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UNIVERSITIY OF CALIFORNIA SAN DIEGO

The interactive effects of time, sex, fiber type, and insulin concentration on insulin-stimulated glucose uptake in mouse skeletal muscle

A thesis submitted in partial satisfaction of the requirements

for the degree of Master of Science

in

Biology

by

Ji Eun Park

Committee in charge:

Professor Simon Schenk, Chair
Professor Randolph Hampton, Co-Chair
Professor Maho Niwa Rosen

2021

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The Thesis of Ji Eun Park is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

DEDICATION

I dedicate this to my parents who have sacrificed everything to get me here today. I also dedicate this to my grandmother who prays for my safety, success, and happiness. Thank you.

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ABBREVIATIONS

ISGU	Insulin-stimulated glucose uptake
IR	Insulin receptor
PI3K	phosphatidylinositol 3-kinase
PKB	Protein kinase B
AS160	Akt substrate of 160 kDa
GLUT4	Glucose transporter 4
2DOGU	2-deoxyglucose uptake
IS-2DOGU	Insulin-stimulated 2-deoxyglucose uptake
T2D	Type 2 diabetes
EDL	extensor digitorum longus
KHB	Krebs-Henseleit buffer
Rab	Rab-related proteins
Ser473	Serine 473
Thr308	Threonine 308
Thr642	Threonine 642
SLC2A4	Solute carrier family 2 member 4
GTP	Guanosine triphosphate

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ABSTRACT OF THE THESIS

The interactive effects of time, sex, fiber type, and insulin concentration on insulin-stimulated glucose uptake in mouse skeletal muscle

by

Ji Eun Park

Master of Science in Biology

University of California San Diego, 2021

Professor Simon Schenk, Chair
Professor Randolph Hampton, Co-Chair

A key metabolic action of insulin is to stimulate skeletal muscle to take up glucose from the systemic circulation. This “insulin-stimulated” glucose uptake is critical during the post-prandial period (i.e. after meal ingestion) as it reduces large excursions in blood glucose concentration; in fact, skeletal muscle accounts for as much as 85% of peripheral glucose uptake after a meal, thereby making it a fundamental

organ to not just glycemic control, but also to overall health. Nevertheless, despite its obvious importance, there is still much unknown about the regulation of insulin-stimulated glucose uptake by skeletal muscle. To study this regulation, the field commonly uses mouse models, as insulin signaling and action in mouse skeletal muscle closely replicates that seen in human skeletal muscle. Remarkably, however, the physiological action of insulin on the dynamics of glucose uptake in mouse skeletal muscle, especially as it relates to the contributions of sex, fiber type, and insulin concentration have not been systematically analyzed. Thus, the objective of this Thesis was to investigate the interactive effect of time, sex, fiber type, and insulin concentration on basal and insulin-stimulated glucose uptake in mouse skeletal muscle. Specifically, we used an *ex vivo* radioactive 2-deoxyglucose uptake (2DOGU) approach and measured basal and insulin-stimulated (insulin concentration: 0.36 nM and 6 nM) glucose uptake for 5, 10, 15, 20, or 30 minutes in paired extensor digitorum longus (EDL) and soleus muscles from 12-15 week old female and male (6nM insulin only) mice. In both the soleus and EDL of female mice, 2DOGU in response to physiological insulin (0.36nM; i.e the circulating insulin concentration typically seen after a meal) was statistically greater than basal at 15 and beyond, but not at 5 or 10 min. In contrast, a supraphysiological insulin concentration (6nM) 2DOGU more rapidly increased 2DOGU above basal, such that it was greater than basal at by 5 minutes in soleus and 10 minutes in EDL, regardless of sex. As it relates to fiber type, the soleus (i.e. primarily slow twitch) had greater insulin-stimulated glucose uptake than the EDL (i.e. primarily fast-twitch), regardless of sex or insulin concentration. Finally, as it relates to sex differences, female mice had greater insulin-stimulated 2DOGU as compared to

male, regardless of fiber type or time point. Taken together, this work demonstrates that in response to physiological insulin there is a delay of ~15 minutes from insulin exposure to when there is a robust increase in insulin-stimulated glucose uptake, and that increasing insulin concentration shortens this biological delay. Overall, this work demonstrates the importance sex, fiber type, insulin concentration and time to the regulation of insulin-stimulated glucose uptake by mouse skeletal muscle.

INTRODUCTION

1.1 Insulin, systemic glucose homeostasis and type 2 diabetes (T2D)

Insulin stimulates glucose uptake in skeletal muscle and adipose tissue (Cartee & Wojtaszewski, 2007; Dugani et al., 2008) and is critical during the post-prandial period (i.e. after meal ingestion). Thus, insulin is critical to maintaining glycemic control. A characteristic feature of the etiology of T2D is an impairment in, or complete loss of, the physiological actions of insulin on insulin target tissues, such as skeletal muscle, liver and adipose tissue (Ralph A. DeFronzo, 2004; Ralph A. DeFronzo & Tripathy, 2009; Ryder et al., 2001). In turn, this "insulin resistance", especially at the level of skeletal muscle, leads to β-cell compensation and ultimately, β-cell failure (R. A. DeFronzo et al., 1985; Dugani et al., 2008; Pendergrass et al., 2007).

T2D is one of the most prevalent non-communicable diseases in the world; in 2014 it was estimated to afflict 460 million people worldwide (Abdul et al., 2020; N. H. Cho et al., 2018; Risk & Collaboration, 2016), and by 2045 this is anticipated to increase to 693 million people (N. H. Cho et al., 2018). Similarly, in the United States, the prevalence of T2D is at worrying levels, with ~10% of the population estimated to have T2D (Centers for Disease Control and Prevention, 2020). Importantly, T2D carries a substantial economic, health care and personal burden. For example, US health care spending directly related to T2D and its associated complications was ~$300 billion in 2017 (Yang et al., 2018). As part of this, T2D is associated with longer hospital stays and a higher cost of hospital stay (Centers for Disease Control and Prevention, 2020; Vamos et al., 2010). Moreover, having T2D increases the risk of

developing other health-related issues, such as coronary heart disease, cancer, and obesity (Facchini et al., 2001; Yau et al., 2012). Perhaps most important is the personal cost and burden of T2D; for instance, T2D is the primary cause of non-traumatic blindness and lower leg amputation (Johannesson et al., 2009; Vamos et al., 2010; Yang et al., 2018; Yau et al., 2012).

As one of the most prevalent "preventable" yet financially and personally costly diseases in the United States and globally, if we are to find approaches to treat or prevent the development of T2D, it is imperative that we first understand the physiological actions of insulin on target tissues. To this end, the primary focus of this Thesis is on skeletal muscle and the regulation of insulin-stimulated glucose uptake by skeletal muscle.

Why focus on skeletal muscle? Skeletal muscle is critical to peripheral glucose uptake and glycemic control, such that skeletal muscle accounts for as much as ~85% of peripheral glucose disposal (Björntorp & Sjöström, 1978; R. A. DeFronzo et al., 1985; Ralph A DeFronzo et al., 1981; Sherwin et al., 1974; Thiebaud et al., 1982; Zorzano et al., 2005); in contrast, adipose tissue only contributes approximately 3-10% (Björntorp et al., 1971; Björntorp & Sjöström, 1978; Kahn, 1996). Notably, this contribution of skeletal muscle to glucose disposal is similar in rats, with ~70% of insulin-stimulated peripheral glucose disposal being into skeletal muscle (Kraegen et al., 1985). Importantly, this % contribution of skeletal muscle to insulin-stimulated glucose uptake is similar in instances of insulin resistance, such as T2D, such that while total skeletal muscle glucose uptake is reduced by 45% in T2D subjects, ~87% of peripheral glucose uptake was into skeletal muscle (R. A. DeFronzo et al., 1985).

Overall, these studies highlight the critical contribution of skeletal muscle to glucose homeostasis.

The clinical importance of insulin timing to glucose control. Diabetic patients are instructed to administer insulin 30 to 60 minutes before a meal to maximize its physiological benefit on glucose homeostasis (Hirsch et al., 2021; Sanlioglu et al., 2013). However, factors such as eating habits, daily schedules and other lifestyle limitations result in only one third of individuals with T2D rigidly follow this insulin dosing schedule, with the remaining majority often missing, mistiming or reducing insulin injections (Brod et al., 2012). Consequently, this poor management of insulin dosing is linked to poor glycemic control (Dibonaventura et al., 2014; Donnelly et al., 2007; Huang et al., 2009; Schaper et al., 2017) and a recurrent or constant fear of hypoglycemia (Cryer et al., 2003; Davies et al., 2013; Farsaei et al., 2014; Peyrot et al., 2010). Thus, by gaining greater insight into the temporal delay between an increase in systemic insulin concentration (e.g. after a meal or exogenous insulin injection) to when there is a significant increase in glucose uptake (above basal), approaches to better manage glycemic control can be developed. To this point, an underlying goal of this Thesis is to better understand this temporal regulation in skeletal muscle.

