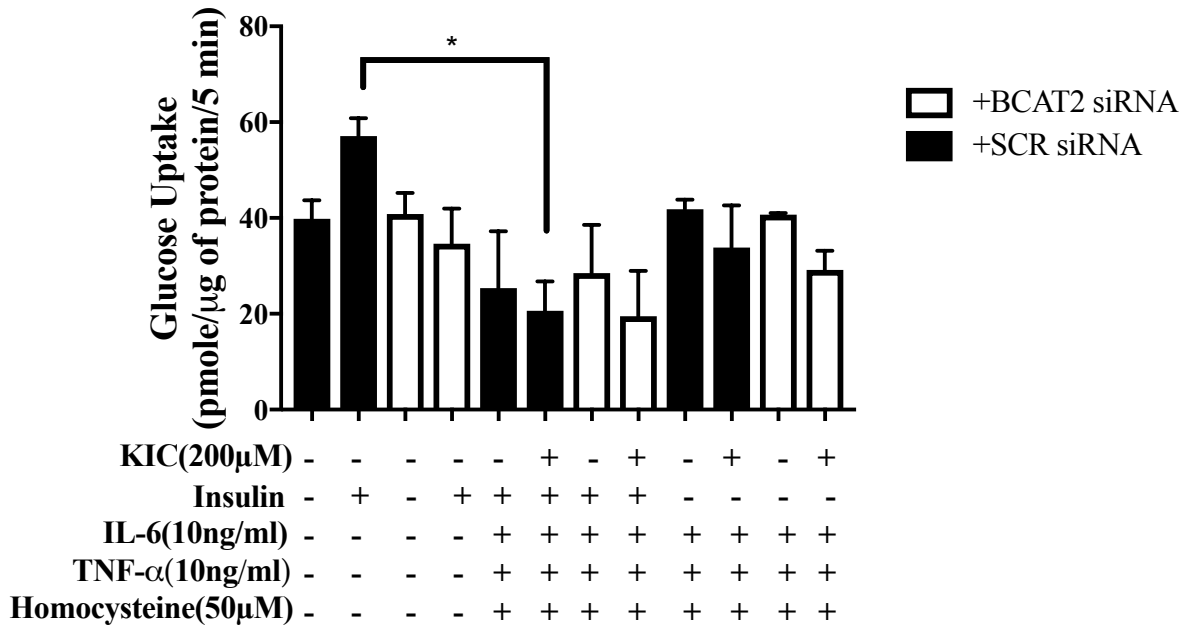
n=3 independent experiments with 3 replicates per experiment. Data presented as Means  $\pm$  SEM. \* (p<0.05)



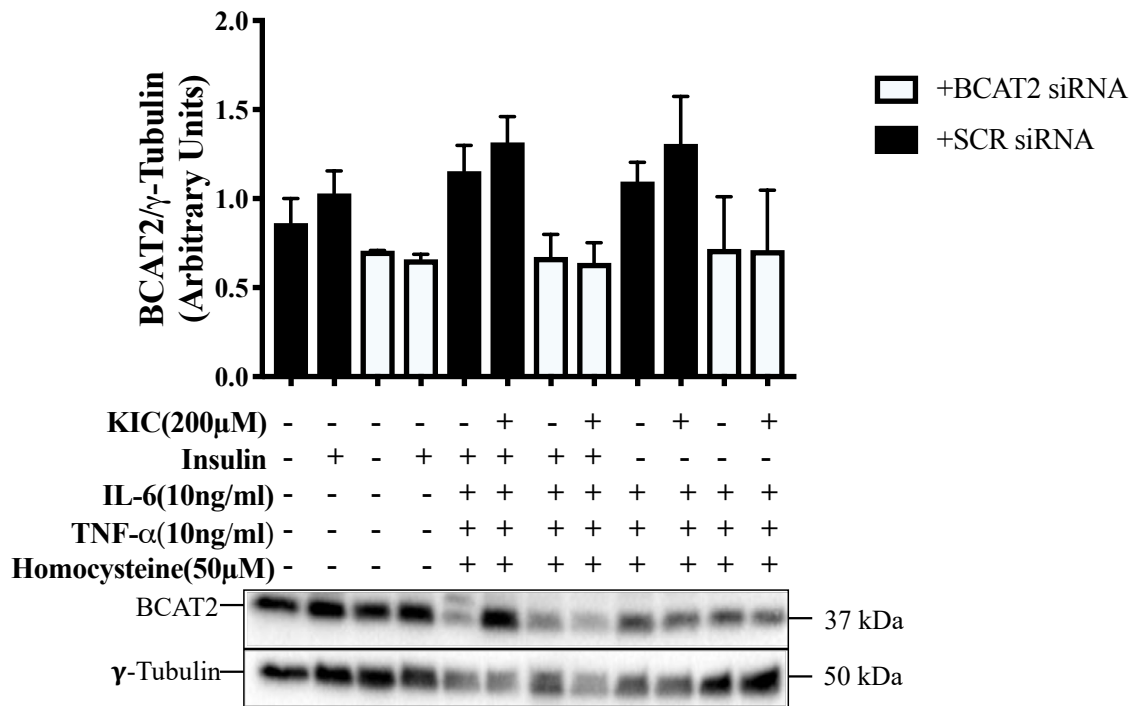
**Figure 8: Effect of pro-inflammatory factors and KIC on insulin-stimulated glucose uptake**

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

a)



b)

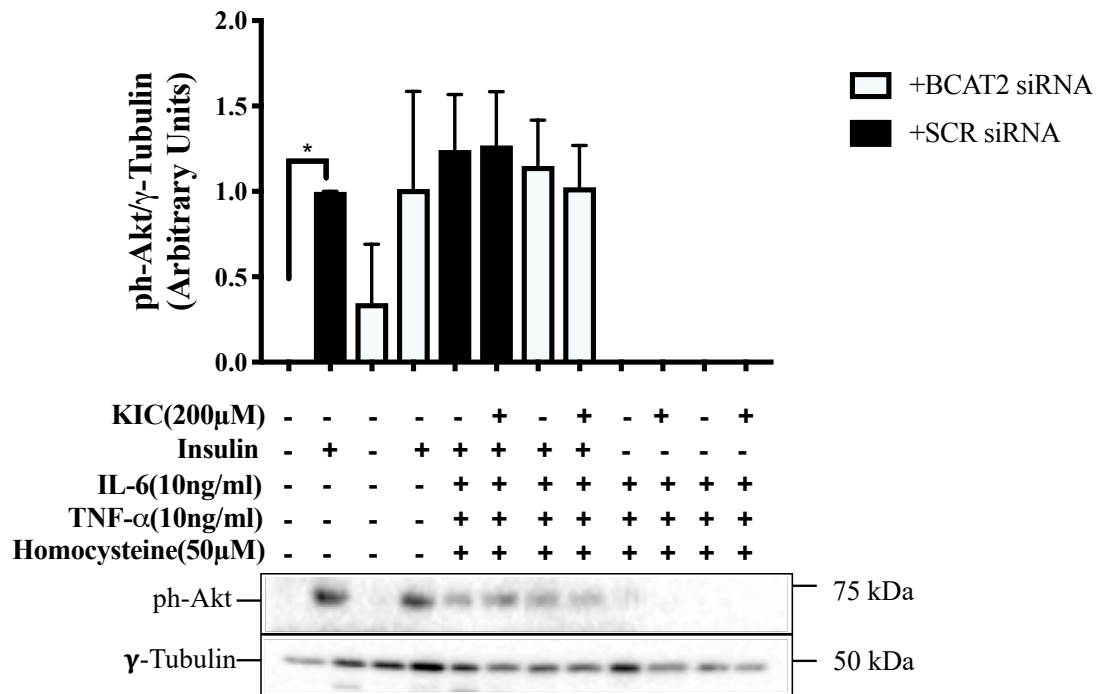


**Figure 9: BCAT2 attenuates reduction of insulin-stimulated glucose uptake in the presence of KIC and inflammation**

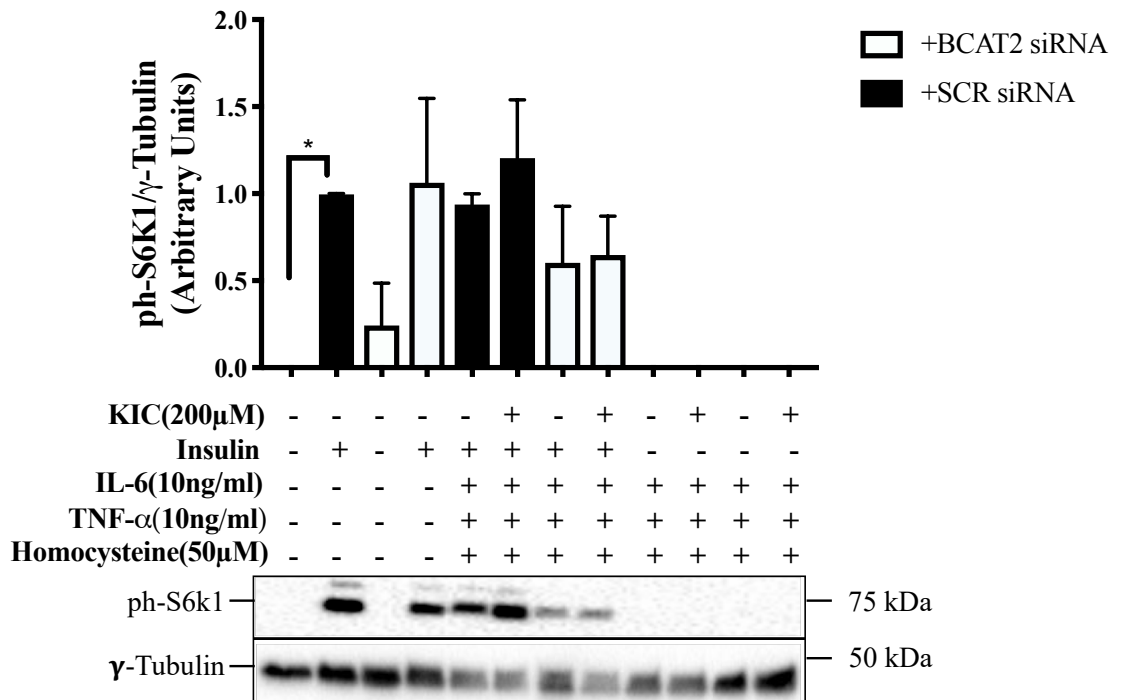
n=3 independent experiments with 3 replicates per experiment. Data presented as Means  $\pm$  SEM. \* (p<0.05)



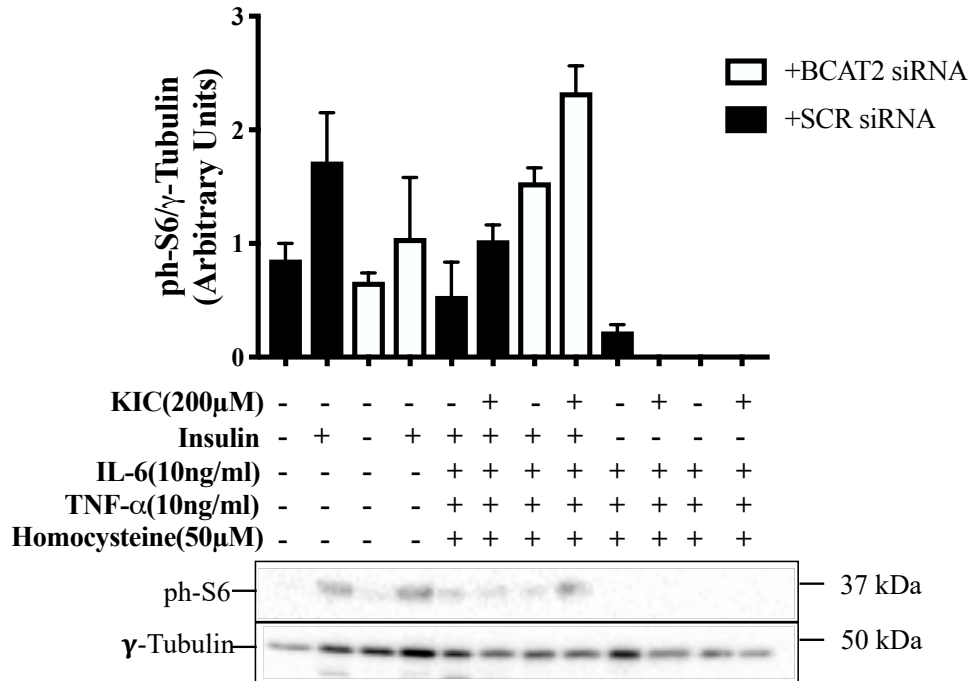
a)



b)

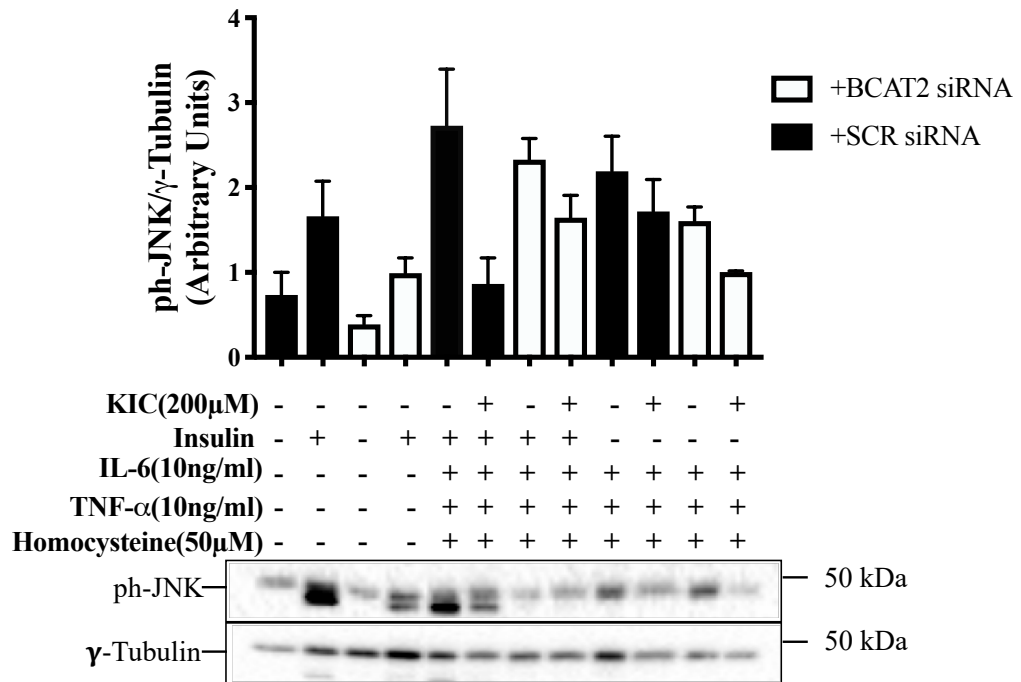


c)



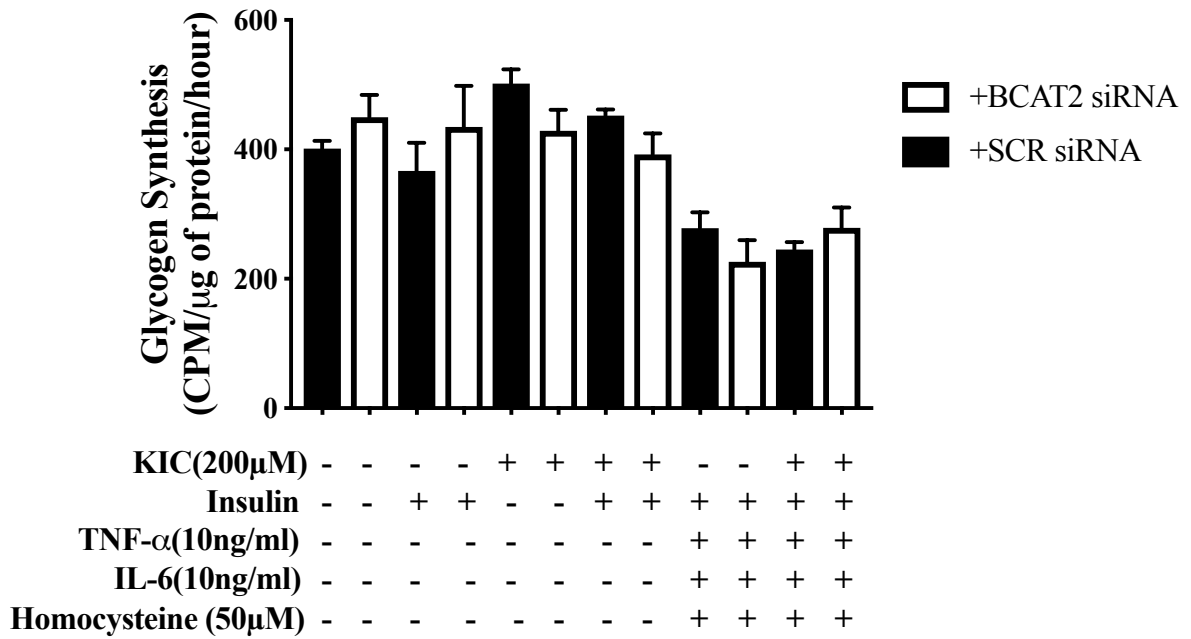
**Figure 10: The effect of a BCAT2 knockdown with KIC and inflammation on insulin signaling**

