Glutamate increases glucose uptake in L6 myotubes in a concentration- and time-dependent manner that is mediated by AMPK

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The majority of studies that have examined the interactions between glutamate, insulin and glucose are *in vivo* and have focused on the insulin response that is elicited when glutamate is elevated in circulation or in a given tissue. Few studies have investigated the effects of glutamate on glucose uptake and handling. Work from our lab suggests that monosodium glutamate ingestion in humans can attenuate rises in blood glucose following a carbohydrate load (75g of Trutol®) compared with administration of a carbohydrate load alone (DiSebastiano et al., 2013); this improvement in glucose handling occurred in absence of changes in serum insulin concentrations. However, the mechanisms responsible for this observation have yet to be investigated. Considering that glutamate is the primary amino acid taken up by the muscle (Graham & MacLean, 1998; Graham et al., 1997) and skeletal muscle is responsible for approximately 85% of whole-body glucose disposal (DeFronzo et al., 1981), this study examined the isolated effects of glutamate on glucose uptake in skeletal muscle to better understand the glucose response that has been observed *in vivo* as a result of glutamate supplementation. The objectives were to: 1) examine the effects of glutamate on glucose uptake in isolated L6 myotubes in a dose and time dependant manner, 2) measure and compare glucose uptake with the provision of leucine and insulin, and 3) investigate and compare the primary mechanisms of glucose handling in skeletal muscle cells in each experimental condition. Differentiated L6 rat muscle cells were treated with increasing doses of glutamate for 1 hr and glucose uptake was assessed by the addition of [^{3}H]-2-Deoxyglucose in HBS for 10 min. Cells treated with 500μM, 1mM, and 2mM significantly increased [^{3}H]-2-DG uptake to 129 ± 7%, 123 ± 5%, and 121 ± 4%, respectively relative to the control

condition ($P<0.05$). To evaluate the effect of incubation time on glucose uptake, cells were treated with 2mM of glutamate for various times ranging from 0 to 120 minutes. Cells treated for 30 minutes resulted in the greatest increase in [^{3}H]-2-DG uptake versus the control condition (143 ± 9%, $P<0.001$), while cells treated for 45 and 60 minutes also significantly increased [^{3}H]-2-DG uptake (125 ± 9% and 129 ± 7% relative to the control, respectively; $P<0.05$). To measure and compare the magnitude of glucose uptake elicited by glutamate to other known stimulators of uptake, cells were treated with leucine, insulin, and a combination of GLU+LEU and GLU+INS. Each treatment significantly elevated [^{3}H]-2-DG uptake relative to the control condition (126 ± 9%, 141 ± 12%, 148 ± 3%, and 148 ± 3%, respectively; $P<0.05$). To investigate the primary mechanisms by which glutamate acts to increase glucose uptake, cells were treated with 2mM of glutamate, leucine, or insulin for 30 minutes with a PI3K inhibitor (LY294002), PKC inhibitor (BMD I), or AMPK inhibitor (Compound C). Compound C and BMD1 reduced glucose uptake in cells treated with glutamate to a magnitude that was similar to that of the control condition (98 ± 2% and 103 ± 4%, respectively; $P<0.05$), whereas cells treated with LY294002 showed significantly greater uptake relative to the control (128 ± 5%, $P<0.05$). In contrast, [^{3}H]-2-DG was blocked in cells treated with leucine or insulin in combination with LY294002. The results from this study suggest that glutamate can increase glucose uptake into L6 myotubes in a dose- and time- dependent manner that is similar in magnitude to leucine and insulin, mediated by AMPK and PKC. This study will provide a basis for future animal and human studies exploring the interactions of glutamate and glucose in skeletal muscle.

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Overview

Glutamate, a non-essential amino acid, has been a relatively understudied amino acid with regards to its role in glucose handling and insulin metabolism, despite that it is the primary amino acid taken up by muscle and is involved in many processes related to oxidative metabolism in skeletal muscle (Graham et al., 2000; Mourtzakis & Graham, 2002; Mourtzakis, et al., 2008; Mourtzakis et al., 2006), glucose and insulin metabolism (Chevassus et al., 2002; Di Sebastiano et al., 2013) and insulin secretion (Bertrand et al., 1992; Bertrand et al., 1995).

Emerging studies over the past 10-15 years have used animal models (Bernard et al., 2012; 2011; Doi et al., 2005; Kleinert et al., 2011) and isolated myocytes (Doi et al., 2003; Nishitani et al., 2002) to show that branched chain and essential amino acids can enhance glucose uptake by skeletal muscle. Interestingly, work from our laboratory (Di Sebastiano et al., 2013) has shown that increased circulating glutamate is associated with an attenuated rise in blood glucose following the ingestion of a carbohydrate drink; this is supported by other studies showing an association between glutamate ingestion and improved glucose handling and clearance (Hosaka et al., 2012; Shi et al., 2014). Studies have shown that skeletal muscle serves as a major sink for glutamate (Graham et al., 2000) as well as glucose (DeFronzo et al., 1981) and it is possible that the glucose response observed in studies that have supplemented glutamate is due to increased peripheral clearance by skeletal muscle. Thus this study was designed to isolate and understand the events that occur in muscle that is exposed to elevated concentrations of glutamate to determine if glutamate itself enhances glucose uptake in skeletal muscle.

My thesis aims to characterize the effects of glutamate on glucose uptake in isolated skeletal muscle cells in a dose- and time-dependant manner, to measure and compare the magnitude of glucose uptake elicited by glutamate compared to leucine and insulin, and to examine primary mechanisms by which glutamate acts to increase glucose uptake in skeletal muscle cells. The findings from my work will provide a basis for future animal and human studies exploring the interactions of glutamate and glucose in skeletal muscle.

Literature Review

Overview of glutamate's role in various physiological processes

Glutamate is a non-essential amino acid that is involved in a variety of physiological and metabolic processes. It serves as the primary excitatory neurotransmitter in the brain (Meldrum, 2000) and both the central and peripheral nervous systems (Brann, 1995; Pin & Duvoisin, 1995). Additionally, it is the primary amino acid that is taken up by skeletal muscle at rest, during exercise, and during exercise recovery (Graham et al., 1997; Graham & MacLean, 1998; Mourtzakis et al., 2006), signifying that it plays important metabolic roles in skeletal muscle.

Glutamate has been shown to be particularly important in skeletal muscle for its metabolic roles in energy provision. It acts as an intermediary amino acid providing substrate and carbons to the tricarboxylic acid (TCA) cycle, supported by work showing that intramuscular glutamate levels significantly decline during prolonged exercise (Mourtzakis et al., 2006). One important entry point for glutamate into the TCA cycle is through the alanine aminotransferase reaction (AAT), which is a transamination reaction that combines glutamate and pyruvate to form the TCA intermediate alpha-ketoglutarate as well as alanine. Alpha-ketoglutarate subsequently continues through a series of reactions that lead to fumarate and malate production within the TCA cycle (Mourtzakis et al., 2008). On the other hand, alanine is released from the muscle and taken up by liver for gluconeogenesis (Mourtzakis & Graham, 2002; Thomassen et al., 1990).

Despite the fact that glutamate is abundantly taken up by skeletal muscle, it is a complex amino acid to study in muscle because it is involved in a variety of pathways,

many being transamination and anaplerotic reactions (Frigerio et al., 2008), thus changes in glutamate occur in a mass action effect and its explicit effects in muscle can be easily missed. To better explore the integrative role of glutamate in skeletal muscle metabolism, various studies have used glutamate supplementation in the form of monosodium glutamate (MSG) to enhance glutamate availability. These studies have successfully increased circulating glutamate concentrations by up to 18 fold from basal levels with the administration of 150mg/kg body weight dose of MSG to participants (Di Sebastiano et al., 2013; Graham et al., 2000; Mourtzakis & Graham, 2002; Stegink et al., 1983a; Stegink et al., 1983b). Graham et al. (2000) showed that elevated glutamate availability in circulation was associated with significantly elevated intramuscular glutamate concentrations at rest; however this was not accompanied with increases in plasma alanine levels (Graham et al., 2000). This work suggests that glutamate may only have significant interactions with pyruvate when it is elevated during exercise to facilitate alanine production. At the onset of exercise, the combination of elevated pyruvate production via increased carbohydrate consumption and enhanced glutamate availability increases pyruvate and glutamate ultilization through the AAT reaction, resulting in increased alanine and alpha-ketoglutarate production(Mourtzakis & Graham, 2002; Thomassen et al., 1990).

These bodies of work have shown that glutamate has an integrative role with carbohydrates as glutamate is linked to many glycolytic and gluconeogenic processes through interactions with pyruvate and alanine (Bertrand et al., 1992; Mourtzakis et al., 2006; 2008; Mourtzakis & Graham, 2002). Even in the central nervous system, glutamate is shown to stimulate sharp and rapid increase in glucose uptake (Loaiza et al., 2003),

implying that glutamate is integral to carbohydrate metabolism across different types of cells and tissues. However, the interplay between glutamate, carbohydrate, and insulin metabolism in skeletal muscle is not clear.

Glutamate can stimulate pancreatic insulin secretion

Insulin is a hormone that is secreted by pancreatic β-cells in response to elevations in blood glucose concentration. In skeletal muscle cells, insulin binds to its receptor on the sarcolemma, resulting in the phosphorylation of the intracellular protein, insulin-receptor subsrate-1 (IRS-1). In the phosphorylated state, IRS-1 will recruit phosphatidyinositide 3-kinase (PI3K), which results in downstream activation of Akt and protein kinase-c (PKC). Various isoforms of the PKC enzyme exist and partake in a variety of different signalling cascades, however it is the atypical PKCs (PKC-λ and PKC-ζ) that are required for insulin-stimulated glucose uptake (Satiel & Kahn, 2001). Activation of these proteins results in the phosphorylation of AS160, which relieves Rab of inhibition from AS160 and allows for the translocation of GLUT-4 transporters to the sarcolemma. Glucose can then be transported from the plasma into the muscle cell via GLUT-4 transporters (Saltiel & Pessin, 2002).

Early work studying the relationship between glutamate and insulin led to the identification of a pancreatic amino acid receptor in Wistar rats (Bertrand et al., 1992). Bertrand et al. were able to show that glutamate can act on an excitatory amino acid receptor of AMPA subtype on the pancreas. When isolated rat pancreas were perfused with glutamate along with glucose at a concentration of 8.3 mM, it was observed that this

stimulated significantly greater insulin release from pancreatic cells than when the cells were perfused with glucose alone (Bertrand et al., 1992). Furthermore, rats supplemented with glutamate orally or intravenously demonstrated enhanced insulin secretion which contributed to improved peripheral glucose clearance and mitigated the hyperglycemic response that was otherwise observed in rats that did not receive glutamate (Bertrand et al., 1995). Thus, there is evidence to suggest that glutamate plays important roles in insulin secretion and glucose homeostasis. Although Bertrand et al (1995) focused on the effects of glutamate on insulin secretion, it is also possible that glutamate may have contributed to improved peripheral glucose clearance by enhanced skeletal muscle glucose uptake.

Human studies identify a complex balance of insulin secretion and insulin clearance with increased circulating glutamate

In addition to the effects that glutamate may have on the pancreas (Bertrand et al., 1992; 1995; Chevassus et al., 2002), human studies involving oral ingestion of MSG support that glutamate may facilitate insulin secretion. Chevassus et al. (2002) investigated glutamate's effects on glucose and insulin metabolism by giving subjects a 10 g dose of MSG simultaneously with a 75 g glucose load. It was found that glutamate, although not statistically significantly, elevated circulating insulin concentrations following the 75 g glucose load when compared to a control trial that received glucose only (Chevassus et al., 2002). Furthermore, a sub-analysis of the insulin and glutamate data revealed that subjects with higher glutamate bioavailability had a significantly

higher insulin response versus the control trial. Approximately half of the participants showed a 34% or greater increase in total glutamate exposure over the course of the MSG trial versus the placebo trial, which correlated to significantly higher serum insulin concentrations. These data support the idea that increased plasma glutamate availability has a stimulatory effect on insulin metabolism (Chevassus et al., 2002).

