LEUCINE-INDUCED IMPAIRMENT OF INSULIN SENSITIVITY IN SKELETAL MUSCLE OF HEALTHY RATS IS REVERSIBLE

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ABSTRACT

High protein diets promote weight loss and the mTORC1/S6K1 pathway is vital to this process as it senses nutrients and regulates muscle mass. However, excess amino acids (AA) regulate a negative feedback loop causing insulin resistance. S6K1, a substrate of mTORC1, has been implicated in this, as mice lacking S6K1 have improved insulin sensitivity. I hypothesize that AA induced insulin resistance in skeletal muscle is reversible, but possibly not under pathological conditions. L6 myotubes were starved for 4 h and re-incubated with leucine (800 μM) and/or insulin (100 nM). An increase in S6K1 (T389) and IRS1 (serine) phosphorylation and suppression of insulin-stimulated glucose uptake was seen in myotubes treated with leucine and insulin ($P<0.01$). However, these effects were reversible. This pattern of S6K1 and IRS1 phosphorylation and glucose metabolism was recaptured in skeletal muscle of healthy rats gavaged with leucine, but not in insulin resistant rats following leucine gavage.

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True education is not for a mere living, but for a fuller and meaningful life.-SSSB

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LIST OF ABBREVIATIONS

4EBP1	eukaryotic translation initiation factor 4E binding protein 1
5' TOP	5' terminal oligopyrimidine tract
AA	amino acid
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ATGL	adipose triglyceride lipase
ATP	adenosine triphosphate
BAD	Bcl-2 associated death promoter
BCAA	branched chain amino acid
Bcl-2	B-cell lymphoma 2
CaMKII	calmodulin-dependent kinase II
EAA	essential amino acids
eEF2	eukaryotic elongation factor 2
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
ERK	extracellular signal-regulated kinase
FBXW7	F-box and WD repeat domain-containing 7
FFA	fee fatty acid
FOXO1	forkhead box protein O1
GβL	G protein β-subunit like protein
GAP	GTPase activating protein
GDP	guanosine diphosphate

GF	growth factor
GLUT4	glucose transporter type-4
GNG	gluconeogenesis
GTP	guanosine-5'-triphosphate
HEK 293	Human Embryonic Kidney 293
HFD	high fat diet
HSL	hormone sensitive lipase
hVps34	human vacuolar protein sorting 34
IGF1	insulin-like growth factor 1
IKKβ	IκB kinase beta
IP	immunoprecipitation
IR	insulin receptor
IRS-1	insulin receptor substrate 1
JNK	c - Jun N-terminal kinase
LKB1	liver kinase B 1
MAP4K3	mitogen-activated protein kinase kinase kinase kinase 3
MARK4	MAP/microtubule affinity-regulating kinase 4
MEF	mouse embryonic fibroblasts
mSin1	mammalian stress-activated protein kinase (SAPK)- interacting protein-1
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
PDCD4	programmed cell death 4
PDK1	Pyruvate dehydrogenase [lipoamide]] kinase isozyme 1
PH	pleckstrin-homology
PI3K	phosphatidylinositol 3-kinase
PIP3	phosphatidylinositol 3,4,5-trisphosphate

PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PRAS40	proline-rich PKB substrate 40kDa
Protor	protein observed with rictor
PTB	Phospho-tyrosine-binding
PTEN	phosphatases and tensin homologue on chromosome 10
PTM	post translational modification
PX	Phox homology
RalA	Ras family small GTP binding protein
Raptor	rapamycin-sensitive adaptor protein of mTOR
Rheb	ras homologue enriched in brain
rictor	rapamycin-insensitive companion of mTOR
RP	regulatory particle
S6K1	ribosomal protein S6 kinase 1
SAPK	stress-activated protein kinase
Ser	serine
SH2	Src homology-2
STK11	serine/threonine kinase 11
T2DM	type II diabetes mellitus
TAK1	TGF-beta activated kinase 1
Thr	threonine
TNF-α	tumor necrosis factor –alpha
TSC	Tuberous sclerosis complex
TOS	Tor signaling

UPS	ubiquitin-proteasome system

1.0 INTRODUCTION

High protein diets have been used in the management of obesity. Although there are no standard definitions of high protein diets, it has been suggested that 25% of energy intake in weight-stable individuals, which is equivalent to 1.6 g/kg ideal body weight of protein is considered high (this is equal to approximately 350g of meat, or 1500g of lentils) [1]. The mechanisms of action of these diets include thermogenesis and the creation of a prolonged feeling of satiety [2]. This leads to other positive metabolic changes, such as an increase in basal metabolic rate, increased loss of fat mass, and increased weight loss while maintaining lean body mass [3]. Thus, a high protein diet is recommended for obese individuals, particularly in assisting with overall weight loss.

Diets consisting mostly of protein have been especially beneficially in the development of skeletal muscle mass. Amino acids are substrates for our body proteins. In addition, amino acids are able to stimulate protein synthesis by activating specific signalling pathways such as the mTOR (Mammalian Target of Rapamycin) pathway, an essential regulator of skeletal muscle growth and proliferation. It is also a key regulator of protein synthesis. The major proteins involved in this process are mTOR Complex 1 and its downstream substrate, S6 kinase 1 (S6K1).

However, more recent evidence suggests that a high protein diet can also lead to the development of insulin resistance. This has been found to be consistent with the increased association between high protein diets and glucose intolerance, insulin resistance and

increased incidence of type 2 diabetes[4,5]. When specific amino acids such as leucine are present as a result of the consumption of high protein diets, mTORC1/S6K1 becomes hyperactive, leading it to impair the insulin signalling pathway via the attenuation of the function of the insulin receptor substrate 1 (IRS1). Consequently, this presents a very interesting **conundrum**: although high protein diets are desirable for obese individuals to assist with weight loss, it is now also known that they can impair insulin sensitivity.

Although many of the studies conducted in the field have examined the effects of leucine on insulin signalling in muscle, the time course of this action has not been examined. The effect of insulin under these scenarios has also not been studied. Thus, the aims of my thesis were to determine the time course of action of leucine in causing this impairment in insulin sensitivity. I also aim to determine whether this insulin resistant state created by excess leucine is one that can be reversed upon removal of the stimulant. Or, is this effect one that is pathological and irreversible? This is a very important point to examine because muscle is a key regulator of protein metabolism and growth in our bodies; as such, impairments in its ability to sense the presence of growth factors or nutrients may lead to severe consequences with respect to muscle metabolism and development. Thus, the study of how insulin signalling in muscle under the influence of high protein diets is regulated is essential.

2.0 REVIEW OF LITERATURE

2.1 Insulin Resistance

Many of the disorders that are responsible for morbidity in societies today develop in association with changes in diet as well as lifestyle. Such disorders include coronary heart disease, obesity, type 2 diabetes, atherosclerosis, kidney disease and lipodystrophy.

A key factor that is central to the occurrence of these diseases as well as their development and progression is insulin resistance. Insulin resistance is a state in which the peripheral tissues become unresponsive to the effects of the hormone insulin, specifically its ability to trigger glucose uptake and metabolism [6]. Insulin is a hormone that regulates carbohydrate, fat and protein metabolism in the body. This is especially remarkable in cells that are responsive to the effects of insulin, such as muscle and adipose tissue. In insulin-resistant individuals, post-prandial levels of insulin will normally increase in order to maintain a normal blood glucose level[7]. In addition, the liver can regulate glucose levels in the blood by reducing its level of glucose production under the presence of insulin. This ability may also become compromised in individuals with insulin resistance.

As mentioned, insulin resistance does not only refer to a reduction in the ability of insulin to lower blood glucose. For example, insulin resistance in fat cells corresponds to a decrease in the uptake of free circulating lipids, as well as increased hydrolysis of triglycerides in the adipose tissue[8,9]. Furthermore, the cells become unresponsive to the

antilipolytic properties of the hormone; this is also a hallmark of obesity and type 2 diabetes[6].This leads to an increase in the amount of free fatty acids that circulates in the plasma, thereby promoting the development of insulin resistance further[9]. Therefore, since insulin resistance can lead to a variety of metabolic and physiological disorders, the study of the causes of insulin resistance is vital.

2.2 Causes of Insulin Resistance

Insulin resistance can result from a complex interplay between obesity, nutrient overload, hyperlipidemia, increased sedentary lifestyle or reduced physical activity, genetics and hypoxia of the adipose tissue[10]. Although increased food intake in conjunction with physical inactivity have been labelled as the two most modifiable risk factors for the development of T2DM, other determining risk factors have also been discovered [11]. Recently, it has been hypothesized that a lack of sleep can contribute to the development of T2DM [12-13]. This can occur under a state of hypoxic stress or obstructive sleep apnea, commonly seen in individuals that are sedentary[219]. The increased formation of reactive oxygen species in this state can lead to the development of insulin resistance[220].

2.2.1 Nutrition

Nutrition can be a key regulator of insulin sensitivity as well as in the development of insulin resistance. When uncontrolled, a nutrient overload can trigger the development and progression of negative physiological mechanisms, all of which work to impair the functions of insulin and its downstream substrates. The two key nutrients that will be

discussed in this thesis are fat and protein. The effects of carbohydrates in the development of insulin resistance will also be discussed in brief.

2.2.1.1 Lipid Surplus/ High Fat Diets

Obesity has become a pandemic that is largely caused by an increased availability of calorie-dense food that is widely available and inexpensive[14]. As a result, there are now more overweight than underweight children in society, and children are more at risk of becoming obese[15-17]. The phenotype of obese individuals is further accentuated by the fact that the body tries to protect any newly formed adipose tissue when faced with deliberate reductions in food intake[17]. The development of obesity is not only associated with the development of insulin resistance but also cardiovascular disease and cancers [18].

One such factor in the development of obesity and subsequently in insulin resistance is a lipid surplus. Insulin resistance associated with lipid accumulation occurs as a result of the inability of the insulin receptor substrates to function as normal; this factor will be discussed in further detail.

Recently, the impact of trans fats on insulin sensitivity has also been examined in detail. Trans fats are a form of unsaturated fatty acids that rarely exist alone in nature. However, they are found in abundance in many of the 'fast foods' and processed foods that are consumed in large amounts in society today[19]. The trans fats differ from the unsaturated fatty acids in that they will contain one double bond in the *trans* configuration, as opposed to the usual *cis* configuration. As a result, this change causes trans fats to be

straight, thereby resembling the structure of unsaturated fatty acids[19-20]. This structural change is also thought to contribute to the toxic properties of trans fats themselves[20]. The consumption of trans fats accounts for approximately 2-3% of total calories consumed by the American population; however, it be recommended that only 1% of caloric intake is composed of trans fats[21-22].

The consumption of trans fat has also been associated with the risk of the development of coronary heart disease[23-25]; it has also been known to intensify and worsen T2DM. This was suggested after studies found that rodents fed trans fat had increased insulin resistance[26-29] and insulin resistance is commonly associated with a number of diet-related chronic diseases. These include T2DM, obesity and hypertension[30-31].

Although adipose tissue is only thought to be responsible for 10% of glucose uptake in the body, it is a major contributing factor to insulin resistance[32]. In fact, insulin resistance initiated in the adipose is known to affect the insulin sensitivity of the muscle and liver[29]. The consumption of dietary fatty acids can play a vital role in the development of insulin resistance, particularly because they are able to alter and affect the fatty acid composition of the structural lipids and lipid membranes in skeletal muscle as well as adipose tissue[33]. Thus, saturated fats are more likely to induce insulin resistance, while poly-unsaturated fatty acids (PUFA) are able to prevent insulin resistance[34].

Other studies have also shown that saturated and trans fatty acids are able to induce insulin resistance in obese individuals[35]. Thus, these fats have proven to be detrimental, regardless of an individual's metabolic state. Another factor that associates saturated fat

consumption to the development of insulin resistance is the generation of by-products associated with those foods that can induce the insulin resistance. One such example is the production of the sphingolipid ceramide[36], a ubiquitous mediator of cellular stress[37]. Ceramides and its derivatives such as sphingosine, work to impair insulin signalling, generate oxidative stress and inhibit glucose uptake and storage, thereby initiating many of the factors that eventually lead to insulin resistance[36].

It has been shown in literature that although ceramides do accumulate in the insulin resistant model, they don't always appear or accumulate in lipid-infused animals, as was shown by Itani et al[38]. What was remarkable to note however, was that it depended on the type of lipid infused into the animal that determined whether the ceramides developed. A study conducted by Holland et al demonstrated that a soy-oil infusion (consisting of unsaturated fatty acids) impaired whole body glucose uptake, increased circulating FFAs and also increased muscle DAG[39]. However, it did not induce the production of ceramides, demonstrating that soy-based fats induce insulin resistance via a ceramide independent mechanism. This is still beneficial, as it prevents any additional negative impairment in metabolism[39]. In contrast, a lard-oil infusion (consisting of saturated fatty acids) increased the serum FFA concentrations, but it also promoted ceramide accumulation in skeletal muscle. Thus, it has been demonstrated that ceramide synthesis is an important component of insulin resistance. This state can arise as a result of lipid accumulation, but specifically the consumption of saturated fatty acids[39].

2.2.1.2 High protein diets

High protein diets have been highly reviewed and accepted in society as an effective means of weight loss. However, what has recently been proven is that a prolonged consumption of these diets can eventually lead to the development of insulin resistance.

A number of studies conducted on rodents have demonstrated that consumption of high protein or high amino acid diets can have detrimental effects. One particular study has shown that supplementation of a high fat diet with the branched chain amino acids reduce food intake and body weight. However, these animals became equally insulin resistant as heavier animals fed a non-supplemented high fat diet [40]. Furthermore, animals that were fed a high fat diet at the same rate as animals fed the high fat/BCAA diet did not become insulin resistant; additionally, the high fat/BCAA diet-induced insulin resistance was selectively reversed by rapamycin, the mTORC1 inhibitor [40]. These findings led to the proposition that the impairment in BCAA metabolism is able to contribute to insulin resistance in an independent manner; this ultimately leads to the development of T2DM[40].

Other studies using rodents have demonstrated that when rats are given a high protein-low carbohydrate diet, the following effects were found: fasting hyperglycemia, excessive basal hepatic glucose production, and peripheral and hepatic insulin resistance[221]. Furthermore, it has also been found that rats fed with a high protein diet tend to have lower glycemia, but higher levels of insulin[222].

Similar results have been found in humans. A study conducted over six months whereby healthy non-obese subjects consumed a high protein diet found higher glucose-stimulated insulin secretion, increased fasting glucose level, impaired suppression of hepatic glucose output by insulin and enhanced gluconeogenesis[41]. In addition, a 1 year study in type 1 diabetic patients revealed that consumption of a high protein diet led to decreased overall insulin sensitivity, and increased glucose production by the liver[42]. Furthermore, studies examining the metabolite profiles of more than 60 metabolites in humans have revealed the branched chain amino acids as predictors of the future development of diabetes [43]. In addition, it was found that the levels of the BCAAs were significantly higher in obese individuals compared to the lean subjects and the high fat/BCAA diet also promoted the activation of inflammatory factors such as the c-Jun N-terminal kinase (JNK), which further promotes the development of insulin resistance [40].

Studies using humans have shown that there is evidence for altered protein metabolism in individuals with type 1 diabetes; however the result in patients with T2DM is still unclear. A study conducted using hyperinsulinemic-euglycemic-isoaminoacidemic clamps in individuals with T2DM showed that men with T2DM have greater insulin resistance of protein metabolism than women, and these results may have implications for dietary protein requirements [44].

However, although an amino acid overload has been implicated in causing insulin resistance, amino acids have also been shown to have positive effects on muscle protein synthesis and growth. Muscle anabolism as a result of amino acid intake is due to a

stimulation of muscle protein synthesis and protein breakdown. Although this holds true even when ingesting whole proteins, it has been found that the ingestion of essential amino acids (EAA) alone is more efficient in stimulating muscle protein synthesis [45]. Essential amino acids make up about 40-45% of the total amino acids in high quality proteins. Thus, it has been suggested that, if given adequate protein consumption, the EAA component should be sufficient to stimulate protein synthesis[45].

Furthermore, BCAA supplementation has reportedly improved age-related disorders such as sarcopenia, insulin resistance, and cardiovascular dysfunction. Studies in mice have demonstrated that diets enriched with BCAA have increased their average lifespan and also increased cardiac and skeletal muscle mitochondrial biogenesis. BCAA supplementation also up regulated the ROS defence system, resulting in decreased oxidative damage in the muscle[46]. Other studies in mice have demonstrated that, in mice made insulin resistant via high fat diet, doubling dietary leucine reversed the metabolic abnormalities and improved insulin sensitivity without altering food intake or weight gain[47]. In mice with obesity-induced insulin resistance, plasma BCAA levels are increased; but this is suggested to promote protein synthesis to preserve the protein loss that occurs in the face of insulin resistance that is caused by obesity[223]. Studies in human subjects with T2DM have shown that circulating amino acid concentrations in post-prandial individuals does not worsen the impairments in glucose disposal [48]. Thus, the proposal to restrict dietary protein in individuals with metabolic disorders was not supported in this study.

2.2.1.3 High Carbohydrate diets

Carbohydrates serve a very important purpose in whole body metabolism. They provide energy, satisfy hunger and also regulate blood glucose levels[49]. Carbohydrates can be classified according to the way they are digested. Firstly, there are those carbohydrates that are rapidly digested and cause a rapid increase in blood glucose. These include foods such as white bread, white rice and cooked potatoes. There are also carbohydrates that take longer to digest, resulting in a more balanced blood glucose level. These foods tend to contain wholegrain products and are higher in fibre [49].

The term 'sugars' is commonly used to indicate mono- and disaccharides and is thus an integral part of a person's caloric intake. Glucose, fructose and sucrose are the most consumed sugars in a diet [50]. Of this, high-sucrose diets have been implicated in the development of hepatic and peripheral tissue insulin resistance in rats. Studies have shown that high-sucrose fed rats are known to be more insulin resistant than high-starch fed rats, particularly due to its effect on the liver, making it increasingly resistant to the suppression of glucose production under insulin stimulation [51]. High-sucrose diets have also been associated with a decrease in insulin-stimulated glucose uptake in muscle of rats fed high-sucrose diets, compared to rats that have been fed high-starch[52]. However, other studies have also demonstrated that there has been no significant difference in muscle 2-deoxyglucose (DG) uptake in rats fed high-starch versus high-sucrose, using a very similar diet and methodology[51]. Another study conducted by Chun et al

demonstrated that rats fed high-starch versus high-sucrose diets were still able to impair insulin stimulated glucose transport in rat muscle to a similar degree. However, the reduction in glucose transport seen in these groups was significantly lower than rats on a chow diet [53]. Thus, although it is clear that high carbohydrate diets of a particular composition can induce insulin resistance, more research needs to be done.

Fructose has also gained considerable attention in the last decade, particularly as a potential contributor to the worldwide rise in overweight and obesity rates[54]. Although the effects of fructose consumption have been quite controversial, it is important to note that the human diet rarely encounters fructose as a single nutrient. Usually, dietary exposure to fructose occurs through the co-ingestion of glucose via sucrose (composed of glucose and fructose) [55]. Regardless, the consumption of fructose has been linked to the metabolic syndrome identifying features; these include dyslipidemia[56,57], insulin resistance[57] and high blood pressure[54]. The muscle metabolic use of fructose is actually very limited; adipose tissue is said to play a greater role in the metabolism of fructose[53]. Fructose is also metabolised in the liver, kidney and intestine [50,58]. Another characteristic of fructose is that fructose does not stimulate insulin secretion or require insulin for the initial stages of its metabolism in the liver. This is potentially beneficial for diabetic patients [59,60] who will already have difficulty in their ability to release or respond to insulin.

Interestingly, the prevailing hypotheses regarding the mechanisms by which fructose promotes the development of these diseases is via its lipogenic nature[61-63]. A recent study

proposed that fructose consumption is able to trigger lipid deposition into visceral adipose tissue, particularly in men. In contrast, the consumption of glucose is able to favour lipid deposition in subcutaneous adipose tissue[57].

Until recently, the classification of carbohydrates as simple or complex varied depending on the size and structure of the carbohydrate itself. This was done in the belief that the size of the carbohydrate would determine the rate of digestion and absorption. However, when carbohydrates of different sizes and structures are digested, they also introduce changes in an individual's blood glucose and insulin response, an effect commonly measured using the glycemic index[64-65]. The speed and level at which an individual's blood glucose rises following the consumption of carbohydrates is described as the glycemic response. Rapidly absorbed carbohydrates that induce a high post-prandial glucose and insulin response are said to have a high glycemic index[66]. A continuous consumption of these carbohydrates can eventually impair insulin sensitivity, as it causes a rapid and ongoing fluctuation in glucose and insulin level in the body. One must also consider the glycemic load of these carbohydrates. A glycemic load indicates the quantity and quality of carbohydrates, calculated as a product of glycemic index of a particular food as well as its carbohydrate content[66]. Thus, a food with a high glycemic index can also have a low glycemic load, depending on the carbohydrate content.

2.2.2 Physical Inactivity

Physical inactivity or sedentary lifestyles have been shown to increase the risk of developing cardiovascular disease and diabetes [67-68]. Insulin resistance that develops in

association with excessive adiposity and physical inactivity has become a vital factor to the rising burden of T2DM in Western cultures [69-70]. Specifically, about 80% of cases of T2DM are associated with obesity and a rise in sedentary lifestyles [71].

Many studies have demonstrated that there is a strong association between the amount of daily physical activity an individual undergoes and a reduced risk of developing T2DM [71]. Dysfunction of the vascular endothelium has also been linked to physical inactivity[72]. Individuals who follow this lifestyle have impaired endothelial vasomotor function compared to those who are physically active [73]. Consequently, insulin resistance and diabetes have also been found to be linked to endothelial dysfunction. This indicates that the endothelial dysfunction caused by physical inactivity can lead to insulin resistance. Previous studies have demonstrated that a short period of physical inactivity has induced insulin resistant in healthy individuals, along with the development of vascular dysfunction such as dyslipidemia and high blood pressure [7,74].

2.2.3 Genetics

An individual's genetic background is also an important determining factor in the susceptibility of pancreatic beta cells to decompensation and the eventual progression to insulin resistance and type 2 diabetes. It has previously been demonstrated that certain individuals in some families will be predisposed to insulin resistance and T2DM from birth. Since insulin resistance has been found in first degree relatives and the offspring of patients with T2DM, it is generally thought that insulin resistance is partly determined by an individual's genetic composition, and is also a factor in the development of T2DM [75].

It has been previously demonstrated that young, healthy offspring of individuals that are already diabetic, commonly exhibit the signs of insulin resistance and impairments of insulin signalling in muscle as well as glycogen synthesis, many years before the onset of T2DM itself [76]. However, recent genome-wide association (GWA) studies have failed to identify any polymorphisms of the genes involved in insulin signalling in these individuals that could contribute to the development of T2DM. Rather, a number of genes involved in the control of insulin secretion have shown polymorphisms that conferred an increased susceptibility to T2DM [75].

Maturity onset diabetes of the young (MODY), a subclass of type 2 diabetes, is a monogenic disease involving mutations of important beta cell transcription factors or metabolic regulatory proteins. These include the hepatocyte nuclear factor-4α (resulting in MODY1), glucokinase (resulting in MODY2), and pancreatic and duodenal homeobox-1 (PDX1, resulting in MODY4) [77]. Although these diseases are characterized by early-onset diabetes, they only represent about 1-2% of the total T2DM cases worldwide. The remaining population of cases comprised of the typical or obesity-induced T2DM occurs due to a 'cluster' of genetic variants that are also influenced by a variety of environmental factors such as over nutrition, obesity and stress.

2.3 Insulin Signalling Pathway (General Overview)

Insulin resistance can manifest itself in three different types of tissues: adipose, liver, and muscle. Thus, it is essential that one understands the mode of insulin signalling in these

tissues as well as the functional relevance of each. It is also important to note that the mechanism of insulin signalling will differ slightly in each tissue.

2.3.1 The Insulin Receptor

The insulin receptor is a heterotetrameric transmembrane protein and is part of the tyrosine kinase receptor family[78]. The tyrosine kinase domain of the insulin receptor becomes active upon binding with a ligand. Thus, the binding of insulin to the insulin receptor results in its autophosphorylation, specifically of several tyrosine residues in the intracellular domain. This action is then able to attract several 'effector' proteins. Naturally, the specificity of the interaction between the effector proteins and the insulin receptor will depend on the specific motif of amino acids adjacent to or surrounding a particular tyrosine residue[78]. Examples of such effector proteins include the Insulin Receptor Substrates 1 and 2 (IRS1, IRS2), the c-Cbl (Cbl) proto-oncogene and the Src-homology-2-containing protein (SHC) [79]. Among these downstream substrates, the IRS proteins have the most important role in controlling metabolic fuel homeostasis[80-81].

2.3.2 Insulin Receptor Substrates

IRS1 is the key substrate of interest for this thesis and is the major IRS protein involved in insulin signalling within skeletal muscle[44]. This is because IRS1 is known for its association with decreasing or increasing blood glucose level[82]. IRS1 is a key mediator of insulin-stimulated glucose uptake and activation of the anabolic pathways in muscle and adipose tissue. However, the anabolic effects of insulin in the liver are mainly mediated

by IRS2[81]. IRS2 is also a critical component of pancreatic β-cell growth, function and development, while IRS1 contributes to the insulin secretory mechanisms[83].

