Retinoic Acid, Insulin, and Cycloheximide Alter Glycogen Homeostasis in Differentiating L6 Myoblasts

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To the Graduate Council:

I am submitting herewith a dissertation written by Aaron Armstrong entitled "Retinoic Acid, Insulin, and Cycloheximide Alter Glycogen Homeostasis in Differentiating L6 Myoblasts." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Nutritional Sciences.

Jiangang Chen, Major Professor

We have read this dissertation and recommend its acceptance:

Guoxun Chen, Ahmed Bettaieb, Ling Zhao, Jiangang Chen, Jun Lin

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)
Dedication

For my wife Tessa, who is so much stronger than I will ever be. And for my daughter Eleanor, may you only ever receive the love we have to offer, and none of the things which make you understand what that means.
Acknowledgements

I reflect on the transformation which I have undergone during my time at the University of Tennessee. I am the beneficiary of immense privilege. I have overcome an unlikely beginning in the heart of destitute southern poverty to arrive where I now stand. It was not within my capability to accomplish this unaided. Thus, my matriculation is evidence of the tremendous patience, generosity, and kindness of my family, my committee, and most of all my beloved wife Tessa and daughter Eleanor.

The metamorphosis of my perspective from potential and promises into a discerning consumer of information capable of solving problems is only more profound when considered within the context of our modern society. Upon my acceptance into UTK I had the privilege of receiving Dr. Guoxun Chen’s mentorship. Unbeknownst to me, his brilliance and generosity required a restructuring of the substance of my character in ways for which I was wholly unprepared. His patience, and my perseverance, enabled enough of his philosophy to imprint upon my reasoning, an inheritance which I can only honor by paying forward. Each conversation we had broadened my view as I saw him suffer for his principles. His mind has shaped my life in ways that I have only begun to discover, and I cannot represent my gratitude for him within the constraints of this document.

I am also grateful for Dr. Jiangang Chen and his willingness to provide mentorship after Dr. Guoxun Chen’s departure. His intensity, tenacity, kindness, and direct demeanor are responsible in no small part for my completion of this endeavor. I am equally fortunate to have been exposed to his mind, if even for a short time. Dr. Ling Zhao has likewise given me her patience, understanding, and guidance as I struggled
with the traumatic birth of my daughter and my self-doubt through the years of hopelessness and frustration. Dr. Ahmed Bettaieb provided his guidance and resources to help me succeed when I became overwhelmed and saw no pathway forward. I also thank Dr. Jun Lin for lending his expertise to my efforts by providing training and direction in my experimental designs.

Finally, I would like to thank everyone else who has helped me arrive at this point. I thank Nichole Steltenkamp for her patience and diligence in ensuring that the Department of Veterans Affairs fulfilled its commitment to my education. I thank my many lab mates who have helped me through the darkest times of my life so far. It has been bittersweet to watch each of them leave in pursuit of the careers which they earned. I thank the department of nutrition, as each faculty and staff member has played a role in my arriving at this precipice.

“If I have seen further, it is by standing on the shoulders of giants.” – Sir Isaac Newton

I have had the privilege of meeting a few giants for myself during this journey.

I thank them for holding me up when I lacked the strength to do so by myself.
Abstract

Glycogen synthesis in skeletal muscle (SM) is responsible for the majority of post-prandial glucose disposal in healthy individuals. In type 2 diabetes mellitus (T2DM), compromised skeletal muscle glycogen synthesis contributes to hyperglycemia. L6 rat SM cells can develop insulin resistance with prolonged insulin stimulation and provide an in vitro model for mechanistic dysfunction in glycogen homeostasis. SM glycogen is synthesized by glycogen synthase (GS) in response to insulin stimulation. Canonically, insulin signaling increases GS activity through inhibition of its Ser641 regulator glycogen synthase kinase-3β (GSK3β) by protein kinase B (AKT). Proximal insulin signaling activates AKT via Ser473 phosphorylation which proceeds to inhibit GSK3β via Ser9 phosphorylation. All-trans-retinoic acid (RA) has been reported to enhance insulin stimulated glycogen accumulation after 6 days of treatment in terminally differentiated L6 myotubules. We hypothesized that RA would also enhance insulin stimulated glycogen accumulation during myoblast differentiation through altered AKT-GSK3β-GS regulation. Differentiating L6 myoblasts treated with RA (0.5 µM) and insulin (10 nM) were harvested after 2 and 6 days of treatment. We reported that RA significantly enhanced insulin stimulated glycogen accumulation after 2 days of treatment without affecting pAKT (Ser473), pGS (Ser641), or pGSK3β (Ser9) expression. After 6 days of treatment, insulin induced glycogen accumulation as well as the expression of both pGS (Ser641) and pGSK3β (Ser9) without additional effects from RA. We proceeded to investigate the alterations of these key glycogen regulators further using the protein synthesis inhibitor cycloheximide (CHX) during a dose response time course study. CHX dramatically induced the expression of pGSK3β
(Ser9) and pGS (Ser641) between 24 and 48 hours, indicating a shift in GS regulation away from GSK3β. CHX (10 µM) also inhibited GSK3β expression, dramatically induced pGSK3β (Ser9) expression, and attenuated RA (0.5 µM) and insulin (10 nM) stimulated glycogen accumulation after 48 hours. In contrast, CHX (10 nM) induced glycogen accumulation after 3 hours of treatment in rat SM ex vivo but did not alter expression of key regulatory proteins. The main findings of this dissertation indicate that the currently understood mechanisms of glycogen homeostasis in SM are far from complete.
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<tr>
<td>1,3BPG</td>
<td>1, 3-bisphosphoglycerate</td>
</tr>
<tr>
<td>9CRA</td>
<td>9-cis-retinoic acid</td>
</tr>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>AS160</td>
<td>AKT substrate of 160 kDa</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>ATRA</td>
<td>all-trans-retinoic acid</td>
</tr>
<tr>
<td>CK</td>
<td>casein kinase</td>
</tr>
<tr>
<td>cAMP</td>
<td>3',5'-cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CaMK</td>
<td>calcium/calmodulin dependent kinase</td>
</tr>
<tr>
<td>CHX</td>
<td>cycloheximide</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
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<td>fetal calf serum</td>
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<tr>
<td>FFA</td>
<td>free fatty acids</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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</tr>
<tr>
<td>G1P</td>
<td>glucose-1-phosphate</td>
</tr>
<tr>
<td>G6P</td>
<td>glucose-6-phosphate</td>
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<td>glyceraldehyde-3-phosphate</td>
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<td>GBE</td>
<td>glycogen branching enzyme</td>
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<tr>
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<td>glycogen debranching enzyme</td>
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<td>GDP</td>
<td>guanosine diphosphate</td>
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<td>muscle specific PP1 glycogen targeting subunit</td>
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<td>GN</td>
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<td>Gx</td>
<td>G-protein subunit</td>
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<td>glycogen phosphorylase</td>
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<td>GYS</td>
<td>gene encoding for glycogen synthase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HK</td>
<td>hexokinase</td>
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<tr>
<td>HS</td>
<td>horse serum</td>
</tr>
<tr>
<td>HSMC</td>
<td>human skeletal muscle cell</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IR</td>
<td>insulin receptor</td>
</tr>
<tr>
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<td>insulin receptor substrate</td>
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<td>LG268</td>
<td>pharmacological retinoid x receptor agonist</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MYF5</td>
<td>myogenic factor 5</td>
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<tr>
<td>MYOD</td>
<td>myoblast determination protein</td>
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<tr>
<td>MYOG</td>
<td>myogenin</td>
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<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
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<tr>
<td>PAX7</td>
<td>transcription factor paired box 7</td>
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<td>PDK1</td>
<td>phosphoinositide-dependent kinase-1</td>
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<tr>
<td>PhK</td>
<td>phosphorylase kinase</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
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<tr>
<td>PIP3</td>
<td>phosphatidylinositol-3,4,5 triphosphate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Term</td>
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<tr>
<td>PKA</td>
<td>cyclic AMP dependent protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase-1</td>
</tr>
<tr>
<td>PYG</td>
<td>gene encoding for glycogen phosphorylase</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>RAC</td>
<td>related-to-protein-kinase A and C</td>
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<tr>
<td>Rac1</td>
<td>Ras-related C3 botulinum toxin substrate 1</td>
</tr>
<tr>
<td>RAE</td>
<td>retinol activity equivalent</td>
</tr>
<tr>
<td>RAR</td>
<td>retinoic acid receptor</td>
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<tr>
<td>RARE</td>
<td>retinoic acid response element</td>
</tr>
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<td>RXR</td>
<td>retinoid x receptor</td>
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<tr>
<td>SLC</td>
<td>solute carrier family</td>
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<tr>
<td>Smad2</td>
<td>SMAD family member 2</td>
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<tr>
<td>S/T</td>
<td>serine/threonine residues</td>
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<tr>
<td>T2DM</td>
<td>type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
</tr>
<tr>
<td>TTNPB</td>
<td>pharmacological retinoic acid receptor agonist</td>
</tr>
<tr>
<td>UCP3</td>
<td>mitochondrial uncoupling protein 3</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>UDP</td>
<td>uridine diphosphate</td>
</tr>
<tr>
<td>UDP-Glc</td>
<td>uridine diphosphate glucose</td>
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Chapter I: Introduction
Glycogen is the primary mechanism for glucose storage in animals. Glycogen accumulation is greatest in the skeletal muscle and liver. It can also be found in kidney, brain, fat, and other tissues to a lesser extent. Glycogen was first described by French scientist Claude Bernard in 1857 in an attempt to identify the fates of each of the dietary nutrients known at the time [1]. Bernard began his research in carbohydrate metabolism by investigating the reducing sugar contents of dogs’ portal and circulating blood, sacrificed during the digestion of a meal [1]. Bernard manipulated the dietary contents of these dogs, providing some only carbohydrates and others only meat [1]. The content of reducing sugars was then measured within the stomach, intestines, and hepatic vein [1]. Bernard discovered that even in the absence of portal sugar due to dietary carbohydrates restriction, an abundance of reducing sugars could still be detected in the hepatic vein, indicating that the liver was producing the reducing sugars [1]. Later, he discovered by accident that the content of reducing sugars increased in liver extracts of the animals as time elapsed [1]. Further experimentation revealed that the liver could produce large quantities of reducing sugars for hours after isolation [1]. Bernard found that lowering the temperature of fresh extracts would yield fewer reducing sugars immediately yet produce more when warmer temperature was applied [1]. This led to Bernard concluding that an intermediate must be responsible for the rapid production of reducing sugars [1]. Subsequently, the sugar producing substance was isolated and named “la matière glycogene” [2]