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



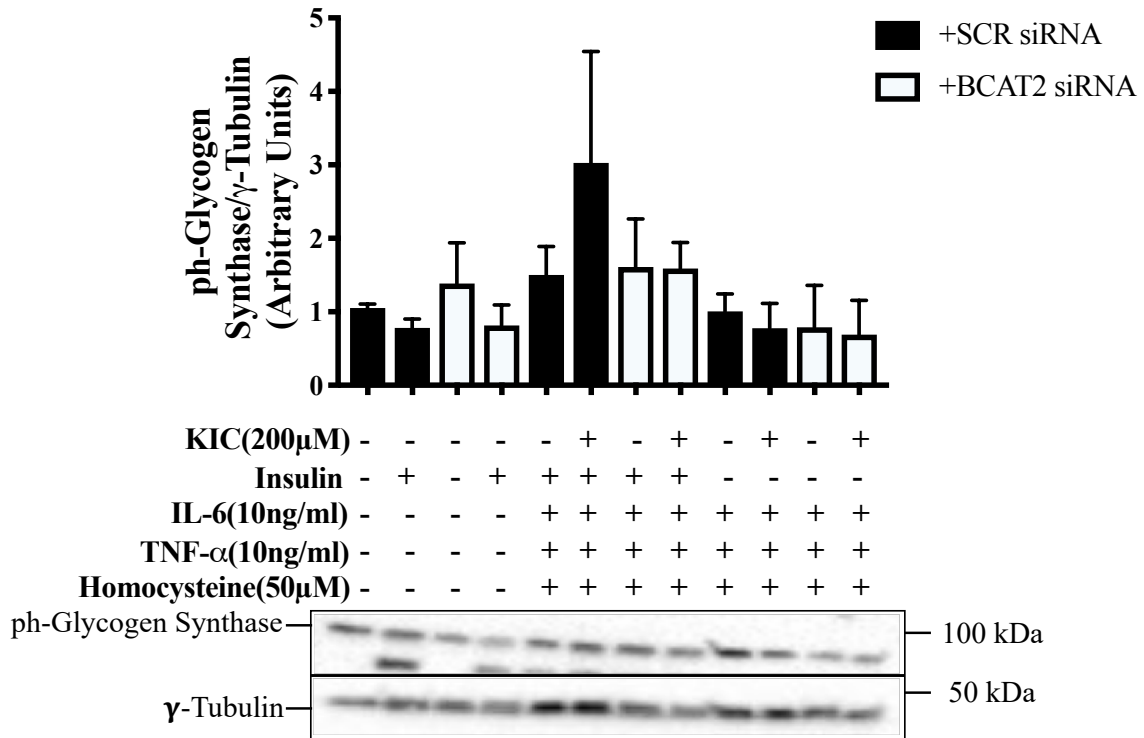
**Figure 11: The effect of KIC and inflammation with and without the presence of BCAT2 on JNK phosphorylation**

n=3 independent experiments with 3 replicates per experiment. Data presented as Means  $\pm$  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

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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<sup>103-105</sup>. 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%<sup>174</sup>. The literature supports this, as high circulating concentrations of KIC has been associated with insulin resistance in humans<sup>172</sup> and animals<sup>173</sup>.

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<sup>204-206</sup>. 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<sup>307</sup> residue on IRS-1 and thus down regulate IRS-1 tyrosine phosphorylation to hinder downstream signaling<sup>56</sup>.

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<sup>194</sup> and insulin resistance<sup>215</sup>.

Hyperhomocysteinemia ranges ~15-50 $\mu$ M in humans<sup>195</sup>. In epidemiological studies

hyperhomocysteinemia has been associated with insulin resistance<sup>196-197</sup>. Homocysteine is a pro-inflammatory factor and has also been displayed to promote inflammation both in vitro and in vivo<sup>198-199</sup>. 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 30 $\mu$ M 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<sup>228-229</sup>. 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<sup>229</sup>, which was consistent with our data displaying a further reduction in S6K1 and S6 phosphorylation at 15 and 50  $\mu$ M 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  $\mu$ M, 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 (50 $\mu$ M). 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)<sup>5,103,118</sup>. 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 established<sup>174,256</sup>.

I established a more robust inflammatory signal with TNF- $\alpha$ , IL-6 and homocysteine co-incubation. Both IL-6 and TNF- $\alpha$  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 uptake<sup>201-203</sup>. 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 muscle<sup>203</sup>.

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  $\gamma$ -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  $\gamma$ -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<sup>612</sup> 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<sup>257</sup>. 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<sup>232</sup>. 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<sup>230</sup>. Other B-vitamins like thiamine and riboflavin increase the activity of BCKDH<sup>231</sup>. 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

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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

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1. Olokoba, A. B., Obateru, O. A., & Olokoba, L. B. (2012). Type 2 diabetes mellitus: a review of current trends. *Oman medical journal*, 27(4), 269-73.
2. The Diabetes Atlas. 4th ed. Brussels, International Diabetes Federation, 2009
3. Lin, Y., & Sun, Z. (2009). Current views on type 2 diabetes. *The Journal of endocrinology*, 204(1), 1-11.
4. Smith, G. I., Yoshino, J., Kelly, S. C., Reeds, D. N., Okunade, A., Patterson, B. W., Klein, S., & Mittendorfer, B. (2016). High-Protein Intake during Weight Loss Therapy Eliminates the Weight-Loss-Induced Improvement in Insulin Action in Obese Postmenopausal Women. *Cell reports*, 17(3), 849-861.
5. Lynch, C. J., & Adams, S. H. (2014). Branched-chain amino acids in metabolic signalling and insulin resistance. *Nature reviews. Endocrinology*, 10(12), 723-36.
6. Gran, P., & Cameron-Smith, D. (2011). The actions of exogenous leucine on mTOR signalling and amino acid transporters in human myotubes. *BMC physiology*, 11, 10.
7. Saxton, R. A., & Sabatini, D. M. (2017). mTOR Signaling in Growth, Metabolism, and Disease. *Cell*, 168(6), 960-976.
8. Teng, K. T., Chang, C. Y., Chang, L. F., & Nesaretnam, K. (2014). Modulation of obesity-induced inflammation by dietary fats: mechanisms and clinical evidence. *Nutrition journal*, 13, 12.
9. Shoelson, S. E., Lee, J., & Goldfine, A. B. (2006). Inflammation and insulin resistance. *The Journal of clinical investigation*, 116(7), 1793-801.
10. Belfiore A, Frasca F, Pandini G, Sciacca L, & Vigneri R.(2009). Insulin receptor isoforms and insulin receptor/insulinlike growth factor receptor hybrids in physiology and disease. *Endocr Rev*, 30, 586–623.
11. Mosthaf, L., Grako, K., Dull, T. J., Coussens, L., Ullrich, A., & McClain, D. A. (1990). Functionally distinct insulin receptors generated by tissue-specific alternative splicing. *The EMBO journal*, 9(8), 2409-13.
12. White, M.F., Livingston, J.N., Backer, J.M., Lauris, V., Dull, T.J., Ullrich, A. & Kahn, C.R. (1988). *Cell*, 54, 641–649.
13. Cai, D., Dhe-Paganon, S., Melendez, P.A., Lee, J. & Shoelson, S.E. (2003). Two New Substrates in Insulin Signaling. RS5/DOK4 and IRS6/DOK5. *J. Biol. Chem.* 278, 25323–25330
14. White, M.F. (2003) Insulin signaling in health and disease. *Science*, 302(5651), 1710–1711
15. Sun, X.J., Crimmins, D.L., Myers, Jr, M.G., Miralpeix, M. & White, M.F. (1993) *Mol. Cell. Biol.* 13, 7418–7428.
16. White M F. (1997). The insulin signalling system and the IRS proteins. *Diabetologia*, 40, 2-17.
17. Araki, E., Lipes, M.A., Patti, M.E., Bruning, J.C., Haag, 3rd, B., Johnson, R.S. & Kahn, C.R. (1994). Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. *Nature*, 372, 186–190.
18. Sun, X.J., Wang, L.M., Zhang, Y., Yenush, L., Myers, Jr, M.G., Glasheen, E., Lane, W.S., Pierce, J.H. & White, M.F. (1995) Role of IRS-2 in insulin and cytokine signalling. *Nature*, 377, 173–177.

19. Lin, X., Taguchi, A., Park, S., Kushner, J. A., Li, F., Li, Y., & White, M. F. (2004). Dysregulation of insulin receptor substrate 2 in beta cells and brain causes obesity and diabetes. *The Journal of clinical investigation*, 114(7), 908-16.
20. De Camilli, P., Emr, S.D., McPherson, P.S. & Novick, P. (1996). Phosphoinositides as regulators in membrane traffic. *Science*, 271, 1533–1539.
21. van der Kaay, J., Batty, I.H., Cross, D.A.E., Watt, P.W. & Downes, C.P. (1997). A novel, rapid, and highly sensitive mass assay for Phosphatidylinositol 3,4,5-Trisphosphate (PtdIns(3,4,5)P<sub>3</sub>) and Its Application to Measure Insulin-stimulated PtdIns(3,4,5)P<sub>3</sub> Production in Rat Skeletal Muscle *in Vivo*. *J. Biol. Chem.*, 272, 5477–5481.
22. Martin, S. S., Haruta, T., Morris, A.J., Klippel, A., Williams, L.T. & Olefsky, J. M. (1996). Activated phosphatidylinositol 3-kinase is sufficient to mediate actin rearrangement and GLUT4 translocation in 3T3-L1 adipocytes. *J Biol Chem* 271, 17605-8.
23. Sarbassov DD, Guertin DA, Ali SM, & Sabatini DM. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*, 307, 1098–1101.
24. Oh WJ, Jacinto E. (2011). mTOR complex 2 signaling and functions. *Cell Cycle*, 10, 2305–2316.
25. Walker, K. S., Deak, M., Paterson, A., Hudson, K., Cohen, P., & Alessi, D. R. (1998). Activation of protein kinase B beta and gamma isoforms by insulin *in vivo* and by 3-phosphoinositide-dependent protein kinase-1 *in vitro*: comparison with protein kinase B alpha. *The Biochemical journal*, 331, 299-308.
26. Sano H, Kane S, Sano E, Miinea CP, Asara JM, Lane WS, Garner CW, & Lienhard GE. (2003). Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *J Biol Chem*, 278, 14599–14602
27. Myers MG Jr, Backer JM, Sun XJ, Shoelson S, Hu P, Schlessinger J, Yoakim M, Schaffhausen B, White MF. (1992). IRS-1 activates phosphatidylinositol 30-kinase by associating with src homology 2 domains of p85. *Proc Natl Acad Sci*, 89, 10350–10354.
28. Shaw LM. (2011). The insulin receptor substrate (IRS) proteins: At the intersection of metabolism and cancer. *Cell Cycle*, 10, 1750–1756.
29. Pearce LR, Komander D, & Alessi DR. (2010). The nuts and bolts of AGC protein kinases. *Nat Rev Mol Cell Biol*, 11, 9–22.
30. Bayascas JR. (2010). PDK1: The major transducer of PI 3-kinase actions. *Curr Top Microbiol Immunol*, 346, 9–29.
31. Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, & Cohen P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase, which phosphorylates and activates protein kinase Ba. *Curr Biol* 7, 261–269.
32. Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB III, Kaestner KH, Bartolomei MS, Shulman GI, & Birnbaum MJ. (2001). Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2(PKB b). *Science*, 292, 1728–1731
33. Duvel K, Yecies JL, Menon S, Raman P, Lipovsky AI, Souza AL, Triantafellow E, Ma Q, Gorski R, & Cleaver S. (2010). Activation of a metabolic gene regulatory network downstream of mTOR complex 1. *Mol Cell*, 39, 171–183.
34. Tzivion G, Dobson M, & Ramakrishnan G. (2011). FoxO transcription factors; Regulation by AKT and 14–3–3 proteins. *Biochim Biophys Acta*, 1813, 1938–1945.
35. Lu M, Wan M, Leavens KF, Chu Q, Monks BR, Fernandez S, Ahima RS, Ueki K, Kahn CR, & Birnbaum MJ. (2012). Insulin regulates liver metabolism *in vivo* in the absence of hepatic Akt and Foxo1. *Nat Med*, 18, 388–395.

36. Kim KH, Song JJ, Yoo EJ, Choe SS, Park SD, & Kim JB. (2004). Regulatory role of glycogen synthase kinase 3 for transcriptional activity of ADD1/SREBP1c. *J Biol Chem*, 279, 51999–52006.
37. Cantley LC, & Neel BG. (1999). New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci*, 96, 4240–4245.
38. Carracedo A, & Pandolfi PP. (2008). The PTEN-PI3K pathway: Of feedbacks and cross-talks. *Oncogene*, 27, 5527–5541.
39. Stiles B, Wang Y, Stahl A, Bassilian S, Lee WP, Kim YJ, Sherwin R, Devaskar S, Lesche R, Magnuson MA, et al. (2004). Liver-specific deletion of negative regulator Pten results in fatty liver and insulin hypersensitivity. *Proc Natl Acad Sci*, 101, 2082–2087.
40. Kurlawalla-Martinez C, Stiles B, Wang Y, Devaskar SU, Kahn BB, & Wu H. (2005). Insulin hypersensitivity and resistance to streptozotocin-induced diabetes in mice lacking PTEN in adipose tissue. *Mol Cell Biol*, 25, 2498–2510.
41. Kahn CR, Flier JS, Bar RS, Archer JA, Gorden P, Martin MM, & Roth J. (1976). The syndromes of insulin resistance and acanthosis nigricans. Insulin-receptor disorders in man. *N Engl J Med*, 294, 739–745
42. Cochran E, Musso C, Gorden P. (2005). The use of U-500 in patients with extreme insulin resistance. *Diabetes Care*, 28, 1240–1244.
43. Taylor SI, Accili D, Cama A, Kadowaki H, Kadowaki T, Imano E, Sierra ML. (1991). Mutations in the insulin receptor gene in patients with genetic syndromes of insulin resistance. *Adv Exp Med Biol*, 293, 197–213.
44. Haruta T, Imamura T, Iwanishi M, Egawa K, Goji K, & Kobayashi M. (1995). Amplification and analysis of promoter region of insulin receptor gene in a patient with leprechaunism associated with severe insulin resistance. *Metabolism*, 44, 430–437.
45. Burguete-Garcia AI, Cruz-Lopez M, Madrid-Marina V, Lopez-Ridaura R, Hernandez-Avila M, Cortina B, Gomez RE, & Velasco-Mondragon E. (2010). Association of Gly972Arg polymorphism of IRS1 gene with type 2 diabetes mellitus in lean participants of a national health survey in Mexico: A candidate gene study. *Metabolism*, 59, 38–45.
46. Martinez-Gomez LE, Cruz M, Martinez-Nava GA, Madrid-Marina V, Parra E, Garcia-Mena J, Espinoza-Rojo M, Estrada-Velasco BI, Piza-Roman LF, & Aguilera P, et al. (2011) A replication study of the IRS1, CAPN10, TCF7L2, and PPARG gene polymorphisms associated with type 2 diabetes in two different populations of Mexico. *Ann Hum Genet*, 75, 612–620.
47. Esposito DL, Li Y, Vanni C, Mammarella S, Veschi S, Della LF, Mariani-Costantini R, Battista P, Quon MJ, Cama A. (2003). A novel T608R missense mutation in insulin receptor substrate-1 identified in a subject with type 2 diabetes impairs metabolic insulin signaling. *J Clin Endocrinol Metab*, 88, 1468–1475.
48. George S, Rochford JJ, Wolfrum C, Gray SL, Schinner S, Wilson JC, Soos MA, Murgatroyd PR, Williams RM, Acerini CL, et al. (2004). A family with severe insulin resistance and diabetes due to a mutation in AKT2. *Science*, 304, 1325–1328.
49. Prudente S, Hribal ML, Flex E, Turchi F, Morini E, De Cosmo S, Bacci S, Tassi V, Cardellini M, Lauro R, et al. (2005). The functional Q84R polymorphism of mammalian Tribbles homolog TRB3 is associated with insulin resistance and related cardiovascular risk in Caucasians from Italy. *Diabetes*, 54, 2807–2811.
50. Prudente S, Scarpelli D, Chandalia M, Zhang YY, Morini E, Del Guerra S, Perticone F, Li