1.1 Insulin signaling and glucose transporter 4 (GLUT4)

Insulin signaling pathway. The binding of insulin to the insulin receptor initiates a series of signaling steps which regulates insulin-stimulated glucose uptake. While a detailed overview of this process is beyond the scope of this Thesis, there are many excellent reviews that cover this topic (Cohen, 2006; da Silva Rosa et al., 2020;

Sylow et al., 2021; Zaid et al., 2008). Briefly, the proteins involved in the binding of insulin and subsequent glucose uptake include the insulin receptor (IR) (Taira et al., 1989; Ullrich et al., 1985), phosphatidylinositol 3-kinase (PI3K) (Endemann et al., 1990; Ruderman et al., 1990), Akt, also referred to as protein kinase B (PKB) (H. Cho et al., 2001; Jiang et al., 2003; N. Sharma et al., 2010; Yeh et al., 1995; Zheng & Cartee, 2016), and Akt substrate of 160 kDa (AS160) (Bruss et al., 2005; Kramer et al., 2006).

As depicted in Figure 1, insulin binds to the alpha-subunit of the IR on the cell surface (Ebina et al., 1985; Kasuga et al., 1982), which stimulates intrinsic tyrosine kinase activity on the beta-subunit (Ebina et al., 1985; Shia & Pilch, 1983; Ullrich et al., 1985). This tyrosine kinase activity recruits insulin receptor substrate (IRS-1) and activates PI3K (Chen et al., 1993; Folli et al., 1992), a kinase essential to insulin-stimulated glucose uptake (Yeh et al., 1995). PI3K activation is followed by the

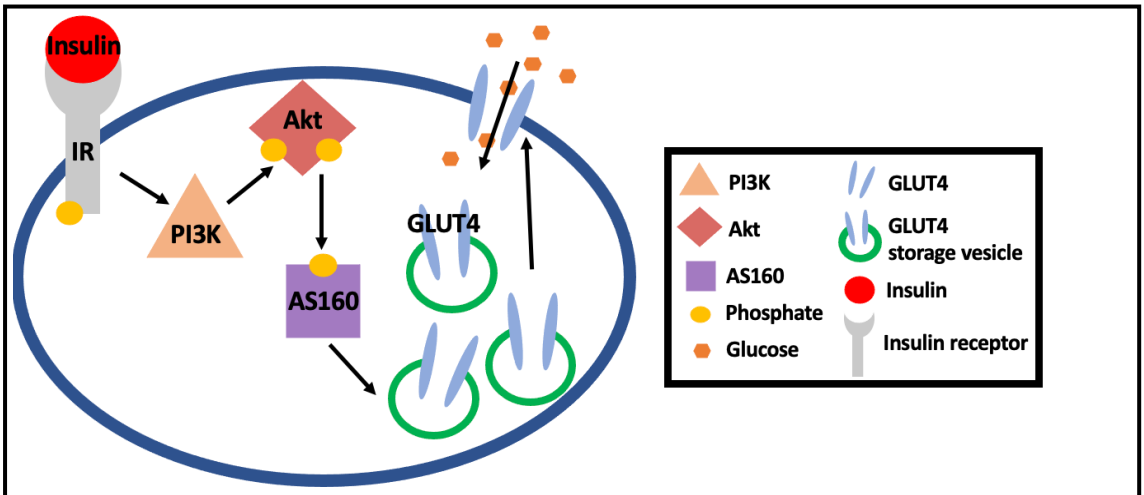

Figure 1. The insulin signaling pathway. The insulin signaling pathway is initiated by the binding of insulin to IR, causing an autophosphorylation of the membrane protein. IR activates PI3K, which brings Akt to the plasma membrane and phosphorylation at Ser473 and Thr308 occurs, activating it. Akt phosphorylates and inactivates AS160, initiating GLUT4 translocation and fusion to the plasma membrane, increasing cellular glucose uptake.

recruitment of Akt to the plasma membrane (Burgering & Coffer, 1995; Molinaro et al., 2019; Zheng & Cartee, 2016) and activation via phosphorylation at Ser473 and Thr308 (Alessi et al., 1996; Hresko & Mueckler, 2005; Sarbassov et al., 2005). Activated Akt phosphorylates AS160 at Thr642, which inactivates AS160 (Kane et al., 2002; Sano et al., 2003; Thong et al., 2007), removing its ability to inhibit Rab GTPases (Jaldin-Fincati et al., 2017; Sun et al., 2010, 2014). This series of signaling events results in the translocation of glucose transporter 4 (GLUT4; also known as SLC2A4 or solute carrier family 2 member 4) to, and fusion with, the plasma membrane, which as elaborated on more below, allows for the facilitated diffusion of glucose into skeletal muscle (Foster et al., 2001; Govers et al., 2004; Ryder et al., 2001).

Insulin and plasma membrane GLUT4. In 1988, James *et* al. was the first group to identify an insulin-sensitive glucose transport protein unique to muscle and adipose tissue (David E. James et al., 1988). As part of this, it was established GLUT4 allows the facilitated diffusion of glucose into the cell (Fujimoto et al., 2019; Kern et al., 1990; Rothman et al., 1995). Indeed, GLUT4 is the most abundant isoform of glucose transporters expressed in skeletal muscle (Deshmukh et al., 2015; Zorzano et al., 2005) and resides both in intracellular GLUT4 storage vesicles and at the plasma membrane (Klip & Marette, 1992). Moreover, the fundamental importance of GLUT4 to insulin-stimulated glucose uptake by skeletal muscle is perhaps best demonstrated by the fact that knockout of GLUT4 in mouse skeletal muscle results in a complete loss of insulin-stimulated glucose uptake (J. K. Kim et al., 2001).

At rest, skeletal muscle glucose uptake is low, GLUT4 primarily resides in an

intracellular location (i.e. away from the plasma membrane) (Foley et al., 2011; Li et al., 2001). With insulin stimulation, however, the levels of GLUT4 at, and fused with, the plasma membrane increases 5- to 30-fold (Coster et al., 2004; Govers et al., 2004; Stöckli et al., 2011); this increase occurs due to three main actions: increased rate of GLUT4 movement to the plasma membrane, decreased rate of GLUT4 endocytosis, and increasing the number of GLUT4 proteins participating in the translocation pathway (Coster et al., 2004; Foster et al., 2001; Li et al., 2001).

Summary. It is clear GLUT4 is essential to insulin-stimulated glucose uptake by skeletal muscle. Insulin stimulates glucose uptake in skeletal muscle and adipose tissue by way of the insulin signaling pathway and subsequent GLUT4 translocation to the plasma membrane. In the next section, I will be discussing the time course of insulin action and glucose uptake.

1.2 Insulin and temporal changes in skeletal muscle glucose uptake

Activation of the insulin signaling pathway over time. Starting with insulin signaling, an *in vivo* human study demonstrated skeletal muscle insulin receptor phosphorylation and PI3K activity significantly increased after 10 minutes of insulin infusion (Wojtaszewski et al., 1997), which was the earliest time point measured. Additionally, phosphorylation of Akt increased ~10 fold and of AS160 ~2 fold compared to basal after 30 minutes of insulin infusion, although it is important to note the authors only measured phosphorylation at 0, 30, and 60 minutes (Pehmøller et al., 2012).

Although *in vivo* studies are necessary to understand the effect of insulin in a

whole-body context, a key limitation of this approach is the lack of control over various physiological factors that potentially affect insulin signaling activation and glucose uptake. As a solution, Song *et al.* performed a series of *ex vivo* experiments on isolated rat epitrochlearis muscle exposed to supraphysiological (120nM) insulin and discovered IR phosphorylation and PI3K activity increased above basal levels after 3 minutes and Akt phosphorylation after 6 minutes, all maintained until the last time point of 40 minutes (Song et al., 1999). Additionally, another group found that supra-physiological (120nM) insulin in rat epitrochlearis increased Akt-Ser473 and AS160 phosphorylation above basal levels at 5 and 10 minutes, but not at the earlier time points of 1 or 2.5 minutes (Bruss et al., 2005).

Insulin-stimulated glucose uptake over time. In human *in vivo* studies, a standard way of measuring skeletal muscle glucose uptake is with the femoral arterio-venous approach. In this approach, subjects are infused with insulin and then femoral artery blood flow is measured, along with blood glucose in the femoral artery and vein, which allows calculation of the glucose uptake across the leg, the majority of which is skeletal muscle (Baron et al., 1994; McConell et al., 2020; Thiebaud et al., 1982). With this method, when measuring glucose uptake in 20 minute intervals after initiating insulin, several studies have demonstrated that a ~3 fold increase in skeletal muscle insulin-stimulated glucose uptake begins 20 minutes after insulin infusion (R. A. DeFronzo et al., 1985; Ralph A DeFronzo et al., 1981; L. Høeg et al., 2009; Thiebaud et al., 1982) and plateaued at 40 minutes onward (R. A. DeFronzo et al., 1985; Ralph A DeFronzo et al., 1981). Similarly, when skeletal muscle glucose uptake was measured at 15 minute intervals, it also increased above basal at 15 minutes after

starting insulin infusion (L. D. Høeg et al., 2011; McConell et al., 2020; Pehmøller et al., 2012; Sjøberg et al., 2017). Notably, whether it increases earlier than 15 min has not been tested.