IRS proteins consist of three key domains; the first is the conserved plekstrin homology (PH) domain located at the amino terminus of the IRS protein[81,84]. This domain is responsible for anchoring the IRS proteins to the membrane phosphoinositides that are in close proximity to the insulin receptor. The PH domain is also flanked by a Phospho-Tyrosine binding (PTB) domain; this functions as a binding site for the NPXY motif located at the juxtamembrane domain of the insulin receptor. [84-85] At the C-terminal region of the IRS proteins, there are multiple tyrosine phosphorylation motifs. These motifs act as a signalling scaffold, providing a docking interface for the SH2 domain-containing proteins, such as the p85α regulatory subunit of phosphatidylinositol 3 kinase (PI3K). SH2 domain proteins are adaptor proteins whose main function is to relay the ligand signal by additional protein-protein interactions. These work to further propagate the growth-promoting effects of insulin[85]. Thus, the phosphorylation of IRS1 leads to its association with the p85 regulatory subunit of PI3K (Fig. 1).

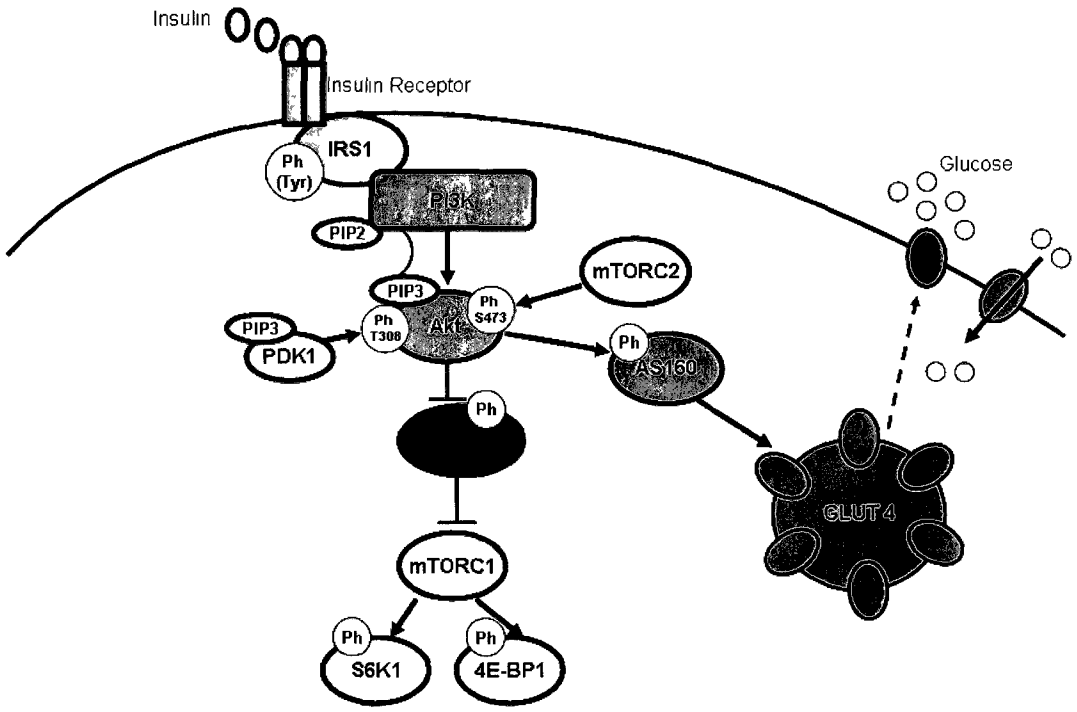


Figure 1. General overview of insulin signalling and the proteins involved in glucose uptake, cell growth and proliferation. The binding of insulin to the insulin receptor leads to its activation, following which it is able to phosphorylate the insulin receptor substrate 1 (IRS1) on tyrosine residues. This allows the interaction of IRS1 with the phosphatidylinositol-3-kinase (PI3K), which facilitates the conversion of the phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 then recruits a number of kinases such as the phosphatidylinositol-dependent protein kinase 1 (PDK1) and Akt to the plasma membrane. PDK1 then phosphorylates Akt; once Akt is activated it phosphorylates the Akt substrate of 160 kD, (AS160) and inhibits its function, thus leading to glucose uptake in the cell.

2.3.3 PI3K/ Akt and downstream targets

As mentioned, PI3-kinase consists of a regulatory subunit that is responsible for binding to the insulin receptor substrates; it also consists of a catalytic subunit responsible for phosphorylation of phosphatidylinositols found in cellular membranes. Thus, after IRS1 associates with the p85 regulatory subunit of PI3K, the p110 catalytic subunit of PI3K is recruited to the plasma membrane. The IRS1/PI3-kinase complex then translocates to internal membranes, which provide abundant substrates for PI3K. PI3K then facilitates the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 is then able to facilitate additional signalling events, such as the recruitment of the serine/threonine kinase, phosphatidylinositol-dependent protein kinase 1 (PDK1), PDK2 and Akt (also known as protein kinase B (PKB)) to the plasma membrane[80]. PDK1 and Akt are then anchored to the plasma membrane via their PH domains[86]. The colocalization of these proteins leads to the activation of Akt by phosphorylation at its Thr 308 residue by PDK1[80, 87] (Fig 1).

Once active, Akt is able to phosphorylate a number of downstream targets that can carry out a variety of functions. For example, Akt can phosphorylate glycogen synthase kinase 3β (GSK3β) in the liver [88] leading to its inactivation as well as FOXO1 (forkhead box O transcription factor), which regulates gene expression [89]. However, it can also phosphorylate AS160 (Akt substrate 160kD). AS160 is an inhibitory protein that prevents translocation of the glucose transporter-4 (GLUT4) by interacting with Rab proteins [80].

Akt phosphorylates the AS160 Rab-GTPase-activating protein, which in turn interacts with the small GTPase RAB10 to facilitate the translocation of GLUT4-containing vesicles to the cell surface. Once GLUT4 is translocated to the membrane, it is then able to assist in the uptake of glucose that exists outside of the cell and metabolize or store it as required[17].

2.4 Defects in Insulin Signalling

The insulin signalling pathway can be attenuated or impaired by various regulators. First, at the level of the insulin receptor (IR), a number of regulators can affect its phosphorylation on tyrosine residues. For example, the autophosphorylation of the IR can be reversed by the protein Tyr phosphatase-1B (PTB1B, also called PTPN1). The Tyr kinase activity of the IR can also be inhibited by the suppressor of cytokine signalling-1 (SOCS1) and SOCS2 [90].

However, the most common defects in the insulin signalling pathway occur at the level of the insulin receptor substrate[91]. Under normal circumstances, IRS1 is phosphorylated on tyrosine residues, leading to its activation. However, the functions of IRS1 can also be mediated by other factors such as protein tyrosine phosphatases, serine phosphorylation and ubiquitination; these factors all serve to impair the function of IRS1, leading to defects in insulin signalling.

An accumulation of lipids in the body (as seen in obese individuals) can lead to insulin resistance through a build-up of diacylglycerols (DAG). This accumulation triggers the

activation of Protein Kinase C (PKC), which is able to phosphorylate IRS1 on serine residues[85].

An overload of amino acids is also able to cause defects in the insulin signalling pathway. Specific amino acids such as leucine are able to directly activate the Mammalian Target of Rapamycin Complex 1 (mTORC1) kinase, which is able to sense nutrients as well as growth factors. Upon its activation, mTORC1 phosphorylates its two downstream targets: S6Kinase 1 (S6K1) and eIF4E-binding protein 1 (4E-BP1). High activity of S6K1 leads to the creation of a negative feedback loop to IRS1, where S6K1 phosphorylates IRS1 on its serine residues and inhibits its function[92] (Fig. 2).

The IRS proteins are targeted for degradation upon their phosphorylation on serine residues[93]. The residues that are targeted include serine 612, 307, 1101 and 636/639. Although earlier studies proposed that degradation of the IRS proteins occurred via calpain (calcium-dependent) proteases, recent evidence has shown that their degradation is regulated by the 26S proteasome complex[85]. Other stimuli such as hyperosmotic or osmotic stress can also trigger degradation. IRS1 can also be degraded by caspases which are activated as a result of apoptotic stimuli[87].

The accumulation of PIP3 can also be impaired by the activity of the lipid phosphatase PTEN (phosphatase and tensin homologue on chromosome-10). PTEN works to convert PIP3 to PIP2. Thus, from this it is clear that research into the elements of the insulin signalling pathway and their regulators provide opportunities for discovery of various

therapies in order to prevent these defects. A study found that knockout of PTPN1 in mice reduced insulin resistance and improved glucose homeostasis [94-95].

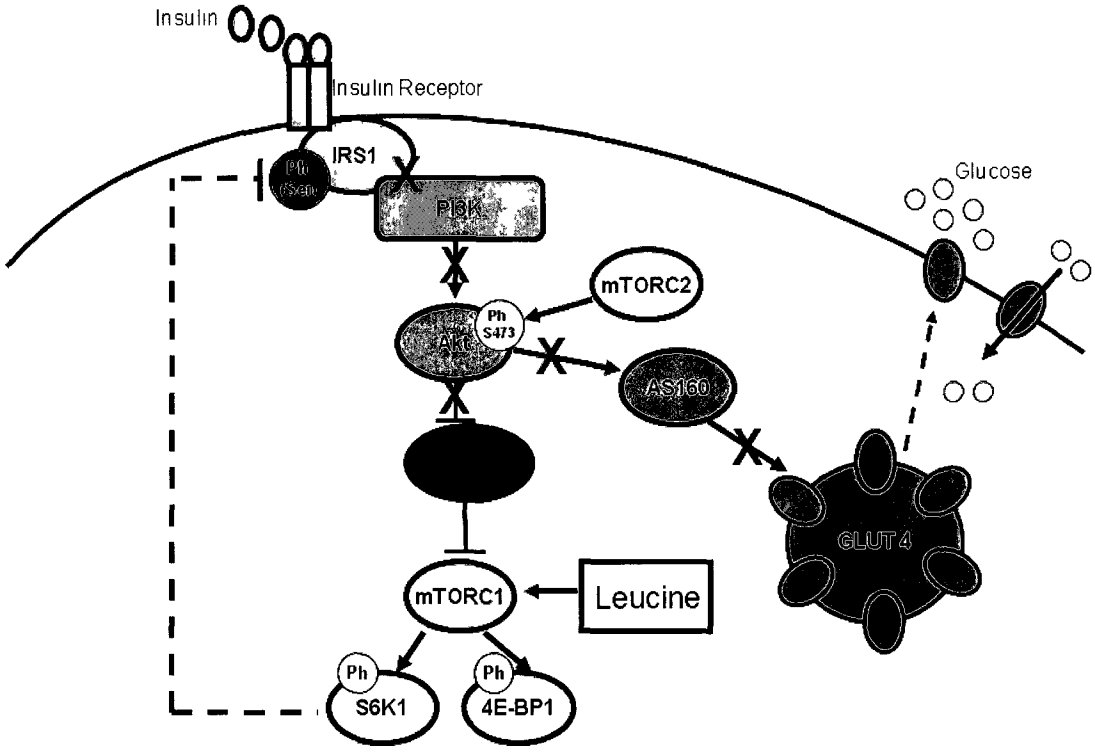


Figure 2. Defects in insulin signalling pathway as a result of amino acid overload. Specifically, leucine is able to directly activate mTORC1, leading to the formation of a negative feedback loop from S6K1 to IRS1, phosphorylating IRS1 on serine residues and inhibiting its function. IRS1: insulin receptor substrate 1, PI3K: phosphatidylinositol 3 kinase, TSC: tuberous sclerosis complex, AS160: Akt substrate of 160 kDa.

2.5 Insulin Sensitivity in Adipose Tissue

Adipose depots have evolved to serve as one of the major energy reserves in the body when glucose becomes limiting[18]. After the ingestion of a meal, insulin is able inhibit lipolysis in adipocytes to a great degree [9].

In adipocytes, the key characteristic of insulin is that it is antilipolytic. Specifically, insulin prevents the release of fatty acids from adipocytes[9]. The insulin signalling cascade in adipocytes involves the activation of the insulin receptor, specifically the tyrosine kinase, as well as the IRS proteins. This leads to further activation of its downstream targets, one being PI3K, and the subsequent production of specific phosphoinositides at the plasma membrane.

Insulin has been proposed to inhibit lipolysis in the adipocyte via the reduction of cyclic AMP (cAMP) levels and thus a reduction in Protein Kinase A (PKA) activity [96]. Under fasting conditions, lipolysis in the adipocyte becomes stimulated as a mode of providing nutrients. Catecholamines such as norepinephrine are able to stimulate lipolysis via the activation of beta-adrenergic receptors, promoting adenylyl cyclase activity and the production of cAMP. cAMP is then able to bind to the regulatory subunits of one of its major downstream targets, protein kinase A (PKA); this triggers the dissociation of the subunits of PKA (RI and RII) [97] and the subsequent activation of the catalytic subunits [96].

Two downstream targets of PKA that are essential for the process of lipolysis are hormone sensitive lipase (HSL) and Perilipin, the major lipid droplet coat protein.

Phosphorylation of HSL allows it to translocate from the cytosol to the lipid droplet; here it acts mainly as a diglyceride lipase. Perilipin is able to work as a protective barrier against lipase activity. Phosphorylation of at least 6 PKA consensus sites triggers a conformational change in perilipin, permitting access to the lipid substrates in the droplet, the recruitment of HSL, and possibly the activation of adipose triglyceride lipase (ATGL). Not only can perilipin block lipolysis in the basal state, it promotes lipolysis when phosphorylated [96].

2.6 Insulin Sensitivity in the Liver

Insulin exerts two dominant functions in the liver: first, it is able to reduce the production of glucose (or gluconeogenesis). This is done by inhibiting hepatic glycogenolysis (or glycogen breakdown to glucose) [98]. It also increases the synthesis of fatty acids and triglycerides (or lipogenesis) [99-101].

In a state of insulin resistance, one of these actions becomes blocked in the liver. In other words, the liver loses its ability to reduce glucose production, but retains its ability to increase the synthesis of fatty acids and triglycerides and enhance lipogenesis. This dual action leads to a lethal combination of hyperglycemia and hypertriglyceridemia that characterizes the diabetic state [99-101].

The hepatic actions of insulin are largely mediated at the transcriptional level. As insulin reduces gluconeogenesis, it is also reducing the transcription of several genes involved in glucose production; these include phosphoenolpyruvate carboxykinase (PEPCK) and

glucose-6-phosphatase. Insulin is able to do this in part by phosphorylating the transcription factor FOXO1, which works to mediate the effects of insulin on PEPCK and glucose-6-phosphate catalytic subunit, the gluconeogenic genes [102].

Thus, it is suggested that since insulin resistance tends to develop in one transcriptional program in the liver, while maintaining sensitivity in another program, the insulin signalling pathway must bifurcate at some point [101]. As mentioned above, the insulin signalling pathway is thought to proceed via the tyrosine phosphorylation of the insulin receptor substrates (IRS1 and/or IRS2), leading to the activation of PI3K and Akt. Thus, it is proposed that the bifurcation of this pathway must occur downstream of Akt [101].

2.7 Insulin Sensitivity in Skeletal Muscle

Quantitatively, skeletal muscle is one of the most important contributors to whole body insulin mediated glucose disposal post-prandially[103]. In humans, the major physiological mechanism responsible for the disposal of ingested glucose is through insulin-stimulated glucose transport into skeletal muscle. The majority of glucose that enters muscle fibres is converted to glycogen [104]. The mechanism of insulin signalling in skeletal muscle is described in sections 2.3.1-2.3.3. Once this signalling occurs, there are key downstream targets in the muscle that activate processes such as muscle growth and protein synthesis.

2.8 Akt, mTORC1 and amino acid induced insulin resistance in skeletal muscle

As mentioned above, the activation of Akt by PI3K leads to the recruitment of GLUT4 protein to the plasma membrane of the cell, thereby allowing for the uptake of glucose to occur. However, Akt is also able to trigger downstream substrates responsible for the growth of muscle. Upon its phosphorylation, Akt is able to phosphorylate its downstream target TSC1/2 (tuberous sclerosis complex 1/2). TSC2 is a GTPase-activating protein for Rheb (Ras homologue enriched in brain), and together with its partner TSC1 (also known as hamartin) forms the heterodimeric tuberous sclerosis complex (TSC) [105]. When active, TSC is able to inhibit the activity of mTORC1 by promoting the conversion of Rheb-GTP to Rheb-GDP. However, when the TSC complex is phosphorylated by Akt, its activity is inhibited, leading mTORC1 to become active. It is suggested that the phosphorylation of TSC2 by Akt would directly suppress its GAP activity toward Rheb [105]. This in part leads to the insulin-induced activation of the mTORC1 axis. One of the well-defined functions of the mTORC1 pathway is the regulation of mRNA translation and protein synthesis.

mTOR is a protein kinase that is involved in the control and regulation of a number of cellular processes, including: protein synthesis, ribosome biogenesis, gene transcription, cell growth, autophagy and overall metabolism [106]. mTOR is the catalytic subunit of two distinct multiprotein complexes, mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2). There are unique accessory proteins that distinguish these complexes. The regulatory-associated protein of mTOR (Raptor) is specific to mTORC1, while the

rapamycin-insensitive companion of mTOR (Rictor) is specific to mTORC2 [107-108]. These accessory proteins function as the scaffolds for the assembly of the complexes, as well as for binding substrates and regulators [109-114]. mTORC1 also has a negative regulator, the 40kDa Pro-rich Akt substrate (PRAS40), while mTORC2 contains the protein observed with Rictor 1 (PROTOR1) as well as PROTOR2, which helps with complex assembly. mTORC2 also contains the mammalian stress-activated map kinase-interacting protein 1 (mSIN1), which targets mTORC2 to the membranes [112,115].

Apart from the mTOR protein both complexes also contain mLST8 (mammalian lethal with Sec 13 protein 8, also called GβL) [112]. They also share the recently identified protein DEP domain-containing mTOR-interacting protein (Deptor). mLST8 functions as a positive regulator of the complex while Deptor functions as a negative regulator [116-117]. It is also very important to note that mTORC1 is a nutrient sensing protein, sensing the availability of growth factors, nutrients and hormones. This largely affects its activity.

2.9 Regulation of mTORC1

mTORC1 works as a signal integrator for four major regulatory inputs: nutrients, growth factors, energy and stress. These inputs can work in a cooperative manner, or antagonistically, thereby enabling the cell to fine-tune its mTORC1 activity. The regulators of mTORC2 remain to be found; however it is suggested that only growth factors regulate this complex[118].

2.9.1 Regulation of mTORC1 by Nutrients

Apart from being the building blocks of proteins, amino acids are the key regulators of mTORC1 activity. For example, removal of amino acids in a medium containing cells can result in a rapid dephosphorylation of the substrates of mTORC1, such as S6K1 and 4E-BP1[106]. Removing single amino acids, particularly leucine and arginine has also been shown to dramatically impair mTORC1 signalling.

There appears to be approximately 4 mechanisms that link amino acids and mTORC1 signalling. The first link occurs via the RAG family of small guanosine triphosphatases (GTP)ases. The presence of amino acids results in the binding of mTORC1 to the RAG proteins. RAG GTPases are heterodimers of either RAGA or RAGB with either RAGC or RAGD. Studies indicate that the Rag complexes reside on the lysosomal surface [106]. In the absence of amino acids, the Rag proteins are in their inactive forms; specifically that RAGA or RAGB are loaded with GDP while RAGC or RAGD contain GTP. However, when amino acids are present, RAGA and RAGB become loaded with GTP and RAGC and RAGD are loaded with GDP [111,118].

Literature reveals that the RAG GTPases are required for the control of mTORC1 by amino acids [111,106]. Under the influence of amino acids, the RAG proteins can target mTORC1 to the lysosomal surface, via the protein complex Ragulator. The Rheb-GTP is also found here; this leads to the activation of mTORC1 and subsequently S6K1[106].

A second mechanism involves the class III PI3K proteins. Once amino acids enter the muscle, an increase in intracellular calcium occurs; this leads to increased binding of calmodulin to a class III PI3K family member, hVps34 (human vacuolar sorting protein 34). Studies have indicated that hVps34 is an intracellular amino acid sensor [119, 120]. The influx of Ca^{2+} caused by the amino acids increases the interaction of Ca^{2+}/CaM (calmodulin) with the hVps34-mTOR complex, thereby activating mTORC1. The interaction between mTORC1 and hVps34 also leads to an increase in PIP3, possibly leading to a conformational change in mTORC1 [121]. More information on the mechanisms behind this action remains to be found.

Another mechanism involved in the activation of mTORC1 by amino acids is the MAP4K3 (mitogens activated protein kinase kinase kinase 3) pathway. MAP4K3 is a kinase that is able to regulate the activity of S6K1 in response to amino acids [122]. Specifically, amino acids are able to induce the phosphorylation of MAP4K3, which in turn leads to the activation of mTORC1 [122, 123]. MAP4K3 is positively regulated by the presence of amino acids, but is not activated by insulin, nor is inhibited by rapamycin. Thus, MAP4K3 appears to work downstream of amino acid sufficiency and upstream of mTORC1 [123].

Finally, the inositol phosphate multikinase (IPMK) has also been shown to affect the ability of amino acids to activate mTORC1. Specifically, IPMK stabilizes the mTOR-Raptor binding in the mTORC1 complex via the amino terminal sequence of IPMK [124]. Furthermore, a study found that depletion of IPMK reduced amino acid-stimulated

mTORC1 activation by approximately 60%; this was comparable to the deletion of a major component of mTORC1, such as raptor [108].

2.9.2 Regulation of mTORC1 by Growth Factors

Growth factors such as insulin are able to regulate the activity of the mTORC1 pathway via the activation of the PI3K/Akt axis. As mentioned previously, the binding of insulin to its receptor activates the PI3K pathway, leading to the phosphorylation and activation of Akt. Akt is then able to phosphorylate and inhibit the TSC1/2 complex [125-126]. Phosphorylation of TSC1/2 by Akt[125-126] leads to the inhibition of the TSC1/2 GAP activity for Rheb; this promotes mTORC1 activation [126].

Growth factors can also signal to mTORC1 using other pathways. For example, the extracellular signal-regulated kinase (ERK) can also phosphorylate TSC2 directly, downstream of the Ras-Raf-MAPK/ERK kinase (MEK)-ERK axis, to inhibit it [127].

2.9.3 Regulation of mTORC1 by Energy

Specific chemical inhibitors of glycolysis and mitochondrial function are able to suppress mTORC1 activity; this is an indicator that mTORC1 is able to sense cellular energy. This is very important to the action and function of mTORC1 as an energy sensor, because mTORC1-driven growth processes consume a large fraction of cellular energy and can therefore be deleterious to starving cells[108,128].

Glycolysis and mitochondrial respiration are responsible for converting nutrients into energy, which is stored in the form of ATP. Under nutrient-deprived conditions such as starvation, there is a drop in cellular ATP levels. mTORC1 is then able to sense this decrease in ATP production using a mechanism centered around the AMP-activated protein kinase (AMPK)[129].

The actions of AMPK are centered on the ratios of AMP to ATP, as they are both allosteric regulators of AMPK. When the ratio of AMP: ATP increases, AMPK is able to activate TSC2, possibly stimulating the GAP activity of the TSC1/2 complex towards Rheb to inhibit mTORC1 signalling. Furthermore, AMPK is also able to phosphorylate Raptor, causing it to bind to 14-3-3 proteins, which leads to the inhibition of mTORC1 via allosteric mechanisms [130].

2.9.4 Stress

mTORC1 activity can be suppressed by stressors, specifically because stressors can affect the levels of ATP in the body. This in turn affects the activity of mTORC1, via the AMP-AMPK axis. Other stressors can also act on mTORC1 through AMPK, using ways not related to impairments in cellular energy. DNA damage can lead to the inhibition of mTORC1 activity via the p53-dependent upregulation of AMPK. Sestrin 1 and 2 are two transcriptional targets of p53 that are implicated in the DNA damage response; it was shown that these targets are able to activate AMPK. Thus, they are able to mediate the p53-dependent suppression of mTORC1 activity upon DNA damage [131].

2.10 Substrates and actions of mTORC1

2.10.1 4E-BP1

mTOR Complex 1 is able to activate two downstream targets: S6 Kinase 1 (S6K1) and the eukaryotic initiation factor (eIF) 4E- binding protein 1 (4E-BP1). These targets are associated with mRNA and are able to regulate mRNA translation initiation, as well as its progression; thus they play a role in regulating the rate of protein synthesis in skeletal muscle. When 4E-BP1 is unphosphorylated it is an inhibitory protein that is able to suppress translation [118]. This protein inhibits mRNA translation and protein synthesis by competing with another eukaryotic mRNA translation initiation factor (eIF4G) for a shared binding site on eIF4E. The binding of 4E-BP1 and eIF4G to eIF4E are mutually exclusive[132]. The binding of 4E-BP1 to eIF4E is dependent upon phosphorylation: while hypophosphorylated forms of 4E-BP1 strengthen the interaction with eIF4E, hyperphosphorylation has the opposite effect. This allows eIF4E to become free to bind with eIF4G, and translation can occur[118].

Phosphorylation of 4EBP1 inhibits its activity. Thus, after being phosphorylated, 4EBP1 dissociates from eIF4E, thereby allowing eIF4E to recruit the translation initiation factor eIF4G to the 5' end of the mRNA [118].

2.10.2 S6K1

The p70 S6 kinase (S6K1) is a member of the A, G and C family of serine/threonine protein kinases [133]. It is responsible for the regulation of protein synthesis, cell size and

proliferation in response to cellular nutritional status, specifically amino acids, and hormonal stimulations[119]. The phosphorylation of S6K1 on its Threonine-389 residue is regulated by mTORC1, which can directly phosphorylate it. Phosphorylation of S6K1 thus leads to the progression of protein synthesis and cell growth. S6K1 is able to promote mRNA translation by phosphorylating or binding multiple proteins, including eIF4B (which affects translation initiation and elongation) [134]. When phosphorylated, eIF4B enhances the activity of eIF4A, an RNA helicase responsible for unwinding the structured 5' untranslated regions (UTRs) of many mRNA[135]. S6K1 also phosphorylates the tumor suppressor, programmed cell death 4 (PDCD4), which usually blocks the association of eIF4A with the translation pre-initiation complex[136, 118].