- R, Powers C, Andreozzi F, et al. (2009). The TRIB3 Q84R polymorphism and risk of early-onset type 2 diabetes. *J Clin Endocrinol Metab*, 94,190–196.
51. Dash S, Sano H, Rochford JJ, Semple RK, Yeo G, Hyden CS, Soos MA, Clark J, Rodin & A, Langenberg C. (2009). A truncation mutation in TBC1D4 in a family with acanthosis nigricans and postprandial hyperinsulinemia. *Proc Natl Acad Sci*, 106, 9350–9355.
  52. Osborn O, Olefsky JM. (2012). The cellular and signaling networks linking the immune system and metabolism in disease. *Nat Med*, 18, 363–374.
  53. Kamei N, Tobe K, Suzuki R, Ohsugi M, Watanabe T, Kubota N, Ohtsuka-Kowatari N, Kumagai K, Sakamoto K, Kobayashi M, et al. (2006). Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. *J Biol Chem*, 281, 26602–26614.
  54. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K, et al. (2006). MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest*, 116, 1494–1505.
  55. Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, Charo I, Leibel RL, & Ferrante AWJr. (2006). CCR2 modulates inflammatory and metabolic effects of highfat feeding. *J Clin Invest*, 116, 115–124.
  56. Aguirre, V., Uchida, T., Yenush, L., Davis, R. & White, M.F. (2000). The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *J. Biol. Chem.* 275, 9047–9054
  57. Akira S, & Takeda K. (2004). Toll-like receptor signalling. *Nat Rev Immunol.*, 4, 499–511.
  58. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. (2006). TLR4 links innate immunity and fatty acid-induced insulin resistance. *J Clin Invest*, 116, 3015–3025.
  59. Kleinridders A, Schenten D, Konner AC, Belgardt BF, Mauer J, Okamura T, Wunderlich FT, Medzhitov & R, Bruning JC. (2009). MyD88 signaling in the CNS is required for development of fatty acid-induced leptin resistance and diet-induced obesity, *Cell Metab*, 10, 249–259
  60. Himes RW, & Smith CW. (2010). Tlr2 is critical for diet-induced metabolic syndrome in a murine model. *FASEB J*, 24, 731–739.
  61. Ferreira LD, Pulawa LK, Jensen DR, & Eckel RH. (2001). Overexpressing human lipoprotein lipase in mouse skeletal muscle is associated with insulin resistance. *Diabetes* 50, 1064–1068.
  62. Schenk S, Saberi M, Olefsky JM. (2008). Insulin sensitivity: Modulation by nutrients and inflammation. *J Clin Invest*, 118, 2992–3002.
  63. Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Gorgun C, Glimcher LH, Hotamisligil GS. (2004). Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science*, 306, 457–461.
  64. Kim JK, Kim YJ, Fillmore JJ, Chen Y, Moore I, Lee J, Yuan M, Li ZW, Karin M, & Perret P. (2001). Prevention of fat induced insulin resistance by salicylate. *J Clin Invest*, 108, 437–446.
  65. Sinha S, Perdomo G, Brown NF, O’Doherty RM. (2004). Fatty acid-induced insulin resistance in L6 myotubes is prevented by inhibition of activation and nuclear localization of nuclear factor κB. *J Biol Chem*, 279: 41294–41301.
  66. Yu C, Chen Y, Zong H, Wang Y, Bergeron R, Kim JK, Cline GW, Cushman SW, Cooney GJ, & Atcheson B. (2002). Mechanism by which fatty acids inhibit insulin activation of



- IRS-1 associated phosphatidylinositol 3-kinase activity in muscle. *J Biol Chem*, 277, 50230–50236.
67. Liu L, Zhang Y, Chen N, Shi X, Tsang B, & Yu BH. (2007). Upregulation of myocellular DGAT1 augments triglyceride synthesis in skeletal muscle and protects against fat induced insulin resistance. *J Clin Invest*, 117, 1679–1689.
  68. Ahmadian M, Duncan RE, Varady KA, Frasson D, Hellerstein MK, Birkenfeld AL, Samuel VT, Shulman GI, Wang Y, & Kang C. (2009). Adipose overexpression of desnutrin promotes fatty acid use and attenuates diet-induced obesity. *Diabetes*, 58, 855–866.
  69. Samuel VT, Petersen KF & Shulman GI. (2010). Lipid-induced insulin resistance: Unravelling the mechanism. *Lancet*, 375, 2267–2277.
  70. Westwick JK, Bielawska AE, Dbaiibo G, Hannun YA, & Brenner DA. (1995). Ceramide activates the stress-activated protein kinases. *J Biol Chem*, 270, 22689–22692.
  71. Teruel T, Hernandez R, & Lorenzo M. (2001). Ceramide mediates insulin resistance by tumor necrosis factor- $\alpha$  in brown adipocytes by maintaining Akt in an inactive dephosphorylated state. *Diabetes*, 50, 2563–2571.
  72. Powell DJ, Hajduch E, Kular G, & Hundal HS. (2003). Ceramide disables 3-phosphoinositide binding to the pleckstrin homology domain of protein kinase B (PKB)/Akt by a PKC $\zeta$ -dependent mechanism. *Mol Cell Biol*, 23, 7794–7808.
  73. Blouin CM, Prado C, Takane KK, Lasnier F, Garcia-Ocana A, Ferre P, Dugail I, & Hajduch E. (2010). Plasma membrane subdomain compartmentalization contributes to distinct mechanisms of ceramide action on insulin signaling. *Diabetes*, 59, 600–610.
  74. Mahadev K, Zilbering A, Zhu L, & Goldstein BJ. (2001). Insulin-stimulated hydrogen peroxide reversibly inhibits protein tyrosine phosphatase 1b in vivo and enhances the early insulin action cascade. *J Biol Chem*, 276, 21938–21942.
  75. Krieger-Brauer HI & Kather H. (1992). Human fat cells possess a plasma membrane-bound H<sub>2</sub>O<sub>2</sub>-generating system that is activated by insulin via a mechanism bypassing the receptor kinase. *J Clin Invest*, 89, 1006–1013.
  76. Chang YC, Chuang LM. (2010). The role of oxidative stress in the pathogenesis of type 2 diabetes: From molecular mechanism to clinical implication. *Am J Transl Res*, 2, 316–331.
  77. Rudich A, Tirosh A, Potashnik R, Hemi R, Kanety H, & Bashan N. (1998). Prolonged oxidative stress impairs insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. *Diabetes*, 47, 1562–1569.
  78. Dokken BB, Saengsirisuwan V, Kim JS, Teachey MK, & Henriksen EJ. (2008). Oxidative stress-induced insulin resistance in rat skeletal muscle: Role of glycogen synthase kinase-3. *Am J Physiol Endocrinol Metab*, 294, E615–E621.
  79. Evans JL, Maddux BA, & Goldfine ID. (2005). The molecular basis for oxidative stress-induced insulin resistance. *Antioxid Redox Signal*, 7, 1040–1052.
  80. Hager SR, Jochen AL, & Kalkhoff RK. (1991). Insulin resistance in normal rats infused with glucose for 72 h. *Am J Physiol*, 260, E353–E362.
  81. Leahy JL, Cooper HE, Deal DA, & Weir DG. (1986). Chronic hyperglycemia is associated with impaired glucose influence on insulin secretion. A study in normal rats using chronic in vivo glucose infusions. *J Clin Invest*, 77, 908–915.
  82. Marshall S, Bacote V, & Traxinger RR. (1991). Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system. Role of hexosamine biosynthesis in the induction of insulin resistance. *J Biol Chem*, 266, 4706–4712.

83. Patti ME, Virkamaki A, Landaker EJ, Kahn CR, & Yki-Jarvinen H. (1999). Activation of the hexosamine pathway by glucosamine in vivo induces insulin resistance of early postreceptor insulin signaling events in skeletal muscle. *Diabetes*, 48, 1562–1571.
84. McClain DA. (2002). Hexosamines as mediators of nutrient sensing and regulation in diabetes. *J Diabetes Complications*, 16, 72–80.
85. McClain DA, Lubas WA, Cooksey RC, Hazel M, Parker GJ, Love DC, Hanover JA. (2002). Altered glycan-dependent signaling induces insulin resistance and hyperleptinemia. *Proc Natl Acad Sci*, 99, 10695–10699.
86. Xia P, Inoguchi T, Kern TS, Engerman RL, & Oates PJ, King GL. (1994). Characterization of the mechanism for the chronic activation of diacylglycerol-protein kinase C pathway in diabetes and hypergalactosemia. *Diabetes*, 43, 1122–1129.
87. Miele C, Riboulet A, Maitan MA, Oriente F, Romano C, Formisano P, Giudicelli J, Beguinot F, & Van Obberghen E. (2003). Human glycated albumin affects glucose metabolism in L6 skeletal muscle cells by impairing insulin-induced insulin receptor substrate (IRS) signaling through a protein kinase C  $\alpha$ -mediated mechanism. *J Biol Chem* 278, 47376–47387.
88. Cassese A, Esposito I, Fiory F, Barbagallo AP, Paturzo F, Mirra P, Ulianich L, Giacco F, Iadicicco C, & Lombardi A. (2008). In skeletal muscle advanced glycation end products (AGEs) inhibit insulin action and induce the formation of multimolecular complexes including the receptor for AGEs. *J Biol Chem*, 283, 36088–36099.
89. Zoncu R, Efeyan A & Sabatini DM. (2011). mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Bio*, 12, 21–35.
90. Sarbassov DD, Ali SM, & Sabatini DM. (2005). Growing roles for the mTOR pathway. *Curr Opin Cell Biol*, 17, 596–603.
91. Wullschlegel S, Loewith R, & Hall MN. (2006). TOR signaling in growth and metabolism. *Cell*, 124, 471–484.
92. Wolfson, RL, & Sabatini DM. (2017). The Dawn of the Age of Amino Acid Sensors for the mTORC1 Pathway. *Cell Metabolism*, 26, 301-309.
93. (2011). mTOR Pathway and mTOR Inhibitors in Cancer Therapy. *British Journal of Clinical Pharmacology*, 71(6), 970.
94. Ruvinsky I, & Meyuhas O. (2006) Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. *Trends Biochem Sci*, 31, 342–348
95. Avruch J, Belham C, Weng Q, Hara K, & Yonezawa K. (2001). The p70 S6 kinase integrates nutrient and growth signals to control translational capacity. *Prog Mol Subcell Biol*, 26, 115–154
96. Showkat, M., Beigh, M. A., & Andrabi, K. I. (2014). mTOR Signaling in Protein Translation Regulation: Implications in Cancer Genesis and Therapeutic Interventions. *Molecular biology international*, 686984.
97. Yoon, M. S., & Choi, C. S. (2016). The role of amino acid-induced mammalian target of rapamycin complex 1(mTORC1) signaling in insulin resistance. *Experimental & molecular medicine*, 48(1), e201. doi:10.1038/emm.2015.93
98. Buerger, C., DeVries, B., & Stambolic, V. (2006). Localization of Rheb to the endomembrane is critical for its signaling function. *Biochem. Biophys. Res. Commun.* 344, 869–880.
99. Saito, K., Araki, Y., Kontani, K., Nishina, H., & Katada, T. (2005). Novel role of the small GTPase Rheb: its implication in endocytic pathway independent of the activation of

- mammalian target of rapamycin. *J. Biochem.*, 137, 423–4.
- Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar- Peled, L., & Sabatini, D.M. (2008). The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* 320, 1496–1501.
100. Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar- Peled, L., & Sabatini, D.M. (2008). The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* 320, 1496–1501.
101. Wolfson, R.L., Chantranupong, L., Saxton, R.A., Shen, K., Scaria, S.M., Cantor, J.R., and Sabatini, D.M. (2016). Sestrin2 is a leucine sensor for the mTORC1 pathway. *Science*, 351, 43–48.
102. Chantranupong, L., Scaria, S.M., Saxton, R.A., Gygi, M.P., Shen, K., Wyant, G.A., Wang, T., Harper, J.W., Gygi, S.P., and Sabatini, D.M. (2016). The CASTOR proteins are arginine sensors for the mTORC1 pathway. *Cell*, 165, 153–164.
103. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, & Lien LF. (2009). A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab*, 9, 311–326.
104. McCormack SE, Shaham O, McCarthy MA, Deik AA, Wang TJ, & Gerszten RE,. (2013) Circulating branched-chain amino acid concentrations are associated with obesity and future insulin resistance in children and adolescents. *Pediatr Obes*, 8, 52–61.
105. Lackey DE, Lynch CJ, Olson KC, Mostaedi R, Ali M, & Smith WH. (2003). Regulation of adipose branched-chain amino acid catabolism enzyme expression and cross-adipose amino acid flux in human obesity. *Am J Physiol Endocrinol Metab*, 304, E1175–E1187.
106. Badoud F, Lam KP, DiBattista A, Perreault M, Zulyniak MA, & Cattrysse B. (2014). Serum and adipose tissue amino acid homeostasis in the metabolically healthy obese. *J Proteome Res*, 13, 3455–3466.
107. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, & McCabe E. (2011) Metabolite profiles and the risk of developing diabetes. *Nat Med*, 17, 448–453.
108. Vandana, S. (2010). Molecular mechanisms of insulin resistance in type 2 diabetes mellitus. *World journal of diabetes*, 1(3), 68-75.
109. Morino, K., Neschen, S., Bilz, S., Sono, S., Tsigiriotis, D., Reznick, R. M., Moore, I., Nagai, Y., Samuel, V., Sebastian, D., White, M., Philbrick, W., ... Shulman, G. I. (2008). Muscle-specific IRS-1 Ser->Ala transgenic mice are protected from fat-induced insulin resistance in skeletal muscle. *Diabetes*, 57(10), 2644-51.
110. McCarthy, M., & Menzel, S. (2001). The genetics of type 2 diabetes. *British journal of clinical pharmacology*, 51(3), 195-9.
111. National Institute of Health. (2009). Insulin Resistance and Pre-diabetes. *Diabetes*, 09.
112. Park, B. S., & Yoon, J. S. (2013). Relative skeletal muscle mass is associated with development of metabolic syndrome. *Diabetes & metabolism journal*, 37(6), 458-64.
113. Jingyi Lu, Guoxiang Xie, & Weiping Jia, Wei Jia. (2013). Insulin resistance and the metabolism of branched-chain amino acids. *Front Med.*, 7, 53-59
114. Cole J.T. (2015) Metabolism of BCAAs. In: Rajendram R., Preedy V., Patel V. (eds) Branched Chain Amino Acids in Clinical Nutrition. *Nutrition and Health*, 2.
115. Kingsbury, J. M., Sen, N. D., & Cardenas, M. E. (2015). Branched-Chain Aminotransferases Control TORC1 Signaling in *Saccharomyces cerevisiae*. *PLoS genetics*, 11(12), e1005714.
116. Xu, G., Kwon, G., Cruz, W.S., Marshall, C.A., McDaniel, M.L. (2001) Metabolic

- Regulation by Leucine of Translation Initiation Through the mTOR-Signaling Pathway by Pancreatic  $\beta$ -Cells. *Diabetes*, 50(2), 353-360.
117. Sengupta, S., Peterson, T. R., & Sabatini, D. M. (2010). Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. *Molecular cell*, 40(2), 310-22.
  118. (2016). The Emerging Role of Branched-Chain Amino Acids in Insulin Resistance and Metabolism. *Nutrients*, 8(7), 405. doi:10.3390/nu8070405
  119. Reitman, A., Schwarz, J., Tomé, D., Kok, F.J., & Mensink, M. (2014). High dietary protein, reducing, or eliciting insulin resistance? *European Journal of Clinical Nutrition*, 68, 973-979.
  120. James O. Hill, Edward L. Melanson & Holly T. Wyatt. (2000) Dietary Fat Intake and Regulation of Energy Balance: Implications for Obesity. *The Journal of Nutrition*, 130, 284-288.
  121. Zierath, J. R., Houseknecht, K. L., Gnudi, L., and Kahn, B. B. (1997). High fat feeding impairs insulin-stimulated GLUT4 recruitment via an early insulin-signaling defect. *Diabetes* 46, 215–223
  122. Carruthers, A., and Melchior, D. L. (1984). Study of the relationship between bilayer water permeability and bilayer physical state. *Biochemistry*, 23, 6901–6911.
  123. Sandra A, Fyler DJ & Marshall SJ. (1984). Effects of lipids on the transport activity of the reconstituted glucose transport system from rat adipocyte. *Biochim Biophys Acta*, 778, 511-515.
  124. Borkman, M., Storlien, L. H., Pan, D. A., Jenkins, A. B., Chisholm, D. J., and Campbell, L. V. (1993) The relation between insulin sensitivity and the fatty-acid composition of skeletal-muscle phospholipids. *N. Engl. J. Med.* 328, 238–244.
  125. R. W. Stevenson, R. K. McPherson, L.M. Persson, P.E. Genereux, A.G. Swick, J. Spitzer, J.J. Herbst, K.M. Andrews, D.K. Kreutter & E.M. Gibbs. (1996). The antihyperglycemic agent englitazone prevents the defect in glucose transport in rats fed a high fat diet. *Diabetes*, 45, 60-66.
  126. Rosholt, M.N., King, P. A., & Horton, E.S. (1994). High-fat diet reduces glucose transporter responses to both insulin and exercise. *Am. J. Physiol.* 266, R95–R101
  127. Reaven, G.M., Hollenbeck, C., Jeng, C.-Y., Wu, M.S., & Chen, Y.-D. (1988). Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM. *Diabetes*, 37, 1020–1024.
  128. Frayne, K.N. (1993). Insulin resistance and lipid metabolism. *Curr. Opin. Lipidol.* 4. 97-204.
  129. McGarry, J.D. (1992). What if Minkowski had been ageusic? An alternative angle on diabetes. *Science*, 258, 766–770.
  130. Boden, G., Chen, X., Ruiz, J., White, J.V., and Rosetti, L. (1994). Mechanism of fatty acid induced inhibition of glucose uptake. *J. Clin. Invest*, 93, 2438–2446.
  131. Perseghin, G., Ghosh, S., Gerow, K., and Shulman, G.I. (1997). Metabolic defects in lean nondiabetic offspring of NIDDM parents: a cross-sectional study. *Diabetes*. 46,1001–1009.
  132. Randle, P.J., Garland, P.B., Hales, C.N., and Newsholme, E.A. (1963). The glucose fatty-acid cycle: its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet.*, 785–789.
  133. Randle, P.J., Garland, P.B., Newsholme, E.A., & Hales, C.N. (1965). The glucose fatty-acid cycle in obesity and maturity onset diabetes mellitus. *Ann. NY Acad. Sci.* 131, 324–333.