Summary. Activation of different components of the insulin signaling pathway occurs at different time points. With that information, it is important to ask at what point in time does insulin-stimulated glucose uptake occur, increase, and plateau. Although *in vivo* human studies suggest that this occurs as early as 10 minutes, there is still much to be done in highly controlled *ex vivo* studies. Additionally, it is just as critical to understand what factors affect this timeline, which will be discussed in the next section.

1.3 Factors affecting when skeletal muscle insulin-stimulated glucose uptake occurs, increases, and plateaus

Insulin concentration. Insulin binding and subsequent intracellular signaling is positively related to the degree of glucose uptake (Bonen et al., 1981; Le Marchand Brustel et al., 1978). Dela *et al.* mapped out that in humans, a 9, 20, and 500 fold increase in insulin results in a 5, 13, and 16 fold increase in leg glucose uptake, respectively (Dela et al., 1992). On a smaller scale, a similar pattern was described in human subjects found, such that a 2, 3.5, and 35 fold increase in insulin resulted in a 2, 12, and 20 fold increase in glucose uptake, respectively (K. J. Mikines et al., 1991). Additionally, *ex vivo* studies in rat skeletal muscle support these *in vivo* results in humans, with low physiological insulin concentrations (0.18-1.2 nM) versus high, supra-physiological insulin concentrations (12-30 nM) resulting in a ~0.8 to 2.5 fold

and ~0.4 to 3 fold increase in slow and fast-twitch muscles, respectively (J. Kim et al., 2006; Naveen Sharma et al., 2011). These studies indicate that the relationship between insulin concentration and quantity of glucose uptake is not linear but logarithmic, where low-dose insulin causes a relatively high increase in glucose uptake compared to no insulin, whilst high-dose insulin results in a smaller, but still significant, increase in glucose uptake compared to low-dose.

Skeletal muscle fiber types. In mammalian skeletal muscle, there are three distinct fiber types classified: the fast-twitch glycolytic (type IIb and IIx [in humans]), fast-twitch oxidative-glycolytic (type IIa), and slow-twitch oxidative (type I) (Gorza, 1990; Peter et al., 1972). Large mammals, including humans, do not have type IIb fibers most likely as a result of an evolutionary change to conserve energy as type IIb fibers are the fastest contracting and most fatigable (Cari M. Tellis, Clark Rosen, Apurva Thekdi, 2004; Stienen et al., 1996). Fast-twitch, which includes the glycolytic and oxidative-glycolytic, and slow-twitch fibers have markedly different metabolic and physiologic contractile properties (Bonen et al., 1981; Close, 1972; Peter et al., 1972). Although the differences in mechanical properties and contractility of fiber types are beyond the scope of this Thesis, there are several excellent reviews which cover this topic (Pette & Staron, 2000; Y. Wang & Pessin, 2013; Yan et al., 2011; Zierath & Hawley, 2004). Common skeletal muscles utilized in mouse studies include the soleus (I: 58%, IIa: 42%, IIb: 0%), which is composed primarily of slow-twitch fibers, versus extensor digitorum longus (EDL) (I: 0%, IIa: 59%, IIb: 41%), which is primarily composed of fast-glycolytic and fast oxidative-glycolytic fibers (Augusto et al., 2004; Burkholder et al., 1994). The size and fiber composition of these muscles make them

for studying the role of fiber type for both *in vivo* and *ex vivo* mouse studies.

A key difference between slow and fast-twitch muscles is the quantity of glucose uptake in response to an insulin concentration ranging from 0.3nM to 130nM, with the former being greater than the latter (Bonen et al., 1981; D. E. James, Burleigh, et al., 1985; D. E. James, Jenkins, et al., 1985; Kern et al., 1990; J. Kim et al., 2006). For example, when comparing glucose uptake at various insulin concentrations, James *et al.* found that a 12-fold increase in systemic insulin (0.324 nM to 4.02 nM) *in vivo* causes a 2 fold and 0.8 fold increase in slow and fast-twitch muscle, respectively (D. E. James, Jenkins, et al., 1985). Conditions such as skeletal muscle insulin resistance do not affect this difference (Pataky, Van Acker, et al., 2019), which supports the presence of an intrinsic, molecular mechanism for fiber type differences. Major factors which contribute to this difference include, but are not limited to, insulin sensitivity (Bonen et al., 1981; Hom & Goodner, 1984; D. E. James, Jenkins, et al., 1985; Song et al., 1999), GLUT4 abundance (Daugaard & Richter, 2001; Henriksen et al., 1990; Kern et al., 1990), and oxidative capacity (Jóhannsson et al., 1996), all of which are greater in slow versus fast-twitch muscles.

Sexual dimorphism. In the 20th century, an overwhelming majority of studies only had male participants until 1993, when the National Institute of Health (NIH) mandated the enrollment of women in clinical trials (Beery & Zucker, 2011). Although both sexes were then included in human studies, ~34% analyzed sex differences, and with no similar initiatives in animal research, only ~15% of studies on animals include both sexes (Beery & Zucker, 2011; Hayes & Redberg, 2008). As male results in both human and animal studies get generalized to the entire population and potential sex

differences are ignored statistically, women's health is negatively impacted due to inaccurate information (Correa-de-Araujo, 2006; Holdcroft, 2007). As of 2021, there has been no significant increase in the number of studies including both sexes with a sex difference analysis since these statistics have been published.

Across multiple populations, men have lower skeletal muscle, but not adipose tissue, insulin sensitivity (Kuhl et al., 2005; Magkos et al., 2010; Nuutila et al., 1995) and a lower abundance of hexokinase II mRNA in vastus lateralis muscle (L. D. Høeg et al., 2011; Houmard et al., 1995), which potentially contribute to their higher rate of glucose homeostasis abnormalities (Yki-Järvinen, 1992). Although understanding the mechanisms of sex hormones and the role they play in contributing to skeletal muscle insulin sensitivity is important, it is beyond the scope of this Thesis. However, there are excellent reviews which cover the subject (Ding et al., 2006; Liu & Sun, 2018; Maric-Bilkan, 2017; Shepard, 2019). These whole-body and molecular differences ultimately contribute to females having ~30% greater insulin-stimulated glucose uptake by skeletal muscle than males in both humans and rodents (L. Høeg et al., 2009; J. Kim et al., 2006; Lundsgaard & Kiens, 2014; Nuutila et al., 1995; Yki-Järvinen, 1992).

Summary. Insulin concentration, muscle fiber type, and sex are all mediating factors to consider when studying insulin-stimulated glucose uptake in skeletal muscle. These differences occur through various components of the insulin signaling pathway, from insulin sensitivity to intracellular proteins such as hexokinase II. As these factors affect key elements essential to insulin signaling, it is critical to have a greater understanding of how they modulate glucose uptake.

1.4 Gaps in knowledge

It has been a hundred years since the discovery of insulin and its fundamental importance to the regulation of glucose homeostasis. Insulin stimulates glucose uptake by binding to the IR at the cell surface and activating an intracellular signaling cascade that results in increased GLUT4 translocation to, and fusion with, the plasma membrane and, consequently increased glucose influx to the cell. Although the timeline of the signaling cascade (ie. IR phosphorylation, PI3K activation, etc.) has been thoroughly studied, there is a lack of systematic and highly-controlled data measured insulin-stimulated glucose uptake in skeletal muscle over time, the most important and final step to the cascade, particularly in *ex vivo* models. Specifically, the number of studies investigating the insulin signaling pathway in mouse models is abundant, yet to our knowledge, no studies have measured temporal changes in glucose uptake in mouse skeletal muscle. There are several studies addressed above which measured glucose uptake over time *in vivo* in humans and rats (Björntorp & Sjöström, 1978; R. A. DeFronzo et al., 1985; Ralph A DeFronzo et al., 1981; L. D. Høeg et al., 2011; Hom & Goodner, 1984; D. E. James, Burleigh, et al., 1985; D. E. James, Jenkins, et al., 1985; Kraegen et al., 1985; McConell et al., 2020; Thiebaud et al., 1982), however these studies do not allow for the precision and control of environmental factors, glucose delivery, insulin exposure, and more. Additionally, insulin concentration, fiber type, and sex are variables that clearly impact insulin-stimulated glucose uptake by skeletal muscle, yet systematic investigation of how they do so, especially in the context of time, has not been described in the literature. Thus, a

clear gap in knowledge in the field is not only how insulin-stimulated glucose uptake by mouse skeletal muscle changes over time, but also, what are the interactive effects of insulin concentration, fiber type, and sex.