2.11 Substrates and actions of mTORC2

The mTORC2 was initially identified as a mediator of the actin cytoskeletal organization, as well as cell polarization[116,137]. It is also responsible for controlling a number of regulators of the cytoskeleton, namely the Rho1 GDP-GTP exchange protein 2 (Rom2), as well as the AGC kinase Ypk2 [118].

More recently, it has been found that mTORC2 is able to phosphorylate and activate Akt, serum- and glucocorticoid- regulated kinase (SGK), and protein kinase C, which are able to regulate cell survival, cell cycle progression and anabolism [137-140]. It was specifically found that mTORC2 phosphorylates Akt at its Ser473 residue in mammals[137-140]. Phosphorylation at this site is then able to prime Akt for further phosphorylation at

Thr308, in the catalytic domain, by 3-phosphoinositide-dependent protein kinase 1 (PDK1). These events together lead to the full activation of Akt[137,141-142].

2.12 Mechanisms of Insulin Resistance in Skeletal Muscle

2.12.1 Nutrient overload (Negative effects of amino acid overload)

The activation of the mTORC1 pathway has also been implicated in the development of insulin resistance and obesity[132]. In these diseases, mTORC1/S6K1 signalling is chronically activated due to an overload of nutrients, particularly amino acids[119, 193]. Leucine, one of the branched-chain amino acids, has been shown to be able to directly activate mTORC1 and stimulate this activation[121]. Studies have shown that this chronic activation promotes the development of insulin resistance. Specifically, the hyperactive S6K1 phosphorylates IRS1 on serine residues 612, 636/639, 1101 and 307, after which IRS1 becomes degraded; the transcription of IRS1 is also impaired under these conditions [10]. This reduction in the abundance of IRS1 attenuates insulin signalling.

It has been shown in the literature that serine phosphorylation of IRS1 can have positive, negative or both positive and negative effects on insulin signalling [10]. The complex regulation of IRS1 can be partly explained by the finding that it is a pattern of phosphorylation that is required to regulate the function of IRS1, rather than the phosphorylation of one particular site. Literature states that the positive regulatory sites on IRS1 may be phosphorylated first and may subsequently protect IRS1 from the phosphorylation of the inhibitory sites, or association with tyrosine phosphatases [10]. The

positive regulatory sites are found on the tyrosine residues: these are Y608, Y895 and Y1172 [145], whereas the inhibitory sites are on serine residues (S307, S612, S636/639, S1101). The inhibitory sites are usually phosphorylated later on and shut down insulin signalling. The inhibitory sites of IRS1, clustered near the PTB domain, are able to disrupt the interactions between IRS1 and IR when phosphorylated. The phosphorylation of those sites also promotes the degradation of IRS1. These sites are also able to negatively regulate the interaction between IRS1 and PI3K, specifically the sites located in the C-terminal tail [10].

2.12.2 Lipid accumulation

Insulin resistance develops due to the accumulation of fatty acid metabolites, specifically diacyglycerols (DAG) within the insulin responsive tissues. However, this discovery was only made after making the connection between DAG accumulation and its effect on protein kinase C (PKC) serine-threonine kinases[17].

PKC can become activated when calcium binds to the C2 domain of the protein, increasing the affinity of the C1 domain for diacylglycerol[146-147]. It has been discovered that phorbol esters can potently activate PKC and impair activation of the insulin receptor in-vitro, via serine phosphorylation of IRS1[148-150]. Thus, it is hypothesized that the accumulation of DAG within the cells can activate PKC to the same degree. In this model, the development of insulin resistance is attributed to defects in the insulin signalling pathway caused by reduced IRS1 tyrosine phosphorylation. Instead, IRS1

serine phosphorylation is increased, leading to inhibition of any further signalling in this pathway.

Surprisingly, accumulation of triglycerides does not exhibit the same effect as the accumulation of diacylglycerols. Studies in mice overexpressing DGAT1 (diacylglycerol acyl transferase 1: this enzyme transfers a fatty acid from fatty acyl coenzyme A to diacylglycerol to make triglycerides) accumulated triglycerides in their muscles[151]. These mice were protected from fat-induced insulin resistance[151]. Insulin resistance caused by lipid accumulation can readily be explained in obese individuals. In these individuals, increased delivery of fatty acids overwhelms the system, inhibiting the cell's ability to oxidize fat or convert diacylglycerol to triacylglycerols [17].

2.12.3 Production of ROS products/ Defects in mitochondrial metabolism

The production of reactive oxygen species is also able to stimulate the development of insulin resistance. Recent evidence reveals that treating 3T3-L1 adipocytes with tumor necrosis factor alpha (TNFα) increases the development of ROS species, resulting in decreased insulin action[146]. However, antioxidant molecules or transgenes encoding ROS scavenging enzymes were able to attenuate the insulin resistance caused as a result of ROS species production. Other supportive human studies have demonstrated that defective mitochondrial fatty acid oxidation, mitochondrial dysfunction and reduced skeletal muscle mitochondria play a role in the pathogenesis of diseases such as insulin resistance and T2DM [152-153].

Reductions in mitochondrial fatty acid oxidation due to mitochondrial dysfunction or reduced mitochondria lead to the production of intracellular fatty acyl CoA and diacylglycerol. The accumulation of these products leads to the activation of PKC, as mentioned previously, leading to inhibition of the insulin receptor substrate and any downstream signalling.

2.12.4 Inflammation

Epidemiological evidence has suggested a strong correlation between inflammation and the development of insulin resistance, dated as early back as the 1950s [154]. Specifically, this correlation has also been associated with disorders such as obesity, indicating that obesity may also share a link. In the last decade however, it has become increasingly clear that obesity and the simultaneous development of inflammation are major contributors to the development of insulin resistance[154].

It has been recognized for the past century that high doses of salicylates such as aspirin have been shown to reverse or reduce insulin resistance and T2DM[155-156]; they also appear to preserve beta cell function[157]. However, insulin resistance caused by high fat diets or obesity is able to trigger the activation of a number of inflammatory markers. One such marker that becomes active is the transcription factor IκB kinase (IKK)-nuclear factor κB (NF-κB) as well as its targets in the liver. However, salicylates have been shown to suppress NF-κB activity[158]. Studies have found that overexpression of a constitutively active version of NF-κB-activating kinase, IκB kinase catalytic subunit

(IKKβ), in the liver of healthy rodents resulted in the development of liver and muscle insulin resistance as well as diabetes[158]. The effect of high fat feeding and IKKβ overexpression also increases the production of interleukin-6 (IL-6), interleukin-β (IL-β) and TNFα in hepatocytes[158]. However, antibody-mediated neutralization of IL-6 in animals on a high fat diet was able to partially restore insulin sensitivity. Furthermore, deletion of IKKβ in the liver of mice protected against diet-induced hepatic insulin resistance, though muscle and adipose insulin resistance continued to develop[158]. Other studies have demonstrated that elevated levels TNFα, IL-6 and IL-8 have been found in diabetic individuals and individuals that have developed insulin resistance [154]. Under conditions of obesity or high fat feeding, the cytokines mentioned above are also able to trigger insulin resistance by inducing the activation of NF-κB-JNK pathway. Once JNK is active, it is able to induce insulin resistance by increasing the serine phosphorylation of IRS1; this is done either directly or via the activation of S6K1 to IRS1, phosphorylating the protein on serine 270 [10, 159].

Overall, there is accumulating evidence that insulin resistance in muscle is at least partly caused by the changes in hormone and cytokine production by the liver, adipose tissue and infiltrating immune cells in response to chronic exposure to lipids and other metabolic fuels[81].

3.0 RATIONALE AND OBJECTIVES

Rationale: The reversibility of insulin signalling has been previously analyzed in the context of high fat diet consumption, along with the addition of exercise. Studies have demonstrated that insulin resistance due to obesity and high fat diets are reversible. Specifically, it has been shown that a reduction of adipose tissue through weight loss is one of the ways that insulin resistance can be reversed [160-161].

In skeletal muscle the reversibility of insulin resistance is beginning to be examined, but only with respect to high fat diets. Furthermore, the time course of this development has not been examined. As stated before, many individuals use protein diets because of the belief that it can increase lean muscle mass and reduce fat mass. In the treatment of obesity, high protein diets are often recommended. However, it has now been shown that high amino acid diets can also contribute to the development of insulin resistance through mTORC1/S6K1 hyperactivation. This induces a negative feedback effect on IRS1. Given that many steps in the insulin signalling pathway are regulated by negative feedback mechanisms, it is likely that the effects of amino acids in regulating mTORC1/S6K1 and down regulating IRS1 signalling is a temporal and reversible event.

Surprisingly, all the studies that have reported amino acid induced signalling did not examine this possibility.

Objectives: 1. To examine the effect of leucine on S6K1-mediated IRS1 serine phosphorylation and insulin stimulated glucose transport in myotubes. The specific

proteins and residues chosen to examine in this time course analysis were S6K1 (T389) and IRS1 (S307, 612, 636/639). This is because S6K1 phosphorylation on this residue is an indicator of whether it is being activated by mTORC1, which would have been activated by leucine. The IRS1 serine residues were chosen on the basis that they would only be phosphorylated by S6K1 under conditions where it is hyperactive. Furthermore, S6K1 has been known to phosphorylate the IRS1 protein on its serine residues. I also aim to determine if amino-acid induced suppression of insulin-stimulated glucose uptake in myotubes is reversible.

2. Examine whether these effects are also seen in-vivo with healthy rats, and whether this is modified in insulin resistant states.

Hypothesis: I hypothesize that, in a normal physiological state, amino acid induced insulin resistance of glucose transport is a transient metabolic response, and is reversible upon amino acid withdrawal. I also hypothesize that in insulin resistant rats, the mechanisms responsible for this reversibility are impaired.

4.0 MATERIALS AND METHODS

Reagents: Fetal Bovine Serum (FBS), Horse Serum (HS), PBS (phosphate buffered saline); Antibiotic-Antimycotic preparations were purchased from Invitrogen Canada (Burlington, ON Canada). Immobilon Western HRP chemiluminescence was obtained from Millipore Corporation (Billerica, MA, U.S.A.). L-leucine (cat #L8912), phosphatase and protease inhibitor cocktails were purchased from Sigma-Aldrich (St. Louis, MO). α-

Eagle's minimal essential medium (AMEM), antibiotic and antimycotic preparation, and trypsin were obtained from Wisent (St Bruno, QC, Canada). Dithiothreitol (DTT) from Research Organics (cat #2190D, Cleveland, OH). L-leucine dehydrogenase was obtained from Calbiochem, Canada (cat# 431525). Glucose solutions and glucose/lactate buffers were obtained from Interscience (Markham, Canada). Scintillation fluid (Ecolite+) was obtained from MP Biomedicals (Solon, OH).

Antibodies: Antibodies to Ph-S6K1 (cat#9234), total S6K1 (cat#9202), Ph-IRS1 Serine 612 (cat#3203), Ser636/639 (cat#2388), Ser307 (cat#2381), total IRS1 (cat#2328), Ph-Akt Threonine 308 (cat#5056), Ph-AS160 Threonine 642 (cat # 4288) and Horseradish Peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit and anti-mouse) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies to γ –tubulin were obtained from Sigma-Aldrich (Oakville, Canada).

Cell culture work: Differentiation experiments were performed as follows: Rat skeletal muscle myoblasts L6 cells were obtained from the American Type Culture Collection. Stocks of the earliest passage cells have been stored and used. The cells were propagated at 37 ° C and 5% CO_2 in humidified atmosphere in complete proliferation medium composed of AMEM supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic (Ab-Am) preparations. After approximately the sixth passage, the cells were plated in 12-well plates at 5.0 x 10^4 cells /well. Forty-eight hours later the medium was changed to a fusion medium that contained AMEM supplemented with 2% horse serum, and 1% Ab-Am.

Incubation-Starvation-Re-incubation Experiments: Myotubes were starved for 4 h in serum-free AMEM, following which cells were either re-incubated in AMEM+800 μM leucine, or RPMI medium (complete starvation medium, free of amino acids, serum and glucose) for 30 minutes. The myotubes were incubated in 800 μM of leucine solution because the plain AMEM media already contains 400 μM of leucine solution. Thus, this amount was doubled in order to measure the effect of leucine on insulin sensitivity. Following 30 minute refeeding, the incubation with leucine continued in either the presence or absence of 100 nM insulin, following which the cells were harvested using lysis buffer at 5 minutes, 30 minutes, 1 h or 2 h post-incubation in insulin. Similarly, the incubation in RPMI continued in either the presence or absence of insulin and harvested at 5 mins, 30 mins, 1 h and 2 h post-incubation in insulin.

In the second experiment, cells were incubated in leucine and insulin overnight. The next day the cells were starved for 4 h in serum-free AMEM and then incubated in either leucine solution or RPMI for 30 minutes, followed by incubation in insulin for an additional 30 minutes. Following the administration of insulin for 30 minutes, cells were harvested using cell lysis buffer and western blot analysis was conducted. ***Harvest***: cells were washed once with PBS and then harvested in lysis buffer containing: 25mM Tris pH 7.5, 1mM EDTA, 2% sodium dodecyl sulfate (SDS) supplemented with protease inhibitor cocktail (10μl/ml), phosphatase inhibitor cocktail (10μl/ml) and 1M DTT (1μl/ml).

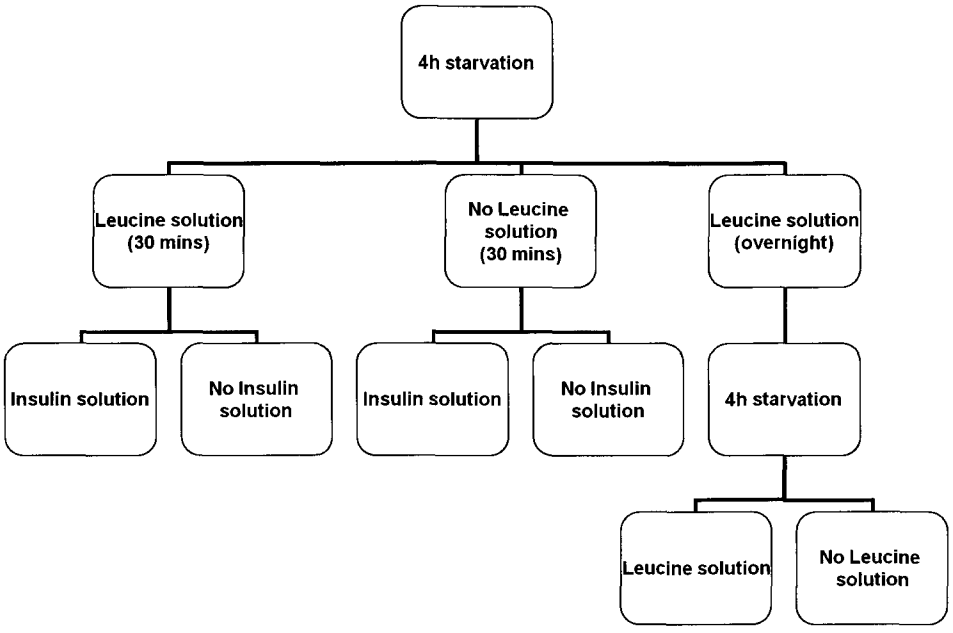


Figure 3. Outline of experiments conducted on myotubes. Myotubes were starved for 4 h and incubated in leucine solution or leucine free solution for 30 minutes. Following this the cells were incubated with or without insulin and harvested at various time points. A third group of myotubes were incubated in leucine and insulin solution overnight and then starved for 4 h the next day (simulating a washout period). They were then re-incubated with either leucine and insulin, or insulin alone, and harvested.

Protein Assay and Western blot determination of IRS1, S6K1 and γ –Tubulin: Protein content was determined using the Pierce BCA Protein Assay Kit (ThermoScientific, Rockford, IL). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare). Membranes were subsequently blocked in 5%

non-fat milk in TBST for 1 h at room temperature. After 1 h, membranes were washed in TBST, and were incubated with the desired primary antibody (1:1000 dilution for rabbit antibody for S6K1 (T389) and IRS1 (S307, 612, 636/639); 1:10000 dilution for mouse antibody for gamma-tubulin) overnight at 4°C. The next day, the membranes were washed (2X quick rinse, 3X 5 minutes at room temperature), and incubated with specific secondary antibodies (1:10000 dilution, goat anti-rabbit for S6K1/IRS1 and goat anti-mouse for gamma tubulin) for 1-3 h at room temperature. The HRP chemiluminescence substrate (Millipore Corporation) and Kodak imaging station (Molecular Imaging System Carestream Health Inc.) were used to visualize the bands. Immunoblot signals were quantified using the Carestream Molecular Imaging software (Version 3, Software, La Jolla CA).

Glucose uptake experiments: Myotubes were treated exactly as mentioned above (see Incubation-Starvation-Re-incubation experiment), following which glucose uptake was measured. Once the myotubes were incubated in their respective re-incubation solutions, all the cells were given 100 nM insulin in either AMEM or RPMI, depending on what medium they were incubated in, for an additional 20 minutes. Following incubation, cells were washed two times with only HEPES buffered saline, after which the cells were incubated in 200 µl of Transport solution (HEPES buffer, 10 µM 2-deoxyglucose, 0.5 µCi/ml [^{3}H]-deoxy) for 5 minutes. Some myotubes were incubated in transport solution supplemented with 10 µM cytochalasin B to control for any glucose uptake through non-specific transport. Following the 5 minute incubation, the transport solution and

cytochalasin B solution was removed and the cells were washed in ice-cold stop solution three times to stop the reaction. To harvest, 500 µl of 0.05 N NaOH was added to each well and the cells were scraped and collected. After the samples were harvested, 100µl of each sample was aliquoted and protein assays were conducted. The remaining 400 µl was combined with 6ml of scintillation fluid (Ecolite+, MP Biomedicals) and radioactivity in each sample was counted and analyzed. Rate of transport was expressed per µg protein.

Animal Studies

Young male Sprague Dawley rats (6 weeks at delivery) ranging from 50-70 g were purchased from Charles River Laboratories Inc. (Quebec, Canada). The rats were left to acclimatize for one week in the animal care facility at York University while being maintained at the standard 12:12-h light-dark cycle at 22–23°C and humidity (50–60%) in a controlled room. Animals were housed approximately 3-6 per cage (depending on body weight and treatment) and provided free access to rat chow (product #D12450B, Research Diets, NJ) and water. After reaching 8 weeks of age, the animals were handled 2-3 times per week in order to reduce any stress due to handling on the day of the experiment. Body mass was also measured during the handling process in order to confirm that animals were growing at their expected rate. All experiments were approved by the York University Animal Care Committee in accordance with the Canadian Council for Animal Care guidelines.

Animal studies using lean animals: Male Sprague-Dawley rats were used for all experiments. The animals used for these experiments weighed ~200 g and were fed a regular chow diet for approximately 2-3 weeks (product #D12450B, Research Diets NJ).

Experimental Outline: Rats were feed-deprived for 18 h and divided into two groups: starved or gavaged with leucine. The first group consisted of starved rats that were gavaged with double distilled water at a dose of 2.4ml/100g body weight. The second set of rats was gavaged with leucine (1g/50ml ddH_2O) also at a dose of 2.4ml/100g body weight; this amount is equivalent to 0.48g leucine/kg or 24ml leucine/kg, and represents a rat's daily leucine consumption. A third group of rats were gavaged with leucine and returned to feed 2 h later. This third group was then starved again overnight and divided into two groups. One group was re-gavaged on Day 2 with water and sacrificed, while the second group was gavaged with leucine and sacrificed at either 30 minutes or 1 h post gavage because these time points showed significant increases in S6K1 and IRS1 serine phosphorylation in myotubes. Following sacrifice, the soleus muscle was collected and used for analysis; this is because the soleus muscle is oxidative and is more insulin sensitive.

Muscle homogenization (buffer): The soleus was hammered into small pieces and a percent of the sample was weighed out for analysis (approximately 40-80mg). Samples were homogenized on ice using 1-1.5mL of homogenization buffer (in mM: 20 HEPES (pH 7.4), 2 EGTA, 50 NaF, 100 KCl, 0.2 EDTA, 50 β-Glycerosphate, supplemented before use with 1 mmol/L DTT, 1 mmol/L benzamidine, 0.5 mmol/L sodium vanadate, and 10 µl/mL of protease and phosphatase inhibitor cocktails, to prevent the degradation

of proteins by proteases). After homogenization, samples were centrifuged at 3800 g for 15 minutes, following which the supernatant was extracted and stored at -80°C. Before storage, approximately 120µl of sample was aliquoted and used for protein assay and western blot analysis, using the method listed above. Immunoblot analysis was conducted and samples were probed for Ph-IRS1 (Ser 612, 307 and 636/639), which would indicate the activation of the negative feedback loop, as well as Ph-S6K1 (Thr 389).

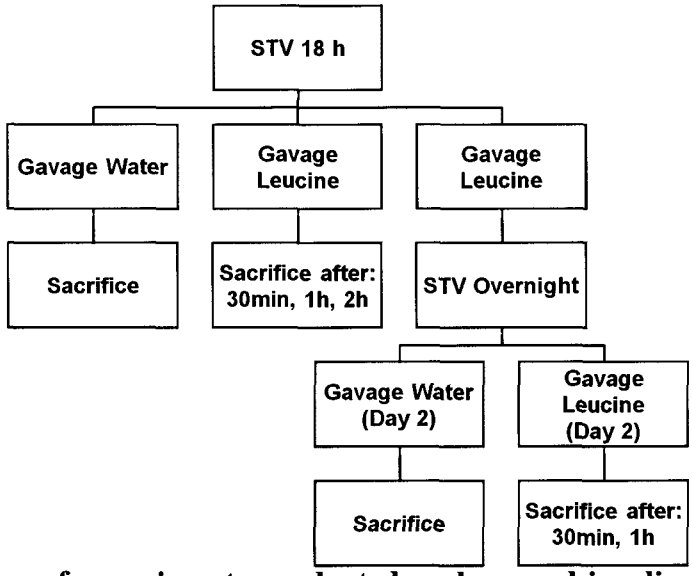


Figure 4. Outline of experiments conducted on lean and insulin resistant rats. Rats were feed-deprived overnight and divided into 3 groups the next day. The first group was gavaged with water and sacrificed, while the second group was gavaged with leucine and sacrificed 30 min, 1 h or 2 h post-gavage. A third group of rats was gavaged with leucine and returned to their chow for 2 h. They were then feed-deprived again overnight (simulating a washout) and divided into two groups again. The first group was gavaged with water and sacrificed, while the second group was gavaged with leucine and sacrificed 30 min or 1 h post-gavage.

Insulin tolerance test and plasma glucose analysis: Rats were feed-deprived for 6 hours. Following the feed-deprivation, animals were gavaged with either ddH_2O or leucine solution (1g leucine/50ml water) at a dose of 2.4ml/100g body weight. Thirty minutes following gavage, blood samples were taken via tail nick and then an intraperitoneal insulin injection was administered at a dose of 0.75U/kg body weight (Insulin obtained from Humulin R, regular human insulin, cat# HI-210, Eli Lilly Canada Inc, Toronto ON). The insulin was diluted from 100U to 2U in 0.9% saline solution). After injection, blood samples were collected at 5, 10, 15, 20, 30, 60, 90 and 120 minutes post-injection. After the blood samples were collected, the samples were centrifuged and the plasma was obtained and frozen at -80°C. Plasma samples were analyzed for glucose concentration using the YSI Glucose/Lactate analyzer.

Branched Chain Amino Acid (BCAA) Assays

BCAA assay was conducted on the plasma samples. In order to do the BCAA assay, a buffer solution containing potassium phosphate (0.1 M), EDTA (2% solution) and β-mercaptoethanol (0.1% solution) was created; this was collectively called 'assay buffer'. The assay buffer was then supplemented with 120 mM nicotinamide adenine dinucleotide (NAD) solution in sodium carbonate buffer (0.1 M) and l-leucine dehydrogenase solution (7.7857 U in 1mg/ml BSA in 25 mM sodium phosphate buffer, pH 7.4). After this, the samples were added for the reaction to begin. In a 96-well plate, the following was added:

Standards	Samples
286 µl assay buffer solution	286 µl assay buffer solution
10 µl 120 mM NAD solution 10 µl l-leucine dehydrogenase solution	10 µl 120 mM NAD solution 10 µl l-leucine dehydrogenase solution
14.3 µl BCAA standards (containing 50% Val, 30% Leu, 20% Iso)	7.15 µl plasma samples

Table 1. BCAA assay proportions. Samples were then detected for fluorescence [162-163].

Animal studies using insulin-resistant animals: Male Sprague-Dawley rats were obtained at 6 weeks of age, acclimatized and handled as mentioned above. After reaching approximately 7 weeks of age, the rats were transferred from the regular chow diet to a high fat diet (product # D12492, Research Diets, NJ). The rats continued to grow on this diet for an additional 6-7 weeks until the rats became insulin resistant (tested using glucose and insulin tolerance tests). The rats also weighed ~550-700g at the time of the experiments. Leucine gavage, ITT and other procedures and assays were conducted as described above for the lean animals.

Statistical Analysis: Data was analyzed using a one-way ANOVA with Bonferroni post-hoc comparisons or Dunnett's multiple comparison tests. Statistical significance was set at $P<0.05$. GraphPad Prism 4.00 software (Version 3, GraphPAD Software, La Jolla CA) and Microsoft Excel was used for these analyses.