In line with these findings, Mourtzakis and Graham (2002) observed significantly elevated serum insulin concentrations relative to baseline measurements in resting young healthy males 30 minutes following a 150 mg/kg body weight dose of MSG that remained elevated for an additional 60 minutes. Furthermore, insulin levels in participants supplemented with MSG had significantly elevated serum insulin levels at various time points throughout the experiment when compared to a placebo trial. This finding was particularly interesting because the observed elevations in insulin concentrations following MSG ingestion were not accompanied by a significant increase in c-peptide (a protein cleaved from pro-insulin to form an active insulin hormone upon its release into circulation) when compared to the control trial. These results suggest that glutamate may be impeding the clearance of insulin from circulation, and that the observed increased insulin concentrations were not related to increased insulin secretion since c-peptide concentrations were not elevated in the same time-frame as insulin (Mourtzakis & Graham, 2002). Unlike previous work, this study did not combine glutamate administration with carbohydrate supplement, suggesting that peripheral insulin clearance may be impeded even when glucose is not ingested. However, it is unclear whether increased insulin concentrations are associated with glutamate-induced insulin secretion from the pancreas or as a result of impaired insulin clearance or both.

To better understand the metabolic interactions between glutamate, insulin, and glucose metabolism, our laboratory employed a study design that staggered MSG and carbohydrate administration in a time-dependent manner (Di Sebastiano et al., 2013). Previous work has shown that simultaneous administration of glutamate and carbohydrates enhances glutamate retention in the gut and attenuates circulating glutamate levels (Stegink et al., 1983b), thus the staggered approached was designed to achieve peak plasma concentrations simultaneously. Plasma glutamate concentrations have been shown to peak 50-60 minutes following MSG capsule ingestion, whereas blood glucose levels begin to rise 15 minutes following ingestion and peak after 20-30 minutes (Petrie et al., 2004). Therefore, we used a study design that administered carbohydrate 30 minutes following glutamate ingestion.

With this approach we could investigate the effects of supplementing glutamate alone, glucose alone, and glutamate in combination with glucose on various metabolic parameters. When analyzing our insulin data across the trials, it was found that supplementing carbohydrates in combination with glutamate did not increase mean serum insulin concentrations in the participants versus when carbohydrates were administered alone. However, a sub-analysis of this data revealed a dichotomous response between subjects; approximately half of our participants demonstrated increased insulin secretion when supplemented with glutamate and carbohydrate versus carbohydrates alone, and approximately half the participants demonstrated decreased insulin secretion (Di Sebastiano et al., 2013). Thus, on average this resulted in no net change in serum insulin concentrations. However, the differences in insulin secretion between the two groups in the sub-analysis were not associated with coinciding differences in plasma glutamate

concentrations between groups. It could be hypothesized that there are genotype differences that make some individuals more responsive and, thus causing them to secrete more insulin or impair its clearance compared to a less sensitive genotype.

There are a few discrepancies in the literature with regards to the insulin response elicited by glutamate. The reason for these discrepancies may be partly attributed to different experimental designs. Di Sebastiano et al. (2013) supplemented MSG and glucose in a time-staggered manner, whereas Chevassus et al. (2002) did not use a staggered design nor did they normalize the MSG dose to each participants' body weight, and Mourtzakis & Graham (2002) did not supplement carbohydrates. While future investigation that further examines this dichotomous response is warranted, a first step would be to examine the isolated effects of glutamate on glucose handling in muscle cells, whereby these confounding factors are removed.

The effects of glutamate on peripheral glucose disposal: does skeletal muscle have a role?

Glutamate has been studied fairly extensively with regards to its effects on insulin secretion and metabolism, however, it is also possible that glutamate may also have a direct effect on glucose uptake and metabolism in peripheral tissue (Di Sebastiano et al., 2013). Our laboratory found that when glutamate and carbohydrate were co-administered, the rise in blood glucose concentrations were attenuated compared with carbohydrate ingestion alone. However, unlike previous human studies, the attenuated rise in blood glucose concentration that was observed when glutamate and glucose were administered

together occurred without any differences in serum insulin concentrations compared to the trial where glucose was administered alone (Di Sebastiano et al., 2013). This implies that glutamate may be having an effect on peripheral tissues to increase glucose clearance. In line with these findings, Hosaka et al. (2012) demonstrated that when glutamate was added in the form of MSG to a lipid-containing meal, participants showed improved postprandial glycemic response versus when the meal was administered without MSG (Hosaka et al., 2012). Furthermore, this improved glycemic response was again observed to occur without any differences in serum insulin concentration between trials. The results from these studies imply that glutamate is enhancing peripheral glucose clearance independently of insulin and that glutamate may be acting on peripheral tissues to enhance glucose uptake.

The findings by DiSebastiano et al. and Hosaka et al. have been supported by a large cohort study following 1056 healthy individuals over a 5 year period to assess associations between MSG intake and hyperglycemia (Shi et al., 2014). Like the conclusions made from the previous MSG studies, Shi et al. found that MSG intake was inversely related to the development of hyperglycemia. In fact, participants in the highest quartile of MSG intake showed the lowest risk of incident of hyperglycemia, even after adjustment for dietary patterns and a number of covariates such as smoking, alcohol consumption, and activity levels among others. DiSebastiano et al. (2013) and Mourtzakis and Graham (2002) were both able to show that MSG ingestion significantly and profoundly increased plasma glutamate availability, thus this study further supports the idea that increased glutamate availability may result in a significant increase in glucose disposal.

Considering that glutamate is the primary amino acid taken up by the muscle (DeFronzo et al., 1981; Graham et al., 1997; Graham & MacLean, 1998), and skeletal muscle is responsible for approximately 85% of whole-body glucose disposal (DeFronzo et al., 1981), one may hypothesize that glutamate is acting on peripheral muscle to enhance glucose uptake into the tissue. Kim et al. (2010) found that homocysteine sulfinic acid (HCSA), an amino acid derivative and selective skeletal muscle glutamate receptor agonist, was able to stimulate enhanced glucose uptake in C2C12 mouse myotubes (Kim et al., 2011). HCSA was shown to bind to a specific glutamate receptor located on these muscle cells, mGluR5, resulting in significant elevations in AMP-activated protein kinase (AMPK) phosphorylation; an energy sensing kinase that acts to increase glucose uptake in skeletal muscle. This work suggests that glutamate may stimulate a similar signaling pathway that results in enhanced glucose uptake by binding to the same mGluR receptor, however the mechanisms by which glutamate is involved in both glucose handing, uptake, and metabolism are unknown. It is not clear whether glutamate acts to enhance the action of insulin in a similar fashion as leucine by acting on proteins involved in the insulin signaling cascade (Nishitani et al., 2002); or by acting through an alternative pathway that enhances glucose uptake independently of insulin.

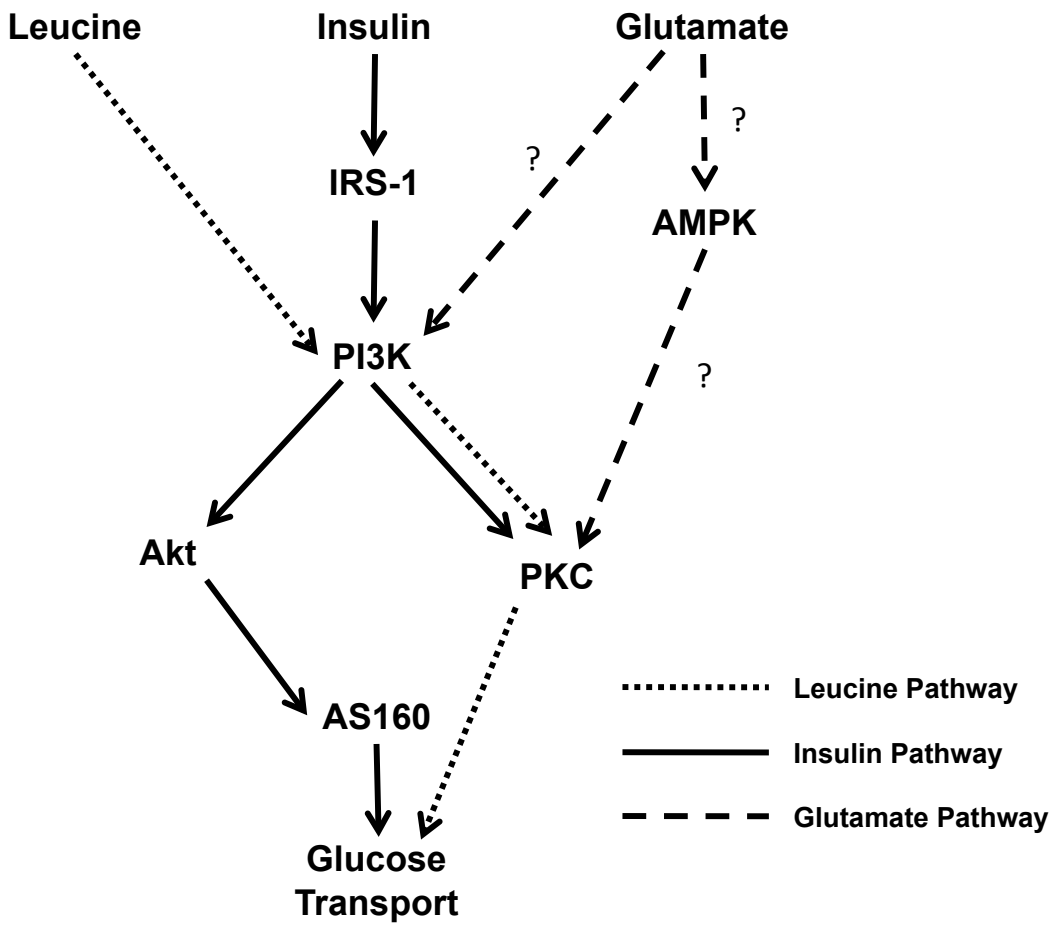

Figure 1. Schematic showing the signalling pathway by which leucine and insulin stimulate glucose transport, and possible mechanisms for glutamate-mediated glucose transport.

The evidence presented in these studies suggests that glutamate can work to enhance glucose uptake into skeletal muscle, but the mechanism by which it takes action remains unknown. It is not known whether glutamate acts to enhance the action of insulin by acting on proteins involved in the insulin signalling cascade, or by acting through an alternative pathway that enhances glucose uptake independently of insulin (Figure 1).

Work has been done investigating the role of other amino acids, specifically the BCAAs, leucine, and isoleucine, on glucose handling in skeletal muscle. Animal work

has shown that supplementing a carbohydrate-rich gavage with 2mM leucine and other amino acids resulted in significantly lower blood glucose concentration when compared to a meal without amino acids; similar to Di Sebastiano et al. (2013), this attenuation in blood glucose occurred without any differences in serum insulin concentration. Further analysis revealed that the amino acid supplement acted to enhance AS160 phosphorylation at the level of the muscle, thus increasing glucose uptake independently of insulin (Bernard et al., 2011). These mechanisms have been investigated further using isolated myocytes, demonstrating that leucine and isoleucine have the ability to significantly increase glucose uptake into skeletal muscle cells through pathways that stimulate PI3K and converge downstream on to the insulin signalling pathway to stimulate glucose uptake (Doi et al., 2003; Nishitani et al., 2002).

In contrast with these findings, the work by Kim et al. (2011) links the glutamate receptor to AMPK and subsequent PKC-ζ activation and suggests that glutamate may bind to its receptor on skeletal muscle to increase AMPK activation and circumvent the insulin signaling pathway to enhance glucose uptake independently of insulin (Kim et al., 2011); however the specific downstream pathways that glutamate may act on have yet to be investigated. Other studies using cell culture models to investigate the effects of compounds such as resveratrol (Park et al., 2007), berberine (Cheng et al., 2006), and tangeretin (Kim, Hur, Kwon, & Hwang, 2012) among others, have outlined an AMPK mediated pathway that is very similar to the one described by Kim et al. (2010) that acts to increase glucose uptake independently of insulin. However, it remains to be determined whether glutamate acts similarly to leucine/isoleucine and increases glucose uptake by enhancing insulin's action and completely circumventing AMPK activation

(Doi et al., 2005), or whether glutamate is able to stimulate glucose uptake in an AMPK mediated fashion.

The work that has been done to this point using MSG supplementation has suggested that there is important interplay between glutamate, glucose, and insulin metabolism *in vivo*. To better understand these interactions, it is necessary to investigate the distinct effects of glutamate in a more isolated manner to better elucidate the specific response of skeletal muscle cells to glutamate exposure.

Rationale

Various studies have examined the glucose and insulin responses that are elicited when glutamate is elevated in circulation (Chevassus et al., 2002; Di Sebastiano et al., 2013; Hosaka et al., 2012). However, considering that glutamate is a primary amino acid taken up by the muscle (Graham et al., 1997; Graham & MacLean, 1998) and skeletal muscle is responsible for approximately 85% of whole-body glucose disposal (DeFronzo et al., 1981), very few studies have investigated the effects of glutamate on glucose uptake and handling by skeletal muscle.