1.5 Research objective and hypothesis of this Thesis

Although mouse models have become the predominant model for studying skeletal muscle insulin action and the etiology of insulin resistance, there is a critical lack of literature on the effect of different biological factors on mouse skeletal muscle glucose uptake. Therefore, the primary objective of this Thesis is to investigate the interactive effect of time, sex, fiber type, and insulin concentration on basal and insulin-stimulated glucose uptake in mouse skeletal muscle. The approach we will use to assess glucose uptake will be the *ex vivo* dual-radioactive tracer approach. With this approach, ^{3}H-2-deoxyglucose will be used to assess glucose uptake by skeletal muscle and ^{14}C-mannitol will be used as a control for glucose uptake that occurs independent of facilitated diffusion. Specifically, the soleus and EDL from each leg will be dissected and incubated *ex vivo*, with one side serving as the "basal" leg (i.e. no insulin) and the contralateral side being exposed to insulin. To address sex differences, we will study male and female wild-type C57BL6 mice. Additionally, a mouse model was chosen as it is a commonly used model organism because insulin signaling and action in mouse skeletal muscle closely replicates that seen in human skeletal muscle. To study fiber type, we will study the soleus (slow-twitch) and EDL (fast-twitch). To study insulin concentration, the "insulin" muscle will be incubated with a physiological (0.36nM) or supraphysiological (6nM) insulin concentration. Across these three factors, we will get

insight into the role of time by studying glucose uptake after an incubation time of 5, 10, 15, 20 and/or 30 minutes; the study design is outlined in Figure 2.

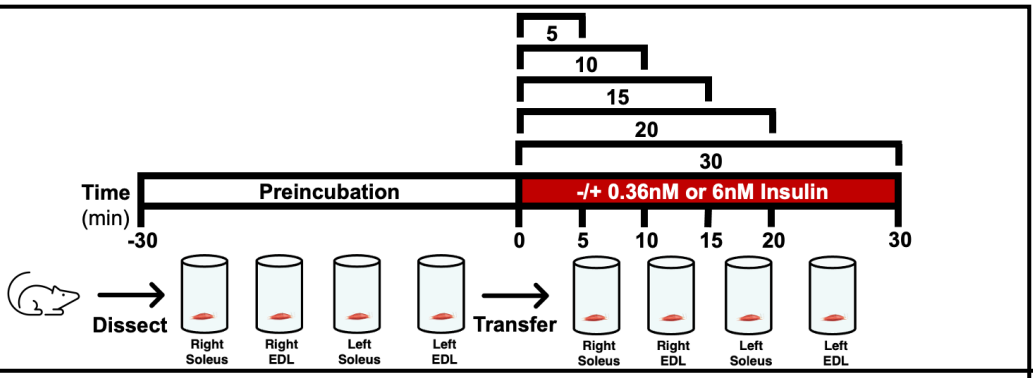

Figure 2. Method of incubation. Muscles will be dissected and immediately transferred into vials individually with pre-incubation solution for 30 minutes; this solution will not contain any insulin. Afterwards, they will be transferred into a second vial with incubation solution with (left) or without (right) insulin. After 5, 10, 15, 20, 30 minutes, muscles with 0.36nM (and corresponding "basal" muscles") will be taken out of the vial, blotted, trimmed, and frozen. After 5, 10, and 20 minutes, 6nM (and corresponding "basal" muscles") will be taken out of the vial, blotted, trimmed, and frozen.

My hypotheses are as follows: *1)* Insulin-stimulated glucose uptake will be greater than basal glucose uptake at 15, 20, and 30 minutes in both muscles based on how human studies show differences in leg glucose uptake starting at 15 minutes (Björntorp & Sjöström, 1978; McConell et al., 2020; Pehmøller et al., 2012); *2)* In both muscle types, glucose uptake will be higher in 6nM versus 0.36nM at 5, 10, and 20 minutes based on the hypothesis a higher insulin concentration will bind to a greater quantity of insulin receptor from the start of insulin exposure, causing a more robust activation of the signaling cascade; *3)* Comparing fiber types, soleus will have greater insulin-stimulated glucose uptake than EDL at 15, 20, and 30 minutes with insulin, again based on the studies mentioned above which found leg glucose uptake begins at 15 minutes. 4) Comparing sexes, glucose uptake will be greater in females than males at

5, 10, and 20 minutes with insulin in soleus and EDL as previous studies found female humans (Nuutila et al., 1995; Yki-Järvinen, 1992) and rats (Hevener et al., 2002; Rattanavichit et al., 2016) have greater insulin-stimulated glucose uptake than males. Additionally, we will use 6nM insulin to compare males and females, and so as mentioned previously in my concentration differences hypothesis, I hypothesize sex differences will be apparent immediately.

RESULTS

Effect of time on 2DOGU in response to a physiological (0.36nM) insulin concentration.

Absolute 2DOGU ($2DOGU^{Absolute}$; i.e. total amount of 2DOGU during the incubation period) with insulin was statistically greater than basal $2DOGU^{Absolute}$ at 15, 20 and 30 minutes in soleus and EDL, but was not different at 5 and 10 min (**Figures 4A and 4C**, respectively). The rate of 2DOGU on a per minute basis (i.e. rate per minute; $2DOGU^{Rate/min}$), which was calculated by dividing 2DOGU by the duration of incubation, was statistically greater with insulin versus basal at 15, 20 and 30 minutes, but not 5 or 10 minutes in the soleus (**Figure 4B**) and at 10, 15, 20, and 30 minutes, but not 5 minutes, in the EDL (**Figure 4D**). The basal $2DOGU^{Rate/min}$ increased at 10 and 15 minutes and plateaued at 20 and 30 minutes in the soleus (**Figure 4B**), while the EDL was not different at 5, 10, 15, 20, and 30 minutes (**Figure 4D**).

Effect of time on 2DOGU in response to a supraphysiological (6nM) insulin concentration.

In response to 6nM insulin, $2DOGU^{Absolute}$ was significantly greater than basal at 10 and 20 minutes, but not different at 5 minutes, in soleus and EDL from male (**Figures 5A and 5C,** respectively) and female (**Figures 5E and 5G**, respectively) mice. The $2DOGU^{Rate/min}$ was significantly greater than basal at 5, 10 and 20 min in the soleus of male and female mice (**Figures 5B and 5D**, respectively), whilst in the EDL it was significantly higher than basal at 10 and 20 minutes in both sexes, but not at 5 minutes (**Figures 5F and 5H**, respectively). Although basal $2DOGU^{Rate/min}$ plateaued

at 10 and 20 minutes in the male soleus (**Figure 5B**) and female soleus and EDL (**Figure 5D and 5H**, respectively), it increased at 10 minutes while plateauing at 20 minutes in male EDL (**Figure 5F**).

Effect of fiber type on insulin-stimulated glucose uptake.

In all mice, we measured temporal changes in $2DOGU^{Absolute}$ in soleus and EDL, thus allowing us to study the effect of fiber type on $2DOGU^{Absolute}$. To analyze if there was an effect of fiber type on insulin-stimulated glucose uptake (IS-$2DOGU^{Absolute}$), which was calculated by subtracting Basal-$2DOGU^{Absolute}$ from Insulin-$2DOGU^{Absolute}$, we compared the EDL versus soleus data from Figures 5 and 6. First, when studying the $2DOGU^{Absolute}$ data using 0.36nM in female mice, there was a main effect of fiber type and time, as well as an interaction. Post-hoc analysis of 0.36nM mice revealed IS-$2DOGU^{Absolute}$ was ~1.4 fold greater in soleus versus EDL at 30 minutes, but not at 5, 10, 15, or 20 minutes (**Figure 6A**). When studying the $2DOGU^{Rate/min}$ data, there was a main effect of fiber type and time, although in the post-hoc analysis there was no effect of fiber type within a given time point (**Figure 6B**). Next, with the $2DOGU^{Absolute}$ data using 6nM in both sexes, there was a main effect of fiber type and time, as well as an interaction in male mice. In female mice, there was a main effect of fiber type and time, but not interaction. Post-hoc analysis of 6nM mice revealed IS-$2DOGU^{Absolute}$ was ~1.8 fold greater at 10 and 20 minutes, but not 5 minutes, in male mice and ~1.4 fold greater at 20 minutes, but not 5 and 10 minutes, in female mice (**Figures 6C and 6E**, respectively). Looking at the $2DOGU^{Rate/min}$ data, there was a main effect of fiber type and time, but no interaction,

in male mice and a main effect of fiber type and time, as well as an interaction, in female mice. Post-hoc analysis revealed that IS-2DOGU$^{Rate/min}$ was greater in soleus than EDL at 10 minutes, but not 5 or 20 minutes, in male mice and at 5 minutes, but not 10 or 20 minutes, in female mice (**Figures 6D and 6F**, respectively).

Comparing the effect of physiological versus maximal insulin concentration on insulin-stimulated glucose uptake.

In female mice we measured temporal changes in 2DOGU^{Absolute} using a physiological and supra-physiological insulin concentration, thus allowing us to study the role of insulin concentration on 2DOGU^{Absolute}. Using a similar method as fiber type differences, IS-2DOGU^{Absolute} values were calculated from Figures 4 and 5 with the soleus and EDL being compared separate from each other. Beginning with the 2DOGU^{Absolute} data, there was a main effect of concentration and time, as well as an interaction, for both the soleus and EDL. Post-hoc analysis found a ~2.5 fold greater IS-2DOGU^{Absolute} in 6nM than 0.36nM at 10 and 20 minutes, but not 5 minutes, for both the soleus and ~2 fold difference in the EDL (**Figures 7A and 7C**, respectively). Next, when analyzing the 2DOGU$^{Rate/min}$ data, there was a main effect of concentration and time, but no interaction, in the soleus and a main effect of concentration and time, as well as an interaction, in the EDL. Post-hoc analysis revealed 6nM had greater IS-2DOGU$^{Rate/min}$ at 5, 10, and 20 minutes in the soleus and at 10 and 120 minutes, but not 5 minutes, in the EDL (**Figures 7B and 7D**, respectively).