5.0 RESULTS

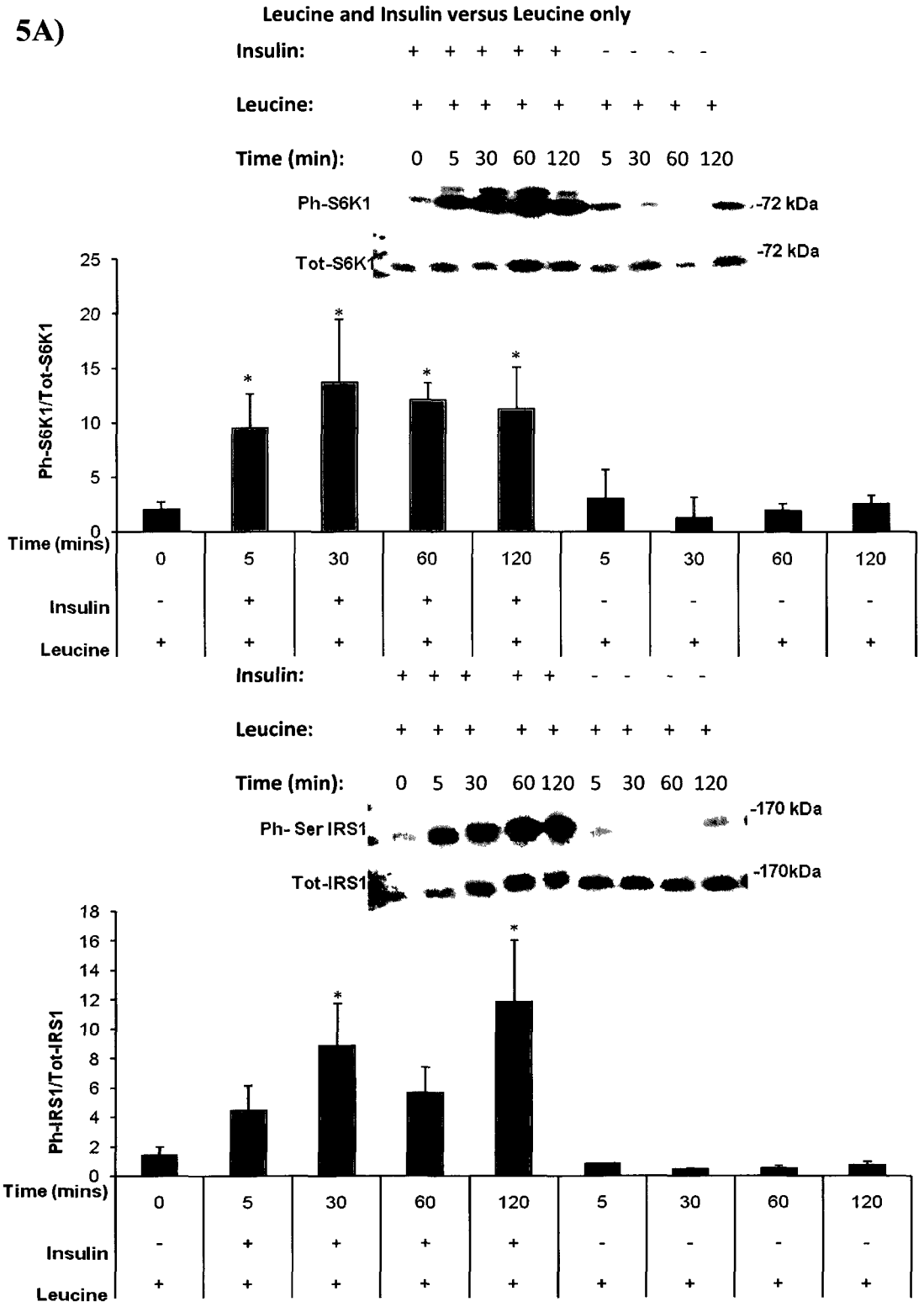
Leucine and insulin have an additive effect on the phosphorylation of S6K1 and IRS1.

It has previously been established that leucine is able to activate S6K1 through the activation of mTORC1. S6K1 is then able to phosphorylate IRS1 on serine residues, leading to its inactivation. It is also known that insulin can activate mTORC1 leading to further activation of its downstream substrates. However, whether this insulin-resistant state created by nutrient/growth factor overload can be reversed remains to be seen. To do this, myotubes were starved and re-stimulated using four different conditions: insulin only, leucine only, leucine and insulin, or no stimulant. When myotubes were starved and re-stimulated with leucine and insulin at a concentration of 800 μM and 100 nM respectively, phosphorylated S6K1 (T389) increased significantly compared to cells that were stimulated with leucine alone or insulin alone (Fig.5A and 5D). Specifically, this effect was time sensitive, with a peak in phosphorylation at 30 minutes post-incubation with leucine and insulin ($P<0.01$). Total S6K1 was also significantly increased when cells were stimulated with leucine and insulin (Fig 5B) and was unchanged when stimulated with insulin alone (Fig 5C).

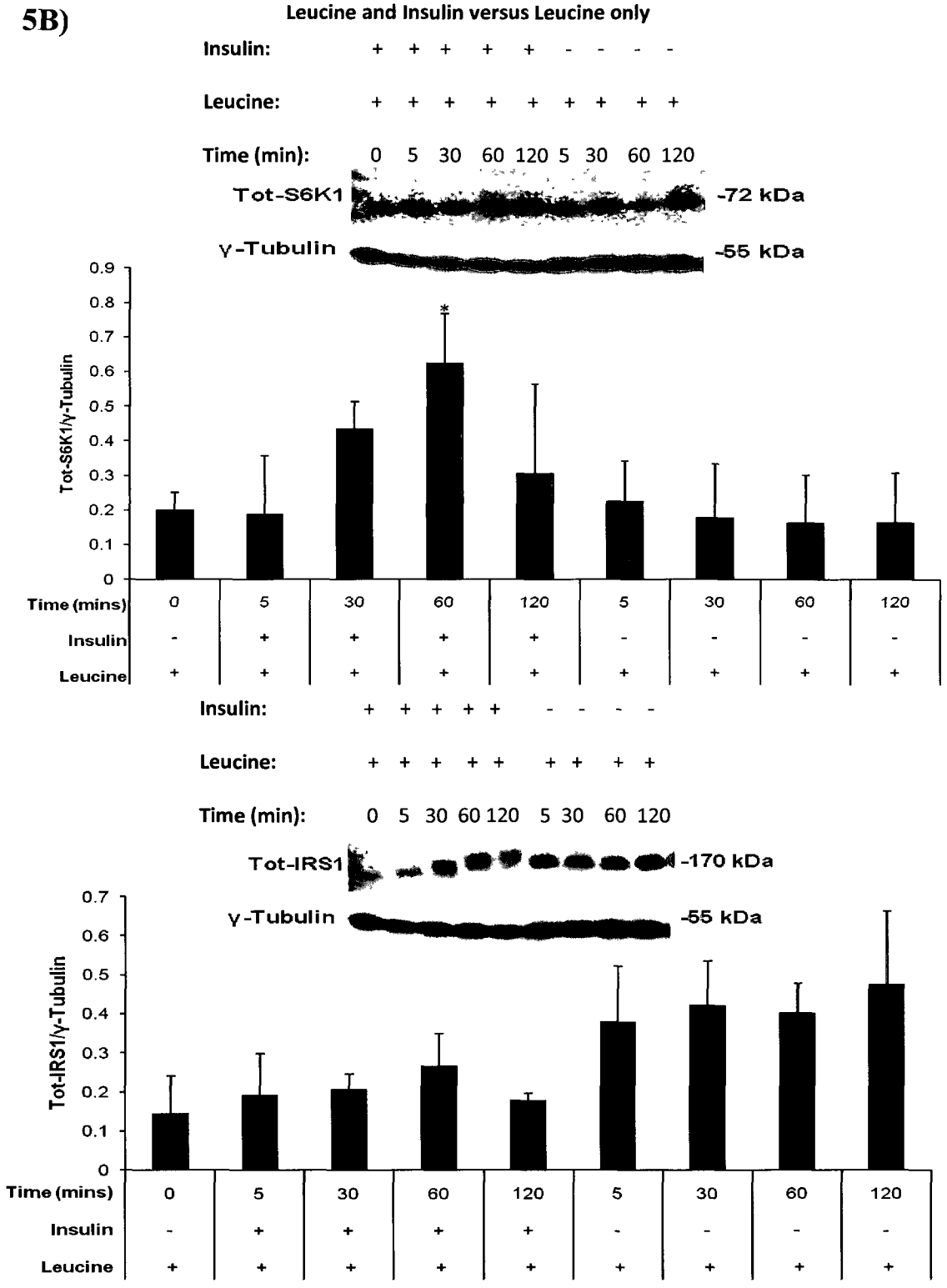
Now that the time course of S6K1 phosphorylation has been determined, as well as the stimulants required to trigger this activation, the next question to answer was whether the time course for IRS1 serine phosphorylation followed the same pattern after activation by the same stimulant. To answer this, an immunoblot analysis was conducted for phospho-

IRS1 using mixed anti-phospho serine antibodies. This would help determine whether the increase in S6K1 phosphorylation is associated with serine 612, 636/639, 307 IRS1 phosphorylation. It was found that, under the influence of leucine and insulin together, IRS1 serine phosphorylation was significantly increased, as compared to the presence of leucine alone, with a peak in expression at 30 minutes post-incubation, and again at 2 h (Fig. 5A). However, Total IRS1 was not significantly changed when cells were incubated in leucine and insulin (5B). Serine phosphorylation was less pronounced when incubation was only with insulin (5D) compared to cells incubated with leucine and insulin. From this, we can conclude that stimulation of mTOR complex 1 by leucine and insulin causes hyperactivation of S6K1. This is associated with increased phosphorylation of IRS1 on serine residues. This is a time sensitive action as was noted with a peak in S6K1 activity at 30 minutes and a similar peak in IRS1 serine expression at 30 minutes and again at 2 h.

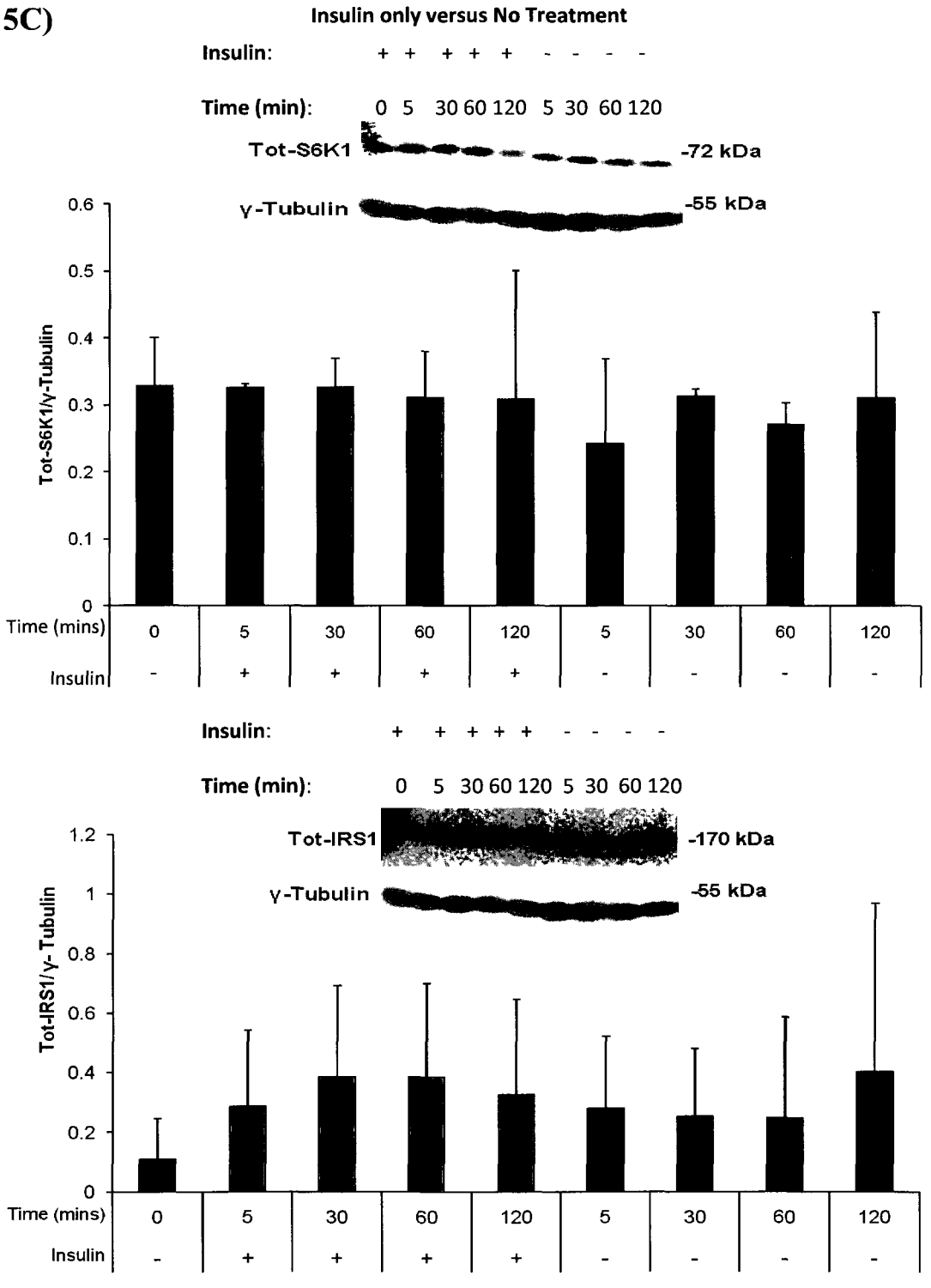
5A)


Leucine and Insulin versus Leucine only
Insulin: + + + + + - - - - -
Leucine: + + + + + + + + +
Time (min): 0 5 30 60 120 5 30 60 120
Ph-S6K1
-72 kDa
Tot-S6K1
-72 kDa
Ph-S6K1/Tot-S6K1
25
20
15
10
5
0
Time (mins) 0 5 30 60 120 5 30 60 120
Insulin - + + + + - - - -
Leucine + + + + + + + + +
Insulin: + + + + + - - - - -
Leucine: + + + + + + + + +
Time (min): 0 5 30 60 120 5 30 60 120
Ph- Ser IRS1
-170 kDa
Tot-IRS1
-170kDa
Ph-IRS1/Tot-IRS1
18
16
14
12
10
8
6
4
2
0
Time (mins) 0 5 30 60 120 5 30 60 120
Insulin - + + + + - - - -
Leucine + + + + + + + + +

5B)


Leucine and Insulin versus Leucine only
Insulin: + + + + + - - - - -
Leucine: + + + + + + + + +
Time (min): 0 5 30 60 120 5 30 60 120
Tot-S6K1
-72 kDa
γ-Tubulin
-55 kDa
Tot-S6K1/γ-Tubulin
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Time (mins) 0 5 30 60 120 5 30 60 120
Insulin - + + + + - - - -
Leucine + + + + + + + + +
Insulin: + + + + + - - - -
Leucine: + + + + + + + + +
Time (min): 0 5 30 60 120 5 30 60 120
Tot-IRS1
-170 kDa
γ-Tubulin
-55 kDa
Tot-IRS1/γ-Tubulin
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Time (mins) 0 5 30 60 120 5 30 60 120
Insulin - + + + + - - - -
Leucine + + + + + + + + +

5C)


Insulin only versus No Treatment
Insulin: + + + + + - - - -
Time (min): 0 5 30 60 120 5 30 60 120
Tot-S6K1
-72 kDa
γ-Tubulin
-55 kDa
Tot-S6K1/γ-Tubulin
0.6
0.5
0.4
0.3
0.2
0.1
0
Time (mins) 0 5 30 60 120 5 30 60 120
Insulin - + + + + - - - -
Insulin: + + + + + - - - -
Time (min): 0 5 30 60 120 5 30 60 120
Tot-IRS1
-170 kDa
γ-Tubulin
-55 kDa
Tot-IRS1/γ-Tubulin
1.2
1
0.8
0.6
0.4
0.2
0
Time (mins) 0 5 30 60 120 5 30 60 120
Insulin - + + + + - - - -

5D)

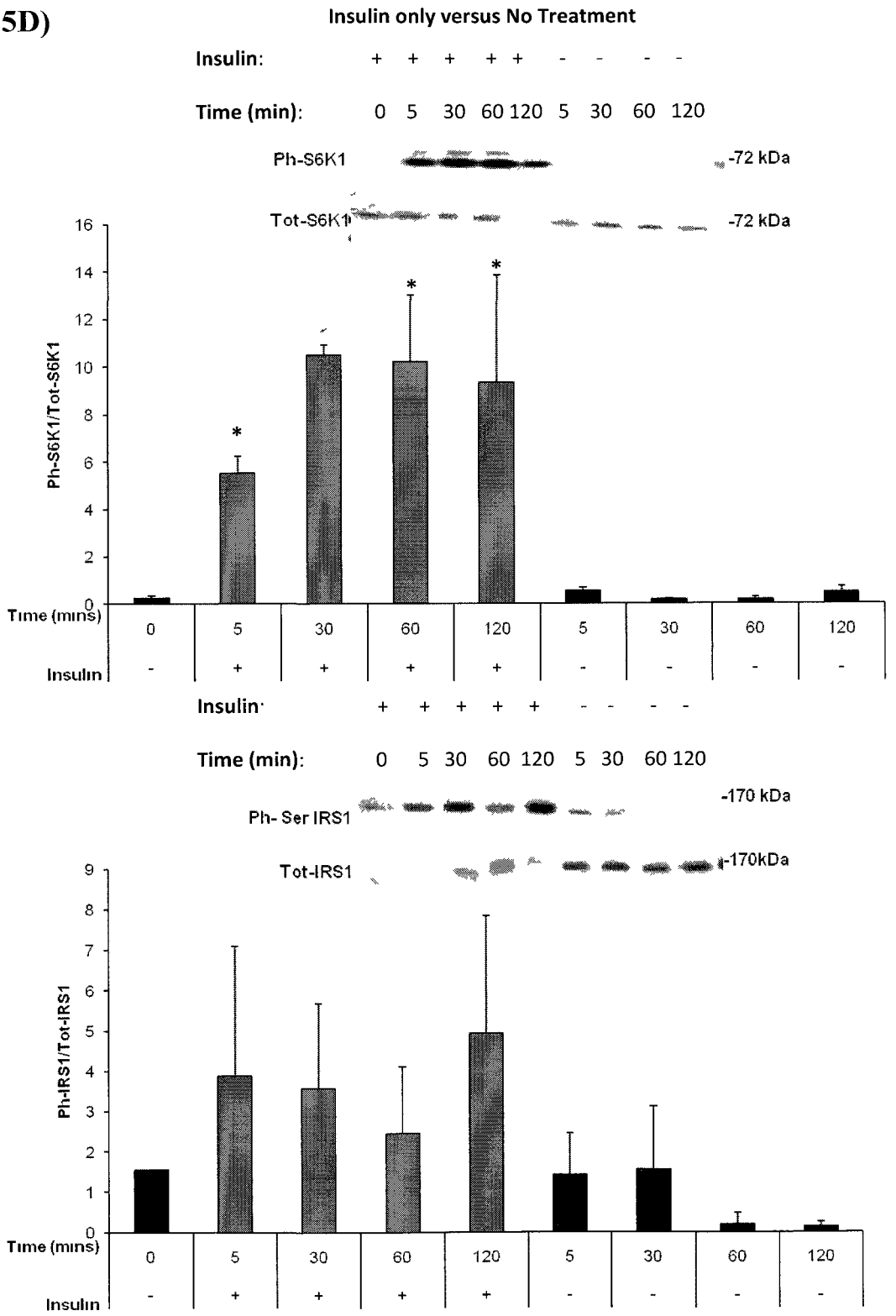

Insulin only versus No Treatment
Insulin: + + + + + - - - - -
Time (min): 0 5 30 60 120 5 30 60 120
Ph-S6K1
-72 kDa
Tot-S6K1
-72 kDa
Ph-S6K1/Tot-S6K1
Time (mins) 0 5 30 60 120 5 30 60 120
Insulin - + + + + - - - -
Insulin: + + + + + - - - -
Time (min): 0 5 30 60 120 5 30 60 120
Ph- Ser IRS1
-170 kDa
Tot-IRS1
-170kDa
Ph-IRS1/Tot-IRS1
Time (mins) 0 5 30 60 120 5 30 60 120
Insulin - + + + + - - - -

Figure 5. Leucine and insulin have an additive effect on the phosphorylation of S6K1 and IRS1. Myotubes were starved for 4 h and incubated in leucine with or without insulin. Time course for phospho and total S6K1 and IRS1 were measured. Values are means ± standard deviation, analyzed using ANOVA with Bonferroni post-hoc. (A) * = $P<0.05$, significantly different from Leucine T0. (B) * = $P<0.05$, significantly different from T0. n=3, experiment has been repeated 2-3 times. (D) * = $P<0.05$, significantly different from T0, T5, T30, T60 and T120.

Threonine 389 phosphorylation of S6K1 and serine phosphorylation of IRS1 in L6 myotubes incubated with leucine is reversible.

Once the time course of S6K1 and IRS1 phosphorylation was determined after starvation and re-feeding over the course of one day, the next question to answer is whether this response is one that is pathological in myotubes. In other words, is the response seen with a nutrient overload one that can be reversed when the nutrients are removed after prolonged stimulation? To examine this, myotubes were incubated in leucine and insulin overnight, and then starved for 4 h the following day. Some myotubes were harvested following the 4 h starvation and subjected to western blot analysis, while others were re-incubated in either leucine and insulin, or RPMI and insulin for 1 h before being harvested. It was found that, following incubation with leucine and insulin overnight and starvation the next day, the level of S6K1 phosphorylation was very low, similar to the levels seen with the starved control on Day 1. When the myotubes were re-stimulated in leucine and insulin for 1 h following starvation on Day 2, the level of S6K1 activity was

again significantly increased, as compared to the incubation in RPMI and insulin for one hour (Figure 6A). A similar pattern of expression was found with IRS1 serine phosphorylation. Specifically, IRS1 phosphorylation was dramatically decreased after starvation on Day 2 compared to cells incubated in leucine and insulin. Levels of serine phosphorylation increased following re-incubation in leucine and insulin, mimicking the pattern that was seen on Day 1 (Fig. 6B). Thus, T389 S6K1 phosphorylation and serine IRS1 phosphorylation in L6 myotubes incubated in leucine and insulin is reversible.

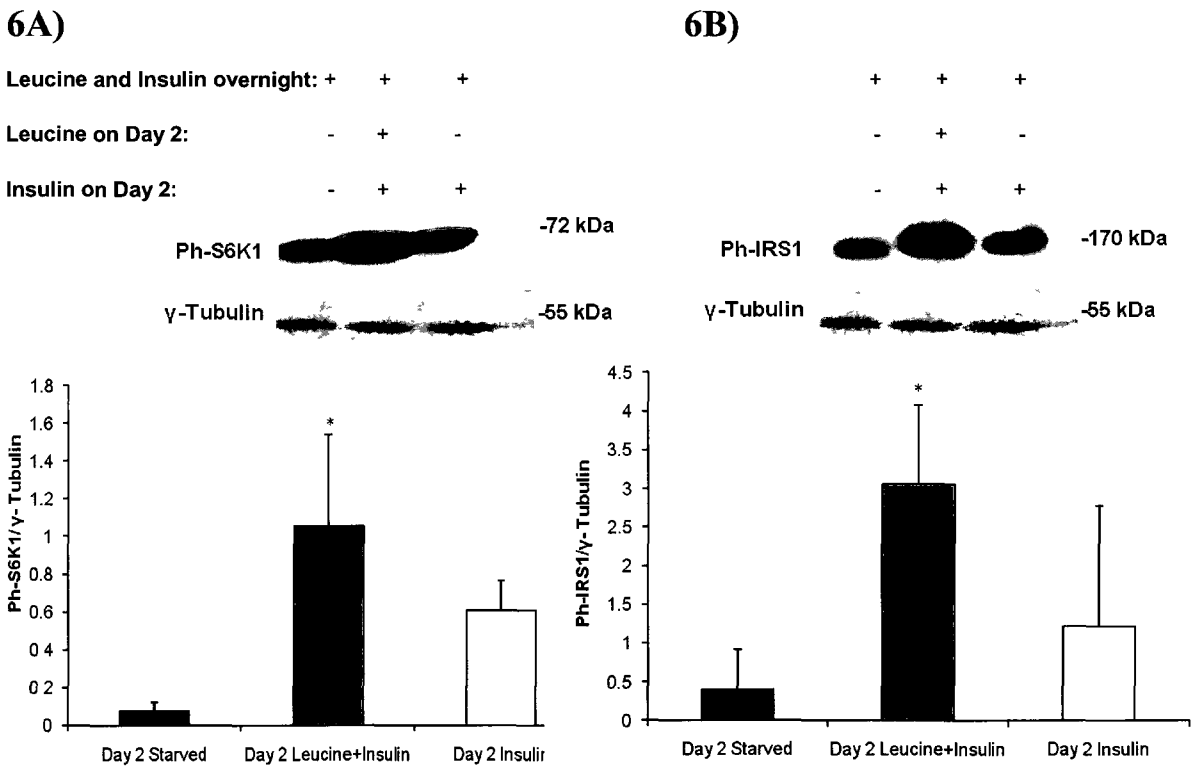


Figure 6. Threonine 389 phosphorylation of S6K1 and serine phosphorylation of IRS1 in L6 myotubes incubated with leucine is reversible. When the myotubes were incubated in leucine solution and insulin overnight, it is expected that S6K1 and IRS1

expression is high. However, when the myotubes were starved the next day to mimic a washout period, and re-incubated with leucine and insulin, the degree of phosphorylation that was seen the previous day was seen again. It was especially remarkable that the myotubes starved on Day 2 showed a significantly decreased level of phosphorylation of both S6K1 and IRS1 (6A and B). Values are means ± standard deviation, analyzed using ANOVA with Bonferroni post-hoc, * = $P<0.05$ significantly different from Day 2 Starved. n=3, experiment has been repeated 2-3 times.