The study of glycogen and carbohydrate storage has been central to the advancement of metabolic sciences. Four Nobel Prize awards have been given in the exploration of glycogen. In 1947 Carl and Gerty Cory received the Nobel Prize in
Physiology or Medicine for identifying the enzymes, equilibriums, and conditions for glycogen synthesis and glycogenolysis [3]. The second Nobel Prize in chemistry was awarded to Luis Leloir in 1970. Dr. Leloir’s group discovered the biological utility of nucleotide interaction regarding carbohydrate usage [4, 5]. The third Nobel Prize in medicine was awarded to Earl Sutherland in 1971. Dr. Sutherland’s group discovered the action of the secondary messenger 3’,5’-cyclic adenosine monophosphate’s (cAMP) initiation of the degradation of glycogen in response to epinephrine [6, 7]. The fourth Nobel Prize in physiology or medicine was awarded to Edmond Fischer and Edwin Krebs in 1992 for the discovery of reversible phosphorylation as a regulatory mechanism in proteins responsible for the mobilization of glucose from glycogen [8, 9]. The study of glycogen metabolism has contributed greatly to the scientific understanding of dynamic phosphorylation states via kinases, allosteric regulation of enzymes, extracellular induction of intracellular mechanisms, and many other biochemical mechanisms critical in the maintenance of homeostasis. Schematic diagrams of the study designs used in Chapter III (Figure 1.1) and Chapter IV (Figure 1.2) can be found in Chapter I Appendix.
References


Appendix

<table>
<thead>
<tr>
<th>Western Blot</th>
<th>Glycogen Assay</th>
<th>Glucose Release from Glycogen Acid Hydrolysis</th>
<th>Colorimetric Analysis</th>
</tr>
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<tbody>
<tr>
<td>BioRad MiniProtein and XRS image Lab</td>
<td><strong>Cell Lysis</strong> 30% KOH, 1 Hour 100°C</td>
<td><strong>Glycogen Precipitation</strong> 70% Ethanol, 1 Hour -20°C, Centrifugation 4°C</td>
<td>Glucose Oxidase</td>
</tr>
<tr>
<td></td>
<td><strong>Glucose Release from Glycogen Acid Hydrolysis</strong> 3M HCl, 1 Hour 100°C</td>
<td><strong>Colorimetric Analysis</strong></td>
<td></td>
</tr>
</tbody>
</table>

**Treatments — Every 2 Days**
- Vehicle Control (-)
- Insulin (10 nM)
- RA (0.5 μM)
- RA + Insulin

**Harvest**
- After 2 days of treatment
- After 6 days of treatment

---

Figure 1.1 Study design used in Chapter III of this dissertation
Figure 1.2 Study design used in Chapter IV of this dissertation
Chapter II: Literature Review
2.1 Overview of the role of glycogen

Glycogen functions as an osmotically neutral glucose reservoir in animals [2]. Glycogen homeostasis is a critical component of cellular and systemic glucose regulation. Glycogen is found in the greatest quantity within the skeletal muscle and the liver [2]. Glycogen is synthesized and degraded in response to cellular energy demands and hormonal signaling. Hepatic glycogen is synthesized postprandially and degraded to release glucose into the blood during the postabsorptive state [2]. Skeletal muscle glycogen is synthesized in primarily in response to insulin stimulation and provides the main non oxidative postprandial blood glucose disposal mechanism [3]. Glycogen is also synthesized within the brain, heart, kidney, adipose, and red blood cells and contribute to systemic homeostasis to a lesser extent than skeletal muscle and liver glycogen [4].