Comparison of insulin-stimulated glucose uptake between male and female mice.

Body weight and fasting blood glucose were greater in male compared to female mice (**Table 1**). Although blood glucose concentrations during the OGTT were not different between the sexes, glucose tolerance as assessed by OGTT AUC was better (i.e. lower AUC) in female compared to male mice (**Figures 3A and 3B**, respectively); notably, this difference was primarily driven by differences in fasting blood glucose concentration. In male and female mice, we measured temporal changes in $2DOGU^{Abolute}$ utilizing a supraphysiological insulin concentration, thus allowing us to study the effect of sex on $2DOGU^{Absolute}$. Similar to fiber type and insulin concentration comparisons, $IS\text{-}2DOGU^{Absolute}$ values were calculated from Figure 5. When analyzing the $IS\text{-}2DOGU^{Absolute}$ data, there was a main effect of sex and time, as well as interaction, in both the soleus and EDL. To elaborate, for the main effect of sex, $p=0.011$ and $p<0.001$ in the soleus and EDL, respectively, suggesting a strong role of sex. Post-hoc analysis $IS\text{-}2DOGU^{Absolute}$ was ~1.5 fold greater in females compared to males at 20 minutes, but not 5 or 10 minutes, in the soleus and EDL (**Figures 8A and 8C**, respectively). With the $2DOGU^{Rate/min}$ data, there was a main effect of sex and time, but no interaction, in soleus and EDL. For the main effect of sex, $p<0.001$ for both muscles, indicating a strong role of sex. Post-hoc analysis demonstrated that females had greater $IS\text{-}2DOGU^{Rate/min}$ than males at 5 and 20 minutes, but not 10 minutes, in the soleus and at 20 minutes, but not 5 or 10 minutes, in the EDL (**Figures 8B and 8D**, respectively).

Table 1. Fasting glucose and body weight were greater in male mice compared to female. Data were analyzed by an unpaired t-test and are presented as Mean±SEM. *, P<0.05, versus male. Unpaired t-test.

	Male	Female
n	54	66
Weight (g)	26.09 ± 0.34	20.58 ± 0.15*
Fasting glucose (mg/dL)	126.4 ± 2.5	119.7 ± 1.8*

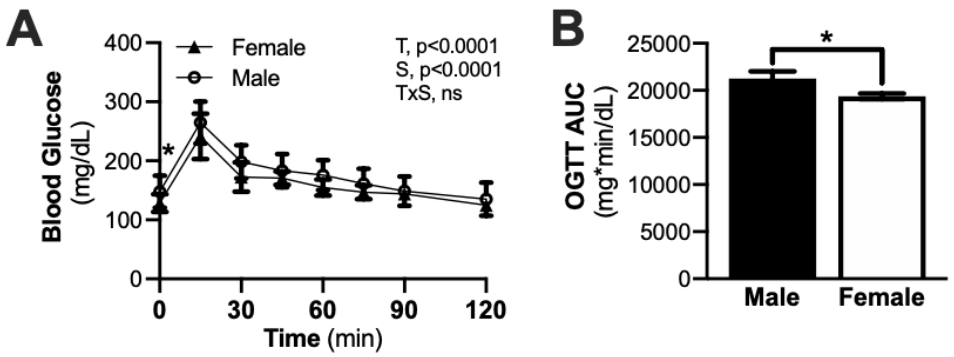

Figure 3. Glucose tolerance is greater in female versus male mice. Blood glucose concentrations **(A)** and area under the curve (AUC; **B**) of male and female mice during an oral glucose tolerance test (OGTT; 2 g/kg). Statistics: Blood glucose concentration data was analyzed by a 2-way ANOVA with multiple comparisons. AUC data was analyzed by an unpaired t-test. *P<0.05, main effect of sex. Male: n=11. Female: n=12. Data are presented as Mean±SEM.

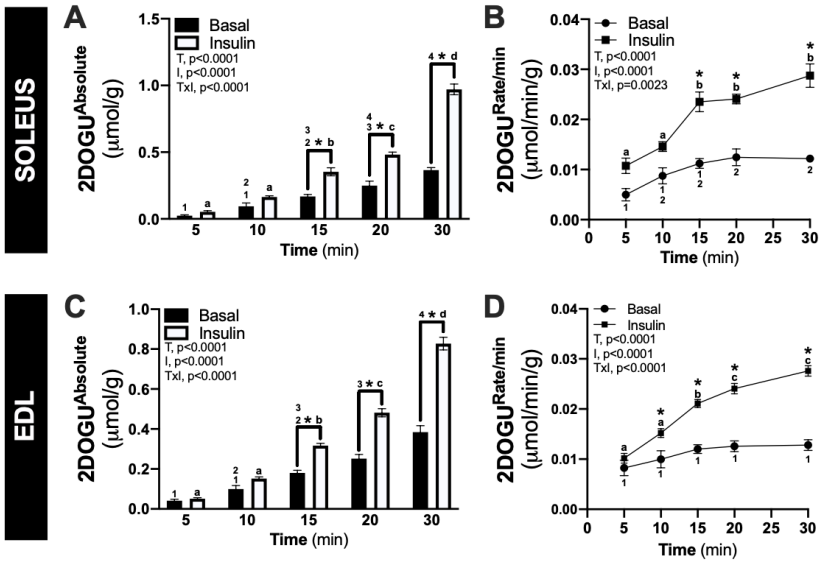

Figure 4. Physiological insulin increases 2DOGU^{Absolute} and 2DOGU^{Minute} at 10, 15, 20, and 30 minutes. Basal 2DOGU^{Absolute} and insulin 2DOGU^{Absolute} paired in the soleus **(A)** and extensor digitorum longus (EDL; **C**) alongside 2DOGU^{Minute} (2DOGU divided by time) in the soleus **(B)** and EDL **(D)** of female mice. Statistics: Data were analyzed by a 2-way ANOVA with multiple comparisons. *P<0.05, main effect of insulin. Different numbers indicate a difference in basal 2DOGU values over time (P<0.05). Different letters indicate a difference in insulin 2DOGU values over time (P<0.05). T, main effect of time; I, main effect of insulin; T x I, interaction between main effects. Soleus: 5 min, n=7; 10 min, n=8; 15 min, n=8; 20 min, n=7; 30 min, n=6. EDL: 5 min, n=6; 10 min, n=8; 15 min, n=8; 20 min, n=8; 30 min, n=7. Data are presented as Mean±SEM.

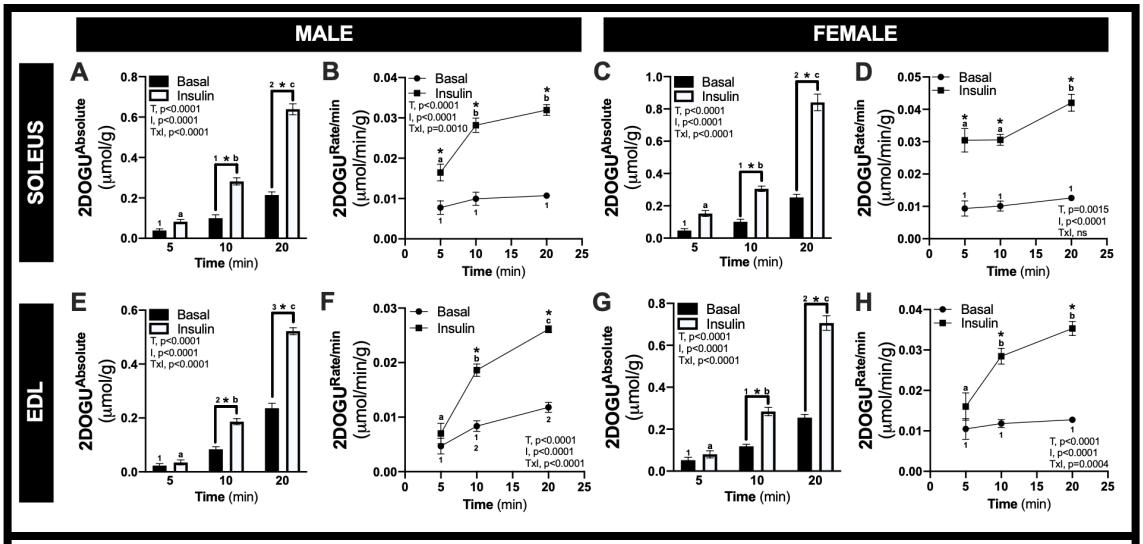

Figure 5. Maximal insulin increases 2DOGU^{Absolute} at 10 minutes and 2DOGU^{Minute} at 5 minutes in soleus and 10 minutes in EDL. Basal 2DOGU^{Absolute} and insulin 2DOGU^{Absolute} paired in the soleus **(A)** and extensor digitorum longus **(E)** of male mice and the soleus **(C)** and EDL **(G)** of female mice alongside basal and insulin 2DOGU^{Minute} of male soleus **(B)** and EDL **(F)** followed by female soleus **(D)** and EDL **(H)** rates. Statistics: Data were analyzed by a 2-way ANOVA with multiple comparisons. *P<0.05, main effect of insulin. Different numbers indicate a difference in basal 2DOGU values over time (P<0.05). Different letters indicate a difference in insulin 2DOGU values over time (P<0.05). T, main effect of time; I, main effect of insulin; T x I, interaction between main effects. Male soleus: 5 min, n=7; 10 min, n=8; 20 min, n=8. Male EDL: 5 min, n=7; 10 min, n=8; 20 min, n=8. Female soleus: 5 min, n=5; 10 min, n=6; 20 min, n=8. Female EDL: 5 min, n=5; 10 min, n=7; 20 min, n=8. Data are presented as Mean±SEM.