Increased circulating glutamate concentrations in human participants may improve glucose handling, independent of insulin. Our laboratory demonstrated that glutamate ingestion was associated with attenuated rises in blood glucose following a carbohydrate load when compared with ingesting carbohydrates alone (Di Sebastiano et al., 2013). Importantly, there were no differences in insulin secretion (as measured by c-peptide) when comparing glutamate ingestion following a carbohydrate load with carbohydrate ingestion alone. Similarly, it has been demonstrated that glutamate infusion during exercise can enhance carbohydrate utilization (Thomassen et al., 1990), supporting the idea that glutamate may enhance glucose uptake and use by skeletal muscle. Furthermore, Hosaka et al (2012) observed enhanced glucose clearance following a lipid-rich meal when ingested with an MSG supplement versus a NaCl supplement of equal sodium content. This finding was observed independent of differences in serum insulin between trials (Hosaka et al., 2012), however the specific

mechanisms responsible for improved glucose handling in conditions where glutamate is elevated are yet to be investigated.

Previous studies using animal models (Bernard et al., 2011; 2012; Kleinert et al., 2011) have used mixed amino acid solutions to examine the effects of a combination of amino acids on glucose handling and insulin signalling. The results from this work have suggested that AS160 phosphorylation may alter glucose uptake into skeletal muscle in the presence of increased amino acid availability. However, these studies primarily used branched-chain and essential amino acids as mixed amino acid solutions, making it difficult to distinguish specific amino acids that primarily contributed to these results. Endogenous insulin was also present, making it difficult to isolate the effects of amino acids on insulin signalling proteins versus insulin itself. Furthermore, glutamate was not included in any of the mixtures and its effect on glucose handling was not investigated.

Interestingly, cell culture work using isolated myotubes has shown that the glutamate receptor agonist homocysteine sulfinic acid can stimulate increased glucose uptake via the activation of AMPK and p38 MAPK (Kim et al., 2011). This work suggests a link between the glutamate receptor present on muscle cells and AMPK signaling activation, implying that when glutamate binds to its receptor on skeletal muscle cells it may stimulate glucose uptake. However, the mechanisms by which glutamate may affect glucose metabolism remain unknown.

The literature suggests that there is potential interplay between glutamate, glucose, and insulin metabolism *in vivo*. Thus to better understand these interactions, it is highly warranted to investigate the distinct effects of glutamate in an isolated *in vitro* manner to observe the response of muscle cells to glutamate exposure with regards to glucose

uptake, the primary mechanisms at work, as well as how these measures compare to other known mediators of glucose uptake.

Study Objectives

1. To examine the effects of glutamate on glucose uptake in isolated L6 myotubes in a dose- and time-dependent manner, independent of the effects of insulin.

2. To measure and compare glucose uptake with the provision of: i) glutamate alone, ii) leucine alone, iii) insulin alone, iv) glutamate in combination with leucine, and v) glutamate in combination with insulin.

3. To investigate and compare the primary mechanisms of glucose handling in skeletal muscle cells in each experimental condition.

Hypotheses

1. Glutamate will increase glucose uptake into skeletal muscle cells compared to the control condition (where cells were not treated with glutamate) in a positive dose-response and positive time-dependent manner. Based on previous work, it is hypothesized that the optimal dose will be 2mM and the optimal treatment time will be 1 hour.

2. Glucose uptake will be: i) increased when cells are treated with glutamate relative to a control condition that does not receive glutamate; ii) increased when cells are treated with leucine, and will be elevated to a similar extent as the glutamate

condition; iii) increased when cells are treated with insulin, and will rise to a greater extent than glutamate or leucine conditions; iv) further increased compared with glutamate or leucine alone when cells are treated with a combination of glutamate and leucine, and v) further increased compared with glutamate or insulin alone when cells are treated with a combination of glutamate and insulin.

3. The mechanisms by which glutamate and leucine stimulate glucose uptake will be different. Glutamate will stimulate an AMPK-dependent pathway, whereas leucine and insulin will stimulate proteins involved in insulin signalling, such as PI3K and PKC.

Manuscript: Glutamate increases glucose uptake in L6 myotubes in a concentration- and time-dependent manner that is mediated by AMPK.

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Introduction

Glutamate is an amino acid that is heavily involved in transamination and anaplerotic reactions related to oxidative metabolism (Mourtzakis et al., 2006; 2008; Mourtzakis & Graham, 2002). However, recent studies have suggested that the role of glutamate extends beyond that of an accessory to energy provision, and that it may play an important role in glucose handling and insulin metabolism, ultimately contributing to glucose homeostasis (Chevassus et al., 2002; Di Sebastiano et al., 2013).

Human studies have identified that increased circulating glutamate concentrations may improve glucose handling, independent of insulin (Di Sebastiano et al., 2013; Hosaka et al., 2012). We recently demonstrated that glutamate ingestion can attenuate rises in blood glucose following a carbohydrate load (Di Sebastiano et al., 2013). Rises in serum insulin concentrations were similar whether carbohydrate was ingested alone or when ingested with glutamate, suggesting that the attenuated rise in glucose during the latter condition occurred independent of insulin-mediated glucose clearance. Similarly, Hosaka et al (2012) demonstrated enhanced glucose clearance following a lipid-rich meal when ingested with an MSG supplement versus a NaCl supplement of equal sodium content. This enhanced glucose clearance occurred in absence of differences in serum

insulin between trials (Hosaka et al., 2012); however the specific mechanisms responsible for improved glucose handling in conditions where glutamate is elevated are yet to be investigated.

Glutamate receptor agonist homocysteine sulfinic acid (HSCA) has been shown stimulate increased glucose uptake via the activation of AMPK and p38 MAPK in isolated myotubes (Kim et al., 2011). This work suggests a link between the skeletal muscle glutamate receptor and AMPK signaling activation; thus, increased circulating glutamate may alter glucose handling in skeletal muscle via increased binding to its receptor and subsequent AMPK activation. However, these hypotheses are yet to be confirmed and the mechanisms by which glutamate may affect glucose metabolism needs to be further investigated.

Considering that glutamate is the primary amino acid taken up by skeletal muscle (Graham et al., 2000; Graham & MacLean, 1998) and skeletal muscle comprises 85% of glucose disposal (DeFronzo et al., 1981), investigating the interplay between glutamate and glucose and the effects glutamate has on glucose handling and insulin signaling is highly warranted. This study aimed to: 1) examine the effects of glutamate on glucose uptake in isolated L6 myotubes in a dose- and time-dependent manner, independent of the effects of insulin, 2) measure the magnitude of glucose uptake with the provision of glutamate and compare with insulin- and leucine-mediated glucose uptake (Bernard et al., 2011; Nishitani et al., 2002), and 3) investigate the primary mechanisms of glucose handling in experimental conditions where L6 myotubes were incubated with glutamate, insulin or leucine.

Methods

Experimental Design

To first investigate the effect of glutamate on glucose uptake, a dose-response experiment was performed to confirm that glutamate can stimulate glucose uptake and to determine optimal glutamate concentrations that will maximize glucose uptake. Subsequently, a time-response experiment was performed to determine the optimal treatment time for glutamate to elicit the greatest magnitude of glucose uptake. Cells were then treated with insulin and leucine to compare the magnitude of uptake elicited by glutamate to these other known stimulators of glucose uptake. Lastly, glucose uptake was assessed in each treatment condition by incubating cells with various inhibitors to elucidate the primary mechanism by which each compound acts to stimulate uptake.

Cell culture

L6 rat muscle myoblasts were seeded in 150mm polystyrene culture dishes (BD Falcon) at a density of approximately 3500 cells/cm^2. Cells were seeded and grown in 20 mL of growth media, consisting of low-glucose Dulbecco's Modified Eagle Medium (DMEM; Hyclone, ThermoFisher) supplemented with 10% Fetal Bovine Serum (Hyclone, ThermoFisher) and 1% penicillin streptomycin (Hyclone, ThermoFisher) and incubated at 37°C and 5% CO_2. Media was changed every 48hrs and cells were washed during each media change with Dulbecco's Phosphate-Buffered Saline (DPBS; Lonza, 10x PBS diluted to 1x in auto-cleaved H_2O). After approximately 3 days, cells would reach 70-80% confluence and were sub-cultured into smaller culture plates for experimentation.

For sub-culturing, growth media was aspirated from the 150mm plates and cells were washed twice with DPBS. Cells were then detached from the plate by adding 1mL of trypsin (ThermoFisher) that was coated evenly over the cells, followed by a 5 minute incubation at 37°C in the CO_2 incubator. Following incubation, cells were re-suspended in growth media and prepped for counting by adding cells into an accuvette containing 10 mL of balanced electrolyte solution (Z-Pak, Beckman Coulter). The concentration of healthy cells in the media was determined using a Z2 Coulter Counter (Beckman Coulter).

For experimentation, L6 rat myoblasts were seeded into 12-well polystyrene culture plates at a density of 50,000 cells/well where they were maintained in growth media for an additonal 2-3 days, until reaching ~90% confluence. Media was then changed to low-glucose DMEM supplemented with 2% horse serum (Hyclone, Thermofisher) and 1% penicillin streptomycin to stimulate differentiation. Cells were maintained in differentiation media for an additional 6-7 days, changing media and washing every 48 hrs, until cells were differentiated myotubes.

Glutamate dose-response experiments

To examine the effects of glutamate on glucose uptake and determine the optimal concentration of glutamate to stimulate maximal glucose uptake, we first treated cells with increasing doses of glutamate to observe the relationship between glutamate exposure and glucose uptake in skeletal muscle.

Prior to treatments, fully differentiated L6 myotubes were washed 2-3x in DPBS before changing media to serum-free DMEM for 4 hrs followed by 1 hr incubation in amino acid-free Earle's Balanced Salts Solution (EBSS; Sigma-Aldrich). The purpose of

this protocol was to wash out the effects of insulin and other compounds in the serum, and the high concentration of amino acids in DMEM, that can effect glucose uptake and mask any effects of the treatments. Cells were then washed at least 2 more times treated with L-glutamic acid (Sigma-Aldrich) in EBSS for 1 hr. The concentrations of glutamate used for the treatments are representative of: i) typical human serum fasting concentration (50μM); ii) approximate human serum glutamate concentrations following a 150mg/kg dose (500μM)(Di Sebastiano et al., 2013); and iii) + iv) supra-physiological glutamate doses to entice a maximal response to the treatment. Immediately following the treatments, cells were washed 2-3x in DPBS and a glucose uptake assay was performed.

Glutamate time-response experiments

To investigate the effect of incubation time on glucose uptake in L6 muscle cells treated with glutamate and to determine the optimal treatment time to stimulate maximal glucose uptake, cells were treated for increasing incubation times. Fully differentiated L6 myotubes were serum-deprived for 4 hrs in DMEM followed by 1 hr in amino acid-free EBSS as previously described. Cells were then washed and treated with 2mM glutamate in EBSS for incubation periods of 0 (control), 15, 30, 45, 60, or 120 minutes. Immediately following each condition, cells were washed 2-3x in DPBS and a glucose uptake assay was performed.

Comparing the effects of glutamate to leucine and insulin

To measure and compare the magnitude of glucose uptake with the provision of glutamate to other known stimulators of skeletal muscle glucose uptake, cells were

treated with leucine and insulin. L6 myotubes were serum deprived for 4 hrs in DMEM followed by 1 hr in amino acid-free EBSS as described. Cells were then washed and treated with either 2mM glutamate or 2mM L-leucine (Sigma-Aldrich) in EBSS, EBSS supplemented with 10µg/mL insulin (Sigma-Aldrich), or a combination of 2mM glutamate and 2mM leucine, or 2mM glutamate and insulin. Cells were incubated for 30 minutes as this has been shown to be the optimal treatment time for glucose uptake for leucine (Nishitani et al., 2002) and for insulin (Klip, Li, & Logan, 1984). Immediately following the treatments, cells were washed 2-3x in DPBS and a glucose uptake assay was performed.

Inhibitor experiments

To investigate and compare the primary mechanisms of glucose handling in skeletal muscle for glutamate, leucine and insulin, select inhibitors were used in combination with various treatments. Fully differentiated L6 myotubes were serum-deprived for 4 hrs in DMEM followed by 1 hr in amino acid-free EBSS and treated with 2mM glutamate, 2mM leucine, or 10µg/mL insulin in EBSS. For each treatment condition, media also contained 10µM LY294002 (Calbiochem), 1µM Bisindolylmalemide I (Calbiochem), or 1µM Compound C (Calbiochem). A control condition was run for each treatment condition in EBSS supplemented with DMSO to an equivalent concentration as the corresponding inhibitor trial.