2.1.1 Glycogen’s role in the brain

Glycogen is found within the brain at 0.1% of the tissue weight [5]. In adults, glycogen within the brain is found almost entirely within astrocytes [5]. Neurons which accumulate glycogen initiate apoptosis and tend to keep glycogen synthetic enzymes inactive [6]. Duran et al. demonstrated the importance of glycogen within the brain using nervous system specific glycogen synthase (GS) knock out mouse models, resulting in glycogen deficient mice in 2013 [7]. The mice deficient in GS exhibited learning deficits, memory formation impairment, and hippocampal alterations compared to control [7]. In 2019 Duran et al. then demonstrated that neuronal glycogen is of critical importance in the development of motor skills, cognition, and hippocampal integrity [8]. These findings suggest that glycogen is maintained within narrow margins within brain tissues. The role
of glycogen within the brain has also been implicated in sensory stimulation, sleep cycles, as well as ischemic events, seizures, and exhaustive exercise [5]

2.1.2 Glycogen’s role in the heart

Glycogen is utilized as an energy source secondary to fatty acid oxidation for cardiomyocytes [9]. Glycogen is synthesized in the fed state and is preferentially degraded for glucose production over glucose import during stress [9, 10]. Goodwin et al. demonstrated that radiolabeled glucose from glycogen was almost entirely oxidized during stimulation with epinephrine and that exogenous glucose was preferentially reduced to lactate after glycolysis [10]. During exercise, as well as hypoxic conditions such as ischemia, glycogen serves as an alternate energy source to fatty acid oxidation [9, 11].

2.1.3 Glycogen’s role in the kidney

The kidneys play a significant role in the regulation of blood glucose homeostasis. The kidneys release glucose into the blood stream that has been resorbed from glomerular filtration as well as synthesized from gluconeogenesis [12]. The kidneys also utilize glycogen as an energy reservoir, providing glucose for energy preferentially in the glomerulus and descending limb of the nephron [12]. Under normal conditions glycogen does not accumulate within the tissues of the kidney [12]. However, pathological glycogen accumulation occurs with hyperglycemia associated with type 2 diabetes mellitus T2DM [12]. The accumulations of glycogen within the cytoplasm and nucleus of tubular epithelial cells of the ascending limb of Henle’s loop and distal convoluted tube induce apoptosis and Armanni-Ebstein lesions to the kidney, which can be fatal [13].
2.1.4 Glycogen’s role in adipose tissue

Glycogen fulfills two major roles within adipose tissue [14]. First, glycogen functions as a source of easily available glucose for carbon skeletons used in triglyceride synthesis [14]. Glucose released from glycogen digestion can enter the glycolytic pathway and the 3 carbon intermediates can be converted to glycerol to serve as scaffolds for fatty acids. Second, glycogen can provide an energy buffer in response to nutritional status [14]. In the fed state, insulin signaling suppresses lipolysis and the release of free fatty acids within the adipocyte [15]. Insulin also stimulates glucose uptake into the adipocyte [16]. This transition between the anabolic processes of glycogenesis, triglyceride synthesis, and fatty acid synthesis and catabolic states such as glycogenolysis, glycolysis, lipolysis participates in the modulation of fatty acid efflux into the bloodstream [16].

2.1.5 Glycogen’s role in erythrocytes

Glycogen does not accumulate within erythrocytes under normal physiological conditions, however glycogen metabolism machinery is present in erythrocytes [17]. Glycogen accumulation presents in erythrocytes of patients with glycogen storage diseases [17]. Mature red blood cells use substrate level phosphorylation via glycolysis as the primary mechanism for energy production rather than oxidative phosphorylation via mitochondrial action [17]. The presence of accumulated glycogen does not result in an increase in lactate production, suggesting that the glycogen is not playing a role in energy homeostasis [17]. The presence of the glycogen metabolic machinery within the erythrocyte has been suggested to be the remnant of the metabolic processes of immature hematopoietic stem cells [17]. It is for these reasons that the presence of
glycogen in erythrocytes is used diagnostically in the assessment of glycogen storage diseases [18].

2.1.6 Glycogen’s role in the liver

Hepatic glucose production results from the liver’s coordination with the pancreas, skeletal muscle, and adipocytes to regulate blood glucose in the postabsorptive state. [19]. In the fed state, abundant glucose from dietary sources stimulates the release of insulin [19]. Glycogen can be synthesized from this excess glucose in the liver providing a glucose disposal mechanism which can account for up to 1/3 of the glucose load per meal. Hepatic glycogen synthesized postprandially also functions as a reservoir of glucose, enabling accelerating hepatic glucose production and sparing gluconeogenic mechanisms between meals [19]. Hepatic glucose production from glycogen is maintained by the catabolic hormones glucagon and epinephrine, as well as the anabolic effects of insulin [20]. The liver contains the greatest concentration of glycogen per gram of tissue. Liver glycogen can be present between 100 µM and 500 µM depending upon the nutritional status, and between 6% and 8% of total organ weight in the fed state [21].

2.1.7 Glycogen’s role in the skeletal muscle

Skeletal muscle glycogen plays two major roles. First, glycogenolysis provides a quick source of glucose for glycolytic energy production during muscle contraction and during response to acute sympathetic stress [19]. Skeletal muscle also functions as the major site of glucose disposal during postprandial conditions, responsible for up to 90% of postprandial glucose disposal [22]. Skeletal muscle constitutes approximately 40% of the total weight of a healthy adult [23]. Due to this, skeletal muscle tissues contain the
greatest absolute quantity of glycogen within the body, though it has less glycogen per gram of tissue when compared to the liver [21]. On average, skeletal muscle contains between 30 µM and 100 µM of glycogen, and between 1% and 2% of total tissue weight is glycogen [21].

2.2 Skeletal muscle glycogen regulation

Glycogen is maintained within a flexible system in skeletal muscle. Glycogen concentration can be increased as an adaptation to physical exercise [22]. In T2DM patients, glycogen concentration and synthesis are significantly decreased compared to healthy counterparts [24, 25]. The flexibility and responsiveness of skeletal muscle glycogen homeostasis serves a critical role in survival and fitness. The adaptation of glycogen concentration can be induced via increased contractile demands on skeletal muscle which quickly exhaust the supply of oxygen required for aerobic oxidative phosphorylation. In this state glycogen is rapidly digested to yield glucose, which can quickly provide energy through the fermentation of pyruvate to lactate, regenerating nicotinamide adenine dinucleotide (NAD)+ for further substrate level phosphorylation. In this way, glycogen content in skeletal muscle can result in preserving contractile function under acute stress for a brief period.

Deficiency of this system can compromise the survival of an organism. From an evolutionary perspective, skeletal muscle glycogen and liver glycogen serve complimentary yet separate roles to meet glucose demand in acute and prolonged settings [26]. Upon receiving stimuli which engage sympathetic fight or flight response and the release of epinephrine, β-adrenergic signaling in the skeletal muscle can produce intracellular glucose-1-phosphate (G1P). This is quickly isomerized to glucose-

12
6-phosphate (G6P) during equilibration for anaerobic energy production [27]. This mechanism can provide a brief yet critical window of skeletal muscle glycolytic energy substrate in the deficit of energy production from aerobic fatty acid oxidation relative to acute demand [28]. During maximal hormonal and contractile stimulation under acute stress, the combined effects of epinephrine, adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate, calcium, and inorganic phosphate can accelerate glycogenolysis from 2 mmol to 40 mmol of glucose released per kilogram of wet weight per minute [29]. This dependency upon a fast response, anaerobic energy substrate reservoir has resulted in the evolutionary optimization of skeletal muscle glycogen hydrolysis and regulation [30]. The fine tuning of this system can be observed in the differences between skeletal muscle glycogen and liver glycogen [22]. Within the skeletal muscle, up to 90% of glycogen stores are located near mitochondria and the sarcoplasmic reticulum in the inter and intramyofibrillar space [31]. This proximity to mitochondria provides rapid transport of glycolytic products to be used anaplerotically and for energy production [32].