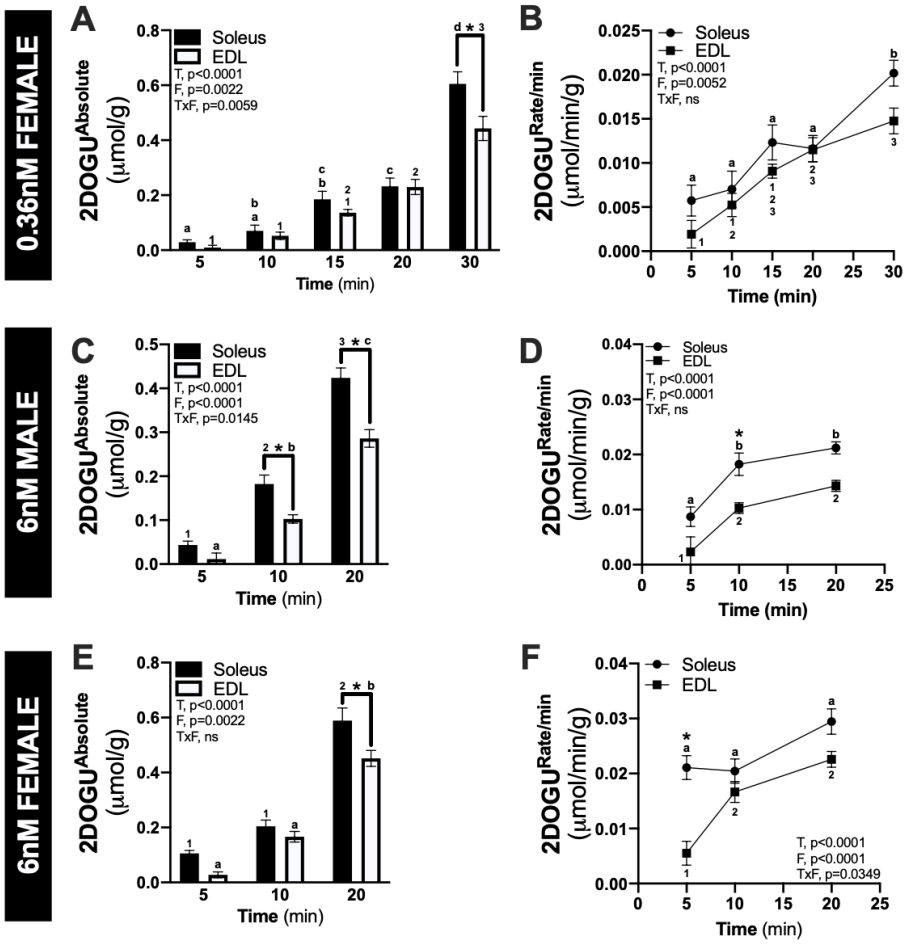

Figure 6. Insulin-stimulated glucose uptake is greater in soleus compared to EDL muscle in male and female mice. Insulin-stimulated 2DOGU (i.e., insulin 2DOGU minus basal 2DOGU; IS-2DOGU) of soleus versus EDL in female mice with physiological **(**0.36nM; **A)** and maximal **(**6nM; **E)** insulin concentration and male mice with maximal insulin **(C)**. IS-2DOGU^{Minute} in soleus and EDL muscle of female mice with physiological **(B)** and maximal **(F)** insulin and male mice with maximal **(D)** insulin. Statistics: Data were analyzed by a 2-way ANOVA with multiple comparisons. *P<0.05, main effect of fiber type. Different numbers indicate a difference I-Stim 2DOGU values over time in the soleus (P<0.05). Different letters indicate a difference in I-Stim 2DOGU values over time in the EDL (P<0.05). T, main effect of time; F, main effect of fiber type; T x F, interaction between main effects. Physiological female soleus: 5 min, n=7; 10 min, n=8; 20 min, n=7. Physiological female EDL: 5 min, n=6; 10 min, n=8; 20 min, n=8. Maximal male soleus: 5 min, n=7; 10 min, n=8; 20 min, n=8. Maximal male EDL: 5 min, n=7; 10 min, n=8; 20 min, n=8. Maximal female soleus: 5 min, n=5; 10 min, n=6; 20 min, n=8. Maximal female EDL: 5 min, n=5; 10 min, n=7; 20 min, n=8. Data are presented as Mean±SEM.

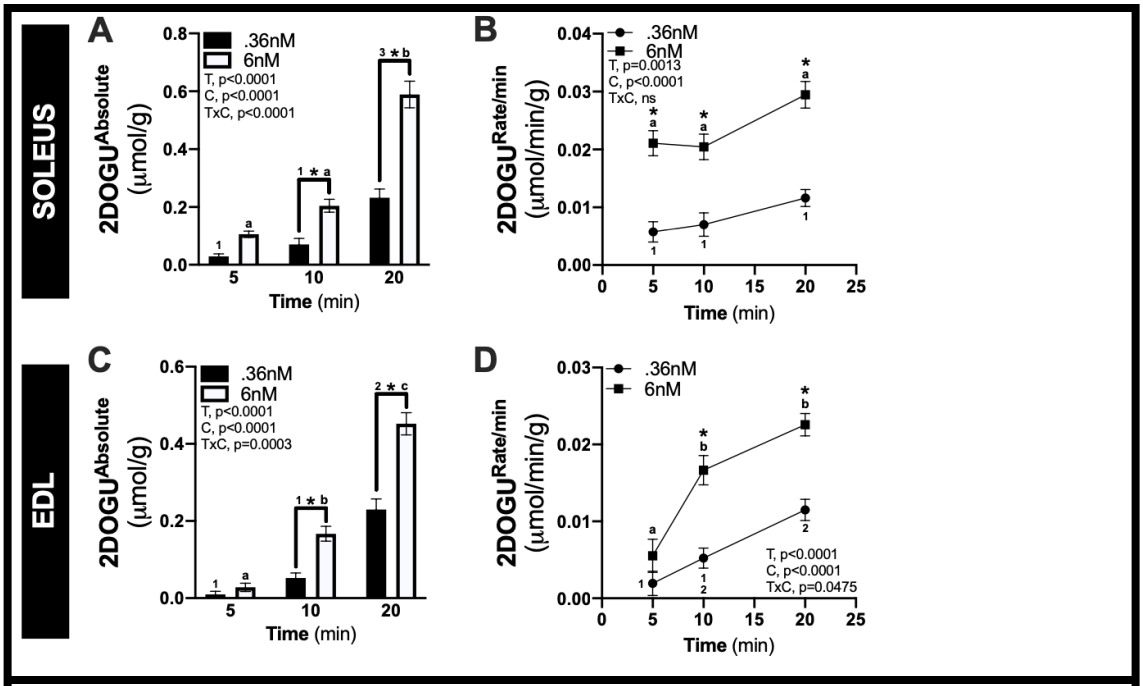

Figure 7. Insulin-stimulated glucose uptake is greater with maximal than physiological insulin concentration in female mice. IS-2DOGU^{Absolute} of physiological versus maximal insulin concentration in soleus **(A)** and EDL **(C)** muscle of female mice alongside IS-2DOGU^{Minute} in soleus **(B)** and EDL **(D)**. Statistics: Data were analyzed by a 2-way ANOVA with multiple comparisons. *P<0.05, main effect of insulin concentration. Different numbers indicate a difference IS-2DOGU values over time with a physiological concentration (P<0.05). Different letters indicate a difference in IS-2DOGU values over time with a maximal concentration (P<0.05). T, main effect of time; C, main effect of concentration; T x C, interaction between main effects. Physiological soleus: 5 min, n=7; 10 min, n=8; 20 min, n=7. Physiological EDL: 5 min, n=6; 10 min, n=8; 20 min, n=8. Maximal soleus: 5 min, n=5; 10 min, n=6; 20 min, n=8. Maximal EDL: 5 min, n=5; 10 min, n=7; 20 min, n=8. Data are presented as Mean±SEM.