2-[^{3}H]-Deoxy-D-glucose uptake assay

Following the indicated treatments, media was poured off and cells were washed 2-3x with DPBS. Cells were then treated with HEPES buffered solution (HBS) containing 140 mM NaCl, 5 mM KCl, 20 mM HEPES, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$, (pH 7.4) and 50μM of 2-[^{3}H]-Deoxy-D-glucose ([^{3}H]-2-DG; Perkin-Elmer) for 10 minutes. Cells were washed 3x with ice-cold 0.9% NaCl (w/v) and lysed in 1mL NaOH. Lysates were added to scintillation cocktail (FisherScientific) and the radioactivity of each sample was assessed by liquid scintillation counting. Non-specific uptake was controlled for in each condition by the addition of 10μM cytochalasin B (CB; Sigma-Aldrich). CB acts to inhibit glucose transporters located on the cell periphery to control for non-specific uptake that does not occur through the GLUTs.

Statistics

All results are reported as means ± standard error of the mean (SEM). Data was analyzed for each experiment using a one-way ANOVA and Tukey's post-hoc analysis, with the exception of the inhibitor experiments that used Dunn's test. Statistical significance was accepted at $P<0.05$. All conditions were run in triplicate and repeated at least 3 times.

Results

Glutamate increases 2-deoxy-glucose uptake in L6 myotubes in a dose-response manner

L6 myotubes treated with 2mM of glutamate resulted in the greatest increase in [^{3}H]-2-DG uptake relative to the control condition (% increase in glucose uptake relative to 0μM treatment: 129 ± 7%, P<0.001, Figure 2). Glutamate treatments of 500μM and 1mM also significantly increased [^{3}H]-2-DG uptake compared to the control condition (121 ± 4%, P=0.005, and 123 ± 5%, P=0.002, respectively), while the 50μM glutamate treatment tended to increase glucose uptake (114 ± 3%, P=0.09) relative to the control condition. Furthermore, there were no statistical differences between glutamate treatment doses, however, using regression analysis, we found that glutamate exposure (50μM-2mM) was positively associated with [^{3}H]-2-DG uptake (R=0.97; P=0.03). Hereafter, for all experiments that were treated with glutamate, 2mM glutamate treatment was used since it resulted in the greatest relative [^{3}H]-2-DG uptake.

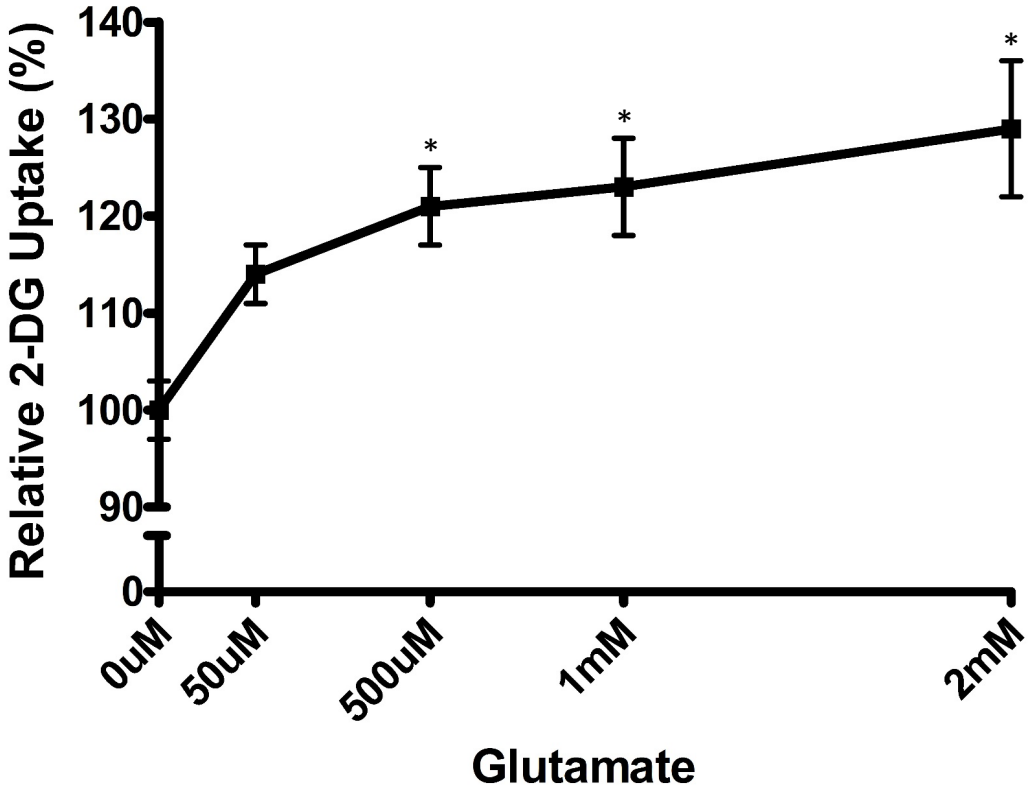

Figure 2. Differentiated L6 muscle cells were exposed to increasing doses of glutamate in EBSS for 1 hr. Glucose uptake using [^{3}H]-2-Deoxyglucose is expressed relative to a control condition that did not receive glutamate. Mean values are presented ± SEM. * indicates mean value was statistically significant versus control ($P<0.05$).

Glucose uptake in the presence of glutamate is time-dependant

L6 myotubes treated with 2mM of glutamate for 30 min resulted in the greatest increase in [^{3}H]-2-DG uptake versus the control condition that did not receive glutamate (143 ± 9%, $P<0.001$, Figure 3) and versus cells treated with glutamate for 15 min (108 ± 8%, $P<0.016$). Compared with control conditions, [^{3}H]-2-DG uptake was also significantly elevated in cells treated for 45 min (125 ± 9%, $P=0.048$) and 60 min (129 ± 7%, $P=0.018$). Cells treated with 2mM glutamate for 15 min and 120 min (117% ± 6%) did not stimulate [^{3}H]-2-DG uptake to an extent that was significantly different from the

control conditions. Hereafter, 30 minutes was used as the incubation time for the 2mM glutamate conditions as it elicited the greatest [^{3}H]-2-DG uptake response.

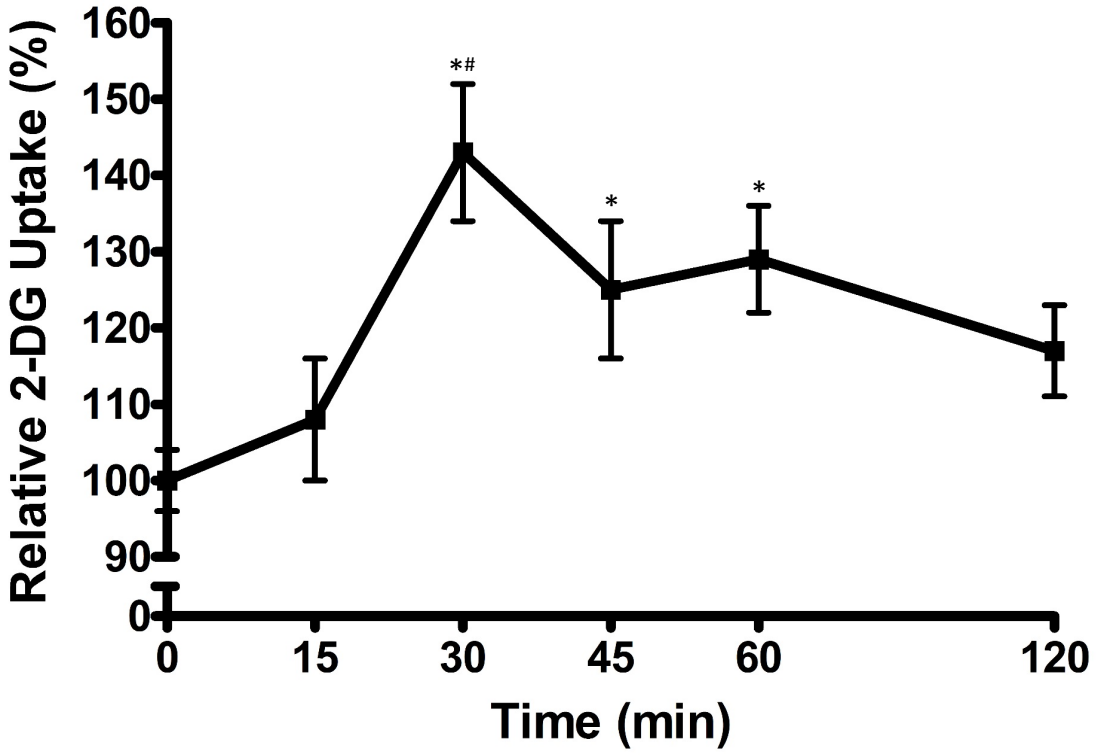

Figure 3. Differentiated L6 muscle cells were exposed to 2mM of glutamate in EBSS for 15, 30, 45, 60, or 120 minutes. Glucose uptake using [^{3}H]-2-Deoxyglucose is expressed relative to a control condition that did not receive glutamate (0 min). Mean values are presented ± SEM. * indicates mean value was statistically significant versus 0 min ($P<0.05$). $^{\#}$ indicates mean value was statistically significant versus 15 min ($P<0.05$).

Glutamate stimulates 2-deoxy-glucose uptake to the same extent as leucine and insulin

Cells treated with glutamate, leucine, and insulin all stimulated significantly higher [^{3}H]-2-DG uptake when compared to the control condition that received no treatment (143 ± 10%, $P<0.001$; 126 ± 9%, $P=0.033$; and 141 ± 12%, $P=0.002$, respectively; Figure 4). Cells were also treated with glutamate in combination with either

leucine or insulin. The combinations of glutamate + leucine and glutamate + insulin elicited the greatest [^{3}H]-2-DG uptake compared with the control condition (148 ± 3%, P<0.001; 148% ± 3%, P<0.001, respectively). However none of the treatment conditions differed significantly from one another.

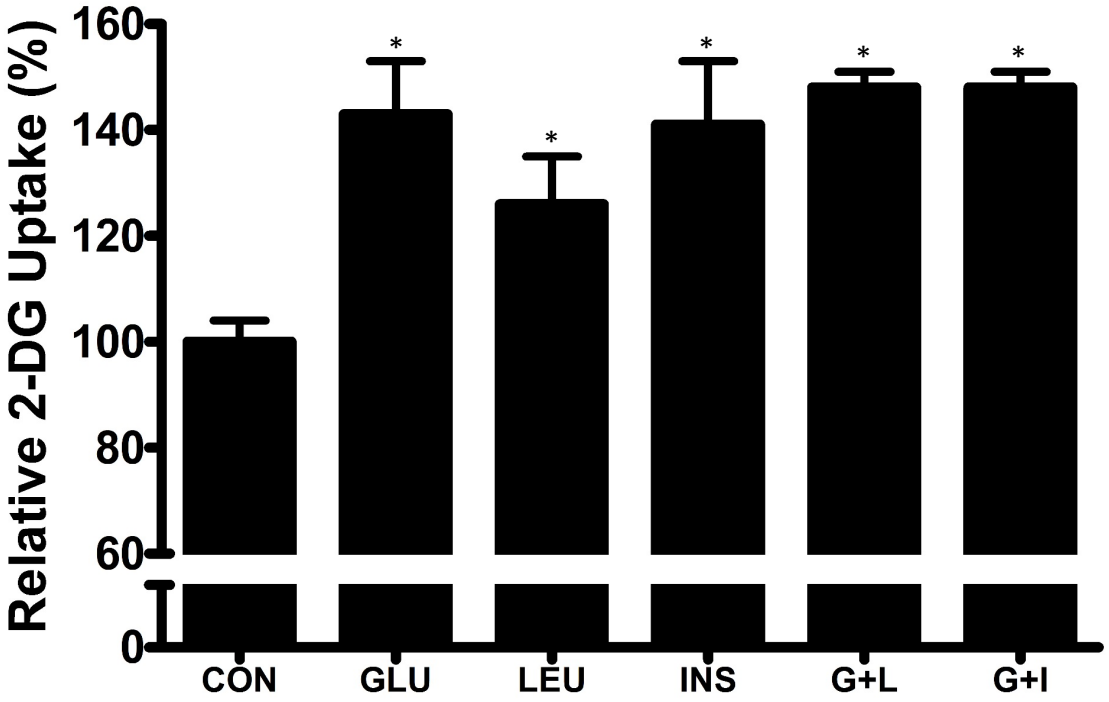

Figure 4. Differentiated L6 muscle cells were exposed to glutamate (GLU), leucine (LEU), insulin (INS), glutamate + leucine (G + L), or glutamate + insulin (G + I) in EBSS for 30min and glucose uptake was assessed using [^{3}H]-2-Deoxyglucose relative to a control trial (CON) that received EBSS alone. Mean values are presented ± SEM. * indicates mean value was statistically significant versus CON (P<0.05).