134. Randle, P.J., Newsholme, E.A., and Garland, P.B. (1964). Regulation of glucose uptake by muscle. Effects of fatty acids, ketone bodies and pyruvate, and of alloxan, diabetes and starvation, on the uptake and metabolic fate of glucose in rat heart and diaphragm muscles. *Biochem. J.* 93, 652–665.
135. Dresner, A., Laurent, D., Marcucci, M., Griffin, M. E., Dufour, S., Cline, G. W., Slezak, L. A., Andersen, D. K., Hundal, R. S., Rothman, D. L., Petersen, K. F., ... Shulman, G. I. (1999). Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *The Journal of clinical investigation*, 103(2), 253-9.
136. Griffin, M.E., et al. (1999). Free fatty acid-induced insulin resistance is associated with activity of protein kinase C theta and alterations in the insulin signaling cascade. *Diabetes*, 48, 1270–1274.
137. Shulman, G. (2000). Cellular mechanisms of insulin resistance. *The Journal of clinical investigation*, 106(2), 171-6.
138. Kahn, B. B., & Flier, J. S. (2000). Obesity and insulin resistance. *The Journal of clinical investigation*, 106(4), 473-81.
139. Neshler R, Karl IE, Kipnis DM. (1985). Dissociation of effects of insulin and contraction on glucose transport in rat epitrochlearis muscle. *Am. J. Physiol.*, 249, 226-232
140. Lund, S., Holman, G. D., Schmitz, O., & Pedersen, O. (1995). Contraction stimulates translocation of glucose transporter GLUT4 in skeletal muscle through a mechanism distinct from that of insulin. *Proceedings of the National Academy of Sciences of the United States of America*, 92(13), 5817-21.
141. Lee AD, Hansen PA, Holloszy JO. (1995). Wortmannin inhibits insulin-stimulated but not contraction-stimulated glucose transport activity in skeletal muscle. *FEBS Lett.* 361, 51-54.
142. Yeh JI, Gulve EA, Rameh L, Birnbaum MJ. (1995). The effects of wortmannin on rat skeletal muscle. Dissociation of signaling pathways for insulin- and contraction-activated hexose transport. *J. Biol. Chem*, 270, 2107-2111
143. Houmard JA, Shinebarger MH, Dolan PL, Leggett-Frazier N, Bruner RK, et al. (1993). Exercise training increases GLUT-4 protein concentration in previously sedentary middle-aged men. *Am. J. Physiol. Endocrinol. Metab.*, 264, 896-901.
144. Houmard JA, Egan PC, Neufer PD, Friedman JE, Wheeler WS, et al. (1991). Elevated skeletal muscle glucose transporter levels in exercise-trained middle-aged men. *Am. J. Physiol. Endocrinol. Metab.* 261, 437-443.
145. Dela F, Ploug T, Handberg A, Petersen LN, Larsen JJ, et al. (1994). Physical training increases muscle GLUT4 protein and mRNA in patients with NIDDM. *Diabetes*, 43, 862-865.
146. Weickert, M. (2012). Nutritional modulation of insulin resistance. *Scientifica*, 2012, 424780.
147. Laplante, M., & Sabatini, D. M. (2009). mTOR signaling at a glance. *Journal of cell science*, 122(Pt 20), 3589-94.
148. Park, B. S., & Yoon, J. S. (2013). Relative skeletal muscle mass is associated with development of metabolic syndrome. *Diabetes & metabolism journal*, 37(6), 458-64.
149. Dobson, M., Ramakrishnan, G., Ma, S., Kaplun, L., Balan, V., Fridman, R., & Tzivion, G. (2011). Bimodal regulation of FoxO3 by AKT and 14-3-3. *Biochimica et biophysica acta*, 1813(8), 1453-64.

150. Astrup, A., Raben, A., & Geiker, N. (2015). The role of higher protein diets in weight control and obesity-related comorbidities. *International journal of obesity (2005)*, 39(5), 721-6.
151. (2011). Emerging perspectives on essential amino acid metabolism in obesity and the insulin-resistant state. *Advances in nutrition (Bethesda, Md.)*, 2(6), 445-56.
152. Menni, C., Fauman, E., Erte, I., Perry, J. R., Kastenmüller, G., Shin, S. Y., Petersen, A. K., Hyde, C., Psatha, M., Ward, K. J., Yuan, W., Milburn, M., Palmer, C. N., Frayling, T. M., Trimmer, J., Bell, J. T., Gieger, C., Mohny, R. P., Brosnan, M. J., Suhre, K., Soranzo, N., & Spector, T. D. (2013). Biomarkers for type 2 diabetes and impaired fasting glucose using a nontargeted metabolomics approach. *Diabetes*, 62(12), 4270-6.
153. McCormack, S. E., Shaham, O., McCarthy, M. A., Deik, A. A., Wang, T. J., Gerszten, R. E., Clish, C. B., Mootha, V. K., Grinspoon, S. K., ... Fleischman, A. (2012). Circulating branched-chain amino acid concentrations are associated with obesity and future insulin resistance in children and adolescents. *Pediatric obesity*, 8(1), 52-61.
154. Mahshid Dehghan, Andrew Mente, Xiaohe Zhang, Sumathi Swaminathan, Wei Li, Viswanathan Mohan, Romaina Iqbal, Rajesh Kumar, Edelweiss Wentzel-Viljoen, Annika Rosengren, Leela Itty Amma, Alvaro Avezum, Jephth Chifamba, Rafael Diaz, Rasha Khatib, Scott Lear, Patricio Lopez-Jaramillo, Xiaoyun Liu, Rajeev Gupta, Noushin Mohammadifard, Nan Gao, Aytakin Oguz, Anis Safura Ramli, Pamela Seron, Yi Sun, Andrzej Szuba, Lungiswa Tsolekile, Andreas Wielgosz, Rita Yusuf, Afzal Hussein Yusufali, Koon K Teo, Sumathy Rangarajan. (2017). Associations of fats and carbohydrate intake with cardiovascular disease and mortality in 18 countries from five continents (PURE): a prospective cohort study. *The Lancet*, 390, 2050–62.
155. Bessesen D.H. (2001). The Role of Carbohydrates in Insulin Resistance. *The Journal of Nutrition*, 131, 2782S-2786S.
156. Zhang X, Tang N, Hadden TJ, and Rish AK. (2011). Akt, FoxO and regulation of apoptosis. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1813, 1978-1986.
157. F. Pérez-Jiménez, J. López-Miranda, M.D. Pinillos, P. Gómez, E. Paz-Rojas, P. Montilla C. Marín, M. J. Velasco, A. Blanco-Molin, J. A. Jiménez Perepérez, and J. M. Ordovas. (2001) A Mediterranean and a high-carbohydrate diet improve glucose metabolism in healthy young persons. *Diabetologia*, 44, 2038-2043.
158. Alexis Ruiz, Erez Dror, Christoph Handschin, Regula Furrer, Joaquin Perez-Schindler, Christoph Bachmann, Susan Treves & Francesco Zorzato. (2018) Over-expression of a retinol dehydrogenase (SRP35/DHRS7C) in skeletal muscle activates mTORC2, enhances glucose metabolism and muscle performance. *Scientific Reports*, 8, 1-14.
159. Henning F. Kramer, Carol A. Witczak, Nobuharu Fujii, Niels Jessen, Eric B. Taylor, David E. Arnolds, Kei Sakamoto, Michael F. Hirshman and Laurie J. Goodyear. (2006). Distinct Signals Regulate AS160 Phosphorylation in Response to Insulin, AICAR, and Contraction in Mouse Skeletal Muscle. *American Diabetes Association*, 55, 2067-2076.
160. Dash, S., Sano, H., Rochford, J. J., Semple, R. K., Yeo, G., Hyden, C. S., Soos, M. A., Clark, J., Rodin, A., Langenberg, C., Druet, C., Fawcett, K. A., Tung, Y. C., Wareham, N. J., Barroso, I., Lienhard, G. E., O'Rahilly, S., Savage, D. B. (2009). A truncation mutation in TBC1D4 in a family with acanthosis nigricans and postprandial hyperinsulinemia. *Proceedings of the National Academy of Sciences of the United States of America*, 106(23), 9350-5.

161. Guertin, D. A., Stevens, D. M., Thoreen, C. C., Burds, A. A., Kalaany, N. Y., Moffat, J., Brown, M., Fitzgerald, K. J. and Sabatini, D. M. (2006). Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. *Dev. Cell*, 11, 859-871.
162. B. Raught, F. Peiretti, A.-C. Gingras et al. (2004). Phosphorylation of eukaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases. *The EMBO Journal*, 23(8), 1761–1769.
163. Yang, H. S., Jansen, A. P., Komar, A. A., Zheng, X., Merrick, W. C., Costes, S., Lockett, S. J., Sonenberg, N., ... Colburn, N. H. (2003). The transformation suppressor Pcd4 is a novel eukaryotic translation initiation factor 4A binding protein that inhibits translation. *Molecular and cellular biology*, 23(1), 26-37.
164. Wang, X., Li, W., Williams, M., Terada, N., Alessi, D. R., & Proud, C. G. (2001). Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. *The EMBO journal*, 20(16), 4370-9.
165. Douen AG, Ramlal T, Cartee GD, Klip A. (1990). Exercise modulates the insulin-induced translocation of glucose transporters in rat skeletal muscle. *FEBS Lett.*, 261, 256-260.
166. Goodyear LJ, Hirshman MF, Smith RJ, Horton ES. (1991). Glucose transporter number, activity and isoform content in plasma membranes of red and white skeletal muscle. *Am. J. Physiol.* 261, 556-561
167. Goodyear LJ, King PA, Hirshman MF, Thompson CM, Horton ED, et al. (1990). Contractile activity increases plasma membrane glucose transporters in absence of insulin. *Am. J. Physiol.* 258, 667-672
168. Hirshman MF, Goodyear LJ, Wardzala LJ, Horton ED, Horton ES. (1990). Identification of an intracellular pool of glucose transporters from basal and insulin-stimulated rat skeletal muscle. *J. Biol. Chem.* 265, 987-991.
169. Garetto LP, Richter EA, Goodman MN, Ruderman NB. (1984). Enhanced muscle glucose metabolism after exercise in the rat: the two phases. *Am. J. Physiol.* 246(9), 471-475
170. Wallberg-Henriksson H, Constable SH, Young DA, Holloszy JO. (1988). Glucose transport into rat skeletal muscle: interaction between exercise and insulin. *J. Appl. Physiol.*, 65, 909-913
171. Zorzano A, Balon TW, Goodman MN, Ruderman NB. (1986). Additive effects of prior exercise and insulin on glucose and AIB uptake by rat muscle. *Am. J. Physiol.* 251(14), 21-26
172. Perng W, Gillman MW, Fleisch AF, Michalek RD, Watkins SM, Isganaitis E, Patti ME, Oken E. (2014). Metabolomic profiles and childhood obesity. *Obesity.* 22, 2570–2578.
173. Giesbertz P, Padberg I, Rein D, Ecker J, Höfle AS, Spanier B, & Daniel H. (2015). Metabolite profiling in plasma and tissues of ob/ob and db/db mice identifies novel markers of obesity and type 2 diabetes. *Diabetologia*, 58, 2133–43,
174. Moghei M, Tavajohi-Fini P, Beatty B, Adegoke OA. (2016). Ketoisocaproic acid, a metabolite of leucine, suppresses insulin-stimulated glucose transport in skeletal muscle cells in a BCAT2-dependent manner. *Am J Physiol Cell Physiol*, 311(3), 518-527.
175. David M. Sabatini. (2017) Twenty-five years of mTOR: Uncovering the link from nutrients to growth. *PNAS*, 114, 11818-11825.
176. Peterson, T. R., Laplante, M., Thoreen, C. C., Sancak, Y., Kang, S. A., Kuehl, W. M., Gray, N. S., & Sabatini, D. M. (2009). DEPTOR is an mTOR inhibitor frequently

- overexpressed in multiple myeloma cells and required for their survival. *Cell*, 137(5), 873-86.
177. Wang, L., Harris, T. E., Roth, R. A. and Lawrence, J. C., Jr. (2007). PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding. *J. Biol. Chem.*, 282, 20036-20044.
  178. Liu YF, Herschkovitz A, Boura-Halfon S, Ronen D, Paz K, LeRoith D, Zick Y. (2004). Serine phosphorylation proximal to its phosphotyrosine binding domain inhibits insulin receptor substrate 1 function and promotes insulin resistance. *Mol Cell Biol* 24, 9668–9681.
  179. Lian K, Du C, Liu Y, Zhu D, Yan W, et al. (2015) Impaired adiponectin signaling contributes to disturbed catabolism of branched-chain amino acids in diabetic mice. *Diabetes*, 64, 49–59
  180. Doisaki M, Katano Y, Nakano I, et al. (2010). Regulation of hepatic branched-chain alpha-keto acid dehydrogenase kinase in a rat model for type 2 diabetes mellitus at different stages of the disease. *Biochem Biophys Res Commun*, 393, 303–307
  181. Bajotto G, Murakami T, Nagasaki M, Sato Y, Shimomura Y. (2009) Decreased enzyme activity and contents of hepatic branched-chain alpha-keto acid dehydrogenase complex subunits in a rat model for type 2 diabetes mellitus. *Metabolism*, 58, 1489–1495.
  182. She, P., Van Horn, C., Reid, T., Hutson, S. M., Cooney, R. N., & Lynch, C. J. (2007). Obesity-related elevations in plasma leucine are associated with alterations in enzymes involved in branched-chain amino acid metabolism. *American journal of physiology. Endocrinology and metabolism*, 293(6), E1552-63.
  183. Jang, C., Oh, S. F., Wada, S., Rowe, G. C., Liu, L., Chan, M. C., Rhee, J., Hoshino, A., Kim, B., Ibrahim, A., Baca, L. G., Kim, E., Ghosh, C. C., Parikh, S. M., Jiang, A., Chu, Q., Forman, D. E., Lecker, S. H., Krishnaiah, S., Rabinowitz, J. D., Weljie, A. M., Baur, J. A., Kasper, D. L., ... Arany, Z. (2016). A branched-chain amino acid metabolite drives vascular fatty acid transport and causes insulin resistance. *Nature medicine*, 22(4), 421-6.
  184. Avogaro A, & Bier DM. (1989). Contribution of 3-hydroxyisobutyrate to the measurement of 3- hydroxybutyrate in human plasma: comparison of enzymatic and gas-liquid chromatography-mass spectrometry assays in normal and in diabetic subjects. *J. Lipid Res.*, 30, 1811–1817.
  185. Giesbertz P, et al. (2015). Metabolite profiling in plasma and tissues of ob/ob and db/db mice identifies novel markers of obesity and type 2 diabetes. *Diabetologia*, 58, 2133–2143.
  186. Mullen E, & Ohlendieck K. (2010) Proteomic profiling of non-obese type 2 diabetic skeletal muscle. *Int. J. Mol. Med.*, 25, 445–458.
  187. Nissen SL, & Sharp RL. (2003). Effect of dietary supplements on lean mass and strength gains with resistance exercise: a metaanalysis. *J Appl Physiol*, 94(2), 651–659
  188. Smith HJ, Mukerji P, & Tisdale MJ (2005) Attenuation of proteasome induced proteolysis in skeletal muscle by b-hydroxy-b-methylbutyrate in cancer-induced muscle loss. *Cancer Res*, 65, 277–283
  189. Eley HL, Russel ST, Tisdale MJ. (2008). Attenuation of depression of muscle protein synthesis induced by lipopolysaccharide, tumor necrosis factor, and angiotensin II by beta-hydroxy-beta-methylbutyrate. *Am J Physiol Endo Metabol*, 295, 1409–1416
  190. Pinheiro C.H., Gerlinger-Romero F., Guimaraes-Ferreira L., de Souza-Jr A.L., Vitzel K.F., Nachbar R.T., Nunes M.T., Curi R. (2012). Metabolic and functional effects of beta-