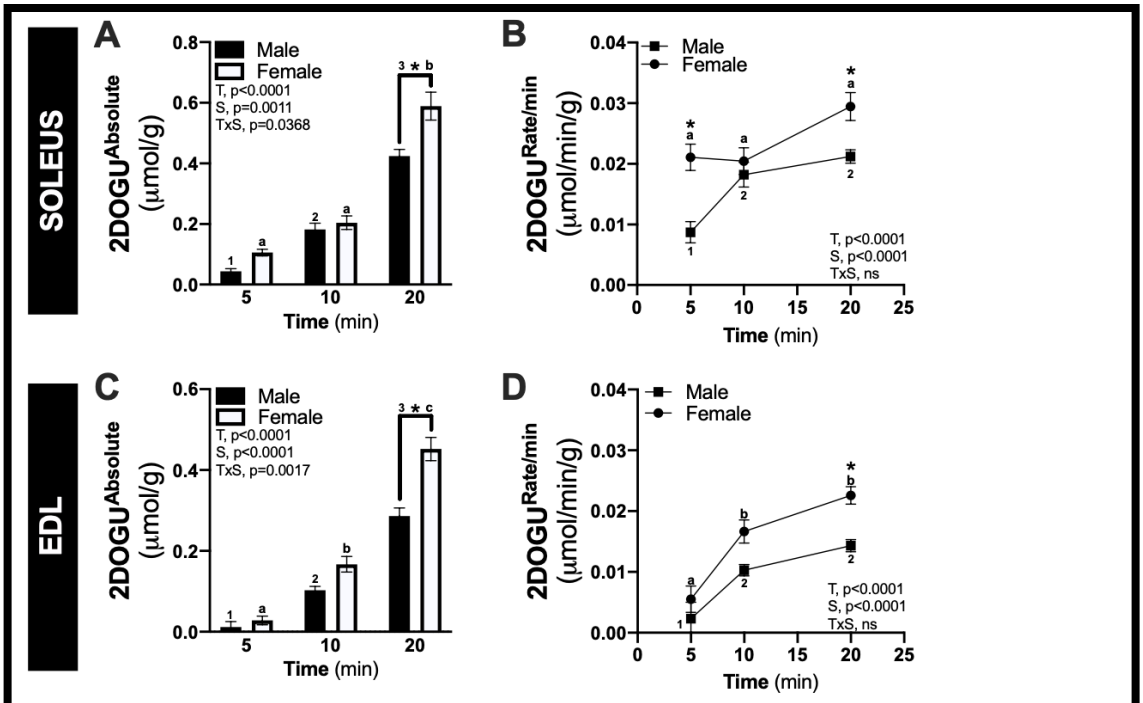

Figure 8. Insulin-stimulated glucose uptake is greater in female mice compared to male. IS-2DOGU^{Absolute} of male versus female mice in soleus **(A)** and EDL **(C)** alongside IS-2DOGU^{Minute} in soleus **(B)** and EDL **(D)**. Statistics: Data were analyzed by a 2-way ANOVA with multiple comparisons. *P<0.05, main effect of sex. Different numbers indicate a difference IS-2DOGU values over time within male mice (P<0.05). Different letters indicate a difference in IS-2DOGU values over time within female mice (P<0.05). T, main effect of time; S, main effect of sex; T x S, interaction between main effects. Male soleus: 5 min, n=7; 10 min, n=8; 20 min, n=8. Male EDL: 5 min, n=7; 10 min, n=8; 20 min, n=8. Female soleus: 5 min, n=5; 10 min, n=6; 20 min, n=8. Female EDL: 5 min, n=5; 10 min, n=7; 20 min, n=8. Data are presented as Mean±SEM.

DISCUSSION

A fundamental role of insulin is its effects on glycemic control during the postprandial period (Kahn, 1996; Le Marchand Brustel et al., 1978; Yau et al., 2012). As part of this role, a key tissue that underlies the glycemic controlling effects of insulin is skeletal muscle, with as much as ~85% of insulin-stimulated peripheral glucose disposal being in skeletal muscle (R. A. DeFronzo et al., 1985; Ralph A DeFronzo et al., 1981; Thiebaud et al., 1982; Zorzano et al., 2005). Considering the overall importance of glycemic control to health (Cramer & Pugh, 2005; Donnelly et al., 2007; Facchini et al., 2001; Schaper et al., 2017), understanding the physiological effects of insulin on skeletal muscle is of great importance. Over the past 30 years, mouse models have developed into the predominant model for studying the biology of skeletal muscle insulin action and, by extension, the etiology of insulin resistance and T2D. Remarkably, however, to our knowledge, no study has systematically studied the individual and interactive effects of time, sex, insulin concentration and fiber type on insulin-stimulated glucose uptake on mouse skeletal glucose uptake, which are all critical variables when considering the biological actions of insulin. Addressing this gap in knowledge, the aim of this Thesis was to study temporal changes in insulin-stimulated glucose uptake in two muscles of differing fiber types (i.e., soleus and EDL) of male and female mice at an insulin concentration of 0.36nM or 6nM. The primary findings from this work were: *1)* insulin-stimulated glucose uptake in skeletal muscle was greater than basal glucose uptake after 15 minutes of 0.36nM insulin and after 10 minutes of 6nM insulin; *2)* slow-twitch soleus had greater insulin-stimulated glucose uptake than fast-twitch EDL at both insulin concentrations and regardless of

sex; *3)* insulin-stimulated glucose uptake was higher after 20 minutes of in with 6nM versus 0.36nM insulin; *4)* insulin-stimulated glucose uptake was greater in female compared to male skeletal muscle, regardless of fiber type.

Insulin increases glucose uptake by skeletal muscle, although this increase is not "instantaneous" (Björntorp & Sjöström, 1978; R. A. DeFronzo et al., 1985; L. Høeg et al., 2009; McConell et al., 2020; Thiebaud et al., 1982). This lag in uptake between an increase in insulin and an increase in glucose uptake is due to the temporal lag between the binding of insulin to the insulin receptor, transduction of this signal via the insulin signaling cascade and ultimately fusion of GLUT4 with the plasma membrane (Jaldin-Fincati et al., 2017; Rothman et al., 1995; Sun et al., 2014). For example, previous studies in humans demonstrated that within 10-15 minutes of initiating an increase in plasma insulin concentration to a physiological (100uU/mL) there is a significant increase in skeletal muscle glucose uptake above basal (Björntorp & Sjöström, 1978; McConell et al., 2020; Pehmøller et al., 2012). In line with this, in this study, which to our knowledge is the first to study temporal changes in basal and insulin-stimulated glucose uptake in skeletal muscle, insulin $2DOGU^{Absolute}$ did not increase above basal until ~10-15 minutes after initiating insulin exposure (but was not higher at 5 minutes), and this effect was mediated by fiber type, sex, and insulin concentration. For example, when studying the effects of insulin concentration, at 0.36nM $2DOGU^{Absolute}$ was greater than basal at 15 minutes in the EDL and soleus, whilst at 6nM this increase occurred at 10 minutes. Together, these data provide important insight into temporal measurement of insulin-stimulated glucose uptake and indicate that an insulin incubation duration of at least 10-15 minutes (depending on the

concentration studied) is needed if trying to discern effects on insulin-stimulated glucose uptake.

Insulin concentration and glucose uptake have a logarithmic relationship, in which insulin increases glucose uptake rapidly at low concentrations and plateaus at high concentrations (Dela et al., 1992; Kari J Mikines et al., 1988; Richter et al., 1989). Our results support a logarithmic relationship as 6nM caused greater glucose uptake than 0.36nM, however the fold difference was not greater than comparing no insulin to 0.36nM. These findings are similar to a previous *ex vivo* study in rat epitrochlearis which found the same relationship between insulin concentration and glucose uptake (Naveen Sharma et al., 2011). However, our study went further to analyze when this difference occurs and we found the rate of 2DOGU (i.e., $2DOGU^{Rate/min}$) was quickly affected by concentration, with differences between 6nM $2DOGU^{Rate/min}$ increasing above 0.36nM as early as 5 minutes.

In rodent skeletal muscle, there are two major fiber types, type I (slow) and type II (fast) which vary in numerous ways including their contractile function, oxidative capacity, and as it relates to this work, insulin sensitivity (Augusto et al., 2004; Buchthal & Schmalbruch, 1970; Close, 1972; Johannesson et al., 2009; Pette & Staron, 2000; L. Wang et al., 2017; Yan et al., 2011; Zierath & Hawley, 2004). In this study, we found that the soleus (i.e., slow) had greater insulin-stimulated glucose uptake than the EDL (i.e., fast), with this difference being present in male and female mice, and with both insulin concentrations. This finding was expected, and is supported by extensive work conducted in rat (D. E. James, Jenkins, et al., 1985; Kern et al., 1990; Pataky, Yu, et al., 2019) and mouse skeletal muscle (Bonen et al., 1981;

H. Cho et al., 2001; J. Kim et al., 2006). Interestingly, by studying changes in glucose uptake at different time points, our study design allows for physiological insight into the underlying reason for the greater $2DOGU^{Absolute}$ in the soleus versus EDL. Specifically, our results reveal a clear main effect of fiber type (regardless of sex or insulin concentration) on the $2DOGU^{Rate/min}$ with it not only being higher in soleus versus EDL, but also with this increase occurring earlier. We interpret this data as suggesting that a reason for the higher 2DOGU in the soleus versus EDL is due to an earlier "ramping up" of 2DOGU. This is likely because slow-twitch fibers have greater insulin sensitivity (Bonen et al., 1981; Hom & Goodner, 1984; D. E. James, Jenkins, et al., 1985) and cellular GLUT4 abundance (Daugaard & Richter, 2001; Henriksen et al., 1990; Kern et al., 1990), contributing to the established insulin-stimulated glucose uptake difference between fiber types (Bonen et al., 1981; D. E. James, Jenkins, et al., 1985; Kern et al., 1990).