In myotubes, the effect of amino acids on IRS1 and insulin-stimulated glucose uptake is reversible.

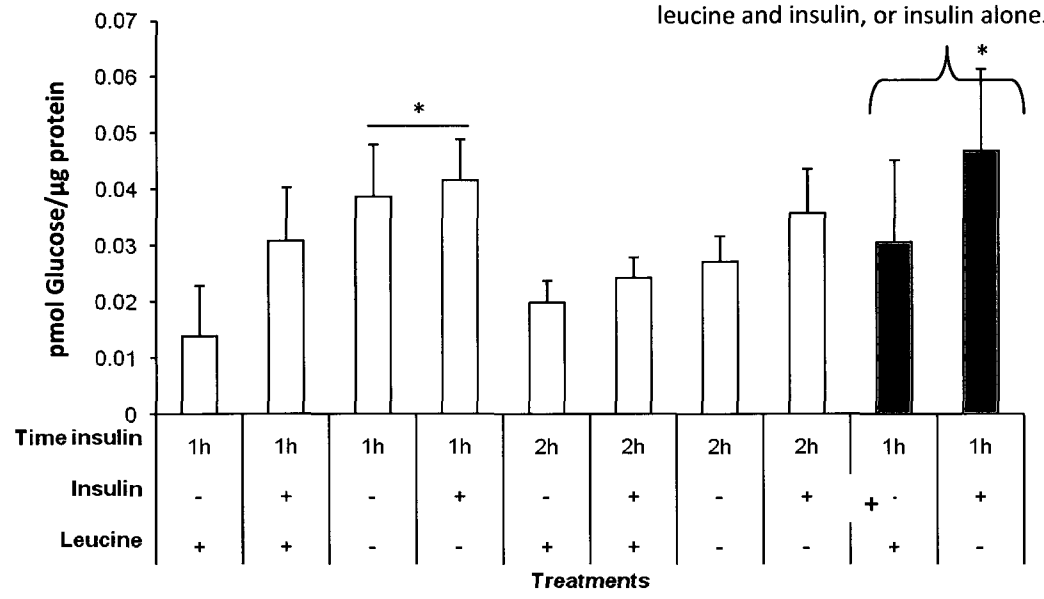
Next, to determine the functional significance of the reversibility of the leucine and insulin-induced regulation of S6K1 and IRS1, we examined the effects of their treatments on glucose uptake. This is because, as mentioned, serine phosphorylation of IRS1 is implicated in insulin resistance. Thus, we measured insulin-stimulated glucose uptake in myotubes following the treatment. Cells treated with leucine and insulin had a significantly decreased amount of glucose uptake (65%), as compared to the groups treated with insulin alone, demonstrating that amino acids suppress insulin-stimulated glucose uptake (Fig. 7A, $P<0.05$). To determine whether this suppression is reversible, myotubes that were treated with leucine and insulin overnight were starved and re-incubated with either leucine and insulin, or insulin alone. Again, insulin-stimulated glucose uptake was significantly higher in the group treated with insulin alone on Day 2, as compared to the group treated with leucine and insulin ($P<0.05$). Insulin-stimulated

glucose uptake on Day 2 was similar to the value seen on Day 1. These results suggest that the insulin-resistant state that is triggered by an amino acid overload can be reversed when the stimulant is removed.

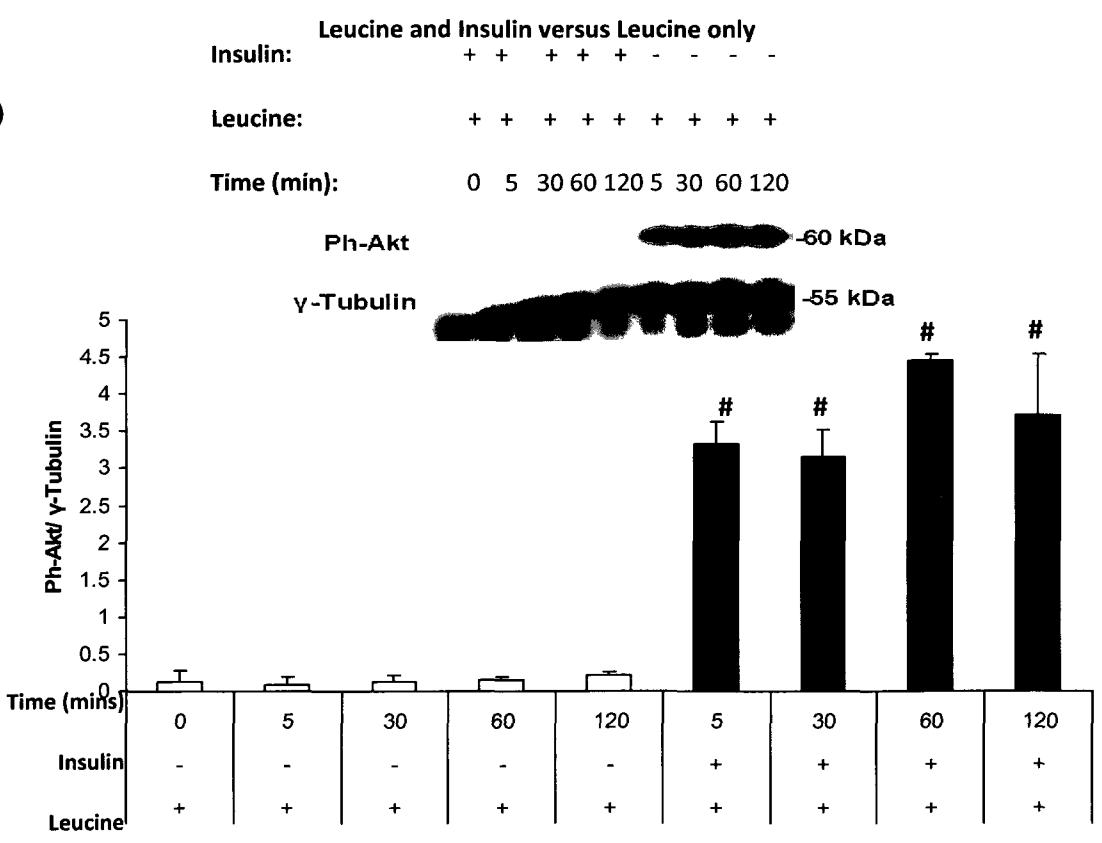
In addition to the knowledge that leucine is able to reversibly impair insulin signalling in myotubes, it also became essential that one examines the impact that leucine and insulin have on the downstream signalling targets of IRS1. Thus, phosphorylation of Akt (Thr 308) and AS160 (Thr 642) were examined. When the cells were treated with leucine and insulin, phosphorylation of Akt was was significantly increased. (7B &C). The presence of leucine alone was not able to significantly increase Akt expression. Similarly, when the cells were treated with leucine and insulin, phosphorylation of AS160 was initially decreased, but then increased 1 h post-incubation. Incubation in leucine alone was not able to activate AS160 to a significant extent. It was also found that, under the presence of insulin, phosphorylation of Akt was significantly increased compared to when the cells were untreated (7C). The phosphorylation of AS160 was also increased under these conditions.

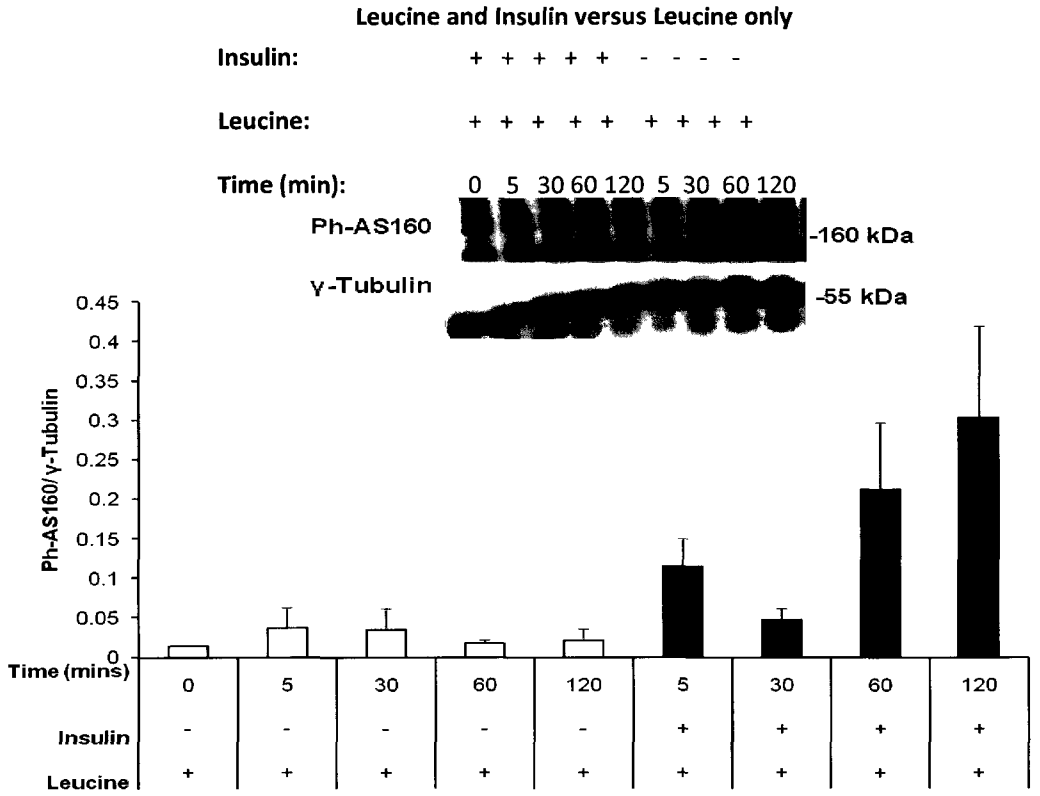
These cells were incubated in leucine and insulin overnight, then starved for 4h the next day (simulating a washout period) and re-stimulated with either leucine and insulin, or insulin alone.

7A)

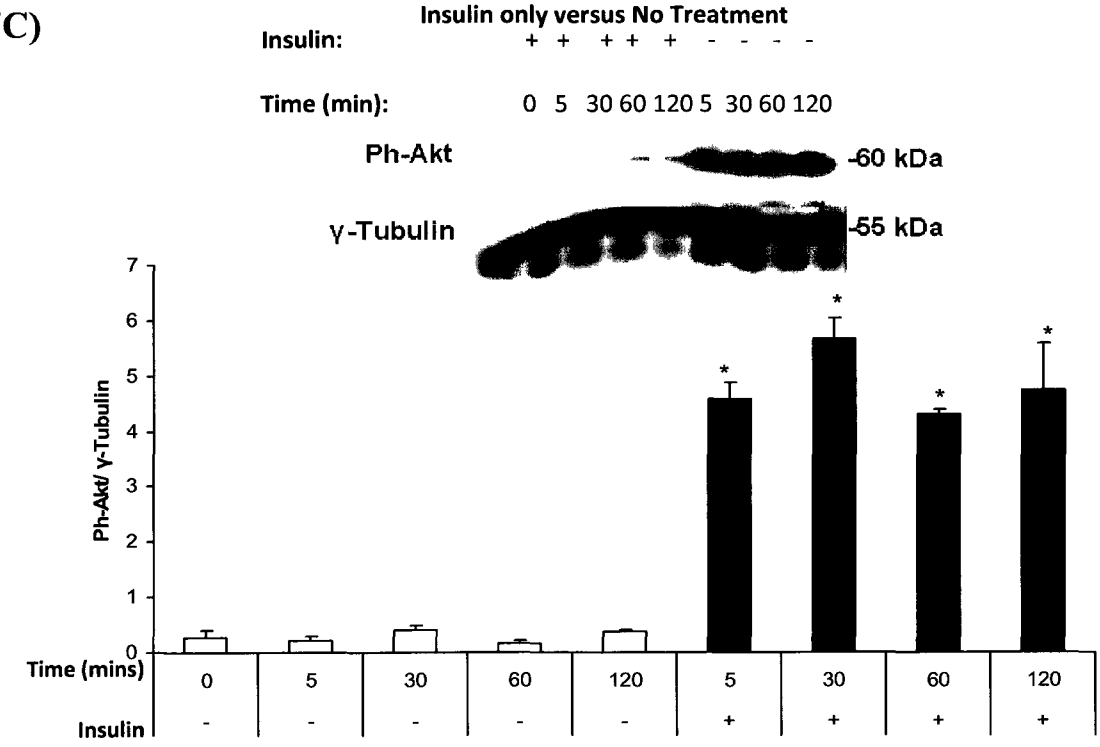


7B)




Leucine and Insulin versus Leucine only
Insulin: + + + + + - - - -
Leucine: + + + + + + + + +
Time (min): 0 5 30 60 120 5 30 60 120
Ph-AS160
-160 kDa
γ-Tubulin
-55 kDa
Ph-AS160/γ-Tubulin
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Time (mins) 0 5 30 60 120 5 30 60 120
Insulin - - - - - + + + +
Leucine + + + + + + + + +

7C)


Insulin only versus No Treatment
Insulin: + + + + + - - - -
Time (min): 0 5 30 60 120 5 30 60 120
Ph-Akt
-60 kDa
γ-Tubulin
-55 kDa
Ph-Akt/ γ-Tubulin
7
6
5
4
3
2
1
0
Time (mins) 0 5 30 60 120 5 30 60 120
Insulin - - - - - + + + +

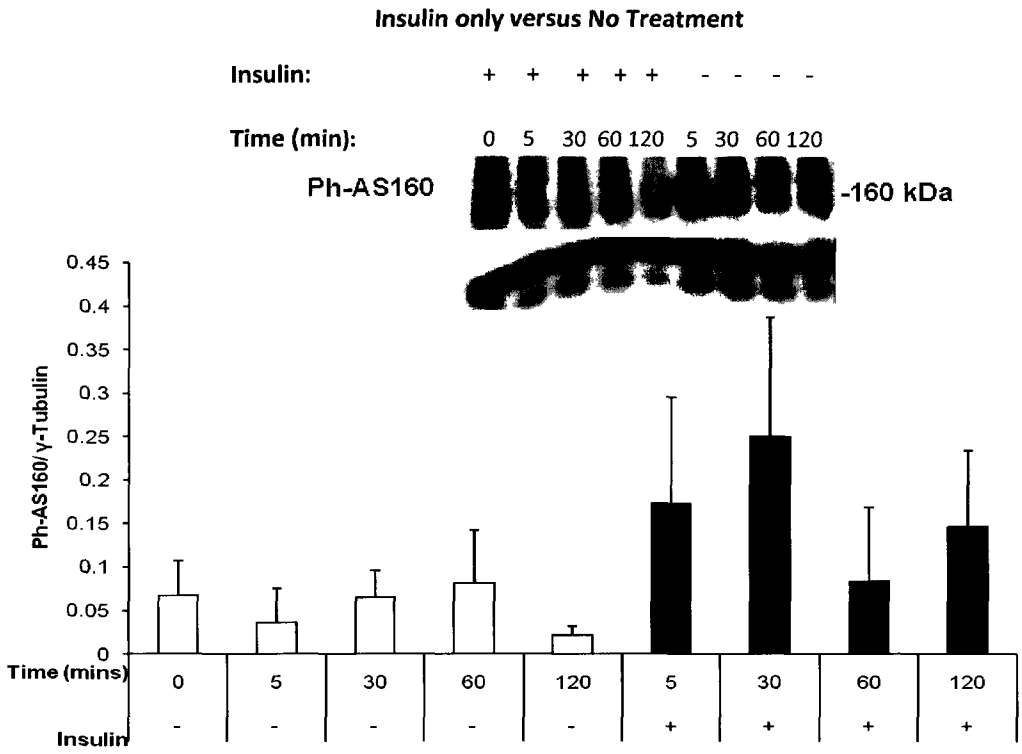


Figure 7. Insulin-stimulated glucose transport in myotubes treated with leucine and/or insulin. (A) Myotubes were starved and re-stimulated with leucine, leucine and insulin, no leucine or insulin alone, for 1 h or 2 h. Insulin-stimulated glucose transport was then measured. Insulin-stimulated glucose uptake was also measured following overnight incubation in leucine and insulin, starvation for 4 h on Day 2, and re-incubation in either leucine and insulin, or insulin alone (represented by the last two bars). Time on x-axis refers to time of incubation in insulin. (B & C) Myotubes were treated exactly as mentioned in Figure 3 and membranes were blotted for Ph-AS160 (Thr 642) and Ph-Akt (Thr 308). Topmost band was quantified for Ph-AS160. Values are (A): means ± standard deviation, analyzed using ANOVA with Bonferroni post-hoc. * = $P<0.01$, significantly

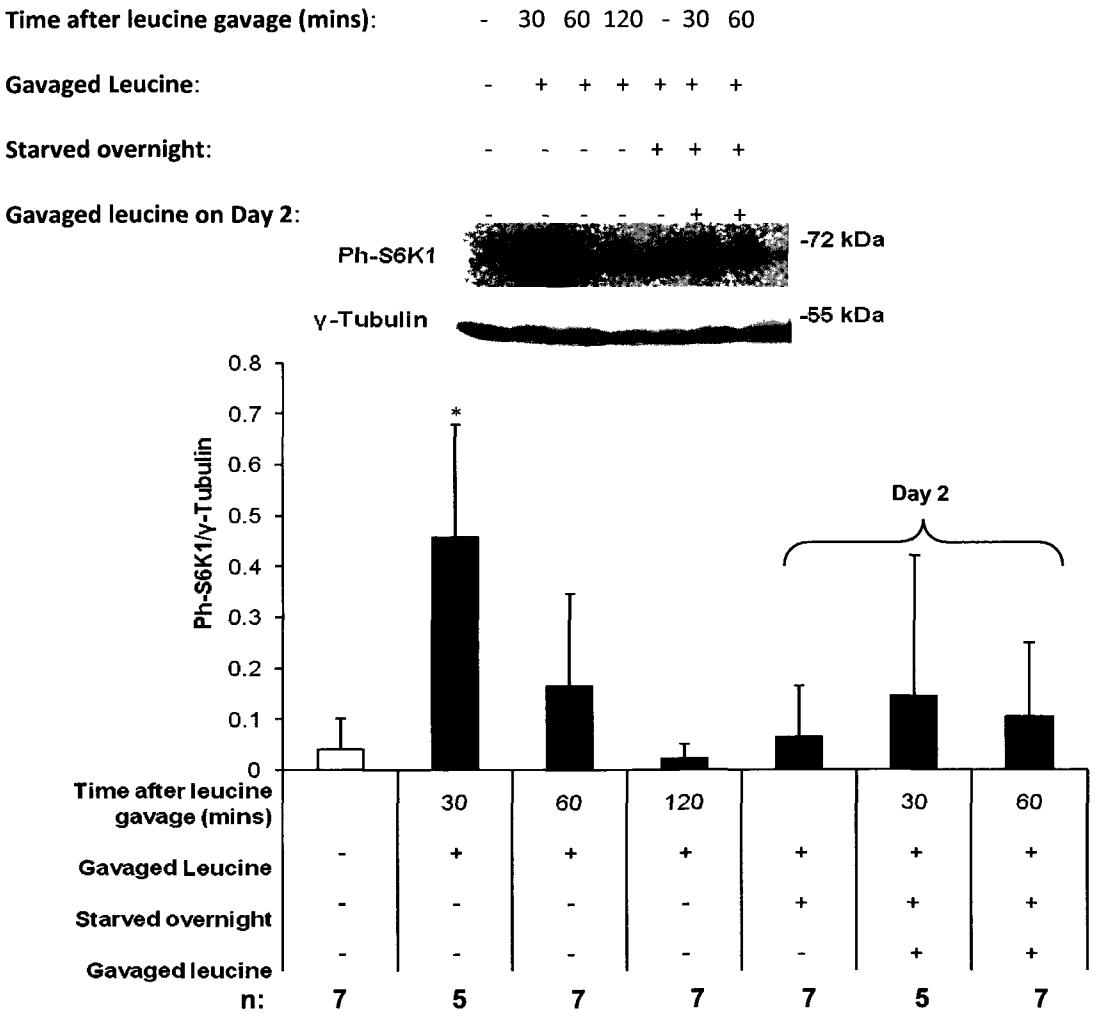
different from Leucine 1 h. (B): means ± standard deviation, analyzed using ANOVA with Dunnett's post-hoc, # = $P<0.05$, significantly different from leucine time 0, leucine 5 min, leucine 30 min leucine 1 h and leucine 2 h. (C): means ± standard deviation, analyzed using ANOVA with Dunnett's post-hoc, * = $P<0.05$ significantly different from T0, T5, T30, T 1h, T 2h.

In lean rats, leucine gavage reversibly increases soleus muscle phosphorylation of S6K1 and IRS1 serine and suppresses whole body insulin sensitivity.

Now that these results have been confirmed in myotubes, we next wanted to determine whether these results could be mimicked in-vivo. To do this, young healthy male Sprague-Dawley rats were given normal rat chow until they reached a weight of 200-220g. Rats were starved for 18 h and either gavaged with double distilled water and sacrificed or gavaged with leucine (1g/50ml water) at a dose of 2.4ml/100g body weight, and sacrificed at 30 minutes, 1 h or 2 h post-gavage. It was found that after gavage with leucine, S6K1 (T389) phosphorylation was dramatically increased, significantly at 30 minutes post-gavage (Fig. 8A, $P<0.05$). Similarly, IRS1 serine phosphorylation was also increased following gavage, with a significant increase at 30 minutes post-gavage (Fig. 8B, $P<0.05$). To determine whether these effects were reversible in healthy rats, a third group of rats was feed-deprived overnight, gavaged with leucine and returned to feed 2 h later, then feed-deprived overnight again for 18 h. They were then re-gavaged with either water or leucine the next day and sacrificed at various time points. The group that was

starved and sacrificed on Day 2 showed an increase in S6K1 (T389) phosphorylation after leucine gavage, followed by a decrease; this was also seen in the level of IRS1 serine phosphorylation. Similar to what was seen on Day 1, rats that were gavaged with leucine on Day 2 showed an increase in S6K1 and IRS1 phosphorylation, but not to a significant degree.

8A)



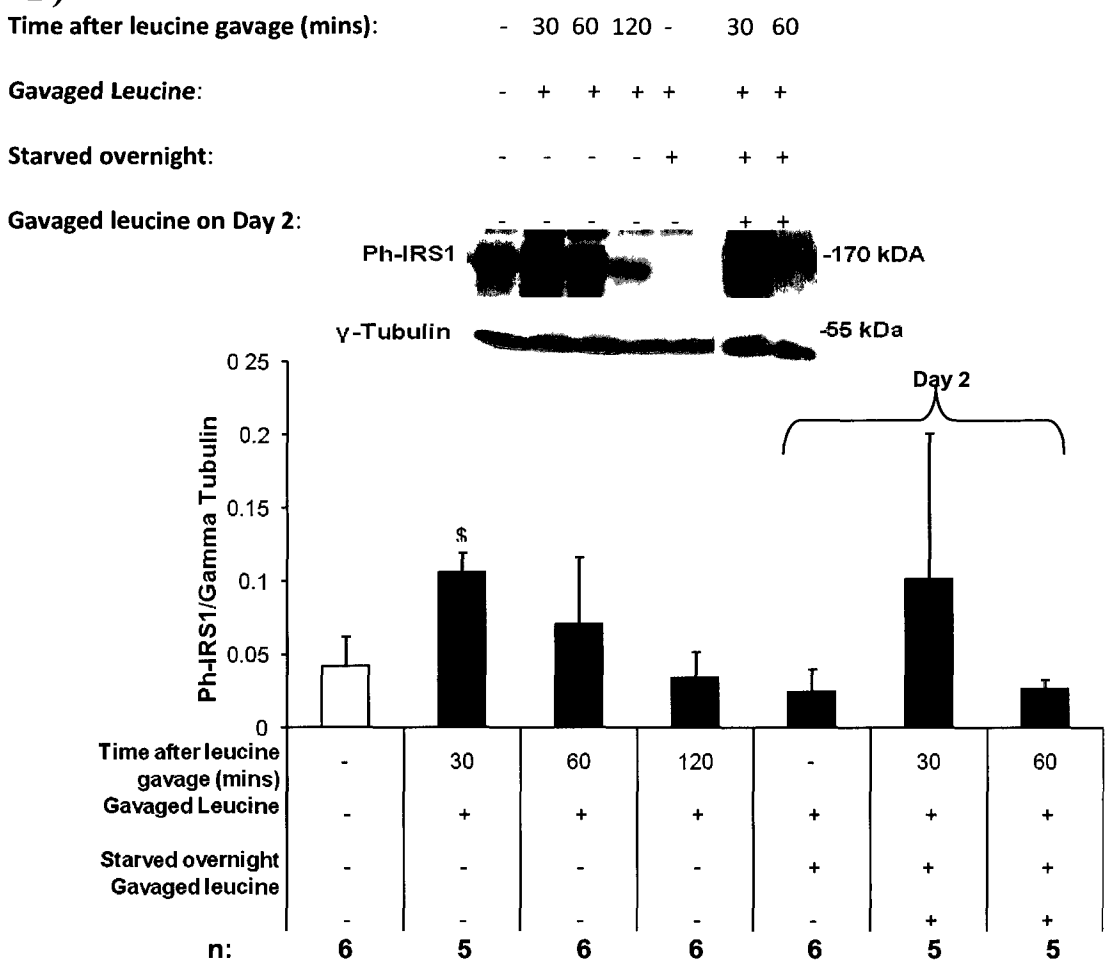


Figure 8. Time course of Ph-S6K1 and IRS1 in skeletal muscle of rats gavaged with leucine. (A & B) When young, healthy rats were starved for 18 h overnight and gavaged with leucine, it was found that S6K1 and IRS1 phosphorylation in skeletal muscle was increased, with a peak in phosphorylation 30 minutes after refeeding. Phosphorylation of S6K1 on Day 2 was similar to the expression seen on Day 1. (A) Values are means ± standard deviation, analyzed using ANOVA with Bonferroni post-hoc, * = $P<0.05$, significantly different from Starved Day 1; n= 5-7. (B) Values are means ± standard

deviation, analyzed using ANOVA with Dunnetts post-hoc, $ = $P<0.05$ significantly different from Starved Day 1, n = 5-6. *IRS1 blot in 8B was run on two gels, so last two treatments were manually placed beside the others.