- hydroxy-beta-methylbutyrate (HMB) supplementation in skeletal muscle. *Eur J. Appl. Physiol.*, 112, 2531–2537
191. Pimentel GD, Rosa JC, Lira FS, et al. (2011)  $\beta$ -Hydroxy- $\beta$ -methylbutyrate (HMB $\beta$ ) supplementation stimulates skeletal muscle hypertrophy in rats via the mTOR pathway. *Nutr Metab (Lond)*. 8(1).
  192. Girón, M. D., Vilchez, J. D., Salto, R., Manzano, M., Sevillano, N., Campos, N., Argilés, J. M., Rueda, R., ... López-Pedrosa, J. M. (2015). Conversion of leucine to  $\beta$ -hydroxy- $\beta$ -methylbutyrate by  $\alpha$ -keto isocaproate dioxygenase is required for a potent stimulation of protein synthesis in L6 rat myotubes. *Journal of cachexia, sarcopenia and muscle*, 7(1), 68-78.
  193. Sharawy, Maha H., El-Awady, Mohammed Shaaban., Megahed, Nirmeen., Gameil, Nariman. (2015). The ergogenic supplement  $\beta$ -hydroxy- $\beta$ -methylbutyrate (HMB) attenuates insulin resistance through suppressing GLUT-2 in rat liver. *Canadian Journal of Physiology and Pharmacology*, 94(5) 488-497.
  194. Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, Graham I. (1991). Hyperhomocysteinemia: an independent risk factor for vascular disease. *N Engl J Med.*, 324, 1149–1155.
  195. Lipton, S. A., Kim, W. K., Choi, Y. B., Kumar, S., D'Emilia, D. M., Rayudu, P. V., Arnelle, D. R., & Stamler, J. S. (1997). Neurotoxicity associated with dual actions of homocysteine at the N-methyl-D-aspartate receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 94(11), 5923-8.
  196. Meigs JB, Jacques PF, Selhub J, Singer DE, Nathan DM, Rifai N, D'Agostino RB Sr, Wilson PW. (2001). Fasting plasma homocysteine levels in the insulin resistance syndrome: the Framingham offspring study. *Diabetes Care*, 24, 1403–1410.
  197. Oron-Herman M, Rosenthal T, & Sela BA. (2003). Hyperhomocysteinemia as a component of syndrome X. *Metabolism*, 52, 1491–1495.
  198. Wang G, Siow YL, O K. (2000). Homocysteine stimulates nuclear factor kappaB activity and monocyte chemoattractant protein-1 expression in vascular smooth-muscle cells: a possible role for protein kinase C. *Biochem J*, 352, 817–826,
  199. Wang G, Woo CW, Sung FL, Siow YL, O K. (2002). Increased monocyte adhesion to aortic endothelium in rats with hyperhomocysteinemia: role of chemokine and adhesion molecules. *Arterioscler Thromb Vasc Biol*, 22, 1777– 1783.
  200. Lee S.J., Lee Y.S., Seo K.W., Bae J.U., Kim G.H., Park S.Y., Kim C.D. (2012). Homocysteine enhances MMP-9 production in murine macrophages via ERK and Akt signaling pathways. *Toxicology and Applied Pharmacology*, 260(1), 89-94.
  201. Stephan Glund, Atul Deshmukh, Yun Chau Long, Theodore Moller, Heikki A.Koistinen, Kenneth Caidahl, Juleen R. Zierath, Anna Krook. (2007). Interleukin-6 Directly Increases Glucose Metabolism in Resting Human Skeletal Muscle. *Diabetes*, 56(6) 1630-1637.
  202. Andrew L. Carey, Gregory R. Steinberg, S. Lance Macaulay, Walter G. Thomas, Anna G. Holmes, Georg Ramm, Oja Prelovsek, Cordula Hohnen-Behrens, Matthew J. Watt, David E. James, Bruce E. Kemp, Bente K. Pedersen, Mark A. Febbraio. (2006). Interleukin-6 Increases Insulin-Stimulated Glucose Disposal in Humans and Glucose Uptake and Fatty Acid Oxidation In Vitro via AMP-Activated Protein Kinase *Diabetes*, 55(10), 2688-2697.

203. Nieto-Vazquez, I., Fernández-Veledo, S., de Alvaro, C., & Lorenzo, M. (2008). Dual role of interleukin-6 in regulating insulin sensitivity in murine skeletal muscle. *Diabetes*, 57(12), 3211-21.
204. Kaneto, H., Katakami, N., Matsuhisa, M., & Matsuoka, T. A. (2010). Role of reactive oxygen species in the progression of type 2 diabetes and atherosclerosis. *Mediators of inflammation*, 2010, 453892.
205. de Luca, C., & Olefsky, J. M. (2007). Inflammation and insulin resistance. *FEBS letters*, 582(1), 97-105.
206. Wei Y, Chen K, Whaley-Connell AT, Stump CS, Ibdah JA, Sowers JR. (2008). Skeletal muscle insulin resistance: role of inflammatory cytokines and reactive oxygen species. *Am J Physiol Regul Integr Comp Physiol*, 294, R673–R680.
207. Qiao L, Goldberg JL, Russell JC, Sun XJ. (1999) Identification of enhanced serine kinase activity in insulin resistance. *J Biol Chem*, 274, 10625–10632.
208. Huang, S., Huang, S., Wang, X., Zhang, Q., Liu, J., & Leng, Y. (2017). Downregulation of lipin-1 induces insulin resistance by increasing intracellular ceramide accumulation in C2C12 myotubes. *International journal of biological sciences*, 13(1), 1-12.
209. Birnbaum MJ (2001). Turning down insulin signaling. *The Journal of clinical investigation*, 108(5), 655-9.
210. Um SH, Frogerio F, Watanabe M, Picard F, Joaquin M, Sticker M, Fumagalli S, Allegrini PR, Kozma SC, Auwerx J, Thomas G. (2004) Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature*, 431, 200–205.
211. Patti M-E & Kahn BB. (2004). Nutrient sensor links obesity with diabetes risk. *Nat Med* 10, 1049–1050.
212. Aguirre V, Werner ED, Giraud J, Lee YH, Shoelson SE, White MF. (2002) Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. *J Biol Chem*, 277, 1531–1537,
213. Wang G, Siow YL, O K. (2000). Homocysteine stimulates nuclear factor kappaB activity and monocyte chemoattractant protein-1 expression in vascular smooth-muscle cells: a possible role for protein kinase C. *Biochem, J* 352, 817–826,
214. Wang G, Woo CW, Sung FL, Siow YL, O K. (2002). Increased monocyte adhesion to aortic endothelium in rats with hyperhomocysteinemia: role of chemo- kine and adhesion molecules. *Arterioscler Thromb Vasc Biol*, 22, 777– 1783.
215. Li Y, Jiang C, Xu G, Wang N, Zhu Y, Tang C, Wang X. (2008) Homocysteine upregulates resistin production from adipocytes in vivo and in vitro. *Diabetes*, 57, 817–827.
216. Jialal I, Kaur H, Devaraj S. (2014). Toll-like receptor status in obesity and metabolic syndrome: a translational perspective. *J Clin Endocrinol Metab.*, 99, 39–48.
217. Maron BA, Loscalzo J. (2009) The treatment of hyperhomocysteinemia. *Annu Rev Med*, 60, 39–54
218. Ciaraldi TP, Carter L, Mudaliar S, Kern PA, & Henry RR.(1998). Effects of tumor necrosis factor-alpha on glucose metabolism in cultured human muscle cells from nondiabetic and type 2 diabetic subjects. *Endocrinology*, 139, 4793–4800
219. del Aguila LF , Claffey KP , Kirwan JP. (1999).TNF-alpha impairs insulin signaling and insulin stimulation of glucose uptake in C2C12 muscle cells.*Am J Physiol Endocrinol Metab* 276, E849-E855.

220. Sishi, B. J. N., & Engelbrecht, A.-M. (2011). Tumor necrosis factor alpha (TNF- $\alpha$ ) inactivates the PI3-kinase/PKB pathway and induces atrophy and apoptosis in L6 myotubes. *Cytokine*, 54(2), 173–184
221. Bray GA & Popkin BM. (1998). Dietary fat intake does affect obesity. *Am J Clin Nutr*, 68, 1157 – 73
222. Curb JD, Marcus EB. (1991) Body fat and obesity in Japanese Americans. *Am J Clin Nutr*, 53, 1552S–5.
223. Blundell JE, Macdiarmid JI. (1997) Passive overconsumption. Fat intake and short-term energy balance. *Ann NY Acad Sci*, 827, 392–407.
224. Sonne-Holm S, Sorensen TIA. (1977) Post-war course of the prevalence of extreme overweight among Danish young men. *J Chronic Dis*, 10, 351-8.
225. Astrup, A., Buemann, B., Western, P., Toubro, S., Raben, A., & Christensen, N. J. (1994). Obesity as an adaptation to a high-fat diet: evidence from a cross-sectional study. *The American Journal of Clinical Nutrition*, 59(2), 350–355.
226. Krachler, B., Eliasson, M., Stenlund, H., Johansson, I., Hallmans, G., & Lindahl, B. (2006). Reported food intake and distribution of body fat: a repeated cross-sectional study. *Nutrition Journal*, 5(1).
227. Winkvist, A., Hultén, B., Kim, J.-L., Johansson, I., Torén, K., Brisman, J., & Bertéus Forslund, H. (2015). Dietary intake, leisure time activities and obesity among adolescents in Western Sweden: a cross-sectional study. *Nutrition Journal*, 15(1).
228. Adegoke, O. A. J., Bates, H. E., Kiraly, M. A., Vranic, M., Riddell, M. C., & Marliiss, E. B. (2014). Exercise in ZDF rats does not attenuate weight gain, but prevents hyperglycemia concurrent with modulation of amino acid metabolism and AKT/mTOR activation in skeletal muscle. *European Journal of Nutrition*, 54(5), 751–759
229. Op den Kamp CM, Langen RC, Snepvangers FJ, de Theije CC, Schellekens JM, Laugs F, Dingemans AM, Schols AM. (2013). Nuclear transcription factor kappaB activation and protein turn- over adaptations in skeletal muscle of patients with progressive stages of lung cancer cachexia. *Am J Clin Nutr*, 98(3) 738-748.
230. Parra, M., Stahl, S., & Hellmann, H. (2018). Vitamin B6 and Its Role in Cell Metabolism and Physiology. *Cells*, 7(7), 84.
231. Li, J., Wynn, R. M., Machius, M., Chuang, J. L., Karthikeyan, S., Tomchick, D. R., & Chuang, D. T. (2004). Cross-talk between Thiamin Diphosphate Binding and Phosphorylation Loop Conformation in Human Branched-chain  $\alpha$ -Keto Acid Decarboxylase/Dehydrogenase. *Journal of Biological Chemistry*, 279(31), 32968–32978
232. Hernández-Alvarez, M. I., Díaz-Ramos, A., Berdasco, M., Cobb, J., Planet, E., Cooper, D., Pazderska, A., Wanic, K., O'Hanlon, D., Gomez, A., de la Ballina, L. R., Esteller, M., Palacin, M., O'Gorman, D. J., Nolan, J. J., ... Zorzano, A. (2017). Early-onset and classical forms of type 2 diabetes show impaired expression of genes involved in muscle branched-chain amino acids metabolism. *Scientific reports*, 7(1), 13850.
233. Boucher, J., Kleinridders, A., & Kahn, C. R. (2014). Insulin receptor signaling in normal and insulin-resistant states. *Cold Spring Harbor perspectives in biology*, 6(1).
234. Copps, K. D., Hançer, N. J., Qiu, W. & White, M. F. (2016). Serine 302 phosphorylation of mouse insulin receptor substrate 1 (IRS1) is dispensable for normal insulin signaling and feedback regulation by hepatic S6 kinase. *J. Biol. Chem*, 291, 8602–8617.
235. Petersen MC, Madiraju AK, Gassaway BM, Marcel M, Nasiri AR, Butrico G, Marcucci MJ, Zhang D, Abulizi A, Zhang XM, et al. (2016). Insulin receptor Thr1160

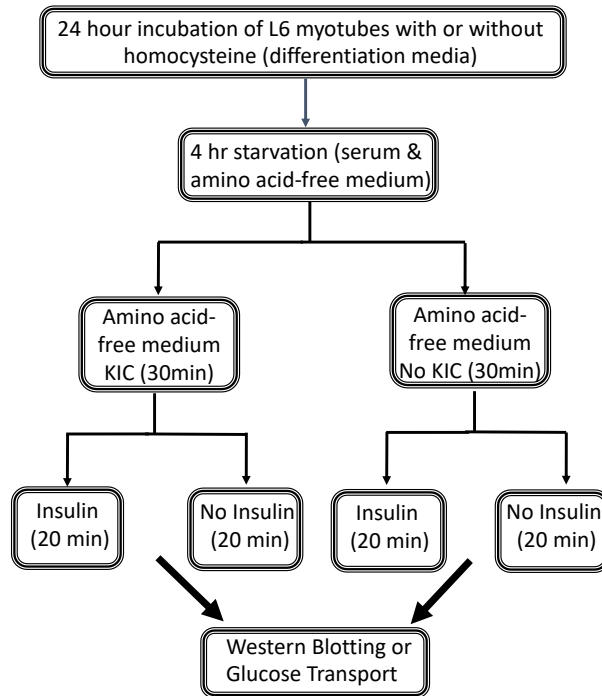
- phosphorylation mediates lipid-induced hepatic insulin resistance. *J. Clin. Invest*, 126, 4361–4371
236. Song, M. S., Salmena, L. & Pandolfi, P. P. (2012). The functions and regulation of the PTEN tumour suppressor. *Nat. Rev. Mol. Cell Biol.*, 13, 283–296
  237. Morley, T. S., Xia, J. Y. & Scherer, P. E. (2015). Selective enhancement of insulin sensitivity in the mature adipocyte is sufficient for systemic metabolic improvements. *Nat. Commun.*, 6, 7906.
  238. Feldhammer, M., Uetani, N., Miranda-Saavedra, D. & Tremblay, M. L. (2013). PTP1B: a simple enzyme for a complex world. *Crit. Rev. Biochem. Mol. Biol.*, 48, 430–445.
  239. Delibegovic M, Bence KK, Mody N, Hong E, Ko HJ, Kim JK, et al. (2007) Improved Glucose Homeostasis in Mice with Muscle-Specific Deletion of Protein-Tyrosine Phosphatase 1B. *Mol Cell Biol*, 27(21), 7727–34.
  240. Delibegovic M, Zimmer D, Kauffman C, Rak K, Hong E-G, Cho Y-R, et al. (2009) Liver-specific deletion of protein-tyrosine phosphatase 1B (PTP1B) improves metabolic syndrome and attenuates diet-induced endoplasmic reticulum stress. *Diabetes*. 58:590–599.
  241. Zheng, X. & Cartee, G. D. (2016) Insulin-induced effects on the subcellular localization of AKT1, AKT2 and AS160 in rat skeletal muscle. *Sci. Rep*, 6, 39230.
  242. Leto, D. & Saltiel, A. R. Regulation of glucose transport by insulin: traffic control of GLUT4. (2012). *Nat. Rev. Mol. Cell Biol.* 13, 383–396.
  243. Vazirani, R. P., Verma, A., Sadacca, L. A., Buckman, M. S., Picatoste, B., Beg, M., Torsitano, C., Bruno, J. H., Patel, R. T., Simonyte, K., Camporez, J. P., Moreira, G., Falcone, D. J., Accili, D., Elemento, O., Shulman, G. I., Kahn, B. B., ... McGraw, T. E. (2016). Disruption of Adipose Rab10-Dependent Insulin Signaling Causes Hepatic Insulin Resistance. *Diabetes*, 65(6), 1577-89.
  244. Bruno, J., Brumfield, A., Chaudhary, N., Iaea, D. & McGraw, T. E. (2016). SEC16A is a RAB10 effector required for insulin-stimulated GLUT4 trafficking in adipocytes. *J. Cell Biol*, 214, 61–76
  245. Hançer, N. J., Qiu, W., Cherella, C., Li, Y., Copps, K. D., & White, M. F. (2014). Insulin and metabolic stress stimulate multisite serine/threonine phosphorylation of insulin receptor substrate 1 and inhibit tyrosine phosphorylation. *The Journal of biological chemistry*, 289(18), 12467-84.
  246. Parker, V.E., Savage, D.B., O’Rahilly, S. & Semple, R.K. (2011) Mechanistic insights into insulin resistance in the genetic era. *Diabet. Med.* 28, 1476–1486.
  247. Sabio, G., & Davis, R. J. (2010). cJun NH2-terminal kinase 1 (JNK1): roles in metabolic regulation of insulin resistance. *Trends in biochemical sciences*, 35(9), 490-6.
  248. Yoneyama Y, Inamitsu T, Chida K, Lemura SI, Natsume T, Maeda T, Hakuno F, Takahashi SI. (2018). Serine phosphorylation by mTORC1 promotes IRS-1 degradation through SCF $\beta$ -TRCP E3 ubiquitin ligase. *iScience*, 5, 1–18.
  249. K.L. Hoehn, C. HohnenBehrens, A.Cederberg, L.E. Wu, N. Turner, T. Yuasa, Y.Ebina, D. E. James. (2008). IRS1-independent defects define major nodes of insulin resistance *Cell Metab.*, 7, 421-433.
  250. Tang, C. Y., Man, X. F., Guo, Y., Tang, H. N., Tang, J., Zhou, C. L., Tan, S. W., Wang, M., Zhou, H. D. (2017). IRS-2 Partially Compensates for the Insulin Signal Defects in IRS-1<sup>-/-</sup> Mice Mediated by miR-33. *Molecules and cells*, 40(2), 123-132.