AMPK and PKC inhibition blocked glucose uptake in cells treated with glutamate

L6 myotubes treated with glutamate and LY294002 (GLU+LY) significantly increased [^{3}H]-2-DG uptake relative to the control condition (128 ± 5%, $P<0.05$; Figure 5a) and to a similar extent as glutamate alone (143 ± 10%). Furthermore, cells treated with GLU+LY demonstrated [^{3}H]-2-DG uptake that was significantly greater than those treated with glutamate and Compound C (GLU+C.C) (98 ± 2%, $P<0.05$). Cells treated with GLU+C.C and glutamate and Bisindolymalemide I (GLU+BMD) (103 ± 4%) were not significantly different from the control trial or each other.

PI3K and PKC inhibition blocked glucose uptake in cells treated with leucine

Cells treated with leucine and Compound C (LEU+C.C) significantly increased [^{3}H]-2-DG uptake relative to the control condition (113 ± 2%, $P<0.05$; Figure 5b) and to a similar extent as leucine alone (126 ± 8%). Furthermore, those cells treated with leucine and LEU+C.C displayed [^{3}H]-2-DG significantly greater than leucine and Bisindolymalemide I (LEU+BMD) (95 ± 2%, $P<0.05$). Cells treated with LEU+BMD and leucine and LY294002 (LEU+LY) (101 ± 3%) were not significantly different from the control trial or each other.

PI3K Inhibition blocked glucose uptake in cells treated with insulin

Cells treated with insulin and Compound C (INS+C.C) or insulin and Bisindolymalemide I (INS+BMD) significantly increased [^{3}H]-2-DG uptake relative to the control condition (134 ± 7% and 116 ± 3%, respectively; $P<0.05$; Figure 5c) and to a similar extent as insulin alone (141 ± 12%). Furthermore, those cells treated with insulin

and INS+CC and displayed [^{3}H]-2-DG significantly greater than insulin and LY294002 (INS+LY) (105 ± 3%, $P<0.05$), which was not different from the control trial that did not receive treatment.

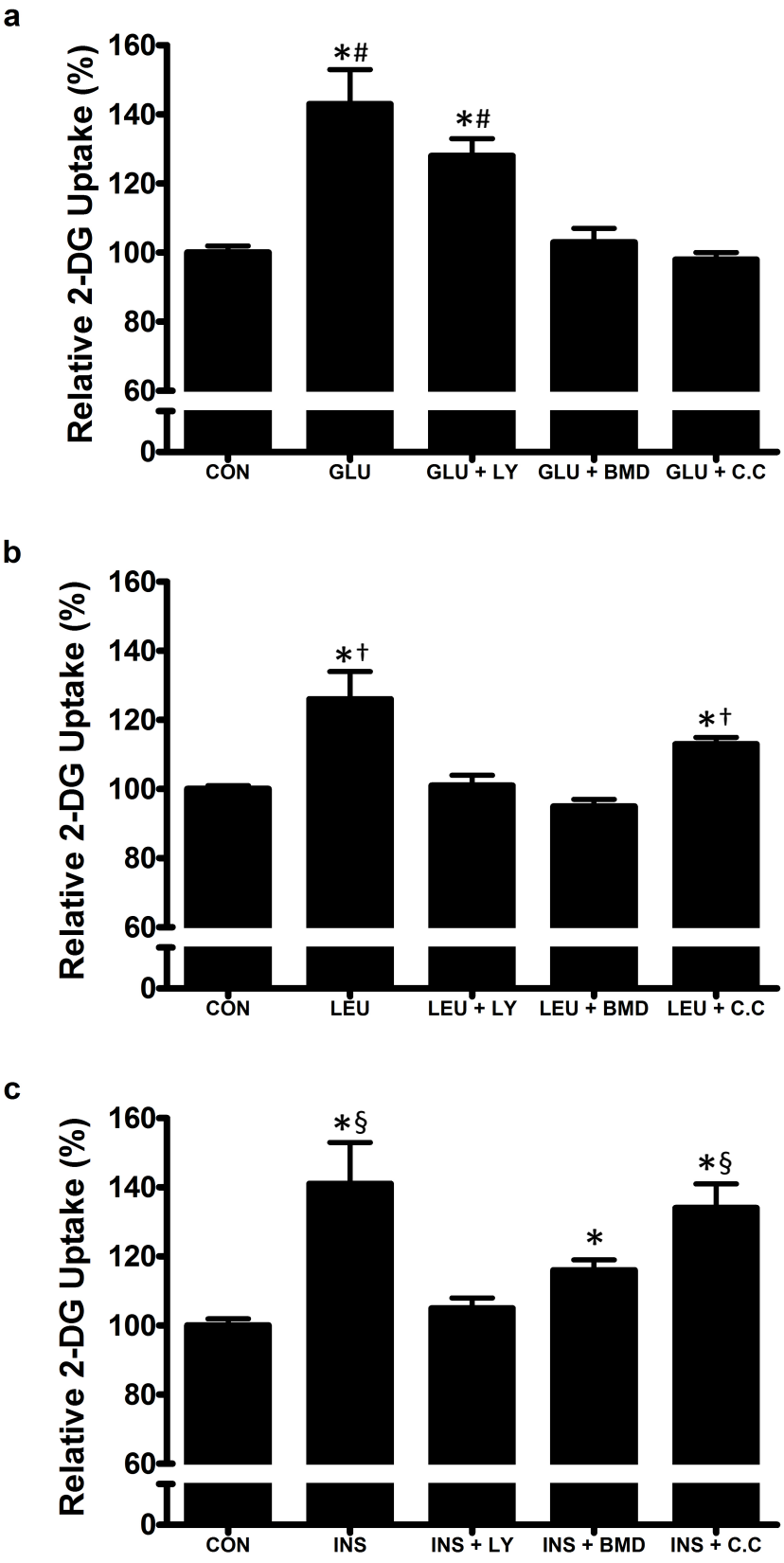
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Figure 5. Differentiated L6 muscle cells were treated with a) glutamate alone (GLU), glutamate and LY294002 (GLU+LY), glutamate and Bisindolymalemide I (GLU+BMD), or glutamate and Compound C (GLU+C.C) in EBSS or for 30min; b) leucine alone (LEU), leucine and LY294002 (LEU+LY), leucine and Bisindolymalemide I (LEU+BMD), or leucine and Compound C (LEU+C.C). in EBSS or for 30min; c) insulin alone (INS), insulin and LY294002 (INS+LY), insulin and Bisindolymalemide I (INS+BMD), or insulin and Compound C (INS+C.C). in EBSS or for 30min. Glucose uptake was then assessed by the addition of [^{3}H]-2-Deoxyglucose in HBS for 10 min and uptake is expressed relative to a control condition (CON) that received EBSS alone. Mean values are presented ± SEM. *indicates mean value was statistically significant versus CON ($P<0.05$); $^{\#}$indicates mean value was statistically significant versus C.C ($P<0.05$); †indicates mean value was statistically significant versus BMD ($P<0.05$); §indicates mean value was statistically significant versus LY ($P<0.05$) for each respective treatment.

Discussion

The current study aimed to examine the effects of glutamate on glucose uptake in isolated L6 myotubes and identify potential underlying mechanisms. We identified that doses of glutamate, that reflected serum concentrations reported in human studies where glutamate was administered, elicited significant increased glucose uptake into skeletal muscle cells. Furthermore, we found that the magnitude of glucose uptake elicited by glutamate is similar to that of leucine and insulin stimulated glucose uptake, and that glutamate acts to increase glucose uptake through AMPK and PKC, which are distinct from leucine- and insulin-mediated glucose uptake (which occurred through PI3K and PKC and PI3K, respectively). This is the first study, to our knowledge, to examine the effects of glutamate exposure on glucose uptake in L6 myotubes, and compare these effects to the magnitude and mechanisms of glucose uptake elicited by leucine and insulin.

Glutamate stimulated skeletal muscle glucose uptake through AMPK and PKC

Exposing L6 myotubes to elevated concentrations of glutamate resulted in a 43% increase in glucose uptake compared with control conditions. The magnitude of glucose uptake elicited with the provision of glutamate was comparable to leucine conditions (126% relative to control) and insulin conditions (140% relative to control). This is the first study to demonstrate increased glucose uptake by L6 muscle cells that were exposed to elevated glutamate concentrations. Previous cell culture studies have shown that branched chain amino acids (BCAAs) can increase glucose uptake 1.3-1.5 fold versus a control and increase glucose uptake to a greater extent than insulin (Doi et al., 2003;

Nishitani et al., 2002). Furthermore cells treated with GLU+LEU or GLU+INS exhibited the greatest increase in glucose uptake (148%), but was not significantly greater than any of the other treatments. This suggests the possibility that glutamate, leucine, and insulin may be partially contributing to glucose uptake through similar pathways or mechanisms, which may explain the lack of synergistic increases in glucose uptake when combining treatments. In contrast, the lack of synergistic effects may be an indication that each treatment maximized the amount glucose transport that was achievable.

Compound C (an AMPK specific inhibitor) effectively blocked glucose uptake in cells treated with 2mM glutamate, indicating that glutamate acts to increase glucose uptake via an AMPK mediated pathway. Kim et al. (2011) showed that C2C12 myotubes treated with mGluR agonist HSCA resulted in significant elevations in glucose uptake and AMPK phosphorylation (Kim et al., 2011). Results from the current study suggest that glutamate acts in a similar fashion to enhance glucose uptake in skeletal muscle cells. Mechanisms of glucose uptake with glutamate exposure were distinct from mechanisms of glucose uptake with insulin and leucine exposure in the manner that they did not act through AMPK. Both insulin- and leucine-treated cells maintained elevated glucose uptake when incubated with Compound C (134% and 113% relative to control, respectively), which is supported by previous work showing BCAA stimulation of glucose uptake acts independently of AMPK (Doi et al., 2005).

Glucose uptake was also blocked (103% relative to control) when cells were incubated with 2mM glutamate and a PKC specific inhibitor (BMD), suggesting that the AMPK pathway stimulated by glutamate acted on PKC. It is likely that PKC was mediated by p38 MAPK, since Kim et al. (2010) and Cheng et al. (2006) showed AMPK

mediated glucose uptake acts on p38 MAPK downstream (Cheng et al., 2006) and subsequently PKC (J. H. Kim et al., 2011). PKC inhibition also blocked glucose uptake in cells treated with 2mM leucine (95% relative to control), however PKC inhibition did not shunt glucose uptake in response to insulin (116% relative to control). These results support previous work showing that leucine works to stimulate glucose uptake through PI3K and PKC. In contrast, insulin, unlike leucine, is able to stimulate uptake despite PKC inhibition through Akt (Iwanaka et al., 2010; Nishitani et al., 2002) (Figure 6). These results suggest that glucose transport was likely maximized when cells were treated with glutamate in combination with insulin or leucine in the previous experiments. Insulin was able to stimulate significant uptake despite PKC inhibition whereas glutamate and leucine did not, implying distinct signalling pathways; however both GLU+INS and GLU+LEU showed almost identical uptake values when given in combination (Figure 4). This indicates that the lack of synergistic effects observed in the previous experiments were due to the fact that each treatment maximized the amount of glucose transport achievable, rather than a similar pathway being activated across all treatments.

A specific PI3K inhibitor (LY294002) blocked glucose uptake (105% relative to control) when cells were treated with insulin or leucine, demonstrating that insulin and leucine each stimulate glucose uptake via PI3K, an upstream regulator in the insulin-signaling pathway (Figure 5), which are in line with findings from Nishitani et al. (2010). However, when myotubes were incubated with 2mM glutamate and LY294002, glucose uptake was still maintained at a level similar to when cells were treated with glutamate alone and significantly higher than the control (128%). Therefore, these data suggest that glutamate works through distinct pathways of insulin, circumventing PI3K signaling to

increase glucose uptake. Interestingly, leucine acts on both PI3K and PKC signaling implying that both glutamate and leucine will activate PKC through distinct pathways. Thus, our present study may help explain our previous findings where we postulated that glutamate supplementation in humans resulted in potentially improved glucose clearance independent of insulin (Di Sebastiano et al., 2013).