Leucine gavage reversibly impairs whole-body insulin sensitivity of glucose and plasma branched chain amino acids in healthy rats.

Next, to determine the functional consequences of these results, an Insulin Tolerance Test (ITT) was conducted. Healthy rats were feed-deprived for 6 h and then either gavaged with water before being given an intraperitoneal insulin (2U/kg) injection at a dose of 0.75U/kg body weight, or gavaged with leucine and given an insulin injection 30 minutes post-gavage. The plasma glucose values of these rats pre-insulin injection were approximately 6.1 ± 1.4 mM. I chose this time because it seems that the peak phosphorylation of these proteins occurs 30 minutes post-gavage. As mentioned, the injection was administered intraperitoneally, and blood samples were collected via tail nick; once before the insulin injection and then after the injection. It was found that rats gavaged with leucine had higher levels of plasma blood glucose than rats that were gavaged with water on Day 1 (Fig. 9A). Specifically, there were significant differences at 15 and 60 minutes post-injection ($P<0.05$). A third group of rats were gavaged with leucine, starved overnight and re-gavaged the next day with water before being administered the insulin tolerance test. It was found that these rats showed similar levels of plasma glucose to the rats that were gavaged with water alone on Day 1 (9A). From this, we are able to conclude that in healthy rats, leucine is able to impair insulin

tolerance. This is an indication that leucine is able to trigger an insulin-resistant state in these rats, perhaps via over activation of S6K1 and subsequent phosphorylation of IRS1 on serine residues. This as we know is able to prevent further signalling of the insulin pathway. However, this state of insulin resistance is reversible when the stimulus is removed. Plasma BCAA was also analyzed and it was found that rats gavaged with leucine had significantly higher plasma BCAA than rats gavaged with water, especially 30 minutes post-gavage (Fig. 9B).

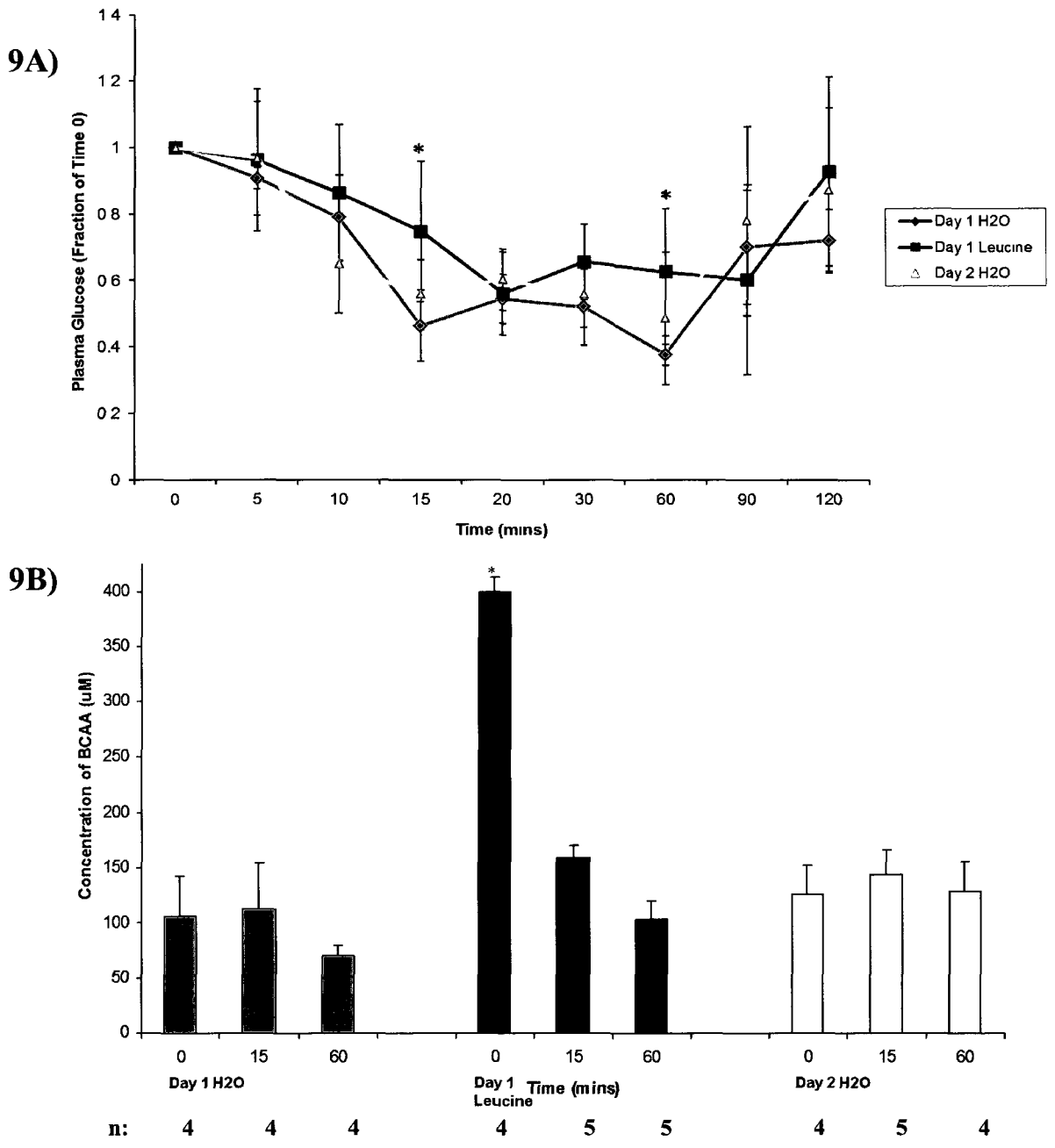


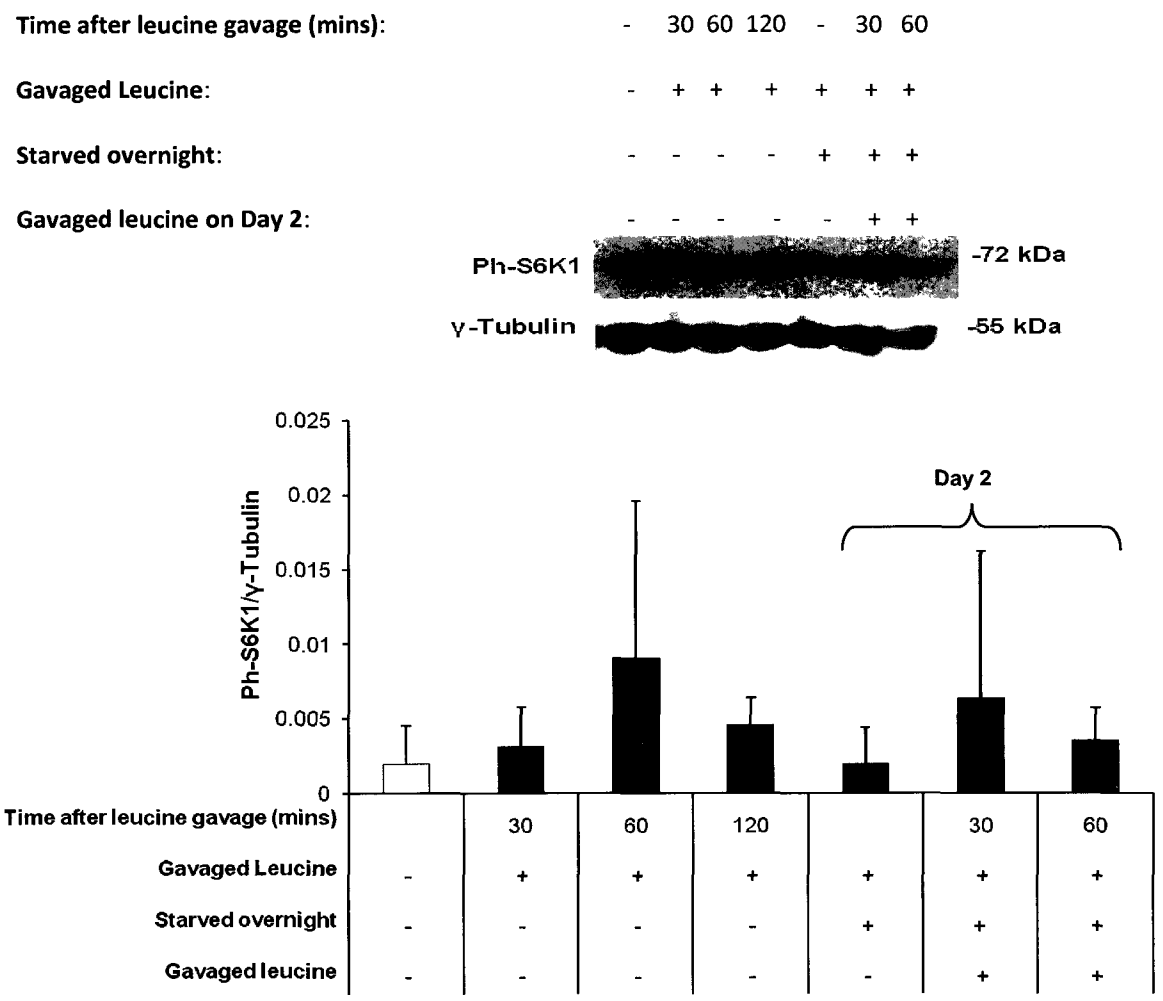
Figure 9. Leucine gavage reversibly impairs whole-body insulin sensitivity of glucose and plasma branched chain amino acids in healthy rats. A: Healthy rats were feed-deprived for 6 h, gavaged with either water or leucine and then given an intraperitoneal insulin injection 30 minutes later. In (A), glucose concentration was

expressed as a fraction of Time 0. Values are means ± standard deviation, analyzed using ANOVA with Bonferroni post-hoc, * = $P<0.05$, significantly different from H_2O Day 1. In (B) values were expressed as means ± standard deviation, analyzed using ANOVA with Dunnett's post-hoc. * = $P<0.05$, leucine gavage on Day 1 was significantly different from water gavage T 60 on Day 1 (n=4-7).

Impairment in insulin signalling and whole body glucose metabolism caused by leucine are not reversed in rats made insulin-resistant via high fat diet.

We next aimed to determine whether this reversibility was also seen in rats that were made insulin resistant. To do this, healthy male Sprague-Dawley rats were obtained and given a high fat diet (60% kcal% fat (lard) diet) for approximately 6-8 weeks. This was done in order to make the rats insulin-resistant. The first group of rats was gavaged with water and sacrificed, while the second group of rats was gavaged with leucine and sacrificed at 30 minutes, 1 h or 2 h post-gavage. S6K1 (T389) and IRS1 (serine) phosphorylation were detected even the muscles of starved rats. As a result, although S6K1 phosphorylation varied at different time points, it was not significantly different from the starved condition (Fig. 10A). The same pattern was seen for IRS1 (Fig. 10B). The third group of rats was feed deprived, gavaged with leucine and returned to their high fat diet 2 h later. They were then feed-deprived again and gavaged with either water or leucine the next day. Again, as seen in animals given leucine on Day 1, the effect of leucine gavage on muscle Ph-S6K1 and Ph-serine IRS1 was not significant.

10A)


Time after leucine gavage (mins): - 30 60 120 - 30 60
Gavaged Leucine: - + + + + + +
Starved overnight: - - - - + + +
Gavaged leucine on Day 2: - - - - - + +
Ph-S6K1
-72 kDa
γ-Tubulin
-55 kDa
Ph-S6K1/γ-Tubulin
0.025
0.02
0.015
0.01
0.005
0
Day 2
Time after leucine gavage (mins)
30 60 120 30 60
Gavaged Leucine + + + + + +
Starved overnight - - - + + +
Gavaged leucine - - - - + +

10B)

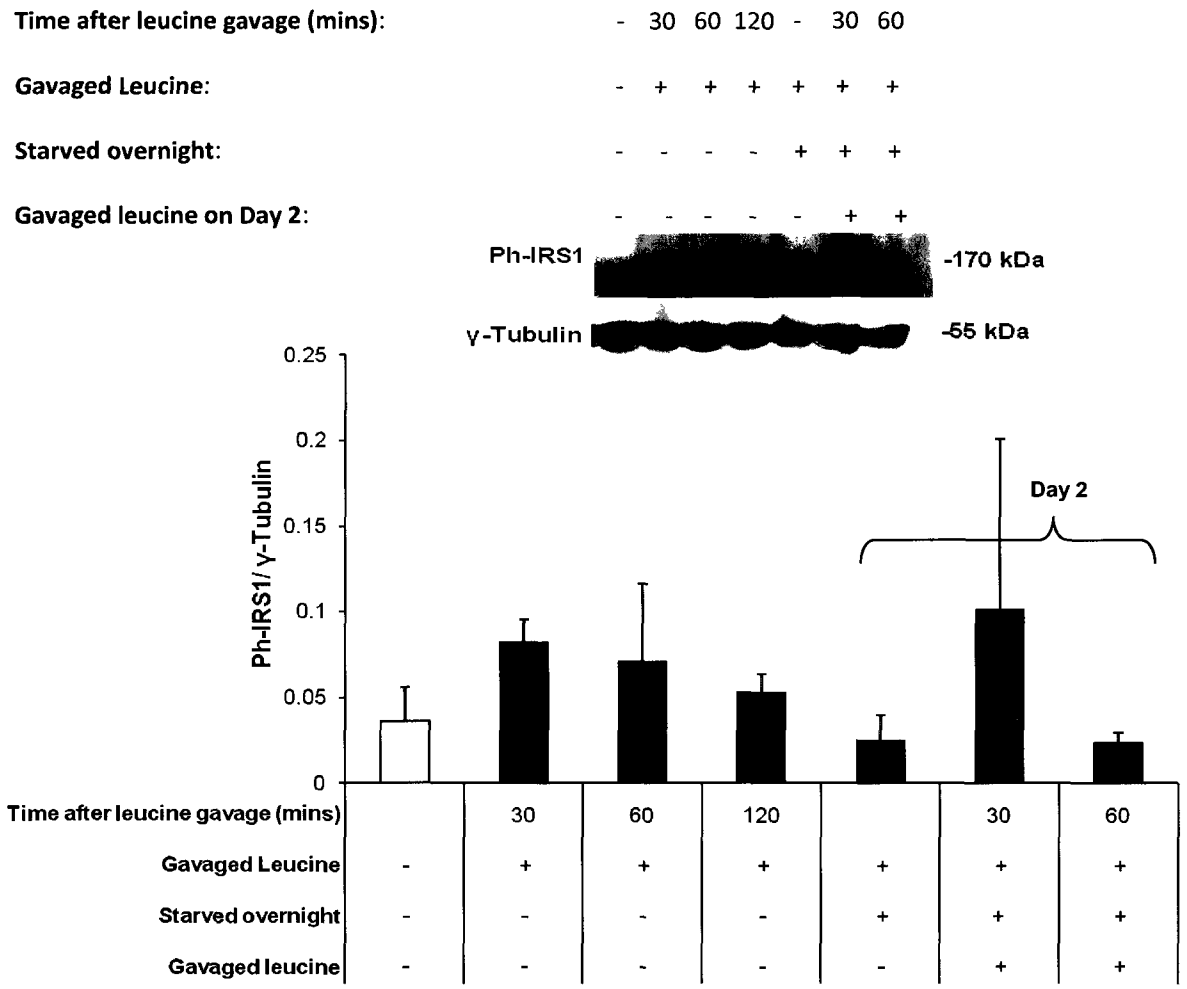


Figure 10. Leucine gavage had no significant effect on the phosphorylation of S6K1 and IRS1 in the skeletal muscle of insulin resistant rats. A & B: Male Sprague-Dawley rats were given a high fat diet for 6-7 weeks until they became insulin resistant. At this point, they were either gavaged with water or leucine and Ph-S6K1 (T389) and Ph-IRS1 (serine) was measured. A third group of rats was gavaged with leucine, feed-deprived for 18 h overnight and re-gavaged with either leucine or water the next day. Ph-

S6K1 and Ph-IRS1 (serine) expression was measured. Values are means ± standard deviation (n=6-7).

Impaired whole body insulin sensitivity and plasma BCAA in insulin resistant rats gavaged with leucine.

The plasma glucose values in these rats pre-insulin injection were higher than the values seen in the lean rats, ranging from 9.1 ± 0.5 Mm, confirming insulin resistance. During the ITT, plasma glucose in rats gavaged with water was consistently lower than the rats gavaged with leucine. There were significant differences at 10, 15, 20, 60 and 90 minutes post-injection ($P<0.05$) between rats gavaged with leucine compared to those gavaged with water (Fig. 11A). Furthermore, plasma glucose did not decrease to a great extent following an injection with insulin. When the rats were gavaged with leucine, allowed to eat overnight, re-starved for 6 h the next day and gavaged with water, plasma glucose did not differ significantly from rats gavaged with either water or leucine on Day 1 (Fig. 9A). To examine whether this leucine gavage impaired insulin sensitivity of whole body amino acid metabolism, changes in plasma branch chain amino acids (BCAA) concentrations were measured during the ITT. This is an indicator that the body is unable to respond to the anabolic effects of insulin, resulting in excess protein breakdown without enough protein synthesis. As expected, rats gavaged with leucine had significantly higher amounts of plasma BCAA in their system, compared to rats gavaged with water. However, rats that were gavaged with water had a great amount of plasma BCAA; in fact the basal plasma BCAA concentration in these rats was as high as the

plasma BCAA found in healthy rats that had been gavaged with leucine (Fig. 11B and 9B).

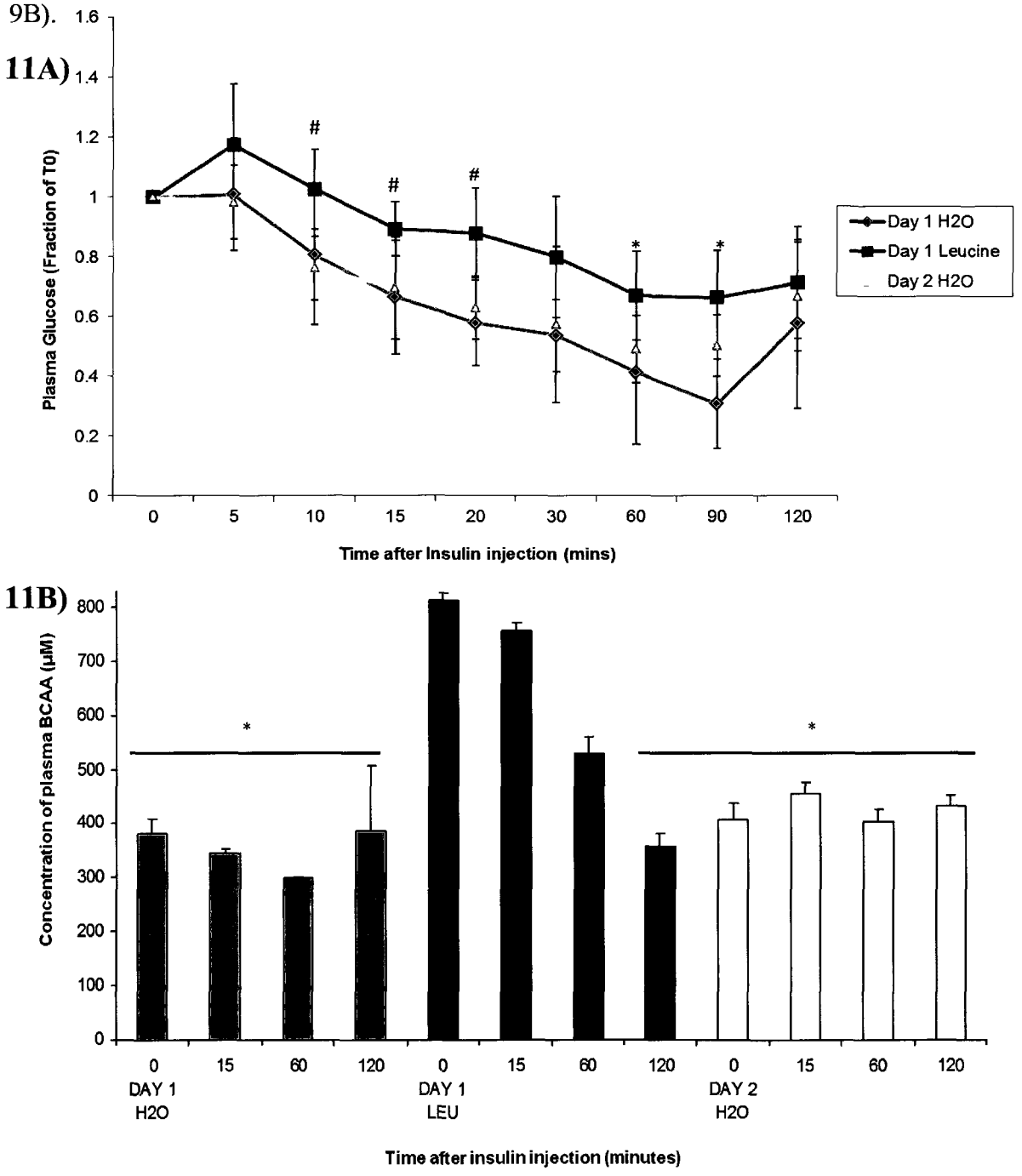


Figure 11. Insulin sensitivity and plasma branched chain amino acid levels in insulin resistant rats. To determine the effect of leucine on whole body insulin sensitivity and

plasma BCAA, the rats were feed-deprived for 6 h and gavaged with either leucine solution or water. Thirty minutes post-gavage, an insulin tolerance test was conducted and plasma glucose and BCAA were measured over time. Values in (A) are expressed as a fraction of Time 0, analyzed using ANOVA with Bonferroni post-hoc, # = $P<0.05$, Leucine Day 1 is significantly different from H_2O Day 1 and H_2O Day 2, * = $P<0.05$, Leucine Day 1 is significantly different from H_2O Day 1 only. Values in (B) are means ± standard deviation, analyzed using ANOVA with Bonferroni post-hoc, * = $P<0.05$, significantly different from Leu T0 and Leu T15(n=8).

6.0 DISCUSSION

The results found indicate a few important points regarding the effect of high protein diets on insulin sensitivity. It was originally shown in the literature that of all the amino acids, leucine is most capable of impairing the insulin signalling pathway and insulin sensitivity in general. This impairment has been demonstrated in both cell cultures as well as animal work.

From my study in myotubes I showed that, upon the addition of leucine and insulin, it took approximately 30 minutes to see a peak in S6K1 (T389) phosphorylation and ultimately IRS1 serine phosphorylation. Furthermore, when the myotubes were stimulated overnight with leucine and insulin, it is expected that S6K1 and IRS1 activity is high. But when the myotubes were re-starved the next day and re-stimulated with leucine and/or insulin, the phosphorylation of S6K1 (T389) and IRS1 (serine) were similar to the previous day. These results were confirmed further when insulin-stimulated

glucose uptake was also measured at this time. In addition, I was able to demonstrate that it also took 30 minutes after leucine gavage to see a peak in S6K1 (T389) phosphorylation in lean Sprague-Dawley rats. This was matched with a similar increase in IRS1 serine phosphorylation. When the leucine stimulus was removed and the rats were feed-deprived overnight, the phosphorylation of S6K1 (T389) and IRS1 (serine) returned to basal levels. However, it was able to increase again when leucine/insulin was re-introduced on Day 2. An insulin tolerance test demonstrated that rats gavaged with leucine had significantly higher plasma glucose and BCAA compared to rats gavaged with water. When the rats were gavaged leucine, returned to feed overnight and then starved the next day and gavaged with water, their plasma glucose during the ITT was not significantly different from the rats gavaged with water on Day 1. This demonstrated that the impairment in insulin sensitivity caused by leucine gavage was in fact reversible in the muscle of healthy rats.

Rats that were made insulin resistant demonstrated that, after leucine gavage, there was an increase in S6K1 (T389) and IRS1 (serine) phosphorylation. However, the increase seen was not significantly different from rats gavaged with water alone. During the ITT, plasma glucose of insulin resistant rats gavaged with leucine did not rise significantly higher than the levels seen in rats gavaged with water alone. However, similar to what was seen in the lean rats, the rats gavaged with leucine also had significantly higher levels of plasma BCAA compared to the rats gavaged with water. When rats gavaged

with leucine were feed-deprived and given water the next day, the plasma BCAA levels reduced to levels seen in rats gavaged with water on Day 1.

The results seen when comparing the plasma BCAA bring forward a number of interesting points to be examined. First, it was found that when the healthy rats were gavaged with leucine, their highest plasma BCAA concentration was reached; however, this value was equivalent to the BCAA concentration seen in the insulin resistant rats that were starved and gavaged with water alone. This raises the question of how high fat diets affect plasma BCAA. Studies conducted by Chevalier et al demonstrate that in overweight and obese individuals with uncontrolled T2DM, there appears to be a blunted whole body anabolic response to insulin[164]. In other words, since insulin stimulates protein synthesis and suppresses proteolysis [165-166], it is expected that in these individuals, proteolysis is increased. It can be suggested that the already high BCAA concentration seen in the insulin resistant rats fed a high fat diet is a consequence of insulin resistance. This is indicative of two things: since the anabolic effects of insulin are impaired, processes such as protein synthesis will also be impaired. Thus, the plasma concentration of free BCAAs will increase, indicating decreased insulin sensitivity. Secondly, insulin also prevents protein breakdown; since this function is also impaired, there is likely to be more protein breakdown in individuals that are insulin resistant. Overall, there is less protein synthesis and more proteolysis.