2.3 Liver glycogen regulation

The spatial arrangement of glycogen within the skeletal muscle provides maximal access for glycogenolytic regulatory enzyme complexes and feedback [33]. This arrangement provides a much greater surface area per quantity of glycogen for hydrolytic interaction than glycogen found within the liver [34]. Glycogen in the liver fulfills a separate metabolic role when considered from an evolutionary perspective. Glucagon signaling induces the glycogenolysis of hepatic glycogen stores in response hypoglycemic conditions [35, 36]. Hepatic glucose production increases as glucose is
released from larger glycogen α-particles in the postabsorptive state [37]. As liver
glycogen depletes, gluconeogenic and ketogenic mechanisms accelerate as skeletal
muscle insulin sensitivity decreases to maintain blood glucose levels which satisfy the
requirements of the central nervous system [38, 39]. In this state, muscle glycogen
remains replete and intramuscular fatty acid accumulation occurs in order to maintain a
state of potential energy for acute response, whereas hepatic glycogen synthesis and
accumulation is dramatically reduced until dietary sources of glucose become available
[22, 40]. Skeletal muscle energy substrate shifts to fatty acid metabolism and glycogen
repletion in the skeletal muscles coincides with insulin sensitivity [41]. Insulin resistance
under these circumstances spares glucose depletion via skeletal muscle from transport
[42]. Should the skeletal muscles not undergo this acute insulin desensitization, the
transport of glucose from the blood can deprive the central nervous system enough to
induce a comatose state from hypoglycemia. These combined effects enable
gluconeogenesis in the liver and kidneys to synthesize enough glucose to provide for
the central nervous system without inducing hypoglycemia under normal circumstances
[43].

Together these two primary systems represent evolutionary adaptations to many
favorable and unfavorable environmental conditions. The inherited flexibility of these
systems, as well as the differential redundant homeostatic functions they fulfill, impress
the importance and necessity of such systems for endurance and survival. The
evolutionary optimizations of this system for different purposes in the skeletal muscle
and the liver are elegant, complex evolutionary solutions stimulated by a lack of control
over food sources. The clearest evidence of the integral nature of this metabolic
buffering system is observed with its malfunction. Mutations which affect members of the glycogen regulatory apparatus can result in glycogen storage diseases (GSD).

2.4 Glycogen metabolism

Glucose flux through glycogen is maintained by a host of anabolic and catabolic enzymes. The arrangement of glucose moieties and their superstructures as well as their associated proteins are functionally optimized for response to internal cellular conditions as well as external signaling. The differing functional roles of glycogen in specific tissues determine its arrangement and regulation through the fine-tuning of its synthetic and degradative mechanisms.

2.4.1 Physical characteristics of glycogen

Glycogen is comprised of branched chains of glucose units and found as particles primarily within the liver and skeletal muscle. Glycogen average size is variable between these two main tissues due to its function. Glycogen within skeletal muscle is found in smaller spherical particles which enables fast degradation for immediate glucose release. Glycogen within the liver is comprised of aggregates of these smaller particles, increasing the spatial efficiency of the particles which allows for greater total glycogen concentration.

2.4.2 Structural arrangement of glucose within glycogen

Glucose moieties can be enzymatically joined via α-1-4-glycosidic linkages to form straight chains seen in amylose and β-1-4-glycosidic linkages seen in cellulose [44]. A second set of branching enzymes can rearrange the α-1-4-glycosidic linkages into α-1-6-glycosidic linkages producing branched the chains seen in glycogen and amylopectin [44]. Glycosidic bonds can be rapidly hydrolyzed to yield glucose and G1P.
Glycogen particle structure is represented in Figure 2.1. All figures in Chapter II can be found in Chapter II Appendix.

The structure of glycogen is variable, consisting of diverse spheroid superstructures with up to 12 concentric tiers of glucose chains [46]. The 12th tier of glucose chains can theoretically occupy 26% of the total space available containing 50% of the total glucose residues within the particle [46]. The glycogen particle must also provide space for the glycogen associated proteins [47]. The synthetic, degradative, and regulatory enzymes must be positioned such that they can be accessed by their respective regulators [46, 48]. The branching of the glucose chains within glycogen particles provides a spatially efficient and accessible way to rapidly degrade glucose chains. This internal spatial configuration of branched glucose chains makes a theoretical 13th tier of glucose chains impossible without structural reorganization of the particle. Melendez-Hevia et al calculated that a 13th tier of glucose chains would require 62% of the total space, exceeding physical limitations [48].

The glucosyl chains of glycogen particles are classified into A-chains and B-chains [34]. A-chains which occupy the outer tiers of the particle through which most glucose flux occurs, and B-chains which occupy the inner tiers [34]. The inner B-chains exhibit an average 13 glucose residues with 2 branch points whereas the A-chains are generally unbranched [34]. B-chain function to enable structural integrity from the inner tiers to the outer tiers, allowing for greater particle size without excessive glucose occupation of the interior of the molecule. This structural arrangement is functionally optimized for the action of glycogen phosphorylase (GYP), providing efficient and rapid hydrolysis of the outer chains without the activity of the glycogen debranching enzyme.
(GDE). This arrangement can enable GYP to release up to 95% of the glucose within the particle [34].

2.4.3 Glycogen particle characteristics

Glycogen particles are classified into $\alpha$-particles and $\beta$-particles according to mass and particle size [49]. Glycogen $\beta$-particles range in size from 10 nm to 44 nm and are present in the skeletal muscle [46, 50, 51]. A $\beta$-particle of glycogen at maximum capacity can consist of approximately 55,000 glucose residues [49]. A $\beta$-particle at maximum capacity would have a mass of $10^7$ kDa and a diameter of approximately 44 nm [46]. However, the average particle size for glycogen $\beta$-particles is 27.3 nm with 3 nm standard deviation, possessing 7 tiers of glucose chains [46, 52]. Accumulating in the sarcoplasm of muscle cells, these $\beta$-particles have been observed via electron micrograph infiltrating the myofibrils between the myofilaments and are associated with the sarcoplasmic reticulum smooth membrane [49, 52].

Glycogen $\alpha$-particles are aggregates of $\beta$-particles and assembled in the liver [4]. Glycogen $\alpha$-particles in liver range in size from 60 nm to 300 nm in diameter [4, 49]. Glycogen $\alpha$-particles have less surface area for the quantity of contained glucose when compared to $\beta$-particles and are not formed within skeletal muscle [46]. The optimum surface area to volume ratio for enzymatic degradation of glycogen by GYP is within the $\beta$-particle size range [53]. The difference in glycogen particle size between the liver and muscle reflects the role glycogen fulfills in each tissue. Muscle glycogen must be available for rapid hydrolysis to provide G1P for glycolytic energy production. G1P released from hepatic glycogen can be dephosphorylated by glucose-1-phosphatase and exits the liver to maintain a constant concentration of blood glucose [54]. Under
normal conditions, blood glucose will not be depleted more rapidly than it can be replenished from hepatic glucose production, even under acute stress. Conditions such as prolonged strenuous exercise during fasting can deplete both hepatic and skeletal muscle glycogen, resulting in hypoglycemia [55].