251. Gagliardi, P.A.; Puliafito, A.; Primo, L. (2017) PDK1: At the crossroad of cancer signaling pathways. *Semin. Cancer Biol*, 48, 27-35.
252. Cartee GD. (2015). Roles of TBC1D1 and TBC1D4 in insulin- and exercise-stimulated glucose transport of skeletal muscle. *Diabetologia*, 58, 19–30
253. Cree-Green, M.; Gupta, A.; Coe, G.V.; Baumgartner, A.D.; Pyle, L.; Reusch, J.E.; Brown, M.S.; Newcomer, B.R.; Nadeau, K.J. (2017). Insulin resistance in type 2 diabetes youth relates to serum free fatty acids and muscle mitochondrial dysfunction. *J. Diabetes Complicat.*, 31, 141–148
254. Li Y, Yang P, Chang Q, Wang J, Liu J, Lv Y, et al. (2017) Inhibitory effect of piceatannol on TNF- $\alpha$  mediated inflammation and insulin resistance in 3T3-L1 adipocytes. *J Agric Food Chem*, 65 (23), 4634–4641.
255. Yamamoto N, Ueda & M, Sato T. (2011) Measurement of glucose uptake in cultured cells. *Curr Protoc Pharmacol*, 1-22.
256. Eva Blomstrand, Jörgen Eliasson, Haåkan K. R. Karlsson & Rickard Köhnke. (2006). Branched-Chain Amino Acids Activate Key Enzymes in Protein Synthesis after Physical Exercise, *The Journal of Nutrition*, 136(1), 269S–273S.
257. Rui L, Yuan M, Frantz D, Shoelson S, & White MF. (2002) SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. *J Biol Chem*, 277, 42394–8.

## 6.0 Appendix

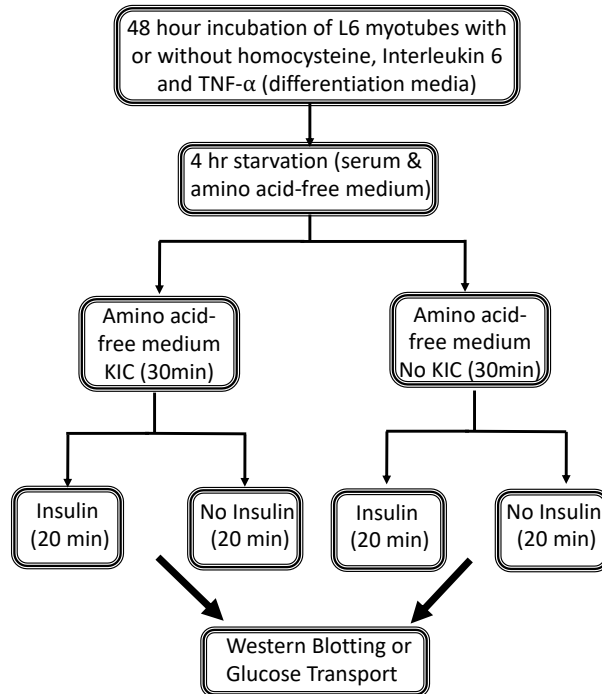
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### A) Experimental Outline for Homocysteine Experiments



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.

## *B) Experimental Outline for Pro-Inflammatory Factors Experiments*



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 MgSO<sub>4</sub> (Bioshop #10034-99-8)
- 1.0 mM CaCl<sub>2</sub> (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 H<sup>3</sup> 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°. 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.

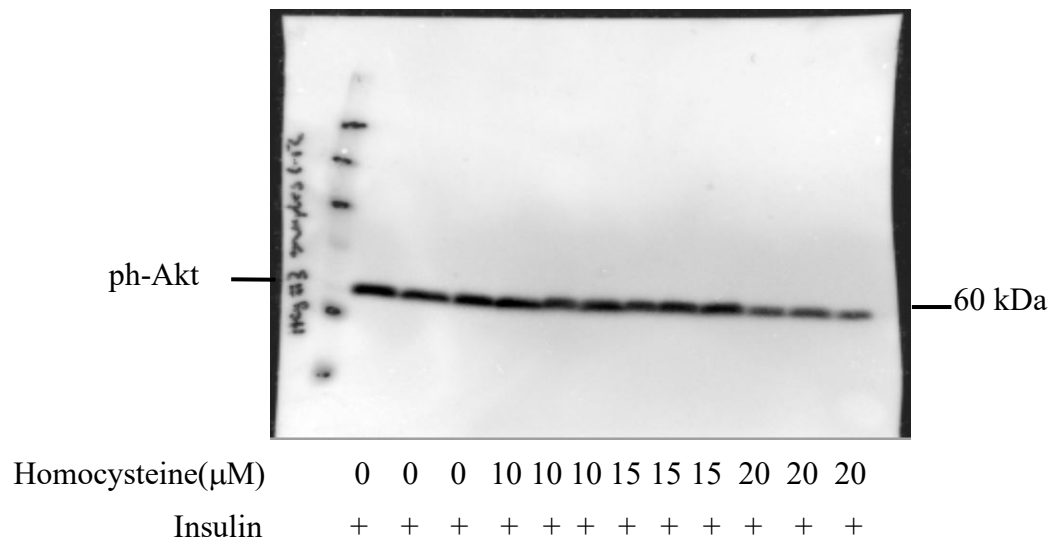
### ***E) Glycogen Synthesis Assay***

- 1) Cells were starved for three hours (2ml/well for 6 well plate)
  - a. Prepare test media containing [ $^{14}\text{C}$ ]-D-Glucose (0.2 $\mu\text{Ci/ml}$ ) – 2  $\mu\text{l}$  of [ $^{14}\text{C}$ ]-D-Glucose (50 $\mu\text{Ci}/0.5\text{ml}$ )/ml of media
- 2) Incubate cells with test media (1ml/well) containing respective conditions, 5.5mM unlabelled glucose and [ $^{14}\text{C}$ ]-D-Glucose to wells for one hour.
- 3) After 1 hour, collect 200  $\mu\text{l}$  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  $\mu\text{l}$  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  $\mu\text{l}$  was removed and put into different Eppendorf tubes.
- 8) Glycogen carrier was added (100  $\mu\text{l}$ ) [stock solution is 25mg/ml]
- 9) 80  $\mu\text{l}$  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  $\mu\text{l}$  of double distilled water.
- 14) Finally, 450  $\mu\text{l}$  of this solution was transferred to scintillation vials containing 3.5 ml of scintillation fluid for counting.

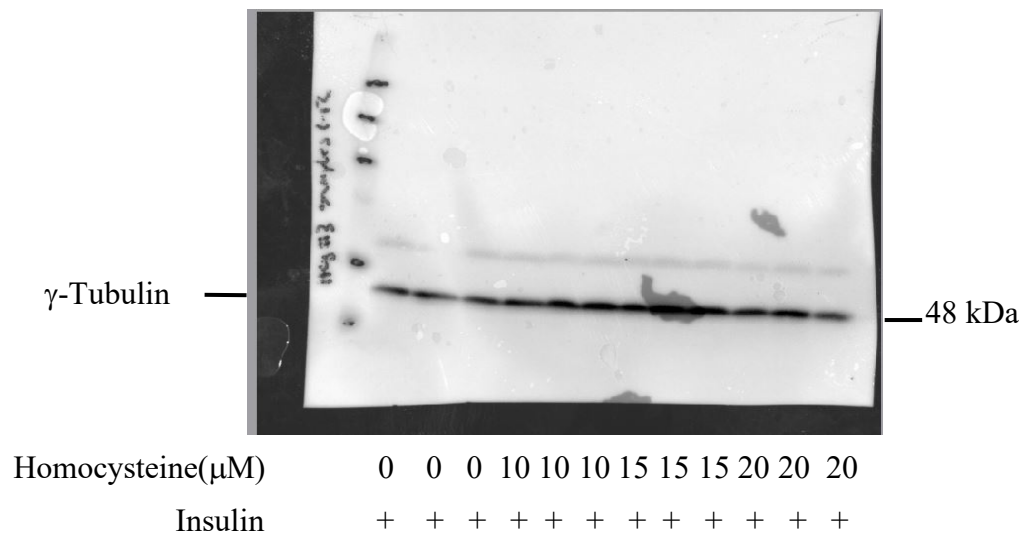
## 7.0 Supplementary Data

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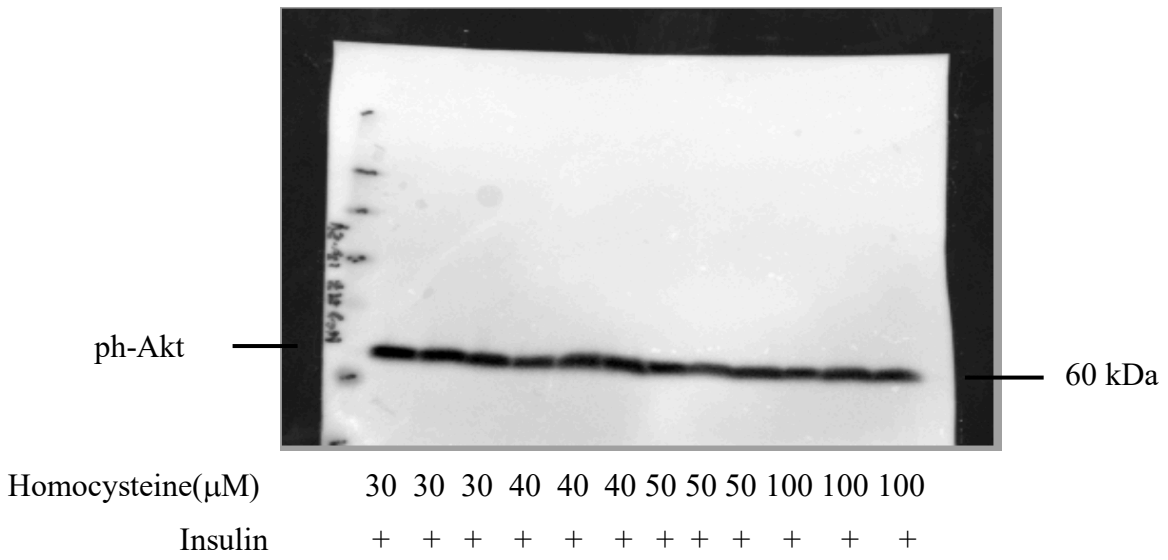
a)



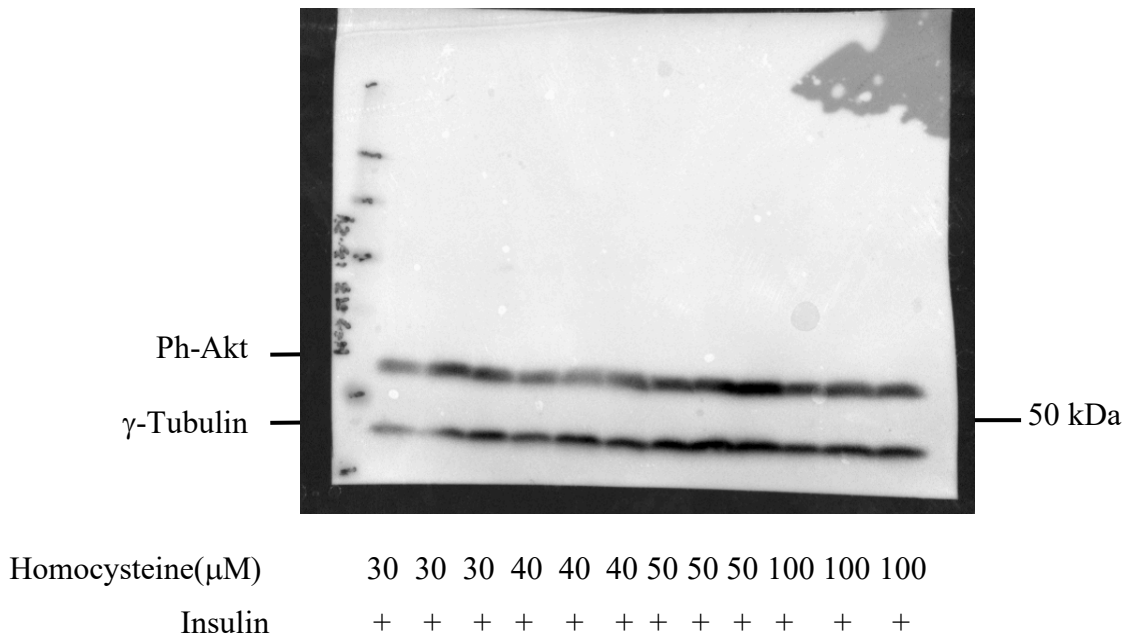
b)



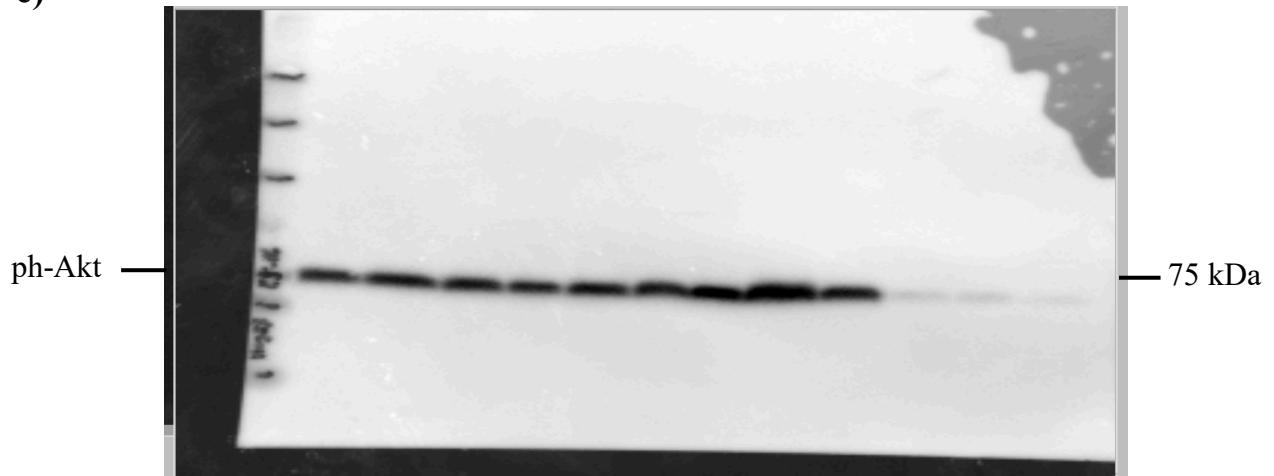
c)



d)

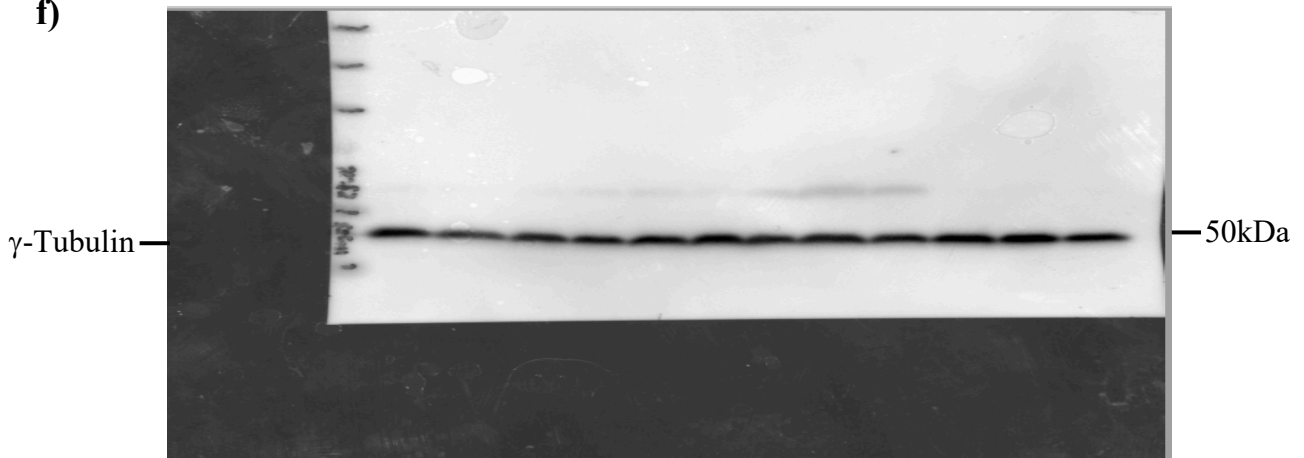


e)



Homocysteine( $\mu$ M)	200	200	200	500	500	500	1000	1000	1000	0	0	0
Insulin	+	+	+	+	+	+	+	+	+	-	-	-

f)

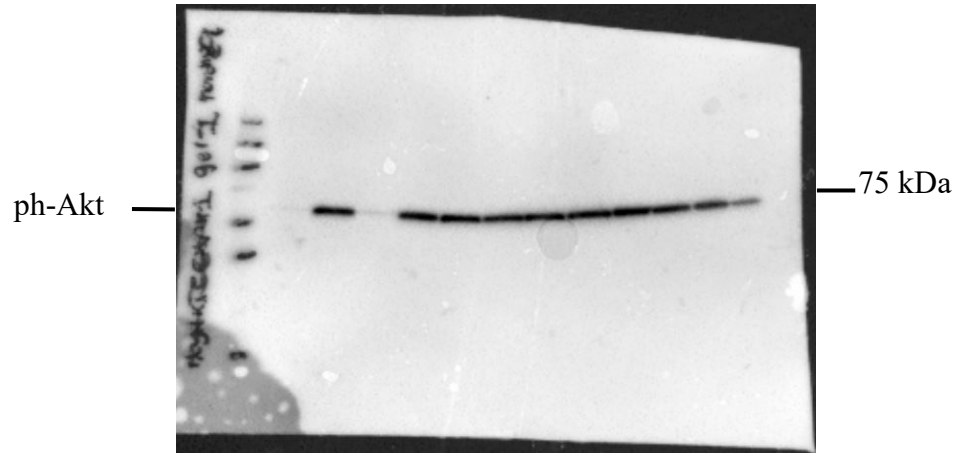


Homocysteine( $\mu$ M)	200	200	200	500	500	500	1000	1000	1000	0	0	0
Insulin	+	+	+	+	+	+	+	+	+	-	-	-