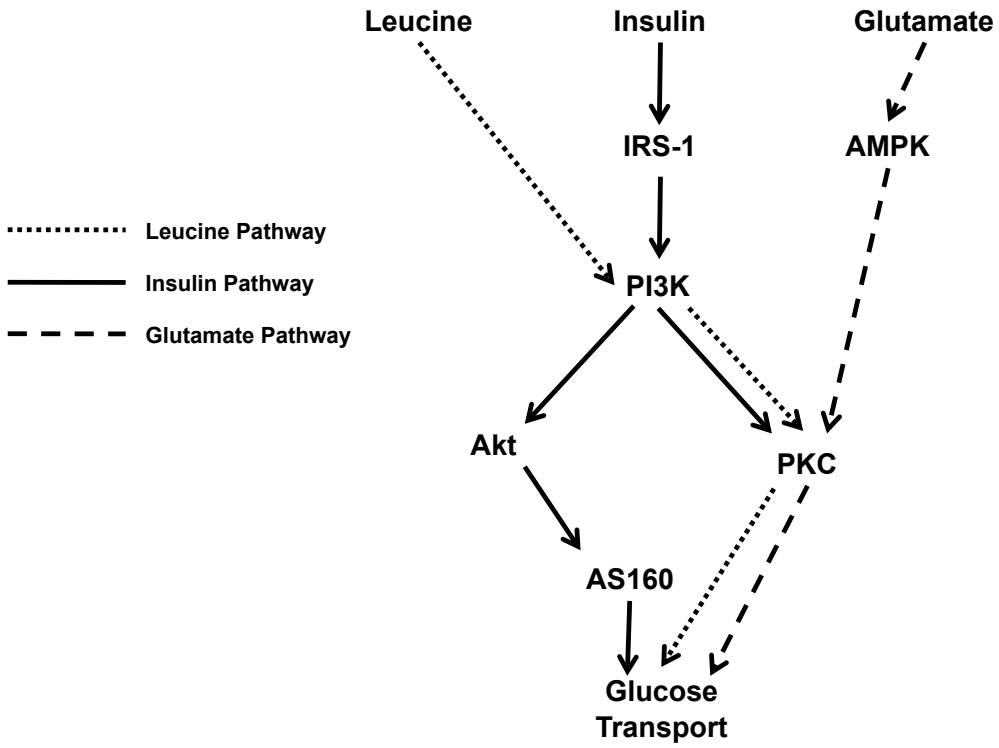

Figure 6. Schematic showing the signaling pathway by which glutamate stimulates glucose uptake in L6 myotubes. Our work with inhibitors shows that glutamate activates AMPK and PKC to augment glucose transport into the muscle cell. This pathway is distinct from leucine and insulin that stimulate glucose transport via PI3K and IRS-1/PI3K dependent mechanisms, respectively.

In summary, glutamate enhanced glucose uptake in L6 muscle cells in a dose- and time-dependant manner. Glutamate exposure augmented glucose uptake in skeletal

muscle to the same extent as leucine and insulin through an AMPK activated pathway and potentially resulting in downstream PKC activation; distinct from the mechanism by which leucine and insulin act to increase glucose uptake via PI3K.

Integrated Discussion

Amino acids play important roles in oxidative metabolism and protein synthesis, degradation, and structure, however much less work has focused on how amino acids influence insulin signalling and glucose metabolism. Most of the studies that have investigated this topic have focused predominantly on amino acid and protein mixtures (Bernard et al., 2011; 2012; Kleinert et al., 2011; Morifuji et al., 2009; Tremblay & Marette, 2001) or solely BCAAs (Doi et al., 2003; 2005; Iwanaka et al., 2010; Nishitani et al., 2002). Despite that it is the primary amino acid taken up by muscle and that it is involved in many processes related to energy metabolism in skeletal muscle, glutamate has been a relatively understudied amino acid with regards to its unique role in glucose handling and insulin metabolism (Graham et al., 2000; Mourtzakis et al., 2006; 2008; Mourtzakis & Graham, 2002). This study demonstrated that increased glutamate exposure in isolated L6 myotubes stimulated an increase in glucose uptake in a dose- and time-dependant manner. This study also showed that glutamate stimulated glucose uptake through AMPK and PKC signaling.

Glutamate increased glucose uptake in a dose- and time-dependant manner

To validate the proposed idea from our human studies that glutamate can increase glucose clearance, we investigated the dose-related responses of glutamate on muscle glucose uptake in an isolated manner using L6 myotubes. We used glutamate concentrations in our dose-response experiments that were representative of circulating concentrations of glutamate reported in various human studies (Di Sebastiano et al., 2013; Graham et al., 1997; Graham & MacLean, 1998; Mourtzakis & Graham, 2002).

We showed that L6 myotubes treated with glutamate concentrations (50μM), reflective of human serum glutamate concentrations in resting and fasting state, did not exhibit glucose uptake that was greater than our control condition. However, when the treatment dosage was increased to 500μM, the muscle cells demonstrated significantly elevated glucose uptake relative to the control (121 ± 4%). These concentrations align with serum concentrations of glutamate observed in previous human studies where 150mg/kg body weight of glutamate supplement was administered to male participants and results in elevated plasma glutamate concentrations to approximately 500μM (Di Sebastiano et al., 2013; Graham et al., 2000; Mourtzakis & Graham, 2002; Stegink et al., 1983a; Stegink et al., 1983b). These glutamate concentrations (~500μM) were associated with an attenuated increase in blood glucose when glutamate was administered with a carbohydrate drink compared to when carbohydrate was administered alone (Di Sebastiano et al., 2013). Thus, our present work suggests that the enhanced glucose clearance (Di Sebastiano et al., 2013; Hosaka et al., 2012) and glycemic control (Shi et al., 2014) observed in humans in response to elevated glutamate exposure may be attributed, in part, to increased peripheral clearance by skeletal muscle.

To further investigate if a dose-response relationship existed, L6 myotubes were exposed to supra-physiological concentrations of glutamate (1mM and 2mM). The supra-physiological treatments did not show significantly greater uptake than 500μM (123% and 129%, respectively), indicating that there may be threshold levels of glutamate concentrations that enhance glucose uptake. Further studies are needed to identify whether there is truly a threshold effect at 500uM or whether progressive increases in glutamate concentrations between 50uM (which represents fasted levels of glutamate

concentrations in humans) and 500uM (which represents the serum glutamate concentration that is associated with 150 mg/kg body weight oral glutamate administration in humans) would incrementally increase glucose uptake. However, a Pearson correlation was performed which demonstrated a positive linear curve indicating that glucose uptake increased in proportion to the glutamate doses used in this study ($R=0.97$; $P=0.03$). Thus, despite no statistical differences between the mean uptake value of each dose, this analysis suggests a progressive effect of glutamate exposure on glucose uptake. Cells exposed to 2mM glutamate showed the greatest uptake, thus this was determined to be the optimal concentration to use for experiments examining the role of glutamate in glucose handling. Furthermore, this concentration has also been shown to be optimal for glucose uptake with BCAAs, specifically leucine and isoleucine (Doi et al., 2003; Nishitani et al., 2002), with glucose uptake diminishing at higher concentrations. Future work should examine whether similar patterns of declining glucose uptake arise with increasing doses of glutamate concentrations.

In addition to the dose-response experiments, a time-response experiment was performed to determine the optimal glutamate exposure-time to treat the myotubes. Thirty minutes was the optimal treatment time, resulting in a 143% increase in glucose uptake versus the control condition, which was used as the treatment time for all subsequent experiments. This time-response is similar to data collected on cells treated with leucine, which found 30 minutes to be the optimal treatment time for glucose uptake (Nishitani et al., 2002). In cells treated with 2mM glutamate, glucose uptake was significantly greater than the control and was maintained 30 - 60 min, but was diminished by the 120 min time-point. Its is possible that glucose uptake started to decline after 30

minutes due to potential increases in intracellular glucose-6-phosphate and glycogen synthesis while glucose is taken up from the treatment media, which potentially act to reduce negative feedback mechanisms for AMPK mediated glucose uptake (Huang & Czech, 2007).

Glucose transporters involvement in glutamate mediated glucose uptake

The protocol used to assess glucose uptake in this study employed the use of a glucose radioisotope. Following each treatment, the radioactivity of each sample was assessed and reflected total glucose uptake. However, we were interested is assessing how glutamate can affect glucose transport specifically through the various glucose transporter (GLUTs) located on the skelatal muscle cell surface.

The L6 cell line expresses 3 isofroms of glucose transporter; GLUT-1, GLUT-3, and GLUT-4 (Klip et al., 1996). In fully differentiated myotubes, GLUT-1 and GLUT-3 are located almost exclusively on the cell surface and are responsible for the majority of basal glucose uptake. In contrast, the GLUT-4 receptor is distributed more evenly between the cell surface and intracellular vesicles, and are translocated to the cell surface in response to insulin (Klip et al., 1996; Mitsumoto & Klip, 1992; Mitsumoto et al., 1991).

In order to control for non-specific glucose uptake, each trial included a sample incubated with cytochalasin B, a GLUT inhibitor was used to correct for any glucose uptake that did not enter the cell through a glucose specific transporter (GLUT-1, 3, or 4). It was found that non-specific glucose uptake accounted for less than 5% of the total uptake across the different conditions, which suggests that glutamate was able to

significantly increase glucose uptake possibly by allosterically stimulating uptake through the GLUT transporters.

Given that the data suggests that glutamate exposure stimulated increased glucose uptake through GLUTs expressed in skeletal muscle, these findings may explain the observations reported by Di Sebastiano et al. (2013); glutamate supplementation in combination with carbohydrate ingestion was associated with an attenuated rise in blood glucose, suggesting that increased circulating glutamate may have improved muscle glucose clearance.

However, it remains unclear whether the increase in glucose uptake that was observed with glutamate supplementation was attributed to increased GLUT-4 translocation to the surface of the skeletal muscle cells, or if glutamate acted to increase the transport activity of GLUT-1, GLUT-3, and/or GLUT-4 already located at the sarcolemma, without increased receptor translocation. It has been shown that L6 myotubes that are exposed to elevated doses of glucose and insulin for up to 24 hrs demonstrate a 40% increase in basal glucose uptake, while GLUT-4 translocation is actually reduced by 50% (Huang et al., 2002). Furthermore, work has shown that AMPK-mediated glucose uptake enhance skeletal muscle glucose uptake in the absence of increased GLUT-4 translocation in L6 myotubes (Naimi et al., 2015). The results from the latter study suggest that because glutamate increased glucose uptake in an AMPK-mediated fashion that uptake was augmented through increased transporter activity; however it is unclear whether glutamate enhances glucose GLUT-4 translocation, or whether glutamate acts to increase the transport activity of the glucose transporters already located on the cell periphery.

Glutamate mediated glucose uptake through AMPK dependant mechanisms

Mechanisms of glucose uptake with glutamate exposure were distinct from mechanisms of glucose uptake with insulin and leucine exposure that act to increase glucose uptake via PI3K. Although cells treated with GLU+LEU or GLU+INS showed the greatest increase in glucose uptake (148%), they were not significantly different than when cells were treated with glutamate, leucine, or insulin alone. However our results suggest that the lack of synergistic effects may be an indication that each treatment maximized the amount of glucose transport that was achievable. Insulin mediated glucose uptake was not blocked by PKC inhibition like it was with leucine and glutamate, therefore we would have expected to see a synergistic uptake response when cells were treated with GLU+INS compared to GLU+LEU, which we did not. To overcome this limitation, future work should measure uptake elicited by glutamate in combination with leucine and/or insulin using lower doses to better explore this synergistic effect without maximizing the transport capacity of the cells.

Compound C, an AMPK specific inhibitor, completely shunted glucose uptake in cells treated with 2mM glutamate, indicating that glutamate acts to increase glucose uptake via an AMPK mediated pathway; thus we determined that glutamate stimulates glucose uptake in skeletal muscle cells via AMPK signalling. Although leucine and insulin treatments maintained significantly elevated uptake versus the control with AMPK inhibition, these treatments showed a less pronounced response when incubated with Compound C compared with experiments where muscle cells were incubated with leucine only and insulin alone (113% and 136% with Compound C versus 126% and 141% alone, respectively). However, it is important to consider that the serum and amino

acid deprivation protocol leading up the glucose uptake assay may have caused an increase in [AMP:ATP] ratio within the cells; a potent activator of AMPK. Thus, the control condition may have achieved slightly enhanced glucose uptake via augmented AMPK signalling, lowering the relative uptake values in leucine and insulin treated cells with Compound C. To overcome this, a control condition receiving Compound C without treatment should be included to control for any basal AMPK activation that could affect the relative uptake values.