2.5 Glycogen synthesis

Glycogen synthesis is accomplished by glycogenin (GN), GS, and glycogen branching enzyme (GBE) beginning with uridine diphosphate-glucose (UDP-Glc) as the substrate [56]. Glycogen synthesis is regulated via hormonal stimulation. Positive hormonal regulators of glycogen include insulin and insulin like growth factor, while negative regulators include epinephrine as well as glucagon in the liver. Glycogen concentration is also regulated internally by energy sensing AMP activated protein kinase (AMPK) sensitivity to ADP/adenosine triphosphate (ATP) ratios [57]. Positive regulation of glycogen synthesis by insulin signaling has been extensively studied and is further elaborated later in this review. A metabolic diagram of glycogen synthesis can be found in Figure 2.2.

2.5.1 Uridine diphosphate glucose pyrophosphorylase

The synthesis of glycogen begins with the production of UDP-Glc which is synthesized by UDP-Glc pyrophosphorylase from uridine triphosphate and G1P [58]. UDP-Glc is the preferred substrate for mammalian glucosyltransferase (GT) enzymes [59]. Testing with each nucleotide diphosphate-glucose revealed that the pyrimidine ring structure is essential for successful binding and transfer of the glucose residues [59]. UDP-Glc is of particular significance in eukaryote carbohydrate metabolism, comprising
up to 55% of the available nucleotide diphosphate-sugar pool and functioning as substrate for at least 270 identified reactions [60].

2.5.2 Glycogenin

GN is the primer for glycogen synthesis and the protein core of the glycogen particle [59]. There are two genes that encode GN in humans [46, 61]. GYG1 is responsible for GN translation in skeletal muscle, brain, kidney, adipose, and other tissues. GYG2 encodes for GN in the liver, heart, and pancreas [46]. Tyr194 serves as the initial glucosyl binding site for glycogen synthesis [62]. Glucose moieties are successively added to GN in α-1-4 glycosidic linkages until a chain of 8-12 glucose units has formed [63]. This initial glucose chain is subsequently used to expand the glycogen particle via branch points and elongation.

2.5.3 Glycogen synthase

GS is responsible for incorporating glucose into glycogen utilizing UDP-Glc as the substrate to form α-1-4 glycosidic linkages for elongation of the glucose chains [64]. GS belongs to the GT-B superfamily [65]. Humans have two genes which encode GS [46]. The GYS1 gene encodes for the isoform in skeletal muscle and other tissues which synthesize glycogen [66]. The GYS2 gene encodes for GS which is present in the liver [67]. The skeletal muscle isoform possesses two additional regulatory sites 1a and 1b over the liver isoform. These sites enable additional regulation from protein kinase A (PKA), which lies within the epinephrine signaling pathway, as well as the calmodulin kinase II (CaMKII). Enhanced regulation from epinephrine signaling enables a greater glycogenolytic effect in response to epinephrine release, liberating glucose for rapid energy production under both aerobic and anaerobic conditions. This increased
sensitivity to epinephrine can enhance survival during sympathetic responses such as fight or flight and exercise. Accelerated responses to energy demands under emergent circumstances can enhance survival, as evidenced by its inherited conservation into the present day. Greater sensitivity to calcium fluctuations is a critical feature of the GYS1 isoform as contractile machinery within skeletal muscle functions via calcium fluctuations.

2.5.4 Glycogen branching enzyme

The GBE is responsible for generating the branched glucosyl chains present in glycogen [68]. The GBE catalyzes a double displacement transglucosylation reaction in which an α-1-4 glucan is cleaved and the nonreducing end is covalently bonded to an aspartate residue within carboxy terminal group in the catalytic region of the GBE [69]. The cleaved glucan is then transferred to the carbon-6 position of an acceptor chain for the generating an α-1-6 glycosidic linkage which functions as a branch point [69]. GBE within animal skeletal muscle belongs to the glycoside hydrolase family 13 [70]. The participation of GBE however is not a rate limiting factor in glycogen synthesis due to the external spatial arrangement of linear A and internal branched B chains [46].

2.6 Glycogen degradation

Like glycogen synthesis, the glycogen degradation within skeletal muscle is likewise regulated via internal energy homeostasis and external hormonal signaling. Glycogen degradation is accomplished primarily by GYP, and to a lesser extent GDE. Analogous to the GBE, the spatial arrangement of the linear A chains enables up to 95% of glucose release by the catalytic action of GYP without participation of GDE. Therefore, GDE is not a rate limiting enzyme in glycogenolysis. Alternatively, glycogen
can be degraded via lysosomal α-amylase activity. Lysosomal degradation does not significantly contribute to the net glycogenolytic products and is utilized for the disposal of modified glycogen resistant to the catalytic action of GYP. Glycogen is enzymatically hydrolyzed to yield free glucose from digesting branch points and G1P from digesting linear chains [4]. Glucose flux through the glycogen particle is accelerated or decelerated via modulation of the activity of both anabolic and catabolic enzymes. This dynamic, finely tuned balance results from localization, complex formation, allosteric and covalent regulator concentrations, and intermediate conformational states with differential substrate binding affinity [71].

2.6.1 Glycogen phosphorylase

GYP functions as a homodimer is the primary enzyme responsible for glycogen degradation [72]. Each catalytic site of GYP contains coenzyme pyridoxal phosphate binding sites [73]. GYP has three isoforms in the human body [72]. The liver isoform is encoded by *PYGL*, muscle by *PYGM*, and the brain isoform *PYGB* [72]. Further description of GYP in this section will refer to the muscle specific isoform unless otherwise noted. GYP only hydrolyzes the α-1-4 glycosidic linkage between the terminal glucose moiety and its predecessor in glucose chains within glycogen to release G1P [72].

GYP is regulated allosterically and covalently. Allosteric regulation by AMP, inorganic phosphate, G1P, and glycogen shift the enzyme into the relaxed (R) conformation [72]. In this state the Ser15 residue is exposed and can be phosphorylated to further induce an active conformational state (a) which leads to increased catalytic activity [73]. Increased concentrations of ATP, G6P, glucose, and purines allosterically
induce the conformational shift to the tense (T) state, blocking the Ser15 phosphorylation site which suppresses catalytic activity [74]. The dephosphorylated GYP remains in a less active state (b) [73]. The activity of GYP is a function of both the phosphorylation of Ser15 as well as the conformational state induced by its allosteric regulators [75]. Phosphorylation of Ser15 is accomplished by phosphorylase kinase (PhK) and dephosphorylation by protein phosphatase 1 (PP1) [46, 73, 76].