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

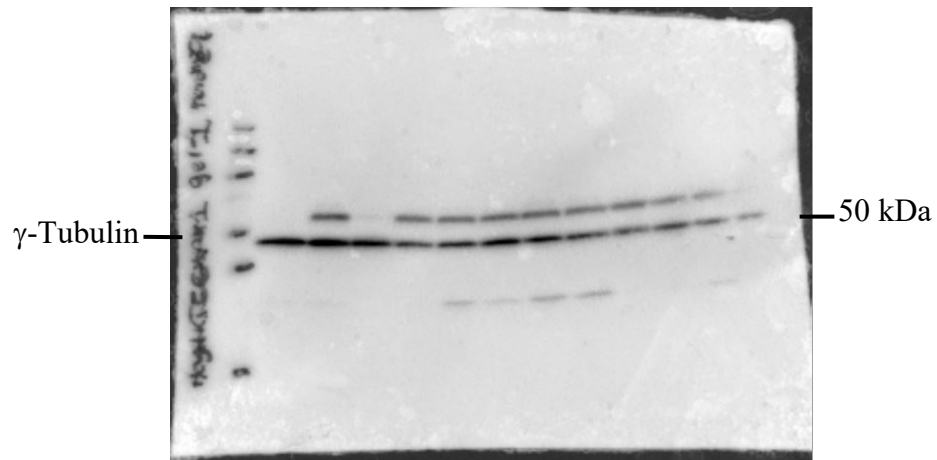
Original blots for ph-Akt (Supplementary Figure S1A, S1C, S1E) and  $\gamma$ -tubulin (Supplementary Figure S1B, S1D, S1F) for Figure 3b.

a)



Homocysteine( $\mu$ M)	0	0	0	0	10	15	50	500	10	15	50	500
Insulin	-	+	-	+	+	+	+	+	-	-	-	-
KIC(200 $\mu$ M)	-	-	+	+	-	-	-	-	+	+	+	+

b)

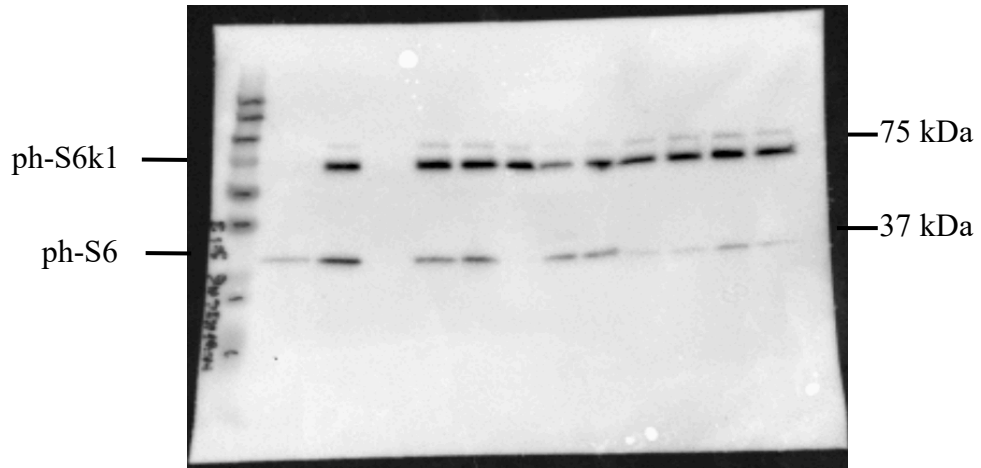


Homocysteine( $\mu$ M)	0	0	0	0	10	15	50	500	10	15	50	500
Insulin	-	+	-	+	+	+	+	+	-	-	-	-
KIC(200 $\mu$ M)	-	-	+	+	-	-	-	-	+	+	+	+

**Figure S2) Effect of homocysteine and KIC on insulin-stimulated Akt phosphorylation**

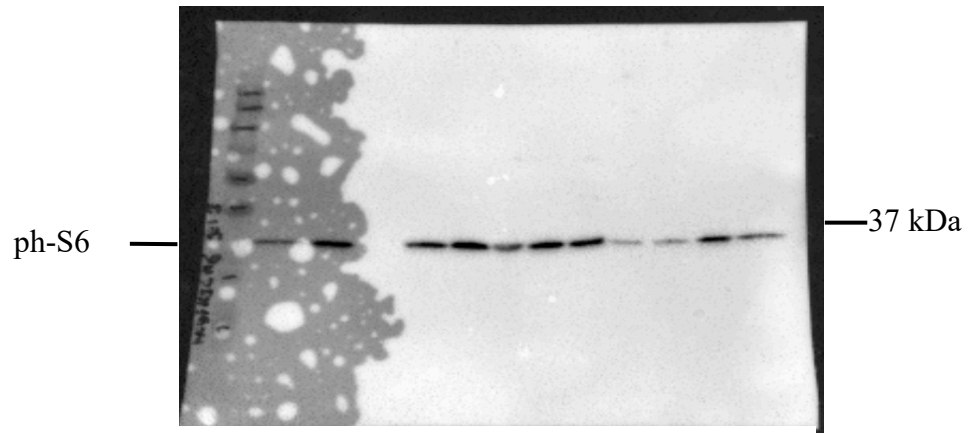
Original blots for ph-Akt (Supplementary Figure S2A) and  $\gamma$ -tubulin (Supplementary Figure S2B) for Figure 5a.

a)



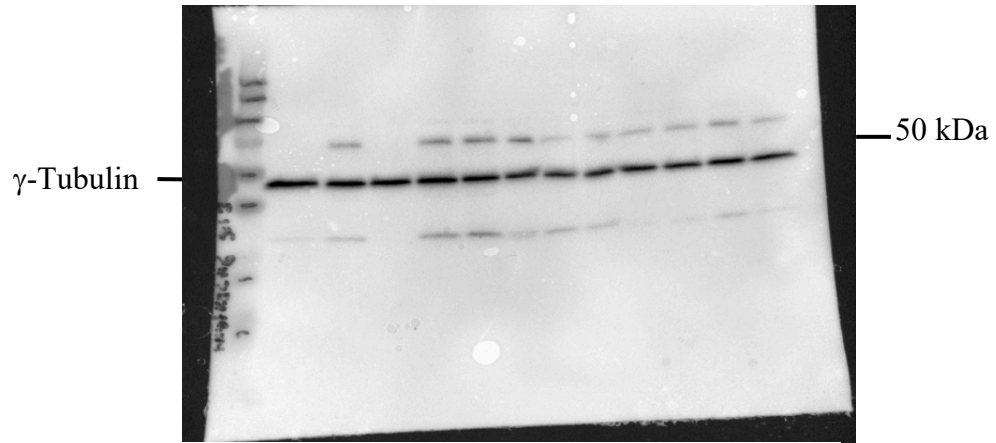
Homocysteine( $\mu$ M)	0	0	0	0	10	15	50	500	10	15	50	500
Insulin	-	+	-	+	+	+	+	+	-	-	-	-
KIC(200 $\mu$ M)	-	-	+	+	-	-	-	-	+	+	+	+

b)



Homocysteine( $\mu$ M)	0	0	0	0	10	15	50	500	10	15	50	500
Insulin	-	+	-	+	+	+	+	+	-	-	-	-
KIC(200 $\mu$ M)	-	-	+	+	-	-	-	-	+	+	+	+

c)



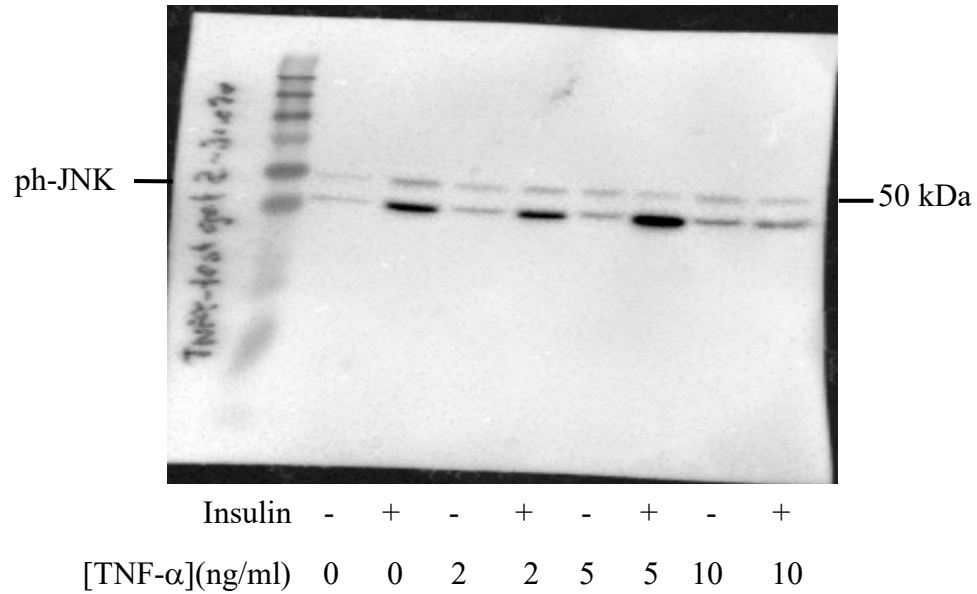
Homocysteine( $\mu$ M)	0	0	0	0	10	15	50	500	10	15	50	500
Insulin	-	+	-	+	+	+	+	+	-	-	-	-
KIC(200 $\mu$ M)	-	-	+	+	-	-	-	-	+	+	+	+

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

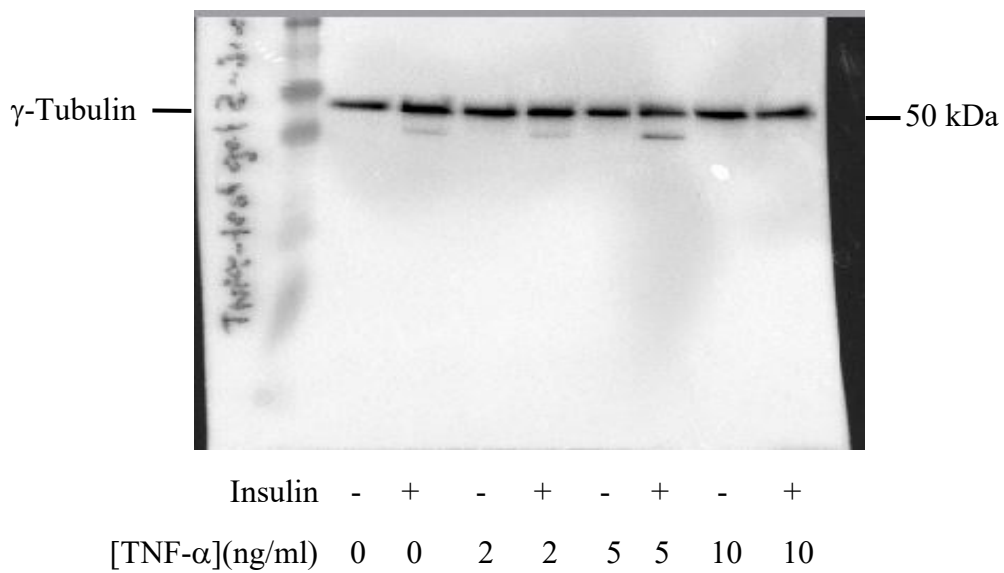
Original blots for ph-S6K1 (Supplementary Figure S3A) and ph-S6 (Supplementary Figure S3B) for Figure 5b and 5c. Original blots for  $\gamma$ -tubulin (Supplementary Figure S3C) was used for both Figure 5b and 5c.



a)



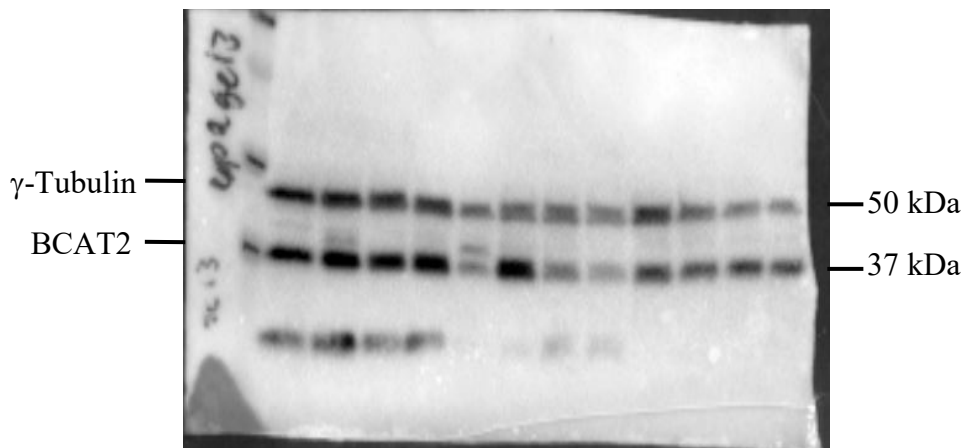
b)



### Figure S4) Effect of TNF- $\alpha$ on JNK phosphorylation

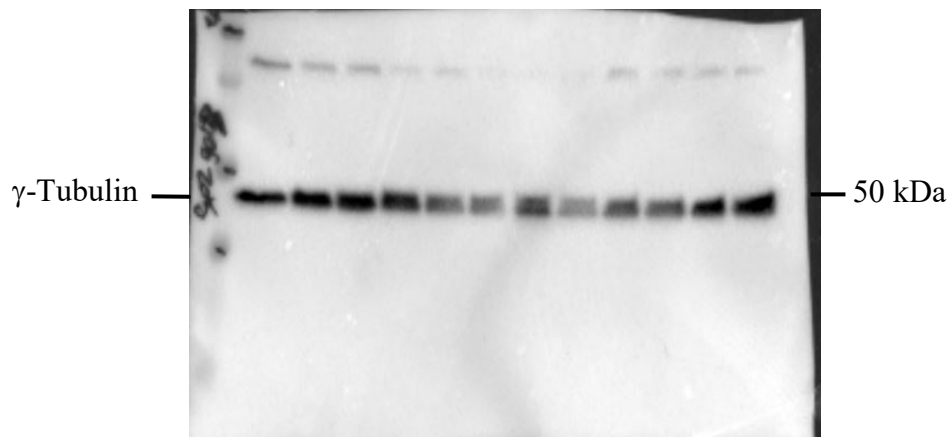
Original blots for ph-JNK (Supplementary Figure S4A) and  $\gamma$ -tubulin (Supplementary Figure S4B) for Figure 6. JNK has two isoforms, 46p and 48p, I chose to quantify 46p throughout, as both have similar functions, but just different structure.

a)



BCAT2 siRNA	-	-	+	+	-	-	+	+	-	-	+	+
Insulin	-	+	-	+	+	+	+	+	-	-	-	-
KIC(200μM)	-	-	-	-	-	+	-	+	-	+	-	+
TNF-α(10ng/ml)	-	-	-	-	+	+	+	+	+	+	+	+
IL-6(10ng/ml)	-	-	-	-	+	+	+	+	+	+	+	+
Homocysteine(50μM)	-	-	-	-	+	+	+	+	+	+	+	+

b)

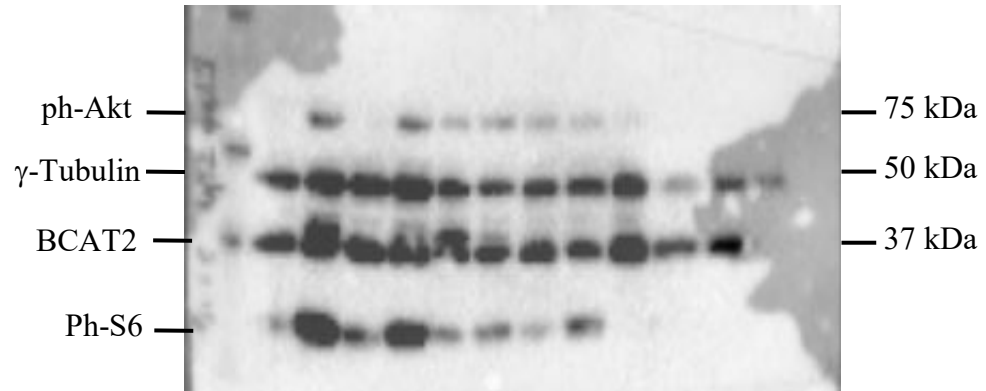


BCAT2 siRNA	-	-	+	+	-	-	+	+	-	-	+	+
Insulin	-	+	-	+	+	+	+	+	-	-	-	-
KIC(200μM)	-	-	-	-	-	+	-	+	-	+	-	+
TNF-α(10ng/ml)	-	-	-	-	+	+	+	+	+	+	+	+
IL-6(10ng/ml)	-	-	-	-	+	+	+	+	+	+	+	+
Homocysteine(50μM)	-	-	-	-	+	+	+	+	+	+	+	+