Glucose uptake was also completely blocked (103% relative to control) when cells were treated with 2mM glutamate and the PKC inhibitor (Bisindolymalemide I; BMD), suggesting that the AMPK pathway stimulated by glutamate acts on PKC. The limitation with this experiment is that BMD is a general PKC inhibitor that will inhibit all isoforms of the protein. This inhibitor was chosen for this experiment because we are investigating mechanisms associated with glutamate and wanted to observe if there is any involvement of PKC before narrowing the investigation to specific isoforms. It has been shown that HCSA will act on the glutamate receptor to increase glucose uptake by stimulating PKC-zeta (Kim et al., 2011), thus it is possible that the same isoform is being stimulated by glutamate. The work done in this study with inhibitors allowed us to identify select primary mechanisms by which glutamate acts to increase glucose uptake in skeletal muscle cells, however it is limited by the fact that we are hypothesizing the order of protein activation in each pathway based on previous work. This limitation could be overcome by performing western blot analyses in combination with inhibitor treatments. For instance, we could western blot to quantify AMPK phosphorylation in myotubes treated with glutamate in combination with BMD1 to determine if AMPK is activated

upstream of PKC. This work would further strengthen the current finding of this study (Figure 6).

Limitations

An L6 rat skeletal muscle cell line was used in this study to examine the effects of glutamate and other treatments on glucose uptake. The use of this cell creates a limitation when comparing and applying the findings from this study to human physiology. Differences in the characteristics of glucose handling in L6 myotubes versus human skeletal muscle cells exist. The C2C12 mouse skeletal muscle cell line has been shown to better mimic human muscle cells with regards to their basal glucose uptake, insulin sensitivity, and insulin stimulated glucose transport; however, an L6 cell line was used in this study due to previous work showing that this cell line demonstrates the greatest fold-increase for insulin-stimulated uptake versus basal uptake when compared to human or mouse cell lines *in vitro* (Sarabia et al., 1990). Because this was a novel study investigating the effects of glutamate on glucose uptake, the L6 cell line was used because it could more clearly answer our foundational research questions potentially eliciting the greatest differences between basal glucose uptake and uptake stimulated by glutamate, insulin, and other treatments used in this study. The degree of differentiation was not quantified in each set of cells, therefore expression of certain signalling proteins, such as Akt and AMPK, and glucose transporters may not have been expressed to their full potential. For example, GLUT-1 is responsible for the majority of glucose uptake in myoblasts, however as the cells differentiate into myotubes, GLUT-4 translocation becomes the primary means for insulin-stimulated glucose uptake (Mitsumoto & Klip,

1992). Comparisons of uptake between control trials and treatment trials were done on cells that were grown and differentiated on the same plate in an identical fashion in an effort to control for these differences.

This study was also limited by the lack of western blotting experiments to confirm the results of the inhibitor experiments. The use of western blotting in combination with the inhibitor experiments could have been used to show that the inhibitors used were targeting the proteins that they were suppose to inhibit. Furthermore, western blots could have been used to confirm that the specific proteins that were determined to be involved in each signalling pathway were phosphorylated significantly above control conditions.

As mentioned previously, the current study is limited by the lack of measurement of GLUT-4 translocation to the cell surface in response to each of the treatments. Thus the current study cannot directly attribute the observed increase in glucose uptake to increased translocation of glucose transporters to the cell surface and the specific mode of glucose uptake, whether it was increased translocation of increased transporter activity, remains unclear.

Lastly, the inhibitor experiments did not include a control condition that received each inhibitor alone. Thus, increased basal activation of signalling proteins, specifically AMPK, was not controlled for. The pre-treatment protocol that deprived cells of serum and amino acids prior to treatments may have increased the basal activation of AMPK due to an increased AMP:ATP ratio. This may have inflated the uptake values associated with the control conditions and subsequently altered the relative uptake in the treatment conditions; caution is needed to interpret the results from the inhibitor data sets.

Conclusions

This is the first study, to our knowledge, demonstrating that glutamate can enhance glucose uptake into skeletal muscle cells in a dose- and time-dependant manner. Glutamate stimulated glucose uptake in skeletal muscle to the same extent as leucine and insulin via an AMPK activated pathway and potentially resulting in downstream PKC activation, whereas both leucine- and insulin-mediated glucose uptake relied on PI3K activation. Furthermore, glutamate acted to increase glucose uptake specifically through the glucose transporters, providing strong evidence for increased glucose clearance by skeletal muscle in response to elevated glutamate concentrations *in vivo*, as human skeletal muscle expresses GLUT-1 and GLUT-4 (Di Sebastiano et al., 2013; Hosaka et al., 2012; Klip et al., 1996).

Future Directions

The dose-response experiments showed an association between glutamate treatment dose and glucose uptake, however none of the treatments differed significantly from each other. In order to better investigate if a true dose-response relationship exists, future studies should include additional glutamate treatments in the physiological range between 50μM and 500μM, as well as additional supraphysiological doses up to approximately 10mM to investigate this relationship further.

Future work is warranted to investigate the effect that glutamate has on glucose transporter translocation and activity to elucidate the mode by which glucose is taken up in response to glutamate beyond the primary signalling pathways. The use of a GLUT-1 and GLUT-4 over-expressing cell lines could be used to perform a translocation assay to

investigate our findings further; this would allow us to answer the research question as to whether glutamate acts to increase glucose uptake by upregulating GLUT-1 and/or GLUT-4 translocation to the cell surface, or by increasing the activity of transporters already located on the periphery.

Future experiments should further examine the glutamate transporters and signalling pathway. This study has identified select primary mechanisms by which glutamate is involved in glucose handling, however the exact mechanistic pathways should be investigated further. By performing western blotting in combination with inhibitor experiments to evaluate the glutamate signalling pathway, it makes it possible establish the order of protein activation, specific isoforms involved, and the involvement of various mediating proteins such as p38 MAPK and AS160 that have been shown to be involved in glucose transport (Cartee & Wojtaszewski, 2007; Kim et al., 2011). Additionally, the use of cell and animal models where the proteins investigated in this study have been knocked out or knocked down could be used in future work to confirm their involvement in the various signalling pathways explored.

Furthermore, work should address the fate of glucose upon entering the muscle cell in response to glutamate. Future experiments should focus on how glutamate affects rates of glycogen synthesis and storage, glucose utilization, and rates of protein synthesis in skeletal muscle cells. The intracellular handling of glucose in response to glutamate may elucidate the underlying purpose for the augmented glucose uptake associated with elevated glutamate exposure in skeletal muscle. This work will provide a basis for future animal and human studies exploring the interactions of glutamate and glucose in skeletal muscle.

Appendix – Methodological Development

Pilot Experiments

The initial pilot work investigating the effect of glutamate on glucose transport in skeletal muscle cells used cultured C2C12 myocytes. Briefly, C2C12 myoblasts were seeded in 150mm polystyrene culture dishes (BD Falcon) at a density of approximately 2500 cells/cm^2. Cells were seeded and grown in 20 mL of growth media, consisting of low-glucose Dulbecco's Modified Eagle Medium (DMEM; Hyclone, ThermoFisher) supplemented with 10% Fetal Bovine Serum (Hyclone, ThermoFisher) and 1% penicillin streptomycin (Hyclone, ThermoFisher). Growth media was changed every 48 hrs and after approximately 3 days, cells would reach 70-80% confluence and were sub-cultured into smaller culture plates for experimentation. For sub-culturing, growth media was aspirated from the 150mm plates and cells were washed twice with DPBS. Cells were then detached from the plate by adding 1mL of trypsin (ThermoFisher) and seeded into 100mm polystyrene culture dishes where they were maintained in growth media for an additional 2 days until reached ~90% confluence. Media was then changed to low-glucose DMEM supplemented with 2% horse serum (Hyclone, Thermofisher) and 1% penicillin streptomycin to stimulate differentiation.

Following 4-5 days of differentiation, cells were treated with an EBSS solution with 5mM glucose (Life Technologies) or EBSS containing 5mM glucose and 2mM glutamate. A sample of each media was collected before treating the cells and after 3 hours of treatment. A glucose peroxidase experiment was performed to assay the glucose concentration of the before and after media samples and glucose consumption was calculated from the difference. This method showed us that glutamate had the ability to

stimulate glucose consumption; however this particular method only assumed glucose uptake, as cellular glucose content was never measured, thus further experimentation was needed.

Fluorescent Experiments

Following the glucose consumption pilot work, we employed the use of a fluorescent glucose uptake kit (Sigma-Aldrich) to further investigate the effect of glutamate on glucose transport, rather than simple consumption. This method involved culturing the cells as previously described, however cells were subcultured into 96-well black flat bottom plates at density of 5000 cells/well. Following differentiation, cells were serum starved for 3 hours in DMEM to wash out the effects of insulin and other compounds, followed by a glucose and amino acid starvation period for 1 hour in Phosphate Buffered Saline (PBS, pH 7.4; Life Technologies). Cells were treated for 1 hour in PBS, PBS with insulin, or PBS with glutamate, containing 1mM of 2-Deoxyglucose (2DG). After treatment, the protocol called for cells to be lysed in extraction buffer, freeze/thawed in liquid nitrogen, heated for 40 minutes. Next, the lysates were collected and the 2-DG taken up by each sample was oxidized to form NADPH; subsequently the fluorescence associated with each sample was analyzed and reflected glucose uptake. The initial work done with this protocol yielded somewhat promising results, however the reliability of this protocol was questionable. Throughout the various attempts to repeat and confirm my initial results, I was unsuccessful and often produced erroneous results. Following several processes in problem-solving, these erratic

results were likely attributed to the extensive sample processing following treatment that may have led to sample degradation.

In an effort to develop a more valid a reliable method to measure glucose uptake, I employed the use of a fluorescent glucose analog, 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG; Life Technologies). For this protocol, cells were cultured as previously described, serum starved for 3 hours in DMEM, glucose deprived for 1 hr in PBS, treated for 1 hr in PBS containing insulin or glutamate, then treated with 50μM 2-NBDG in the treatment media. Following which, cells were washed, lysed, and read in the plate reader. The fluorescence associated with each sample was indicative of glucose uptake. Initially, results showed to be extremely inconsistent due to the fact that the PBS solution used to starve and treat the cells did not contain magnesium and calcium; thus the extended incubation time in PBS associated with this protocol caused cells to lose their adhesion to the plate and lose viability. This problem was fixed by changing the treatment media to DPBS containing $MgSO_4$ and $CaCl_2$ (Life Technologies). However, even after the protocol had been optimized for cell viability, the results still proved to be somewhat unusable due to the low fluorescent detection in the samples and relatively high background fluorescence associated with the assay. Thus it became clear that a much more sensitive method was needed to properly assess and compare glucose uptake across different treatment conditions.

Radioisotope Protocol Development

In order to assess glucose uptake with the sensitivity that is needed to make definite conclusions regarding the role of glutamate, leucine, and insulin in glucose

uptake, I employed the use of a radioisotope 2-[^{3}H]-Deoxy-D-glucose ([^{3}H]-2-DG; Perkin-Elmer). After reviewing the literature, I decided to change from the C2C12 mouse cell line to the L6 rat muscle cell line. The L6 cell was more common in literature investigating glucose uptake, and has also shown to be more sensitive to insulin stimulation (Sarabia et al., 1990). *L6 Cells were cultured as described in the Methods Section.* Originally, the radioisotope protocol was similar to what was used for the 2-NBDG assay. Cells were serum and glucose deprived in DPBS containing $MgSO_4$ and $CaCl_2$ for 3 hrs, then treated for 1 hr in the same DPBS containing glutamate, leucine, or insulin. However, this protocol proved to be problematic due to the fact cells were deprived of nutrients for 4 hours, which induced apoptosis and a loss of cells. In order to overcome this, I developed a protocol that was a hybrid of methods used in previous work investigating the role of amino acids in glucose handling (Peyrollier et al., 2000; Tremblay & Marette, 2001). Essentially, cells were serum deprived in DMEM for 4 hrs to adequately wash out the effects of insulin and other compounds in the serum. This was followed by a 1 hr incubation in Earle's Balanced Salts Solution (EBSS; Sigma-Aldrich) to wash out any effects that the high concentration of amino acids in DMEM may have on glucose uptake, while still providing the cells with glucose to maintain viability. Furthermore, this solution was buffered by sodium bicarbonate and had the buffering capacity to maintain a pH of 7.4 even after the addition of L-glutamic acid to the solution, which immediately dissociates to glutamate and would otherwise decrease pH in solution. This protocol proved to maintain the viability of cells being treated while also having the sensitivity and reliability needed to make concrete conclusions regarding the role of glutamate in glucose handling.