It has long been known that insulin sensitivity is higher in skeletal muscle from female compared to males, and is true in human and rodent models (Kuhl et al., 2005; Magkos et al., 2010; Nuutila et al., 1995). For example, in humans, physiological insulin (90 uU/mL) caused muscle glucose metabolism in females to be ~1.3 fold greater than in males (Yki-Järvinen, 1992). In rats, females had ~1.4 fold greater *in vivo* whole-body glucose disposal rate than males (Hevener et al., 2002), whilst in the rat soleus muscle, insulin-stimulated glucose uptake in response to a supraphysiological concentration (2mU/mL) was ~1.75 fold greater in females versus males (Rattanavichit et al., 2016). Additionally, in mice, insulin-stimulated glucose uptake was significantly greater in both the soleus and EDL of females versus males

(J. Kim et al., 2006). In line with these studies, we found that $2DOGU^{Absolute}$ was greater in the soleus and EDL of female versus male mice. Interestingly, the underlying physiological driver of this difference appears to be due to the fact that $2DOGU^{Rate/min}$ increases faster in females than males.

While we did not address this in this Thesis, an important consideration of this work is the potential molecular mechanisms that underlie these aforementioned differences we find in 2DOGU, be it absolute uptake or the rate of uptake. Ultimately, the abundance of GLUT4 fused with the plasma membrane drives skeletal muscle glucose uptake, and as such, it is reasonable to surmise that insulin-stimulated plasma membrane GLUT4 abundance (i.e., GLUT4 fused with the plasma membrane) is greater in soleus versus EDL, female versus male, 6 versus 0.36nM, and over time. However, a key question, of course, is how is there more GLUT4 fused with the plasma membrane? First, insulin signaling is requisite to GLUT4 translocation to the plasma membrane (Coster et al., 2004; Foster et al., 2001; Govers et al., 2004). Thus, the rate of transduction of insulin signaling is likely a fundamental point of control. Coupled with insulin signaling, considering there are multiple steps the regulate GLUT4 mobilization to, and fusion with, the plasma membrane, it possible that one or more steps related to the dynamics of GLUT4 retention/release from its intracellular location, GLUT4 translocation to the plasma membrane, GLUT4 docking and fusion with the plasma membrane, and/or GLUT4 retention in the plasma membrane, are important.

It is important to discuss potential limitations of this work. Our longest incubation timepoint was 30 minutes. While this is a well-established and common *ex*

vivo incubation duration to measure insulin-stimulated glucose uptake of rodent skeletal muscle (Henriksen et al., 1990; Kern et al., 1990; Pataky, Van Acker, et al., 2019; Rattanavichit et al., 2016; N. Sharma et al., 2010; Naveen Sharma et al., 2011), it is possible a longer incubation period is required to achieve peak $2DOGU^{Absolute}$ or $2DOGU^{Rate/min}$. As the 30 minute timepoint with physiological insulin showed an increase in glucose uptake compared to 20 minutes, our results suggest a longer maximal incubation period would be beneficial in investigating when glucose uptake plateaus. Additionally, although measurements of glucose uptake are important for overall physiological function and changes, we did not measure insulin signaling pathway proteins or steps important to GLUT4 mobilization and trafficking. Assessment of proteins such as phosphorylated IR or phosphorylated Akt (e.g., Serine 473 and Threonine 308) will provide insightful information about when and how quickly peak activity occurs (Alessi et al., 1996; N. Sharma et al., 2010; Song et al., 1999), and by extension, insight into the effects of insulin concentration, fiber type, and sex on whether differences in $2DOGU^{Absolute}$ or $2DOGU^{Rate/min}$.

In conclusion, this work provides an important physiological "map" of the interactive effect of time, insulin concentration, fiber type, and sex on insulin-stimulated glucose uptake in mouse skeletal muscle. This is significant, as the laboratory mouse is by far the most common research model for the mechanistic study of insulin action, yet to date, no study has systematically detailed insulin-stimulated glucose uptake in mouse skeletal muscle. Importantly, not only does this work confirm previously demonstrated effects of sex, fiber type and insulin concentration on absolute glucose uptake by skeletal muscle, it identifies important effects of these

parameters in response to insulin on the rate of glucose uptake. In terms of practical application, these data demonstrate that studies investigating glucose uptake should at least be for ~10-15 minutes in duration, as this is the earliest time point at which we found a significant increase demonstrate insulin-stimulated glucose uptake. Finally, it will be of great interest of future work to assess the interrelationship between temporal changes in the activation of insulin signaling and the amount and rate of insulin-stimulated glucose uptake by skeletal muscle, and how this is differentially affected by sex, fiber type and insulin concentration.

MATERIALS AND METHODS

Animals

All studies were conducted in male and female C57BL/6NJ mice (The Jackson Laboratory, Stock No: 005304) that were 12-15 weeks old. Mice were housed (12:12-h light-dark cycle) at room temperature (~21°C) in a conventional vivarium facility and had ad libitum access to chow (catalog no. 7912, irradiated; Envigo Teklad) and water. Procedures were carried out with the approval of, and in accordance with, the Animal Care Program and Institutional Animal Care and Use Committee at the University of California, San Diego.

Oral glucose tolerance test

Following a 4 h fast, fasting blood glucose concentration was measured via the tail vein. Then, mice were orally gavaged with dextrose (2 g/kg) and blood glucose concentration was measured at 15, 30, 45, 60, 75, 90, and 120 min after gavage. Blood glucose was measured using a handheld glucose meter (Ascensia Contour, Bayer HealthCare, Mishawaka, IL). Area under the curve (AUC) was calculated using Prism 9 (GraphPad Software, La Jolla, CA) with 0 mg/dl used as the baseline.

Ex vivo 2-deoxy glucose uptake (2DOGU)

Following a 4 h fast, mice were anesthetized (ketamine, 25mg/kg; acepromazine, 1mg/kg; xylazine, 2 mg/kg) via intraperitoneal injection. Soleus and extensor digitorum longus (EDL) muscles from both legs were dissected and transferred to individual flasks containing oxygenated (95% O_2, 5% CO_2) Krebs-

Henseleit buffer (KHB) with 2 mM sodium pyruvate and 6 mM mannitol (PreInc-KHB) for 30 minutes at 35°C. Subsequently, muscles were transferred to a second flask with a solution that included KHB containing 1 mM 2DG, 9 mM mannitol, [^{14}C]-mannitol (0.053 mCi/mmol; American Radiolabeled Chemical [ARC]) and [^{3}H]-2DG (3 mCi/mmol; ARC) (Inc-KHB), with muscles from one side being incubated in physiological (0.36 nM) or supra-maximal (6 nM) insulin (HumulinR, Eli Lilly and Company) and the contralateral side being incubated without insulin. The duration of incubation in Inc-KHB was 5, 10, 15, 20, or 30 minutes. Immediately after the incubation period, the muscles were blotted on filter paper, trimmed, rapidly frozen in liquid nitrogen and stored (–80°C). 2DOGU was calculated as previously described (Martins et al., 2019; McCurdy & Cartee, 2005; Schenk et al., 2011). To account for cumulative 2DOGU over time, rate values were calculated by dividing 2DOGU by the duration of incubation in minutes.

Muscle homogenization

Soleus and EDL were homogenized (Bullet Blender Tissue Homogenizer, Next Advance #BT24M) in 500µL of ice-cold homogenization buffer (50 mm Tris, pH 7.5, 250 mm sucrose, 1 mm EDTA, 1 mm EGTA, 1% Triton X-100, 50 mm NaF, 1 mm $NaVO_2$ $Na_2(PO_4)_2$, and 0.1% DTT) containing phosphatase inhibitor cocktail (PIC) 2 (MilliporeSigma #P5726), PIC 3 (MilliporeSigma #P0044), Complete (MilliporeSigma #11836170001), 1 mM trichostatin A (Cell Signaling #9950S), 1M nicotinamide (MilliporeSigma #N0636), and 1 mM Pefabloc SC PLUS (MiliporeSigma #11873601001). After homogenization, muscles were rotated for 2

hours at 4°C followed by centrifugation (14,167 g) for 20 minutes at 4°C. The supernatant was collected and stored at -80°C for counting for 2DOGU.

Statistics

Statistical analyses were performed using Prism 9 (GraphPad Software Incorporated, La Jolla, CA, USA). All data were analyzed using an unpaired Student's t-test, ordinary 1-way analysis of variance (ANOVA) or 2-way ANOVA followed by a Tukey's post-hoc test for multiple comparisons, with significant differences at $P < 0.05$. All data are expressed as mean±SEM.

This thesis, in whole, is currently being prepared for submission for publication of the material. Park, Ji E.; Schenk, Simon. The thesis author was the primary investigator and author of this material.

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