The regulation of GYP by multiple mechanisms allows it to be rapidly activated in response to external and internal stimuli [72]. In skeletal muscle, the activation of GYP by Ser15 phosphorylation can be achieved independent of allosteric regulation by AMP [77]. This enables extracellular neuronal and hormonal signaling to induce muscle contraction without first satisfying a necessary conformational shift by its allosteric regulators, a prerequisite in the brain and liver isoforms [77]. For example, PhK functions as a tetramer consisting of α, β, and δ, as well as one catalytic subunit γ. PKA phosphorylates Ser1018 on the α subunit and Ser26 β subunit of PhK after activation of the calmodulin binding domains in the α, β, and δ subunits [78]. Inhibition of PP1 is also induced by PKA phosphorylation of Ser67 in the muscle specific glycogen targeting PP1 subunit G_M (G_M) [79]. This in turn, destabilizes the association of PP1 with the glycogen metabolic enzymes, preventing dephosphorylation and deactivation of GYP by PP1. The multifunctional roles of PP1 and PhK involved in the complimentary management of glycogen synthesis and glycogen degradation highlight the critical role that subcellular localization plays in glycogen metabolism. A diagram of GYP regulation can be found in Figure 2.3.
2.6.2 Glycogen debranching enzyme

Enzymatic glycogen degradation is accomplished by the combined action of GYP and GDE [80]. Hydrolysis of α-1-4 glycosidic linkages by GYP is stopped four glucose residues before each α-1-6 branch point within the glucose chain [80], under such situation, GDE is required for subsequent steps of glycogen degradation. GDE exhibits α-1-4 → α-1-4 glucosyltransferase activity as well as α-1-6 amyloglucosidase activity [80]. GDE transfers a maltotriosyl group to the non-reducing end of a neighboring chain, creating an α-1-4 glycosidic linkage [80]. GBE then hydrolyzes the remaining α-1-6 glycosidic linkage releasing free glucose [80]. The elimination of the branch point by GDE enables GYP to resume its catalytic activity and further digest the linear glucose chains.

2.6.3 Lysosomal digestion

Glycogen hydrolysis can also occur in the lysosome [4]. Glycogen which has not been entirely degraded by the action of GYP and GDE can be degraded by α-1-4 glucosidase within autophagic vacuoles [4]. It is unclear how digestion of the α-1-6 branch points occur with lysosomal degradative enzyme species [4].

2.7 Insulin stimulated glycogen synthesis in skeletal muscle

Insulin stimulation is the primary mechanism for the initiation of glycogen synthesis. A diagram of skeletal muscle role in systemic insulin signaling can be found in Figure 2.4. Insulin sensitivity is modulated by skeletal muscle based on the availability of dietary carbohydrates and cellular energy demand in skeletal muscle. Glycogen is maintained in skeletal muscle in the postabsorptive state as an immediate source of energy for emergent conditions.
2.7.1 Skeletal muscle physiology

The primary role of glycogen within the skeletal muscle is to provide a quick source of energy during stress [19]. The skeletal muscle also functions as the major site of glucose disposal during postprandial conditions. The skeletal muscle constitutes approximately 40% of the total weight of a healthy adult [23]. Due to this, skeletal muscle tissues contain the greatest absolute quantity of glycogen within the body [21]. On average, skeletal muscle contains between 30 µM and 100 µM of glycogen, and between 1% and 2% of total tissue weight is glycogen [21].

Skeletal muscle contraction is achieved via coordination of local calcium and ATP concentrations [81]. Acetylcholine released from neurons interacts with receptors in the neuromuscular junction inducing the increase of cytosolic calcium concentration from extracellular influx and intracellular efflux from the sarcoplasmic reticulum [81]. Calcium serves as the cofactor for actin-myosin binding and subsequent conformation change of the myosin motor unit is responsible for the mechanical work accomplished across the length of the muscle fiber [82]. After tetanic concentric actuation of the actin-myosin units is achieved, calcium is displaced via sarcoplasmic reticulum calcium ATPase pumps [82]. This enabling unbinding of myosin from actin and conformational resetting of the myosin motor unit [83]. This cycle is repeated throughout the skeletal muscle fiber until ATP is exhausted and tetanic contraction remains or neuronal stimulation ceases. This dynamic calcium homeostasis provides the necessary thermodynamic environment for actin-myosin contractile binding as well as ATP resetting of the myosin motor domain [84]. Calcium sensitivity enables GS tandem regulation to maintain glucose availability for glycolytic ATP production.
Skeletal muscle consists of two contractile fiber types, fast and slow twitch [23]. Slow twitch muscle fibers have a high oxidative capacity and tend to utilize more fatty acids as energy substrate [23]. Fast twitch muscle fibers on the other hand, have higher contractile strength and tend to rely more on substrate level phosphorylation for energy production through glycolysis during stress [23]. Increased contractile demands on skeletal muscle quickly exhaust the supply of oxygen required for oxidative phosphorylation. In this state, glycogen is rapidly hydrolyzed to yield G1P. G1P can then be isomerized to G6P by phosphoglucomutase and proceed into the glycolytic pathway. G1P, which enters glycolysis via glycogen hydrolysis, does not need initial phosphorylation by hexokinase (HK). The increase availability of G1P combined with the inhibition of peripheral biosynthetic pathways thermodynamically favors the isomerization of G1P to G6P.

Dephosphorylation of 1, 3-bisphosphoglycerate (1,3BPG) to 3-phosphoglycerate is catalyzed by phosphoglycerate kinase. This dephosphorylation occurs twice per glucose molecule. This can yield 2 phosphate groups which can be used to regenerate ATP. Two phosphate groups are also released in the dephosphorylation of phosphoenolpyruvate to pyruvate via pyruvate kinase. Since G6P was isomerized from G1P the net ATP potentially produced from substrate level phosphorylation of glucose from hydrolyzed glycogen is three rather than the two from unphosphorylated glucose transported into the cell. Two molecules of NADH are also produced during the oxidation of glyceraldehyde-3-phosphate (GA3P) to 1, 3BPG catalyzed by GA3P dehydrogenase. The NADH produced via this mechanism can yield 3 molecules of ATP.
each via oxidative phosphorylation within the mitochondria under aerobic conditions [85].

Under anaerobic conditions, NAD+ can be regenerated by reduction of pyruvate to lactate in the cytosol by lactate dehydrogenase. This fermentation mechanism enables glycolysis to proceed in the absence of oxygen until the rate of ATP resynthesis from ADP and an additional phosphate becomes unfavorable with increasing acidity. Under these conditions the concentration of ADP can be correlated with the concentration of hydrogen ions, and ATP resynthesis becomes inversely proportional to the concentration of hydrogen ions [86]. In this way, glucose released from glycogen allows production of ATP to support contractile function under acute stress for a brief period.

2.7.2 Insulin signaling in skeletal muscle

Insulin released from pancreatic β-cells enters general circulation, acting as the ligand for the insulin receptor (IR). Proximal insulin signaling can be summarized as interactions of the IR with insulin and the insulin receptor substrates (IRS), as well as the phosphorylation events which induce conformation change in each [87]. Participants involved in signal transduction beyond direct IR interaction can be considered distal insulin signaling, beginning with phosphoinositide-3-kinase (PI3K) within this context [87].

The IR is a receptor tyrosine kinase and functions as a tetrameric integral membrane receptor with two extracellular α domains and two membrane spanning β domains [88]. Insulin binds to an extracellular domain at the surface of the cell membrane and induces a conformational change in its intracellular domain structure
This shift enables autophosphorylation of Tyr1162, Tyr1158, and Tyr1163 of the insulin receptor, inducing further conformational changes which permits the subsequent autophosphorylation of Tyr972, enabling the recruitment of IRS1 and IRS2. IRS1 is primarily responsible for the metabolic response of insulin signaling in skeletal muscle.