The results found with the myotubes were able to support the literature with regard to the effects of amino acids on glucose uptake. Although the other BCAA's were able to

stimulate S6K1, they were not able to activate its expression to the same degree as leucine alone [167]. One possibility that still remains to be examined is what dose of leucine is enough to stimulate the insulin resistant effects that were shown in our study. In the cells, the concentration of basal leucine in the media was simply doubled, while in the animals, an amount of leucine representing their daily protein consumption was administered to the animal. However, it may be possible that a smaller amount can still trigger this response.

The results found in this thesis were novel in that they were able to show that this negative impairment in insulin sensitivity caused by leucine can in fact be reversed upon removal of the stimulant. This is important because no other study has examined the physiological reversibility of this effect, nor the time course of this action. It is especially remarkable to note the fact that this reversible physiological regulation can occur without the assistance of any exercise or physical activity. The results seen with the insulin resistant rats are also interesting in that they demonstrate that, in rats that are already insulin resistant, adding more leucine does not significantly alter the metabolic state they are already in, particularly with respect to S6K1 (T389) and IRS1 (serine) phosphorylation and whole body insulin sensitivity. This is because, in these rats, the level of phosphorylated S6K1 and IRS1 is already expected to be quite high.

Although the leucine was able to create a state of insulin resistance in both myotubes and healthy rats, what possibilities can explain the reversibility that was seen in these results that were found in both myotubes and whole skeletal muscle?

One possibility is that the S6K1 phosphatase, PP2A-B' becomes activated upon removal of the stimulant leucine. The main effect causing the impairment in the ability of IRS1 to phosphorylate its downstream targets is the negative feedback loop from S6K1 to IRS1[103]. Since we now know that S6K1 hyperactivation and IRS1 serine phosphorylation can be reversed, one possible mechanism in this reversal could be an increase in the activation of PP2A-B'[103]. De-phosphorylation of S6K1 would prevent any further activation of the negative feedback loop to IRS1, thereby preventing any further impairments to the insulin signalling pathway. But what about the IRS1 proteins that have already been phosphorylated on their serine residues? Since the IRS1 proteins would still be phosphorylated on serine residues, they will continue to impair the insulin signalling pathway.

It is already known from literature that once IRS1 is phosphorylated on serine residues, it is also degraded [62, 30]. Thus, degradation of these proteins would attenuate the inhibition of insulin signalling caused by IRS1 serine phosphorylation. To correct for this possibility during the examination of the time course and to confirm that this degradation does not affect the results, total IRS1 and gamma tubulin were also examined. It was found that total IRS1 protein was lower in myotubes stimulated with leucine and insulin, in comparison to cells stimulated with insulin alone, or leucine alone (S1). Overall, the general assumed mechanism of the reversal would be that PP2A-B' dephosphorylates S6K1[168] which inactivates the negative feedback loop. Then any IRS1 that was

phosphorylated on serine residues will become degraded, thereby allowing for the synthesis of new IRS1 proteins.

Another contributing factor that might aid in the reversibility of the negative feedback loop, in conjunction with the above method, is muscle contraction. This of course, is only applicable to the animal model and will not be a significant factor as the rats wre not subjected to exercise. Once the leucine was gavaged, a significant increase in S6K1 and IRS1 serine phosphorylation was immediately noticed over time. However, once the rats were feed deprived again overnight, their muscle contraction can potentially become a factor in repairing the impairment in insulin sensitivity. This is because rats are nocturnal, meaning that they will undergo most of their physical activity at night. As a result, the movement of the rats triggers muscle contractions in their bodies, leading to the activation of specific proteins in the muscle, such as the liver dependent kinase 1 (LKB1)[169] and the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [170]. As we know, contraction and insulin are the two biggest stimulators of glucose uptake in skeletal muscle [170]. What has been suggested is that during muscle contraction, LKB1 and CaMKII are important mediators[171]. Under conditions in which energy is low, LKB1 will become active; it then phosphorylates one of its direct substrates, AMPK. AMPK then phosphorylates TSC2 on specific serine sites, and TSC2 is then able to inhibit mTORC1 indirectly via Rheb. Thus, this may have contributed to the reversibility in insulin impairment that was seen, but only to a minimal degree.

Recent findings have suggested that contraction and insulin-stimulated signals actually converge at the level of AS160 (also known as TBD1D4), which then allows for translocation of the GLUT4 vesicles[172]. Although very little is known about the mechanisms involved in the transport of the GLUT4 vesicles to the cell surface membrane, it is suggested that the cytoskeleton and its components are key regulators.

Myo1c is a single-headed actin-associated molecular motor protein, belonging to the class 1 myosin family. This protein has been shown to be expressed in skeletal muscle and involved in the uptake of glucose in skeletal muscle. Thus, upon contraction by skeletal muscle, LKB1 and CaMKII trigger the activation of AS160, which assists in the activation of F-actin and the subsequent activation of Myo1c[172]. The activation of these motor proteins then recruits more GLUT4 vesicles to the cell surface membrane and glucose uptake occurs. Overall, in healthy rats, it assumed that the work of phosphatases along with muscle contractile factors are both required in order to reverse the impairment in insulin signalling, and promote glucose uptake in skeletal muscle. These results were also matched in the insulin tolerance tests conducted on the rats. However, as mentioned the muscle contraction factor would have only aided to a minimal degree.

In the rats made insulin-resistant via high fat diet, a different mechanism of action is proposed. Since the rats were not given a high protein diet during their development, it can be assumed that these rats became insulin resistant possibly via an accumulation of DAG, leading to activation of PKC[86-87]. PKC is then able to phosphorylate IRS1 on serine residues, leading to impairments in overall insulin signalling. Although this

negative mechanism of action could have potentially been reversed upon removal of the diet and physical activity, it was not possible due to the fact that the diet was administered over a period of ~6-7 weeks and the rats were not allowed to exercise. Once the rats became insulin resistant, the experiments were conducted. When leucine was gavaged, S6K1 and IRS1 phosphorylation still increased, but not significantly. It is proposed that the leucine was able to hyperactivate S6K1, leading to the development of the negative feedback loop. This would cause IRS1 serine phosphorylation, on top of the IRS1 serine phosphorylation that occurred via DAG accumulation and PKC activation; however the increase in the phosphorylation of these proteins did not occur to a significant degree.

Once the leucine was removed from the system, the S6K1 phosphatase PP2A-B' potentially becomes active. This would lead to de-phosphorylation of S6K1 and the subsequent inhibition of the negative feedback loop. Any IRS1 proteins phosphorylated on serine residues would also become degraded. However, the accumulation of lipids would not prevent a complete attenuation of IRS1 proteins phosphorylated on serine residues. Furthermore, when leucine was administered to the rats and plasma glucose was examined 30 minutes later, the plasma glucose was found to be higher in these rats, but again, not a significant degree.

Overall, there are a number of key points to take away from these findings. First, that although consumption of high amino acid diets have been associated with the development of insulin resistance and impaired whole body insulin sensitivity, they are

not permanent lasting effects in the short term. This was especially remarkable in skeletal muscle of healthy rats. Furthermore, rats that were already insulin resistant were also able to consume high amino acid diets. This was associated with the same increase in S6K1 (T389) and IRS1 (serine) phosphorylation and plasma glucose that was seen in the lean rats. However, the increases were not to a significant degree, and also did not last permanently. From this it can be concluded that even insulin resistant individuals can consume high protein diets and reap the benefits that they can provide, without any additional impairments in their insulin sensitivity and metabolism.

7.0 SUMMARY

Although high protein diets are known to promote weight loss and increase lean body mass, there is evidence that they can also cause insulin resistance when consumed continuously. However, we now know that this state of insulin resistance caused by high protein diets (and represented by increased amino acid intake in this study) can in fact be reversed upon removal of the stimulant. However, in individuals that are already insulin resistant, this reversibility does not occur to the same extent as it would in healthy individuals. When examining these results in terms of the general population at large, it can be said that any insulin resistance caused by short term high protein diet intake is temporal and reversible. This is seen in healthy subjects. It is thus safe for these individuals to consume this type of diet and reap the associated health benefits. In people that are already insulin resistant, there appears to be no lasting detrimental effects to the use of this diet as for a short term diet management plan.

8.0 FUTURE DIRECTIONS

- It is known that exercise also increases the number of GLUT4 transporters that translocate to the surface membrane; thus, another question to ask might be: after these rats become insulin resistant, can the introduction of exercise assist in reversing this state via the recruitment of more GLUT4 transporters? Another factor that should be implemented in conjunction with this change is a change in diet. Rats that are already insulin resistant could either be placed on: a) an exercise regimen, b) a protein diet, or c) both. It may tell us something about reversing the insulin resistance caused by diet, and whether introducing other factors can do something about it.

- Examining the activity of PP2A-B' in healthy versus insulin resistant individuals may also be interesting. In other words, does the activity of PP2A-B' differ in healthy rats gavaged with leucine versus insulin resistant rats gavaged with leucine? In theory, the activation of PP2A-B' should be diminished in insulin resistant rats, as a lack of IRS1 activity would indicate decreased activation of the protein Akt, which is known to activate mTORC1 by inhibiting TSC1/2. A lack of mTORC1 activation would mean less phosphorylation of S6K1, meaning less activity of PP2A-B'.

- Consequently, it may also be worthwhile to examine the changes in the activity or differences in activation of GLUT4 in healthy versus insulin resistant rats. It is

already known that in insulin resistant individuals, there are impairments in insulin signalling and mostly likely less recruitment of GLUT4 in skeletal muscle. However, to what degree is GLUT4 activity decreased? Does this change when exercise is later introduced as a factor, as mentioned above?

- Examining the differences in Ph-Akt (T308) phosphorylation between lean rats and rats that are already made insulin resistant. Any differences in the pattern of Akt phosphorylation would be able to tell us more about how the insulin signalling pathway is affected.

9.0 REFERENCES

1-11,14-15,17-70,72-218

1. Eisenstein, J., Roberts, S.B., Dallal, G. & Saltzman, E. High-protein weight-loss diets: are they safe and do they work? A review of the experimental and epidemiologic data. *Nutr Rev* **60**, 189-200 (2002).
2. Westerterp-Plantenga, M.S., Rolland, V., Wilson, S.A. & Westerterp, K.R. Satiety related to 24 h diet-induced thermogenesis during high protein/carbohydrate vs high fat diets measured in a respiration chamber. *Eur J Clin Nutr* **53**, 495-502 (1999).
3. Layman, D.K., *et al.* Dietary protein and exercise have additive effects on body composition during weight loss in adult women. *J Nutr* **135**, 1903-1910 (2005).
4. Schulze, M.B., Manson, J.E., Willett, W.C. & Hu, F.B. Processed meat intake and incidence of Type 2 diabetes in younger and middle-aged women. *Diabetologia* **46**, 1465-1473 (2003).
5. Song, Y., Manson, J.E., Buring, J.E. & Liu, S. A prospective study of red meat consumption and type 2 diabetes in middle-aged and elderly women: the women's health study. *Diabetes Care* **27**, 2108-2115 (2004).
6. Koves, T.R., *et al.* Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab* **7**, 45-56 (2008).
7. Hamburg, N.M., *et al.* Physical inactivity rapidly induces insulin resistance and microvascular dysfunction in healthy volunteers. *Arterioscler Thromb Vasc Biol* **27**, 2650-2656 (2007).

8. Delarue, J. & Magnan, C. Free fatty acids and insulin resistance. *Curr Opin Clin Nutr Metab Care* **10**, 142-148 (2007).
9. Duncan, R.E., Ahmadian, M., Jaworski, K., Sarkadi-Nagy, E. & Sul, H.S. Regulation of lipolysis in adipocytes. *Annu Rev Nutr* **27**, 79-101 (2007).
10. Tanti, J.F. & Jager, J. Cellular mechanisms of insulin resistance: role of stress-regulated serine kinases and insulin receptor substrates (IRS) serine phosphorylation. *Curr Opin Pharmacol* **9**, 753-762 (2009).
11. Sherwin, R.S., *et al.* Prevention or delay of type 2 diabetes. *Diabetes Care* **27 Suppl 1**, S47-54 (2004).
12. Horne, J. Short sleep is a questionable risk factor for obesity and related disorders: statistical versus clinical significance. *Biol Psychol* **77**, 266-276 (2008).
13. Spiegel, K. Sleep loss as a risk factor for obesity and diabetes. *Int J Pediatr Obes* **3 Suppl 2**, 27-28 (2008).
14. Harris, J.L., Pomeranz, J.L., Lobstein, T. & Brownell, K.D. A crisis in the marketplace: how food marketing contributes to childhood obesity and what can be done. *Annu Rev Public Health* **30**, 211-225 (2009).
15. Mendez, M.A., Monteiro, C.A. & Popkin, B.M. Overweight exceeds underweight among women in most developing countries. *Am J Clin Nutr* **81**, 714-721 (2005).
16. Sherry, B., Mei, Z., Scanlon, K.S., Mokdad, A.H. & Grummer-Strawn, L.M. Trends in state-specific prevalence of overweight and underweight in 2- through 4-year-old children from low-income families from 1989 through 2000. *Arch Pediatr Adolesc Med* **158**, 1116-1124 (2004).
17. Samuel, V.T., Petersen, K.F. & Shulman, G.I. Lipid-induced insulin resistance: unravelling the mechanism. *Lancet* **375**, 2267-2277 (2010).
18. Carnevalli, L.S., *et al.* S6K1 plays a critical role in early adipocyte differentiation. *Dev Cell* **18**, 763-774 (2010).
19. Koppe, S.W., Elias, M., Moseley, R.H. & Green, R.M. Trans fat feeding results in higher serum alanine aminotransferase and increased insulin resistance compared with a standard murine high-fat diet. *Am J Physiol Gastrointest Liver Physiol* **297**, G378-384 (2009).
20. Roach, C., *et al.* Comparison of cis and trans fatty acid containing phosphatidylcholines on membrane properties. *Biochemistry* **43**, 6344-6351 (2004).
21. Allison, D.B., *et al.* Estimated intakes of trans fatty and other fatty acids in the US population. *J Am Diet Assoc* **99**, 166-174; quiz 175-166 (1999).
22. Mozaffarian, D., Katan, M.B., Ascherio, A., Stampfer, M.J. & Willett, W.C. Trans fatty acids and cardiovascular disease. *N Engl J Med* **354**, 1601-1613 (2006).
23. Ascherio, A., *et al.* Dietary fat and risk of coronary heart disease in men: cohort follow up study in the United States. *BMJ* **313**, 84-90 (1996).
24. Oh, K., Hu, F.B., Manson, J.E., Stampfer, M.J. & Willett, W.C. Dietary fat intake and risk of coronary heart disease in women: 20 years of follow-up of the nurses' health study. *Am J Epidemiol* **161**, 672-679 (2005).
25. Oomen, C.M., *et al.* Association between trans fatty acid intake and 10-year risk of coronary heart disease in the Zutphen Elderly Study: a prospective population-based study. *Lancet* **357**, 746-751 (2001).

26. Hu, F.B., *et al.* Diet, lifestyle, and the risk of type 2 diabetes mellitus in women. *N Engl J Med* **345**, 790-797 (2001).
27. Ibrahim, A., Natrajan, S. & Ghafoorunissa, R. Dietary trans-fatty acids alter adipocyte plasma membrane fatty acid composition and insulin sensitivity in rats. *Metabolism* **54**, 240-246 (2005).
28. Meyer, K.A., Kushi, L.H., Jacobs, D.R., Jr. & Folsom, A.R. Dietary fat and incidence of type 2 diabetes in older Iowa women. *Diabetes Care* **24**, 1528-1535 (2001).
29. van Dam, R.M., Rimm, E.B., Willett, W.C., Stampfer, M.J. & Hu, F.B. Dietary patterns and risk for type 2 diabetes mellitus in U.S. men. *Ann Intern Med* **136**, 201-209 (2002).
30. Hauner, H. Insulin resistance and the metabolic syndrome-a challenge of the new millennium. *Eur J Clin Nutr* **56 Suppl 1**, S25-29 (2002).
31. Reaven, G.M. & Chen, Y.D. Role of insulin in regulation of lipoprotein metabolism in diabetes. *Diabetes Metab Rev* **4**, 639-652 (1988).
32. Smith, U. Impaired ('diabetic') insulin signaling and action occur in fat cells long before glucose intolerance--is insulin resistance initiated in the adipose tissue? *Int J Obes Relat Metab Disord* **26**, 897-904 (2002).
33. Storlien, L.H., *et al.* Diet composition and insulin action in animal models. *Br J Nutr* **83 Suppl 1**, S85-90 (2000).
34. Storlien, L.H., *et al.* Influence of dietary fat composition on development of insulin resistance in rats. Relationship to muscle triglyceride and omega-3 fatty acids in muscle phospholipid. *Diabetes* **40**, 280-289 (1991).
35. Christiansen, E., Schnider, S., Palmvig, B., Tauber-Lassen, E. & Pedersen, O. Intake of a diet high in trans monounsaturated fatty acids or saturated fatty acids. Effects on postprandial insulinemia and glycemia in obese patients with NIDDM. *Diabetes Care* **20**, 881-887 (1997).
36. Summers, S.A. & Nelson, D.H. A role for sphingolipids in producing the common features of type 2 diabetes, metabolic syndrome X, and Cushing's syndrome. *Diabetes* **54**, 591-602 (2005).
37. Hannun, Y.A. & Obeid, L.M. The Ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind. *J Biol Chem* **277**, 25847-25850 (2002).
38. Itani, S.I., Ruderman, N.B., Schmieder, F. & Boden, G. Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha. *Diabetes* **51**, 2005-2011 (2002).
39. Holland, W.L., *et al.* Inhibition of ceramide synthesis ameliorates glucocorticoid-, saturated-fat-, and obesity-induced insulin resistance. *Cell Metab* **5**, 167-179 (2007).
40. Newgard, C.B., *et al.* A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab* **9**, 311-326 (2009).
41. Linn, T., *et al.* Effect of long-term dietary protein intake on glucose metabolism in humans. *Diabetologia* **43**, 1257-1265 (2000).
42. Linn, T., Geyer, R., Prassek, S. & Laube, H. Effect of dietary protein intake on insulin secretion and glucose metabolism in insulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* **81**, 3938-3943 (1996).

43. Wang, T.J., *et al.* Metabolite profiles and the risk of developing diabetes. *Nat Med* **17**, 448-453 (2011).
44. Pereira, S., Marliss, E.B., Morais, J.A., Chevalier, S. & Gougeon, R. Insulin resistance of protein metabolism in type 2 diabetes. *Diabetes* **57**, 56-63 (2008).
45. Ferrando, A.A., *et al.* EAA supplementation to increase nitrogen intake improves muscle function during bed rest in the elderly. *Clin Nutr* **29**, 18-23 (2010).
46. D'Antona, G., *et al.* Branched-chain amino acid supplementation promotes survival and supports cardiac and skeletal muscle mitochondrial biogenesis in middle-aged mice. *Cell Metab* **12**, 362-372 (2010).
47. Macotela, Y., *et al.* Dietary leucine--an environmental modifier of insulin resistance acting on multiple levels of metabolism. *PLoS One* **6**, e21187 (2011).
48. Bassil, M., *et al.* Hyperaminoacidaemia at postprandial levels does not modulate glucose metabolism in type 2 diabetes mellitus. *Diabetologia* **54**, 1810-1818 (2011).
49. Govindji, A. The role of carbohydrates in a healthy diet. *Nurs Stand* **21**, 56-64; quiz 66 (2006).
50. Laville, M. & Nazare, J.A. Diabetes, insulin resistance and sugars. *Obes Rev* **10 Suppl 1**, 24-33 (2009).
51. Storlien, L.H., Kraegen, E.W., Jenkins, A.B. & Chisholm, D.J. Effects of sucrose vs starch diets on in vivo insulin action, thermogenesis, and obesity in rats. *Am J Clin Nutr* **47**, 420-427 (1988).
52. Podolin, D.A., Gayles, E.C., Wei, Y., Thresher, J.S. & Pagliassotti, M.J. Menhaden oil prevents but does not reverse sucrose-induced insulin resistance in rats. *Am J Physiol* **274**, R840-848 (1998).
53. Chun, M.R., *et al.* Differential effects of high-carbohydrate and high-fat diet composition on muscle insulin resistance in rats. *J Korean Med Sci* **25**, 1053-1059 (2010).
54. Tappy, L. & Le, K.A. Metabolic effects of fructose and the worldwide increase in obesity. *Physiol Rev* **90**, 23-46 (2010).
55. Dekker, M.J., Su, Q., Baker, C., Rutledge, A.C. & Adeli, K. Fructose: a highly lipogenic nutrient implicated in insulin resistance, hepatic steatosis, and the metabolic syndrome. *Am J Physiol Endocrinol Metab* **299**, E685-694 (2010).
56. Le, K.A., *et al.* Fructose overconsumption causes dyslipidemia and ectopic lipid deposition in healthy subjects with and without a family history of type 2 diabetes. *Am J Clin Nutr* **89**, 1760-1765 (2009).
57. Stanhope, K.L., *et al.* Consuming fructose-sweetened, not glucose-sweetened, beverages increases visceral adiposity and lipids and decreases insulin sensitivity in overweight/obese humans. *J Clin Invest* **119**, 1322-1334 (2009).
58. Van den Berghe, G. Fructose: metabolism and short-term effects on carbohydrate and purine metabolic pathways. *Prog Biochem Pharmacol* **21**, 1-32 (1986).
59. Adams, S.H., Stanhope, K.L., Grant, R.W., Cummings, B.P. & Havel, P.J. Metabolic and endocrine profiles in response to systemic infusion of fructose and glucose in rhesus macaques. *Endocrinology* **149**, 3002-3008 (2008).
60. Thomsen, C., *et al.* Differential effects of saturated and monounsaturated fatty acids on postprandial lipemia and incretin responses in healthy subjects. *Am J Clin Nutr* **69**, 1135-1143 (1999).

61. Adeli, K. & Lewis, G.F. Intestinal lipoprotein overproduction in insulin-resistant states. *Curr Opin Lipidol* **19**, 221-228 (2008).
62. Bonen, A., Dohm, G.L. & van Loon, L.J. Lipid metabolism, exercise and insulin action. *Essays Biochem* **42**, 47-59 (2006).
63. Dresner, A., *et al.* Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J Clin Invest* **103**, 253-259 (1999).
64. Jenkins, D.J., *et al.* Glycemic index of foods: a physiological basis for carbohydrate exchange. *Am J Clin Nutr* **34**, 362-366 (1981).
65. Ludwig, D.S. Glycemic load comes of age. *J Nutr* **133**, 2695-2696 (2003).
66. Salmeron, J., *et al.* Dietary fiber, glycemic load, and risk of non-insulin-dependent diabetes mellitus in women. *JAMA* **277**, 472-477 (1997).
67. Blair, S.N., *et al.* Physical fitness and all-cause mortality. A prospective study of healthy men and women. *JAMA* **262**, 2395-2401 (1989).
68. Manson, J.E., *et al.* A prospective study of exercise and incidence of diabetes among US male physicians. *JAMA* **268**, 63-67 (1992).
69. Leahy, J.L. Pathogenesis of type 2 diabetes mellitus. *Arch Med Res* **36**, 197-209 (2005).
70. Zimmet, P., Alberti, K.G. & Shaw, J. Global and societal implications of the diabetes epidemic. *Nature* **414**, 782-787 (2001).
71. Venables, M.C. & Jeukendrup, A.E. Physical inactivity and obesity: links with insulin resistance and type 2 diabetes mellitus. *Diabetes Metab Res Rev* **25 Suppl 1**, S18-23 (2009).
72. Widlansky, M.E., Gokce, N., Keaney, J.F., Jr. & Vita, J.A. The clinical implications of endothelial dysfunction. *J Am Coll Cardiol* **42**, 1149-1160 (2003).
73. DeSouza, C.A., *et al.* Regular aerobic exercise prevents and restores age-related declines in endothelium-dependent vasodilation in healthy men. *Circulation* **102**, 1351-1357 (2000).
74. Benjamin, E.J., *et al.* Clinical correlates and heritability of flow-mediated dilation in the community: the Framingham Heart Study. *Circulation* **109**, 613-619 (2004).
75. Vaag, A. To be predisposed to insulin resistance and type 2 diabetes. *J Physiol* **586**, 2421 (2008).
76. Vaag, A., Henriksen, J.E. & Beck-Nielsen, H. Decreased insulin activation of glycogen synthase in skeletal muscles in young nonobese Caucasian first-degree relatives of patients with non-insulin-dependent diabetes mellitus. *J Clin Invest* **89**, 782-788 (1992).
77. Stoehr, J.P., *et al.* Genetic obesity unmasks nonlinear interactions between murine type 2 diabetes susceptibility loci. *Diabetes* **49**, 1946-1954 (2000).
78. Goalstone, M.L. & Draznin, B. Insulin signaling. *West J Med* **167**, 166-173 (1997).
79. Jornayvaz, F.R., Samuel, V.T. & Shulman, G.I. The role of muscle insulin resistance in the pathogenesis of atherogenic dyslipidemia and nonalcoholic fatty liver disease associated with the metabolic syndrome. *Annu Rev Nutr* **30**, 273-290 (2010).
80. Sano, H., *et al.* Rab10, a target of the AS160 Rab GAP, is required for insulin-stimulated translocation of GLUT4 to the adipocyte plasma membrane. *Cell Metab* **5**, 293-303 (2007).