**Figure S5) BCAT2 attenuates reduction of insulin-stimulated glucose uptake in the presence of inflammation and KIC**

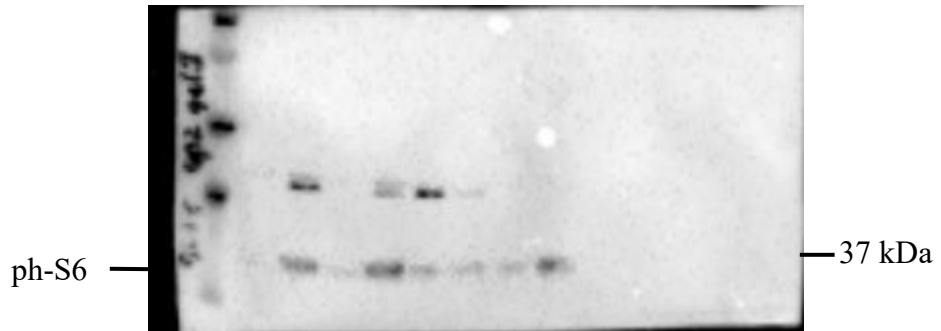
Original blots for BCAT2 (Supplementary Figure S5A) and  $\gamma$ -tubulin (Supplementary Figure S5B) for Figure 9b.

a)



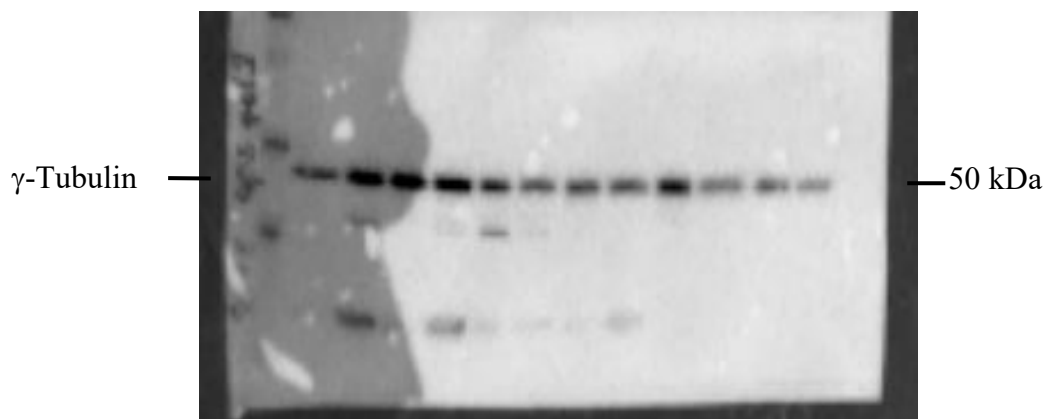
BCAT2 siRNA	-	-	+	+	-	-	+	+	-	-	+	+
Insulin	-	+	-	+	+	+	+	+	-	-	-	-
KIC(200μM)	-	-	-	-	-	+	-	+	-	+	-	+
TNF-α(10ng/ml)	-	-	-	-	+	+	+	+	+	+	+	+
IL-6(10ng/ml)	-	-	-	-	+	+	+	+	+	+	+	+
Homocysteine(50μM)	-	-	-	-	+	+	+	+	+	+	+	+

b)



BCAT2 siRNA	-	-	+	+	-	-	+	+	-	-	+	+
Insulin	-	+	-	+	+	+	+	+	-	-	-	-
KIC(200μM)	-	-	-	-	-	+	-	+	-	+	-	+
TNF-α(10ng/ml)	-	-	-	-	+	+	+	+	+	+	+	+
IL-6(10ng/ml)	-	-	-	-	+	+	+	+	+	+	+	+
Homocysteine(50μM)	-	-	-	-	+	+	+	+	+	+	+	+

c)

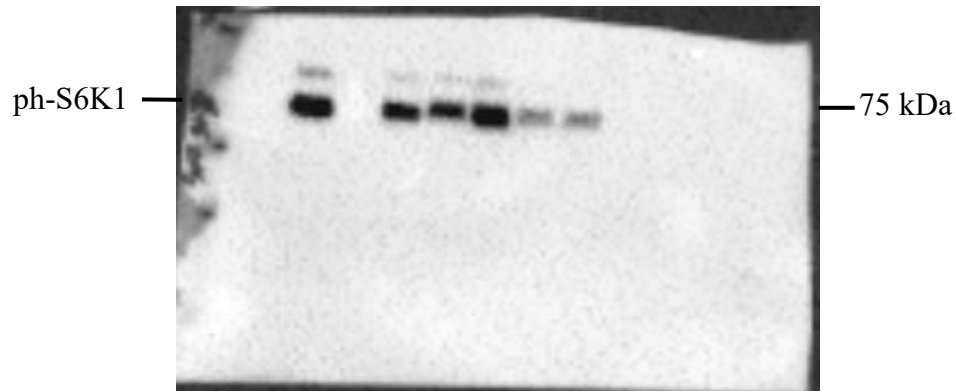


BCAT2 siRNA	-	-	+	+	-	-	+	+	-	-	+	+
Insulin	-	+	-	+	+	+	+	+	-	-	-	-
KIC(200μM)	-	-	-	-	-	+	-	+	-	+	-	+
TNF-α(10ng/ml)	-	-	-	-	+	+	+	+	+	+	+	+
IL-6(10ng/ml)	-	-	-	-	+	+	+	+	+	+	+	+
Homocysteine(50μM)	-	-	-	-	+	+	+	+	+	+	+	+

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

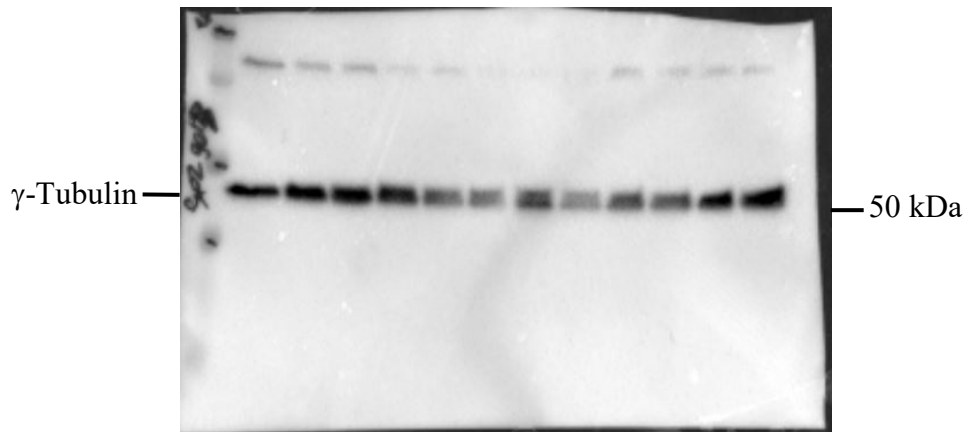
Original blots for ph-Akt (Supplementary Figure S6A) and ph-S6 (Supplementary Figure S6B) for Figure 10a and 10c respectively. Original blots for  $\gamma$ -tubulin (Supplementary Figure S6C) was used in Figure 10a and 10c.

a)



BCAT2 siRNA	-	-	+	+	-	-	+	+	-	-	+	+
Insulin	-	+	-	+	+	+	+	+	-	-	-	-
KIC(200μM)	-	-	-	-	-	+	-	+	-	+	-	+
TNF-α(10ng/ml)	-	-	-	-	+	+	+	+	+	+	+	+
IL-6(10ng/ml)	-	-	-	-	+	+	+	+	+	+	+	+
Homocysteine(50μM)	-	-	-	-	+	+	+	+	+	+	+	+

b)

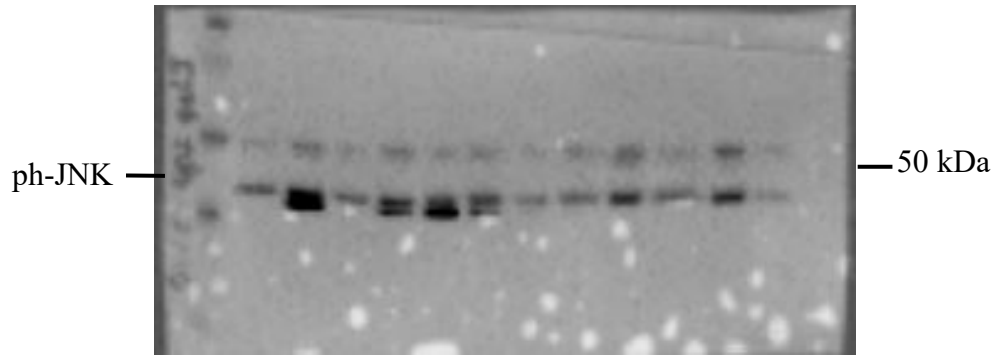


BCAT2 siRNA	-	-	+	+	-	-	+	+	-	-	+	+
Insulin	-	+	-	+	+	+	+	+	-	-	-	-
KIC(200μM)	-	-	-	-	-	+	-	+	-	+	-	+
TNF-α(10ng/ml)	-	-	-	-	+	+	+	+	+	+	+	+
IL-6(10ng/ml)	-	-	-	-	+	+	+	+	+	+	+	+
Homocysteine(50μM)	-	-	-	-	+	+	+	+	+	+	+	+

**Figure S7) The effect of a BCAT2 knockdown with KIC and inflammation on insulin signaling**

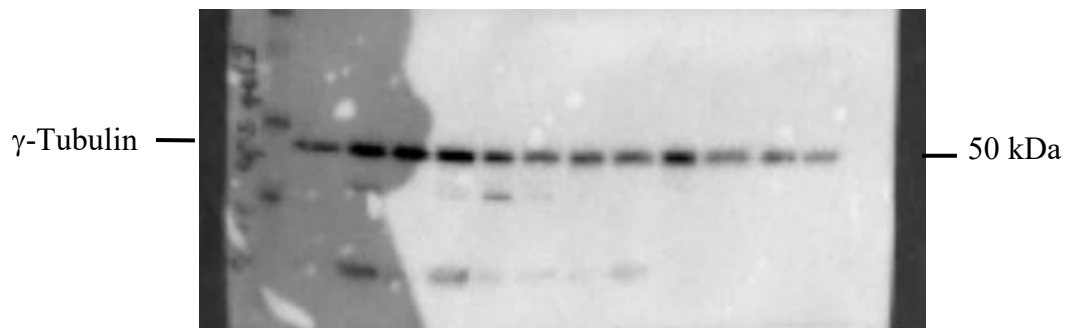
Original blots for ph-S6K1 (Supplementary Figure S7A), and  $\gamma$ -tubulin (Supplementary Figure S7B) for Figure 10b.

a)



BCAT2 siRNA	-	-	+	+	-	-	+	+	-	-	+	+
Insulin	-	+	-	+	+	+	+	+	-	-	-	-
KIC(200μM)	-	-	-	-	-	+	-	+	-	+	-	+
TNF-α(10ng/ml)	-	-	-	-	+	+	+	+	+	+	+	+
IL-6(10ng/ml)	-	-	-	-	+	+	+	+	+	+	+	+
Homocysteine(50μM)	-	-	-	-	+	+	+	+	+	+	+	+

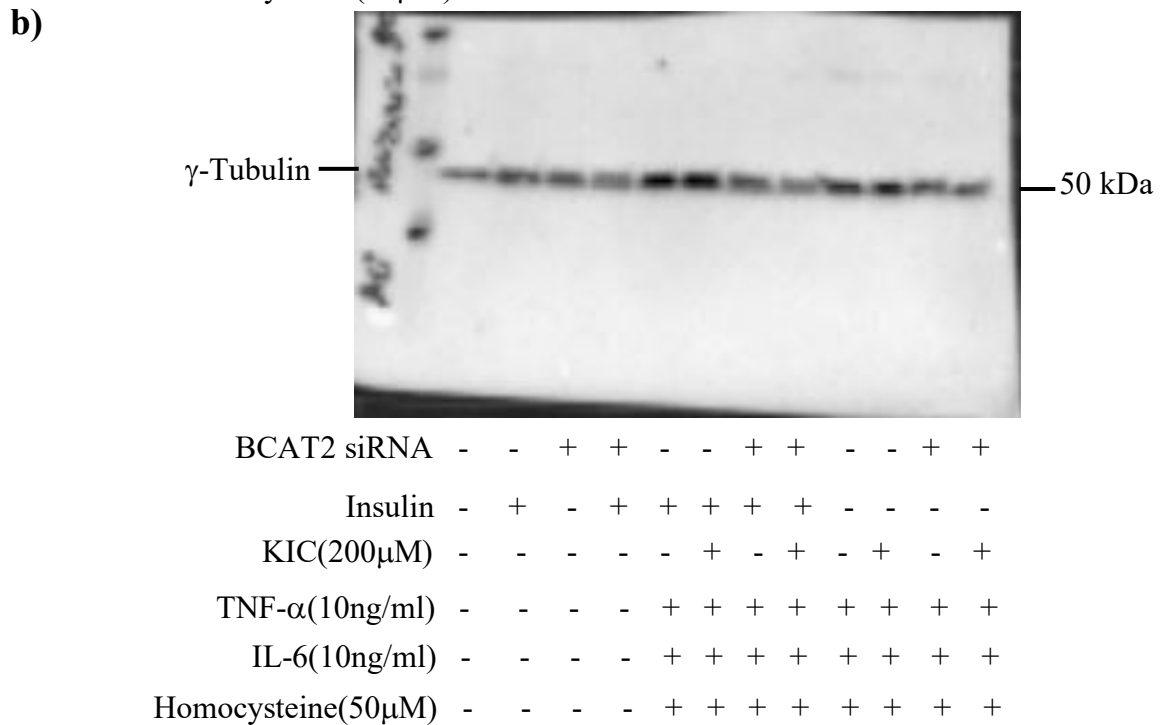
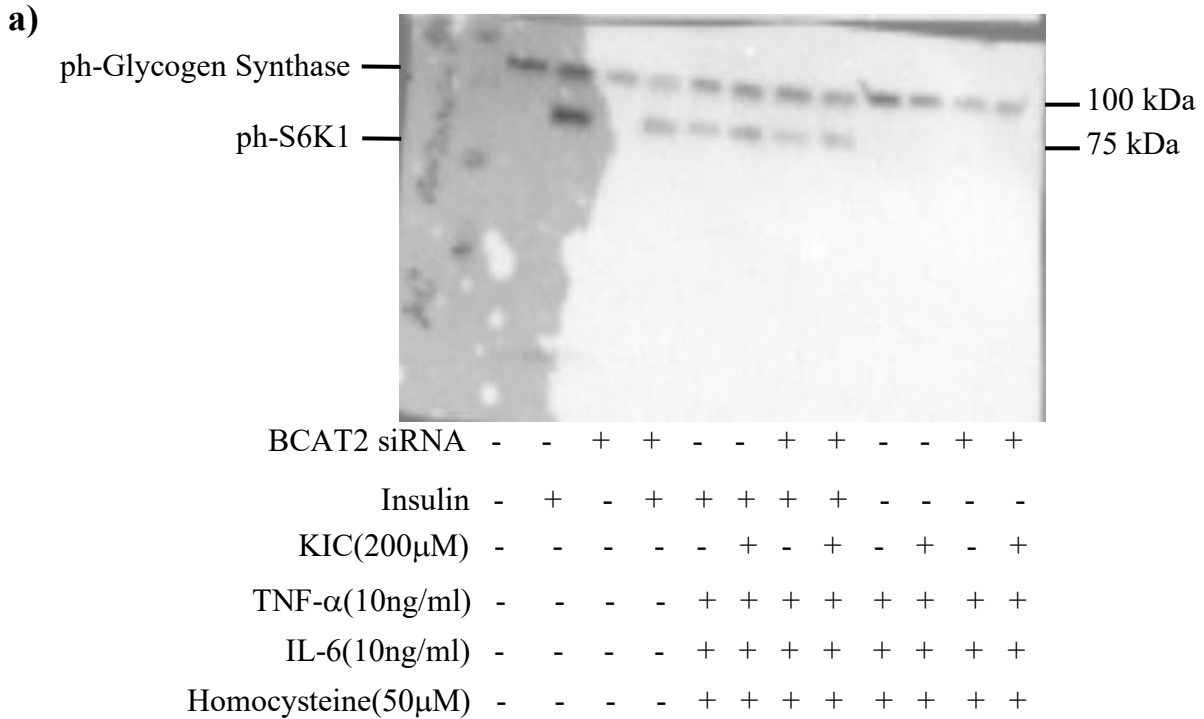
b)



BCAT2 siRNA	-	-	+	+	-	-	+	+	-	-	+	+
Insulin	-	+	-	+	+	+	+	+	-	-	-	-
KIC(200μM)	-	-	-	-	-	+	-	+	-	+	-	+
TNF-α(10ng/ml)	-	-	-	-	+	+	+	+	+	+	+	+
IL-6(10ng/ml)	-	-	-	-	+	+	+	+	+	+	+	+
Homocysteine(50μM)	-	-	-	-	+	+	+	+	+	+	+	+

**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  $\gamma$ -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.



**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  $\gamma$ -tubulin (Supplementary Figure S9B) for Figure 13.