References

Bernard, J. R., Liao, Y.-H., Doerner, P. G., Ding, Z., Hsieh, M., Wang, W., et al. (2012). An amino acid mixture is essential to optimize insulin-stimulated glucose uptake and GLUT4 translocation in perfused rodent hindlimb muscle. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *113*(1), 97–104. doi:10.1152/japplphysiol.01484.2011

Bernard, J. R., Liao, Y.-H., Hara, D., Ding, Z., Chen, C.-Y., Nelson, J. L., & Ivy, J. L. (2011). An amino acid mixture improves glucose tolerance and insulin signaling in Sprague-Dawley rats. *American Journal of Physiology. Endocrinology and Metabolism*, *300*(4), E752–60. doi:10.1152/ajpendo.00643.2010

Bertrand, G., Gross, R., Puech, R., Loubatières-Mariani, M. M., & Bockaert, J. (1992). Evidence for a glutamate receptor of the AMPA subtype which mediates insulin release from rat perfused pancreas. *British Journal of Pharmacology*, *106*(2), 354–359.

Bertrand, G., Puech, R., Loubatières-Mariani, M. M., & Bockaert, J. (1995). Glutamate stimulates insulin secretion and improves glucose tolerance in rats. *The American Journal of Physiology*, *269*(3 Pt 1), E551–6.

Brann, D. W. (1995). Glutamate: a major excitatory transmitter in neuroendocrine regulation. *Neuroendocrinology*, *61*(3), 213–225.

Cartee, G. D., & Wojtaszewski, J. F. P. (2007). Role of Akt substrate of 160 kDa in insulin-stimulated and contraction-stimulated glucose transport. *Applied Physiology, Nutrition, and Metabolism*, *32*(3), 557–566. doi:10.1139/H07-026

Cheng, Z., Pang, T., Gu, M., Gao, A.-H., Xie, C.-M., Li, J.-Y., et al. (2006). Berberine-stimulated glucose uptake in L6 myotubes involves both AMPK and p38 MAPK. *Biochimica Et Biophysica Acta (BBA) - General Subjects*, *1760*(11), 1682–1689. doi:10.1016/j.bbagen.2006.09.007

Chevassus, H., Renard, E., Bertrand, G., Mourand, I., Puech, R., Molinier, N., et al. (2002). Effects of oral monosodium (L)-glutamate on insulin secretion and glucose tolerance in healthy volunteers. *British Journal of Clinical Pharmacology*, *53*(6), 641–643.

DeFronzo, R. A., Jacot, E., Jequier, E., Maeder, E., Wahren, J., & Felber, J. P. (1981). The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes*, *30*(12), 1000–1007.

Di Sebastiano, K. M., Bell, K. E., Barnes, T., Weeraratne, A., Premji, T., & Mourtzakis, M. (2013). Glutamate supplementation is associated with improved glucose metabolism following carbohydrate ingestion in healthy males. *The British Journal of Nutrition*, 1–8. doi:10.1017/S0007114513001633

Doi, M., Yamaoka, I., Fukunaga, T., & Nakayama, M. (2003). Isoleucine, a potent plasma glucose-lowering amino acid, stimulates glucose uptake in C2C12 myotubes. *Biochemical and Biophysical Research Communications*, *312*(4), 1111–1117.

Doi, M., Yamaoka, I., Nakayama, M., Mochizuki, S., Sugahara, K., & Yoshizawa, F. (2005). Isoleucine, a blood glucose-lowering amino acid, increases glucose uptake in rat skeletal muscle in the absence of increases in AMP-activated protein kinase activity. *Journal of Nutrition*, *135*(9), 2103–2108.

Frigerio, F., Casimir, M., Carobbio, S., & Maechler, P. (2008). Tissue specificity of mitochondrial glutamate pathways and the control of metabolic homeostasis. *Biochimica Et Biophysica Acta (BBA) - Bioenergetics*, *1777*(7), 965–972. doi:10.1016/j.bbabio.2008.04.031

Graham, T. E., & MacLean, D. A. (1998). Ammonia and amino acid metabolism in skeletal muscle: human, rodent and canine models. *Medicine & Science in Sports & Exercise*, *30*(1), 34–46. doi:10.1097/00005768-199801000-00006

Graham, T. E., Sgro, V., Friars, D., & Gibala, M. J. (2000). Glutamate ingestion: the plasma and muscle free amino acid pools of resting humans. *American Journal of Physiology. Endocrinology and Metabolism*, *278*(1), E83–9.

Graham, T. E., Turcotte, L. P., Kiens, B., & Richter, E. A. (1997). Effect of endurance training on ammonia and amino acid metabolism in humans. *Medicine & Science in Sports & Exercise*, *29*(5), 646–653.

Hosaka, H., Kusano, M., Zai, H., Kawada, A., Kuribayashi, S., Shimoyama, Y., et al. (2012). Monosodium glutamate stimulates secretion of glucagon-like peptide-1 and reduces postprandial glucose after a lipid-containing meal. *Alimentary Pharmacology & Therapeutics*, *36*(9), 895–903. doi:10.1111/apt.12050

Huang, C., Somwar, R., Patel, N., Niu, W., Török, D., & Klip, A. (2002). Sustained exposure of L6 myotubes to high glucose and insulin decreases insulin-stimulated GLUT4 translocation but upregulates GLUT4 activity. *Diabetes*, *51*(7), 2090–2098.

Huang, S., & Czech, M. P. (2007). The GLUT4 glucose transporter. *Cell Metabolism*, *5*(4), 237–252. doi:10.1016/j.cmet.2007.03.006

Iwanaka, N., Egawa, T., Satoubu, N., Karaike, K., Ma, X., Masuda, S., & Hayashi, T. (2010). Leucine modulates contraction- and insulin-stimulated glucose transport and upstream signaling events in rat skeletal muscle. *Journal of Applied Physiology*, *108*(2), 274–282. doi:10.1152/japplphysiol.00420.2009

Kim, J. H., Lee, J. O., Lee, S. K., Moon, J. W., You, G. Y., Kim, S. J., et al. (2011). The Glutamate Agonist Homocysteine Sulfinic Acid Stimulates Glucose Uptake through the Calcium-dependent AMPK-p38 MAPK-Protein Kinase C zeta Pathway in Skeletal Muscle Cells. *Journal of Biological Chemistry*, *286*(9), 7567–7576. doi:10.1074/jbc.M110.149328

Kim, M. S., Hur, H. J., Kwon, D. Y., & Hwang, J.-T. (2012). Tangeretin stimulates glucose uptake via regulation of AMPK signaling pathways in C2C12 myotubes and improves glucose tolerance in high-fat diet-induced obese mice. *Molecular and Cellular Endocrinology*, *358*(1), 127–134. doi:10.1016/j.mce.2012.03.013

Kleinert, M., Liao, Y.-H., Nelson, J. L., Bernard, J. R., Wang, W., & Ivy, J. L. (2011). An amino acid mixture enhances insulin-stimulated glucose uptake in isolated rat epitrochlearis muscle. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *111*(1), 163–169. doi:10.1152/japplphysiol.01368.2010

Klip, A., Li, G., & Logan, W. J. (1984). Induction of sugar uptake response to insulin by serum depletion in fusing L6 myoblasts. *The American Journal of Physiology*, *247*(3 Pt 1), E291–6.

Klip, A., Volchuk, A., He, L., & Tsakiridis, T. (1996). The glucose transporters of skeletal muscle. *Seminars in Cell & Developmental …*.

Loaiza, A., Porras, O. H., & Barros, L. F. (2003). Glutamate triggers rapid glucose transport stimulation in astrocytes as evidenced by real-time confocal microscopy. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience*, *23*(19), 7337–7342.

Meldrum, B. S. (2000). Glutamate as a neurotransmitter in the brain: review of physiology and pathology. *Journal of Nutrition*, *130*(4S Suppl), 1007S–15S.

Mitsumoto, Y., & Klip, A. (1992). Development regulation of the subcellular distribution and glycosylation of GLUT1 and GLUT4 glucose transporters during myogenesis of L6 muscle cells. *Journal of Biological Chemistry*, *267*(7), 4957–4962.

Mitsumoto, Y., Burdett, E., Grant, A., & Klip, A. (1991). Differential expression of the GLUT1 and GLUT4 glucose transporters during differentiation of L6 muscle cells. *Biochemical and Biophysical Research Communications*, *175*(2), 652–659.

Morifuji, M., Koga, J., Kawanaka, K., & Higuchi, M. (2009). Branched-chain amino acid-containing dipeptides, identified from whey protein hydrolysates, stimulate glucose uptake rate in L6 myotubes and isolated skeletal muscles. *Journal of Nutritional Science and Vitaminology*, *55*(1), 81–86.

Mourtzakis, M., & Graham, T. E. (2002). Glutamate ingestion and its effects at rest and during exercise in humans. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *93*(4), 1251–1259. doi:10.1152/japplphysiol.00111.2002

Mourtzakis, M., Graham, T. E., González-Alonso, J., & Saltin, B. (2008). Glutamate availability is important in intramuscular amino acid metabolism and TCA cycle intermediates but does not affect peak oxidative metabolism. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *105*(2), 547–554. doi:10.1152/japplphysiol.90394.2008

Mourtzakis, M., Saltin, B., Graham, T., & Pilegaard, H. (2006). Carbohydrate metabolism during prolonged exercise and recovery: interactions between pyruvate dehydrogenase, fatty acids, and amino acids. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *100*(6), 1822–1830. doi:10.1152/japplphysiol.00571.2005

Naimi, M., Tsakiridis, T., Stamatatos, T. C., Alexandropoulos, D. I., & Tsiani, E. (2015). Increased skeletal muscle glucose uptake by rosemary extract through AMPK activation. *Applied Physiology, Nutrition, and Metabolism*, *40*(4), 407–413. doi:10.1139/apnm-2014-0430

Nishitani, S., Matsumura, T., Fujitani, S., Sonaka, I., Miura, Y., & Yagasaki, K. (2002). Leucine promotes glucose uptake in skeletal muscles of rats. *Biochemical and Biophysical Research Communications*, *299*(5), 693–696.

Park, C. E., Kim, M.-J., Lee, J. H., Min, B.-I., Bae, H., Choe, W., et al. (2007). Resveratrol stimulates glucose transport in C2C12 myotubes by activating AMP-activated protein kinase. *Experimental & Molecular Medicine*, *39*(2), 222–229. doi:10.1038/emm.2007.25

Petrie, H. J., Chown, S. E., Belfie, L. M., Duncan, A. M., McLaren, D. H., Conquer, J. A., & Graham, T. E. (2004). Caffeine ingestion increases the insulin response to an oral-glucose-tolerance test in obese men before and after weight loss. *American Journal of Clinical Nutrition*, *80*(1), 22–28.

Peyrollier, K., Hajduch, E., Blair, A. S., Hyde, R., & Hundal, H. S. (2000). L-leucine availability regulates phosphatidylinositol 3-kinase, p70 S6 kinase and glycogen synthase kinase-3 activity in L6 muscle cells: evidence for the involvement of the mammalian target of rapamycin (mTOR) pathway in the L-leucine-induced up-regulation of system A amino acid transport. *Biochemical Journal*, *350 Pt 2*, 361–368.

Pin, J. P., & Duvoisin, R. (1995). The metabotropic glutamate receptors: structure and functions. *Neuropharmacology*, *34*(1), 1–26.

Saltiel, A. R., & Kahn, C. R. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature*, *414*(6865), 799–806. doi:10.1038/414799a

Saltiel, A. R., & Pessin, J. E. (2002). Insulin signaling pathways in time and space. *Trends in Cell Biology*, *12*(2), 65–71.

Sarabia, V., Ramlal, T., & Klip, A. (1990). Glucose uptake in human and animal muscle cells in culture. *Biochemistry and Cell Biology*.

Shi, Z., Taylor, A. W., Yuan, B., Zuo, H., & Wittert, G. A. (2014). Monosodium glutamate intake is inversely related to the risk of hyperglycemia. *Clinical Nutrition (Edinburgh, Scotland)*, *33*(5), 823–828. doi:10.1016/j.clnu.2013.10.018

Stegink, L. D., Baker, G. L., & Filer, L. J. (1983a). Modulating effect of Sustagen on plasma glutamate concentration in humans ingesting monosodium L-glutamate. *American Journal of Clinical Nutrition*, *37*(2), 194–200.

Stegink, L. D., Filer, L. J., & Baker, G. L. (1983b). Effect of carbohydrate on plasma and erythrocyte glutamate levels in humans ingesting large doses of monosodium L-glutamate in water. *American Journal of Clinical Nutrition*, *37*(6), 961–968.

Thomassen, A., Bøtker, H. E., Nielsen, T. T., Thygesen, K., & Henningsen, P. (1990). Effects of glutamate on exercise tolerance and circulating substrate levels in stable angina pectoris. *The American Journal of Cardiology*, *65*(3), 173–178.

Tremblay, F., & Marette, A. (2001). Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. *Journal of Biological Chemistry*.