IRS1, once recruited to the IR through via Src homology (SH) domain binding, undergoes multi-site phosphorylation which results in the recruitment of heterotetrametric PI3K containing both p110α/β catalytic subunits as well as p85α/β regulatory subunits. PI3K catalyzes the conversion of phosphatidylinositol-4,5 bisphosphate to phosphatidylinositol-3,4,5 triphosphate (PIP3). PIP3 is capable of recruiting proteins with pleckstrin homology binding domains, namely protein kinase B (AKT) and phosphoinositide-dependent kinase-1 (PDK1). Recruited AKT in proximity to PDK1 undergoes phosphorylation in its catalytic domain at Thr308. Further phosphorylation by mechanistic target of rapamycin complex 2 within its C-terminal regulatory domain at Ser473 activates AKT. Activated AKT in turn dissociates from the PI3K complex and proceeds to regulate cytosolic substrates glycogen synthase kinase-3β (GSK3β), PP1, and AKT substrate of 160 kDa (AS160).

### 2.7.3 Signal transduction through AKT

AKT participates in insulin signal transduction in the skeletal muscle. AKT is also referred to as related-to-protein-kinase A and C (RAC) as well as protein kinase B (PKB). AKT has 3 isoforms, AKT 1, 2, and 3 corresponding to PKB α, β, and γ respectively in human physiology. Further mention of AKT in this review will refer to
AKT2 unless otherwise stated, the isoform which is responsible for the metabolic effects of insulin signaling in skeletal muscle glucose homeostasis [91]. AKT serves as a metabolic hub transducing the membrane bound PI3K regulatory complex to the cytosolic metabolic glycogen regulatory machinery. AKT activity is canonically correlated with positive regulation of glycogen synthesis through a myriad of regulatory interactions. AKT directly phosphorylates GSK3β at Ser9 leading to a reduction in GSK3β activity, a main negative regulator of glycogen synthesis. Insulin stimulated glucose uptake by skeletal muscle is also mediated through AKT signal transduction in parallel with glycogen synthesis through its direct activation via phosphorylation of AS160 [88]. AS160 mediates glucose transporter 4 (GLUT4) vesicle trafficking and membrane fusion proceeds through AKT activation and Rho guanosine triphosphate (GTP) cyclohydrolase Ras-related C3 botulinum toxin substrate 1 (Rac1) coordination, enabling the import of extracellular glucose for utilized in glycogen synthesis. A diagram of insulin regulation of cytosolic substrate can be found in Figure 2.5.

AKT also participates in PP1 regulation of glycogenesis [92]. The regulatory feedback apparatus between PP1 and AKT is accomplished by phosphorylation, localization, and complex formation. The catalytic subunit of PP1 does not exhibit direct substrate specificity and achieves its many roles through substrate targeting and regulatory subunits [93]. PP1 can negatively regulate AKT via dephosphorylation dependent upon peripheral regulatory conditions such as complex proximity, subunit specificity, and isoform affinity [94]. However, AKT can positively regulate PP1 via phosphorylation of regulatory subunits, enhancing PP1 activity resulting in hepatic glycogen accumulation [95]. The regulatory interactions between AKT and PP1 in
glycogen metabolism are not fully understood, however the insulin signal transduction resulting from AKT involves positive regulation of PP1 in skeletal muscle.

2.7.4 Glycogen synthase kinase-3β role in glycogen homeostasis

Humans have two subtypes of GSK3, GSK3α and GSK3β [96]. The GSK3 subtypes share 98% sequence homology in the catalytic domain yet differ significantly at the amino and carboxy termini with 36% sequence difference, sharing 85% overall sequence homology [97, 98]. GSK3 belongs to the highly conserved CMGC group within the human kinome along with eight other families and retains functionality most similar to cyclin directed kinases and mitogen activated protein kinases [99]. The role of GSK3 in physiology and pathophysiology can only be considered within the highly specific configuration of circumstances that GSK3 occupies. GSK3 is expressed in almost all cell types and tissues and exerts influence over many homeostatic and pathophysiological processes which are outside the scope of this review [100]. Within the context of skeletal muscle glycogen synthesis, GSK3β activity has been negatively correlated with glycogen synthesis via direct phosphorylation of GS [46].

GSK3β constitutively active serine-threonine kinase whose regulatory effects are dependent upon a substrate binding domain and a kinase domain [101]. The S/T-X-X-X-S/T pattern of phosphorylation enables GSK3 to phosphorylate several sequential serine/threonine residues within GS at Ser652, Ser648, Ser644, and Ser640 [100]. GSK3β can be regulated via substrate priming, where the S/T-X-X-X-S/T motif of a target substrate only becomes structurally available after phosphorylation by a peripheral regulator [102]. Phosphorylation of Ser9 by AKT induces the N-terminus transition from trans to cis conformation, enabling the N-terminal tail to function as a
pseudosubstrate. Known regulators of GSK3β at Ser9 can be found in Table 1.1 within the Chapter I Appendix. This results in the N-terminus occupying the binding site of the substrate binding domain. A diagram of GSK3β conformational regulation can be found in Figure 2.6.

In addition to phosphorylation by its direct regulators GSK3β activity can be influenced via its incorporation into protein complexes with its target substrates and regulators. Suzuki et al. demonstrated that GSK3β can create its own priming condition by phosphorylating the catalytic α subunit of AMPK by phosphorylating the Thr479 residue in a AKT-Pi3K dependent manner [103]. Suzuki et al. demonstrated that AKT inhibits AMPK activity in conjunction with GSK3β in vivo by first phosphorylating Ser485 of the α subunit of AMPK [103]. However, phosphorylation of Ser485 by AKT is unable to inhibit the activity of AMPK without subsequent phosphorylation at Thr479 by GSK3β. GSK3β can then proceed to phosphorylate Ser475 and Thr471, resulting in the inhibition of AMPK activity [103]. In this way GSK3β can act as both an AMPK inhibitor as well as an inhibitor of glycogen synthesis, representing an apparent dissonance between anabolic and catabolic complimentary regulation. The nuanced negotiation of GSK3β’s participation in these pathways is a partial function of its ability to form complexes with different regulators and target substrates.

2.7.5 Glycogen synthase regulation in glycogen homeostasis

GS functions in a tetrameric configuration subject to both covalent and allosteric regulation [104, 105]. The most impactful allosteric regulators of GS are G6P and ATP [106, 107]. G6P has been demonstrated to increase the activity of GS via interaction at six arginine residues: Arg579, Arg580, Arg582, Arg586, Arg588, and Arg591 [108, 109].
The activation of GS by G6P induces a change in the tetrameric conformational state which exposes the catalytic binding site to UDP-Glc [105]. In contrast, GS is inhibited by increased concentration of ATP [110]. ATP competitively inhibits both the G6P allosteric binding sites as well as the UDP-Glc catalytic binding site [107, 111]. Due to the multiple mechanisms by which ATP interacts with GS, its inhibitory effects cannot be eliminated by increasing the concentration of G6P [64, 107, 110]. Therefore, ATP inhibition takes precedence over G6P activation in the allosteric regulation of GS.