81. Muoio, D.M. & Newgard, C.B. Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. *Nat Rev Mol Cell Biol* **9**, 193-205 (2008).
82. Chakraborty, C., Agoramoorthy, G. & Hsu, M.J. Exploring the evolutionary relationship of insulin receptor substrate family using computational biology. *PLoS One* **6**, e16580 (2011).
83. Taguchi, A. & White, M.F. Insulin-like signaling, nutrient homeostasis, and life span. *Annu Rev Physiol* **70**, 191-212 (2008).
84. White, M.F. Insulin signaling in health and disease. *Science* **302**, 1710-1711 (2003).
85. Lin, Y. & Sun, Z. Current views on type 2 diabetes. *J Endocrinol* **204**, 1-11 (2010).
86. Yoshizaki, H., Mochizuki, N., Gotoh, Y. & Matsuda, M. Akt-PDK1 complex mediates epidermal growth factor-induced membrane protrusion through Ral activation. *Mol Biol Cell* **18**, 119-128 (2007).
87. Malagelada, C., Jin, Z.H. & Greene, L.A. RTP801 is induced in Parkinson's disease and mediates neuron death by inhibiting Akt phosphorylation/activation. *J Neurosci* **28**, 14363-14371 (2008).
88. Hahn-Windgassen, A., *et al.* Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity. *J Biol Chem* **280**, 32081-32089 (2005).
89. Greer, E.L. & Brunet, A. FOXO transcription factors at the interface between longevity and tumor suppression. *Oncogene* **24**, 7410-7425 (2005).
90. Lazar, D.F. & Saltiel, A.R. Lipid phosphatases as drug discovery targets for type 2 diabetes. *Nat Rev Drug Discov* **5**, 333-342 (2006).
91. Hoehn, K.L., *et al.* IRS1-independent defects define major nodes of insulin resistance. *Cell Metab* **7**, 421-433 (2008).
92. Gual, P., Le Marchand-Brustel, Y. & Tanti, J.F. Positive and negative regulation of insulin signaling through IRS-1 phosphorylation. *Biochimie* **87**, 99-109 (2005).
93. Zhande, R., Mitchell, J.J., Wu, J. & Sun, X.J. Molecular mechanism of insulin-induced degradation of insulin receptor substrate 1. *Mol Cell Biol* **22**, 1016-1026 (2002).
94. Delibegovic, M., *et al.* Improved glucose homeostasis in mice with muscle-specific deletion of protein-tyrosine phosphatase 1B. *Mol Cell Biol* **27**, 7727-7734 (2007).
95. Xue, B., *et al.* Protein-tyrosine phosphatase 1B deficiency reduces insulin resistance and the diabetic phenotype in mice with polygenic insulin resistance. *J Biol Chem* **282**, 23829-23840 (2007).
96. Choi, S.M., *et al.* Insulin regulates adipocyte lipolysis via an Akt-independent signaling pathway. *Mol Cell Biol* **30**, 5009-5020 (2010).
97. Cheadle, C., *et al.* Regulatory subunits of PKA define an axis of cellular proliferation/differentiation in ovarian cancer cells. *BMC Med Genomics* **1**, 43 (2008).
98. Wahren, J. & Ekberg, K. Splanchnic regulation of glucose production. *Annu Rev Nutr* **27**, 329-345 (2007).
99. Matsumoto, M., Han, S., Kitamura, T. & Accili, D. Dual role of transcription factor FoxO1 in controlling hepatic insulin sensitivity and lipid metabolism. *J Clin Invest* **116**, 2464-2472 (2006).
100. Shimomura, I., *et al.* Insulin selectively increases SREBP-1c mRNA in the livers of rats with streptozotocin-induced diabetes. *Proc Natl Acad Sci U S A* **96**, 13656-13661 (1999).

101. Li, S., Brown, M.S. & Goldstein, J.L. Bifurcation of insulin signaling pathway in rat liver: mTORC1 required for stimulation of lipogenesis, but not inhibition of gluconeogenesis. *Proc Natl Acad Sci U S A* **107**, 3441-3446 (2010).
102. Valenti, L., *et al.* Increased expression and activity of the transcription factor FOXO1 in nonalcoholic steatohepatitis. *Diabetes* **57**, 1355-1362 (2008).
103. DeFronzo, R.A. Insulin resistance, lipotoxicity, type 2 diabetes and atherosclerosis: the missing links. The Claude Bernard Lecture 2009. *Diabetologia* **53**, 1270-1287 (2010).
104. Bouskila, M., *et al.* Allosteric regulation of glycogen synthase controls glycogen synthesis in muscle. *Cell Metab* **12**, 456-466 (2010).
105. Sengupta, S., Peterson, T.R. & Sabatini, D.M. Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. *Mol Cell* **40**, 310-322 (2010).
106. Wang, X. & Proud, C.G. mTORC1 signaling: what we still don't know. *J Mol Cell Biol* (2010).
107. Hara, K., *et al.* Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell* **110**, 177-189 (2002).
108. Kim, D.H., *et al.* mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* **110**, 163-175 (2002).
109. Nojima, H., *et al.* The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. *J Biol Chem* **278**, 15461-15464 (2003).
110. Pearce, L.R., *et al.* Identification of Protor as a novel Rictor-binding component of mTOR complex-2. *Biochem J* **405**, 513-522 (2007).
111. Sancak, Y., *et al.* The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* **320**, 1496-1501 (2008).
112. Sancak, Y., *et al.* PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. *Mol Cell* **25**, 903-915 (2007).
113. Schalm, S.S., Fingar, D.C., Sabatini, D.M. & Blenis, J. TOS motif-mediated raptor binding regulates 4E-BP1 multisite phosphorylation and function. *Curr Biol* **13**, 797-806 (2003).
114. Wullschleger, S., Loewith, R., Oppliger, W. & Hall, M.N. Molecular organization of target of rapamycin complex 2. *J Biol Chem* **280**, 30697-30704 (2005).
115. Vander Haar, E., Lee, S.I., Bandhakavi, S., Griffin, T.J. & Kim, D.H. Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. *Nat Cell Biol* **9**, 316-323 (2007).
116. Loewith, R., *et al.* Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell* **10**, 457-468 (2002).
117. Peterson, T.R., *et al.* DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. *Cell* **137**, 873-886 (2009).
118. Zoncu, R., Efeyan, A. & Sabatini, D.M. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* **12**, 21-35 (2011).
119. Byfield, M.P., Murray, J.T. & Backer, J.M. hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase. *J Biol Chem* **280**, 33076-33082 (2005).
120. Nobukuni, T., *et al.* Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proc Natl Acad Sci U S A* **102**, 14238-14243 (2005).

121. Gulati, P., *et al.* Amino acids activate mTOR complex 1 via Ca2+/CaM signaling to hVps34. *Cell Metab* **7**, 456-465 (2008).
122. Findlay, G.M., Yan, L., Procter, J., Mieulet, V. & Lamb, R.F. A MAP4 kinase related to Ste20 is a nutrient-sensitive regulator of mTOR signalling. *Biochem J* **403**, 13-20 (2007).
123. Yan, L., *et al.* PP2A T61 epsilon is an inhibitor of MAP4K3 in nutrient signaling to mTOR. *Mol Cell* **37**, 633-642 (2010).
124. Kim, S., *et al.* Amino acid signaling to mTOR mediated by inositol polyphosphate multikinase. *Cell Metab* **13**, 215-221 (2011).
125. Inoki, K., Li, Y., Zhu, T., Wu, J. & Guan, K.L. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol* **4**, 648-657 (2002).
126. Potter, C.J., Pedraza, L.G. & Xu, T. Akt regulates growth by directly phosphorylating Tsc2. *Nat Cell Biol* **4**, 658-665 (2002).
127. Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P. & Pandolfi, P.P. Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. *Cell* **121**, 179-193 (2005).
128. Dennis, P.B., *et al.* Mammalian TOR: a homeostatic ATP sensor. *Science* **294**, 1102-1105 (2001).
129. Hardie, D.G. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol* **8**, 774-785 (2007).
130. Inoki, K., *et al.* TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. *Cell* **126**, 955-968 (2006).
131. Feng, Z., Zhang, H., Levine, A.J. & Jin, S. The coordinate regulation of the p53 and mTOR pathways in cells. *Proc Natl Acad Sci U S A* **102**, 8204-8209 (2005).
132. Le Bacquer, O., *et al.* Elevated sensitivity to diet-induced obesity and insulin resistance in mice lacking 4E-BP1 and 4E-BP2. *J Clin Invest* **117**, 387-396 (2007).
133. Um, S.H., D'Alessio, D. & Thomas, G. Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. *Cell Metab* **3**, 393-402 (2006).
134. Holz, M.K., Ballif, B.A., Gygi, S.P. & Blenis, J. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell* **123**, 569-580 (2005).
135. Raught, B., *et al.* Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases. *EMBO J* **23**, 1761-1769 (2004).
136. Dorrello, N.V., *et al.* S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. *Science* **314**, 467-471 (2006).
137. Sarbassov, D.D., *et al.* Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol* **14**, 1296-1302 (2004).
138. Facchinetti, V., *et al.* The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. *EMBO J* **27**, 1932-1943 (2008).
139. Garcia-Martinez, J.M. & Alessi, D.R. mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). *Biochem J* **416**, 375-385 (2008).

140. Ikenoue, T., Inoki, K., Yang, Q., Zhou, X. & Guan, K.L. Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. *EMBO J* **27**, 1919-1931 (2008).
141. Guertin, D.A., *et al.* 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 (2006).
142. Soukas, A.A., Kane, E.A., Carr, C.E., Melo, J.A. & Ruvkun, G. Rictor/TORC2 regulates fat metabolism, feeding, growth, and life span in Caenorhabditis elegans. *Genes Dev* **23**, 496-511 (2009).
143. Mauvoisin, D., *et al.* Role of the PI3-kinase/mTor pathway in the regulation of the stearoyl CoA desaturase (SCD1) gene expression by insulin in liver. *J Cell Commun Signal* **1**, 113-125 (2007).
144. Bachar, E., *et al.* Glucose amplifies fatty acid-induced endoplasmic reticulum stress in pancreatic beta-cells via activation of mTORC1. *PLoS One* **4**, e4954 (2009).
145. Amoui, M., Craddock, B.P. & Miller, W.T. Differential phosphorylation of IRS-1 by insulin and insulin-like growth factor I receptors in Chinese hamster ovary cells. *J Endocrinol* **171**, 153-162 (2001).
146. Dries, D.R., Gallegos, L.L. & Newton, A.C. A single residue in the C1 domain sensitizes novel protein kinase C isoforms to cellular diacylglycerol production. *J Biol Chem* **282**, 826-830 (2007).
147. Stahelin, R.V., *et al.* Diacylglycerol-induced membrane targeting and activation of protein kinase Cepsilon: mechanistic differences between protein kinases Cdelta and Cepsilon. *J Biol Chem* **280**, 19784-19793 (2005).
148. Anderson, C.M. & Olefsky, J.M. Phorbol ester-mediated protein kinase C interaction with wild-type and COOH-terminal truncated insulin receptors. *J Biol Chem* **266**, 21760-21764 (1991).
149. Lewis, R.E., Cao, L., Perregaux, D. & Czech, M.P. Threonine 1336 of the human insulin receptor is a major target for phosphorylation by protein kinase C. *Biochemistry* **29**, 1807-1813 (1990).
150. Pillay, T.S., Whittaker, J. & Siddle, K. Phorbol ester-induced downregulation of protein kinase C potentiates insulin receptor tyrosine autophosphorylation: evidence for a major constitutive role in insulin receptor regulation. *Biochem Soc Trans* **18**, 494-495 (1990).
151. Liu, L., *et al.* Upregulation of myocellular DGAT1 augments triglyceride synthesis in skeletal muscle and protects against fat-induced insulin resistance. *J Clin Invest* **117**, 1679-1689 (2007).
152. Maechler, P. & Wollheim, C.B. Mitochondrial function in normal and diabetic beta-cells. *Nature* **414**, 807-812 (2001).
153. Morino, K., *et al.* Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. *J Clin Invest* **115**, 3587-3593 (2005).
154. de Luca, C. & Olefsky, J.M. Inflammation and insulin resistance. *FEBS Lett* **582**, 97-105 (2008).

155. Yuan, M., *et al.* Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. *Science* **293**, 1673-1677 (2001).
156. Kim, J.K., *et al.* Prevention of fat-induced insulin resistance by salicylate. *J Clin Invest* **108**, 437-446 (2001).
157. Chen, M. & Robertson, R.P. Effects of prostaglandin synthesis inhibitors on human insulin secretion and carbohydrates tolerance. *Prostaglandins* **18**, 557-567 (1979).
158. Cai, D., *et al.* Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med* **11**, 183-190 (2005).
159. Zhang, J., Gao, Z., Yin, J., Quon, M.J. & Ye, J. S6K directly phosphorylates IRS-1 on Ser-270 to promote insulin resistance in response to TNF-(alpha) signaling through IKK2. *J Biol Chem* **283**, 35375-35382 (2008).
160. Fukuda, M., *et al.* Effects of very-low-calorie diet weight reduction on glucose tolerance, insulin secretion, and insulin resistance in obese non-insulin-dependent diabetics. *Diabetes Res Clin Pract* **7**, 61-67 (1989).
161. Petersen, K.F., *et al.* Reversal of nonalcoholic hepatic steatosis, hepatic insulin resistance, and hyperglycemia by moderate weight reduction in patients with type 2 diabetes. *Diabetes* **54**, 603-608 (2005).
162. Beckett, P.R., *et al.* Spectrophometric assay for measuring branched-chain amino acid concentrations: application for measuring the sensitivity of protein metabolism to insulin. *Anal Biochem* **240**, 48-53 (1996).
163. Chevalier, S., Gougeon, R., Kreisman, S.H., Cassis, C. & Morais, J.A. The hyperinsulinemic amino acid clamp increases whole-body protein synthesis in young subjects. *Metabolism* **53**, 388-396 (2004).
164. Chevalier, S., Marliss, E.B., Morais, J.A., Lamarche, M. & Gougeon, R. Whole-body protein anabolic response is resistant to the action of insulin in obese women. *Am J Clin Nutr* **82**, 355-365 (2005).
165. Tessari, P. Effects of insulin on whole-body and regional amino acid metabolism. *Diabetes Metab Rev* **10**, 253-285 (1994).
166. Tischler, M.E., Desautels, M. & Goldberg, A.L. Does leucine, leucyl-tRNA, or some metabolite of leucine regulate protein synthesis and degradation in skeletal and cardiac muscle? *J Biol Chem* **257**, 1613-1621 (1982).
167. Stadlbauer, K., *et al.* The effects of amino acids on glucose metabolism of isolated rat skeletal muscle are independent of insulin and the mTOR/S6K pathway. *Am J Physiol Endocrinol Metab* **297**, E785-792 (2009).
168. Hahn, K., *et al.* PP2A regulatory subunit PP2A-B' counteracts S6K phosphorylation. *Cell Metab* **11**, 438-444 (2010).
169. Woods, A., *et al.* LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol* **13**, 2004-2008 (2003).
170. Huang, H.Y., Liao, J. & Sacks, M.S. In-situ deformation of the aortic valve interstitial cell nucleus under diastolic loading. *J Biomech Eng* **129**, 880-889 (2007).
171. Jessen, N. & Goodyear, L.J. Contraction signaling to glucose transport in skeletal muscle. *J Appl Physiol* **99**, 330-337 (2005).
172. Toyoda, T., *et al.* Myo1c regulates glucose uptake in mouse skeletal muscle. *J Biol Chem* **286**, 4133-4140 (2011).

173. Abdul-Ghani, M.A., *et al.* Mitochondrial reactive oxygen species generation in obese non-diabetic and type 2 diabetic participants. *Diabetologia* **52**, 574-582 (2009).
174. Adochio, R., Leitner, J.W., Hedlund, R. & Draznin, B. Rescuing 3T3-L1 adipocytes from insulin resistance induced by stimulation of Akt-mammalian target of rapamycin/p70 S6 kinase (S6K1) pathway and serine phosphorylation of insulin receptor substrate-1: effect of reduced expression of p85alpha subunit of phosphatidylinositol 3-kinase and S6K1 kinase. *Endocrinology* **150**, 1165-1173 (2009).
175. Bassil, M., *et al.* Postprandial hyperaminoacidaemia overcomes insulin resistance of protein anabolism in men with type 2 diabetes. *Diabetologia* **54**, 648-656 (2011).
176. Bernard, J.R., *et al.* An amino acid mixture improves glucose tolerance and insulin signaling in Sprague-Dawley rats. *Am J Physiol Endocrinol Metab* **300**, E752-760 (2011).
177. Binda, M., *et al.* The Vam6 GEF controls TORC1 by activating the EGO complex. *Mol Cell* **35**, 563-573 (2009).
178. Bloch-Damti, A. & Bashan, N. Proposed mechanisms for the induction of insulin resistance by oxidative stress. *Antioxid Redox Signal* **7**, 1553-1567 (2005).
179. Boura-Halfon, S., *et al.* A novel domain mediates insulin-induced proteasomal degradation of insulin receptor substrate 1 (IRS-1). *Mol Endocrinol* **24**, 2179-2192 (2010).
180. Cybulski, N., Polak, P., Auwerx, J., Ruegg, M.A. & Hall, M.N. mTOR complex 2 in adipose tissue negatively controls whole-body growth. *Proc Natl Acad Sci U S A* **106**, 9902-9907 (2009).
181. Evans, J.L., Goldfine, I.D., Maddux, B.A. & Grodsky, G.M. Are oxidative stress-activated signaling pathways mediators of insulin resistance and beta-cell dysfunction? *Diabetes* **52**, 1-8 (2003).
182. Evans, J.L., Maddux, B.A. & Goldfine, I.D. The molecular basis for oxidative stress-induced insulin resistance. *Antioxid Redox Signal* **7**, 1040-1052 (2005).
183. Gwinn, D.M., *et al.* AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol Cell* **30**, 214-226 (2008).
184. Loh, K., *et al.* Reactive oxygen species enhance insulin sensitivity. *Cell Metab* **10**, 260-272 (2009).
185. Long, Y.C., Cheng, Z., Copps, K.D. & White, M.F. Insulin receptor substrates Irs1 and Irs2 coordinate skeletal muscle growth and metabolism via the Akt and AMPK pathways. *Mol Cell Biol* **31**, 430-441 (2011).
186. Luo, J., Manning, B.D. & Cantley, L.C. Targeting the PI3K-Akt pathway in human cancer: rationale and promise. *Cancer Cell* **4**, 257-262 (2003).
187. Oi, Y., *et al.* Allylthiamindisulfide and related compounds enhance thermogenesis with increasing noradrenaline and adrenaline secretion in rats. *J Nutr Sci Vitaminol (Tokyo)* **45**, 643-653 (1999).
188. Pinzani, M., Rosselli, M. & Zuckermann, M. Liver cirrhosis. *Best Pract Res Clin Gastroenterol* **25**, 281-290 (2011).
189. Soliman, G.A., *et al.* mTOR Ser-2481 autophosphorylation monitors mTORC-specific catalytic activity and clarifies rapamycin mechanism of action. *J Biol Chem* **285**, 7866-7879 (2010).

190. Stefan, N., Staiger, H. & Haring, H.U. Dissociation between fatty liver and insulin resistance: the role of adipose triacylglycerol lipase. *Diabetologia* **54**, 7-9 (2011).
191. Taniguchi, C.M., Emanuelli, B. & Kahn, C.R. Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* **7**, 85-96 (2006).
192. Tremblay, F., *et al.* Overactivation of S6 kinase 1 as a cause of human insulin resistance during increased amino acid availability. *Diabetes* **54**, 2674-2684 (2005).
193. Tremblay, F., Lavigne, C., Jacques, H. & Marette, A. Role of dietary proteins and amino acids in the pathogenesis of insulin resistance. *Annu Rev Nutr* **27**, 293-310 (2007).
194. Um, S.H., *et al.* Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* **431**, 200-205 (2004).
195. Workeneh, B.T. & Mitch, W.E. Review of muscle wasting associated with chronic kidney disease. *Am J Clin Nutr* **91**, 1128S-1132S (2010).
196. Yang, L., Li, P., Fu, S., Calay, E.S. & Hotamisligil, G.S. Defective hepatic autophagy in obesity promotes ER stress and causes insulin resistance. *Cell Metab* **11**, 467-478 (2010).
197. Yang, Q., Inoki, K., Ikenoue, T. & Guan, K.L. Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. *Genes Dev* **20**, 2820-2832 (2006).
198. Yip, W.K., Leong, V.C., Abdullah, M.A., Yusoff, S. & Seow, H.F. Overexpression of phospho-Akt correlates with phosphorylation of EGF receptor, FKHR and BAD in nasopharyngeal carcinoma. *Oncol Rep* **19**, 319-328 (2008).
199. Layman, D.K., *et al.* A moderate-protein diet produces sustained weight loss and long-term changes in body composition and blood lipids in obese adults. *J Nutr* **139**, 514-521 (2009).
200. Wei, Y., *et al.* Skeletal muscle insulin resistance: role of inflammatory cytokines and reactive oxygen species. *Am J Physiol Regul Integr Comp Physiol* **294**, R673-680 (2008).
201. Newgard, C.B. & McGarry, J.D. Metabolic coupling factors in pancreatic beta-cell signal transduction. *Annu Rev Biochem* **64**, 689-719 (1995).
202. Henquin, J.C., Ravier, M.A., Nenquin, M., Jonas, J.C. & Gilon, P. Hierarchy of the beta-cell signals controlling insulin secretion. *Eur J Clin Invest* **33**, 742-750 (2003).
203. Nenquin, M., Szollosi, A., Aguilar-Bryan, L., Bryan, J. & Henquin, J.C. Both triggering and amplifying pathways contribute to fuel-induced insulin secretion in the absence of sulfonylurea receptor-1 in pancreatic beta-cells. *J Biol Chem* **279**, 32316-32324 (2004).
204. Petersen, K.F., Dufour, S., Befroy, D., Garcia, R. & Shulman, G.I. Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med* **350**, 664-671 (2004).
205. Teff, K.L., *et al.* Dietary fructose reduces circulating insulin and leptin, attenuates postprandial suppression of ghrelin, and increases triglycerides in women. *J Clin Endocrinol Metab* **89**, 2963-2972 (2004).
206. Brozinick, J.T., Jr., Roberts, B.R. & Dohm, G.L. Defective signaling through Akt-2 and -3 but not Akt-1 in insulin-resistant human skeletal muscle: potential role in insulin resistance. *Diabetes* **52**, 935-941 (2003).
207. Henry, R.R., Abrams, L., Nikoulina, S. & Ciaraldi, T.P. Insulin action and glucose metabolism in nondiabetic control and NIDDM subjects. Comparison using human skeletal muscle cell cultures. *Diabetes* **44**, 936-946 (1995).

208. Vollenweider, P., Menard, B. & Nicod, P. Insulin resistance, defective insulin receptor substrate 2-associated phosphatidylinositol-3' kinase activation, and impaired atypical protein kinase C (zeta/lambda) activation in myotubes from obese patients with impaired glucose tolerance. *Diabetes* **51**, 1052-1059 (2002).
209. Sandra, A., Fyler, D.J. & Marshall, S.J. Effects of lipids on the transport activity of the reconstituted glucose transport system from rat adipocyte. *Biochim Biophys Acta* **778**, 511-515 (1984).
210. Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J. & Cantley, L.C. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. *Mol Cell* **10**, 151-162 (2002).
211. Garami, A., *et al.* Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. *Mol Cell* **11**, 1457-1466 (2003).
212. Inoki, K., Li, Y., Xu, T. & Guan, K.L. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev* **17**, 1829-1834 (2003).
213. Karlsson, H.K. & Zierath, J.R. Insulin signaling and glucose transport in insulin resistant human skeletal muscle. *Cell Biochem Biophys* **48**, 103-113 (2007).
214. Kim, J.Y., *et al.* High-fat diet-induced muscle insulin resistance: relationship to visceral fat mass. *Am J Physiol Regul Integr Comp Physiol* **279**, R2057-2065 (2000).
215. Pagliassotti, M.J., Shahrokhi, K.A. & Moscarello, M. Involvement of liver and skeletal muscle in sucrose-induced insulin resistance: dose-response studies. *Am J Physiol* **266**, R1637-1644 (1994).
216. Adegoke, O.A., *et al.* Fed-state clamp stimulates cellular mechanisms of muscle protein anabolism and modulates glucose disposal in normal men. *Am J Physiol Endocrinol Metab* **296**, E105-113 (2009).
217. Bennet, W.M., Connacher, A.A., Scrimgeour, C.M., Jung, R.T. & Rennie, M.J. Euglycemic hyperinsulinemia augments amino acid uptake by human leg tissues during hyperaminoacidemia. *Am J Physiol* **259**, E185-194 (1990).
218. Hillier, T.A., Fryburg, D.A., Jahn, L.A. & Barrett, E.J. Extreme hyperinsulinemia unmasks insulin's effect to stimulate protein synthesis in the human forearm. *Am J Physiol* **274**, E1067-1074 (1998).
219. Young, T., Sahar, E., Nieto, F.J. et al. Predictors of sleep-disordered breathing in community-dwelling adults: The Sleep Heart Health Study. *Arch Intern Med* **162,** 893-900 (2002).
220. Valko, M., Leibfritz, D., Moncol, J et al. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* **39**, 44-84 (2007).
221. Rossetti, L. Rothman, D., DeFronzo, R. et al. Effect of dietary protein on in vivo insulin action and liver glycogen repletion. *Am J Physiol.* **257**, E212-E219 (1989).
222. Siegel, E., Wollheim, C. Renold, A. et al. Beneficial effects of low-carbohydrate, high-protein diets in long-term diabetic rats. *Metabolism* **29**, 421-428 (1980).
223. She, P., Van Horn, C., Reid, T., et al. Obesity-related elevations in plasma leucine are associated with alterations in enzymes involved in branched-chain amino acid metabolism. *Am J Physiol Endocrinol Metab* **293**, E1552-E1563 (2007).