GS activity is also modulated by phosphorylation [64]. GS phosphorylation generally inhibits enzyme activity. Four clustered regions in the GS protein purified from rabbit skeletal muscle were identified which can be phosphorylated to regulate GS activity [112, 113]. A diagram of GS cluster organization can be found in Figure 2.7. Each of the four clustered phosphorylation regions contain a primary serine residue [46]. The primary residue must be phosphorylated before the next residue within the cluster be conformationally available for subsequent phosphorylation, a configuration termed hierarchical phosphorylation by Roach et al. in 1990 [112]. Nine serine residues across these four regions have been experimentally identified in vivo to be phosphorylated as a part of the covalent regulatory apparatus of GS [64]. The layered configuration of this regulation, as well as the kinases which interact with each residue, enable complex regulation of enzymatic activity in response to a wide range of intracellular and extracellular conditions [114]. However, mutation of any single serine residue is unable to eliminate enzyme activity, demonstrating that activity of the enzyme is the result of at least two or more phosphorylation sites [64]. It is worth noting that the mechanism by which phosphorylation affects enzyme activity does not depend solely on
the conformational changes which result in exposure of allosteric and catalytic binding sites. Phosphorylation of different sites also strongly correlates with the subcellular location of the GS through its association with surrounding protein structures [21, 64, 115]. In rabbit and human skeletal muscle GS, phosphorylation of sites 2, 3a, 3a + 3b, and 1a have been associated with its localization to the perinuclear region and cross-striations under basal conditions [116-118]. However, during exercise stimulated conditions GS localizes to the glycogen particles in preparation for resynthesis of glycogen which was hydrolyzed during exercise [117, 118]. Phosphorylation of Ser7 at site 2 and Ser10 at site 2a is associated with GS localization to the inter-myofibrillar clusters under both basal and exercise stimulation [117, 118]. Likewise, phosphorylation of Ser710 at site 1b results at GS localization to the cross-striations under both basal and exercise stimulation [117, 118]. Importantly, the location and activity of GS are directed not only by phosphorylation, but in concert with allosteric regulation and to a lesser degree substrate availability [111].

2.7.6 **Hormonal regulation of glycogen synthase activity**

GS activity can be inhibited via external signaling. The major mechanism through which this occurs in the skeletal muscle is epinephrine signaling [119]. The regulation of glycogen metabolism is an evolutionarily significant mechanism by which the stress response enables an increased concentration of blood glucose for metabolic needs. During stress, epinephrine is released from the adrenal glands into general circulation. Epinephrine release results in a rise in blood glucose [120]. Epinephrine accomplishes this by stimulating glycogenolysis to increase hepatic glucose production, enabling the liver to transport released glucose into the bloodstream. Another mechanism by which
glucose rises in response to epinephrine is by decreasing skeletal muscle glucose uptake. Epinephrine signaling stimulates skeletal muscle glycogenolysis, inhibits glycogen synthesis, and decreases skeletal muscle transport of glucose by inactivating membrane GLUT4 as well as increasing the intracellular concentration of G6P [121]. Inhibition of the activity of GS by extracellular stimuli is largely accomplished by G-protein coupled receptor (GPCR) mediated activation of PKA. GPCRs mediate intracellular responses to environmental stimuli in eukaryotes [122].

GPCRs have an extracellular ligand binding site, seven transmembrane domains, and an internal cytoplasmic region [123]. GPCRs consist of approximately 800 unique receptors divided into three families [124]. G-proteins that are coupled to the cytoplasmic region of this receptor form a stable complex consisting of Gα, Gβ, and Gγ subunits [123]. G-protein subunits stabilize into inactive heterotrimers while bound to guanosine diphosphate (GDP) [125]. The binding of the ligand to the receptor triggers a conformational change, repositioning the transmembrane domain 6 to expose the cytoplasmic region for interaction [123]. Intracellular G-protein binding domains also undergo conformational changes in response to receptor reorganization [123]. This rearrangement of the intracellular components releases bound GDP and enables GTP binding in regulatory sites [122, 123]. GTP binds to the Gα subunit induces its dissociation from the Gβγ subunits [126]. The dissociated Gα subunit can then activate adenylyl cyclase (AC) to catalyze the conversion of ATP to cAMP and pyrophosphate [127]. In mammals there are nine membrane bound isoforms of AC1-AC9 as well as one cytosolic isoform [128]. Each membrane bound isoform of AC has two transmembrane domains, one cytoplasmic domain, and one C-terminal domain [128].
Membrane bound isoforms of AC can be activated by the Gα subunit after its dissociation from the GPCR complex [129]. The soluble isoform of AC can be activated independently from GPCR by bicarbonate anions and divalent calcium ions [129].

The complex regulatory apparatus of GS enables the rate of glycogen synthesis to proceed in intermediate states of activity [111]. Phosphorylation of GS at Ser697 of site 1a, Ser710 at site 1b, and Ser7 at site 2 by PKA inhibits GS activity [64, 111]. This provides an avenue by which glycogenesis may be suppressed in response to epinephrine via GPCR mediated signal transduction [130, 131]. The direct suppression of GS activity via phosphorylation by PKA works in conjunction with glycogen degradation [79]. Dephosphorylation of GS can be catalyzed by PP1 resulting in the increase of GS activity and subsequent increase in glycogen synthesis [79]. The activity of PP1 in the regulation of glycogen metabolism is accomplished by its association with glycogen targeting subunits [132]. Four isoforms of these glycogen specific PP1 targeting subunits have been identified and are responsible for localizing glycogen metabolic enzymes to the glycogen particle [132]. Muscle specific PP1 G₉ enables conditional regulation of PP1 interactions with glycogen associated proteins [132]. Direct binding of G₉ to GS enables the formation of a GS-G₉-PP1 complex resulting in the dephosphorylation of GS by PP1 [132]. In contrast, phosphorylation at both Ser48 and Ser67 of the PP1 G₉ by PKA signaling, stimulated by epinephrine, induces dissociation of G₉ from the complex [132]. This dissociation leads to the delocalization of PP1 from GS [132], resulting in GS remaining phosphorylated [132].

The hierarchical phosphorylation mechanism by which GS increases its activity is also critically gated by casein kinases (CK) [133]. Phosphorylation of site 5 by CK2
induces conformational shifts which enable structural compatibility with and subsequent phosphorylation by GSK3β at sites 4, 3c, 3b, and 3a, suppressing glycogen synthesis [133]. Phosphorylation sites near the NH2-terminus can be phosphorylated by PKA and AMPK as part of the energy sensing apparatus and response to energy demands [133]. The phosphorylation of sites 1a and 1b induce conformational compatibility for CK1 to phosphorylate site 2a, resulting in access to further regulation by GSK3β [133]. These complex relationships enable glycogen synthesis to be accelerated or decelerated in response to regulation by critical regulators within peripheral energy homeostasis pathways.

GS activity is stimulated by insulin signaling in the skeletal muscle through AKT signal transduction, primarily through the inhibition of GSK3β. Phosphorylation of Ser640, Ser644, Ser648, and Ser652 within cluster 2 of GS by GSK3β results in the greatest inhibitory influence of GSK3β on the activity of GS [46]. Insulin signaling in the postprandial state is the hormonal initiator for glycogen repletion in skeletal muscle [134]. Insulin stimulation of skeletal muscle provides the environment for GS activation through AKT signaling through two other critical mechanisms as well, glucose transport and PP1 activation. AKT activation of AS160 induces glucose transport into the skeletal muscle, providing glucose substrate for HK phosphorylation to G6P [135]. G6P is a potent allosteric activator of GS, and is also substrate for phosphoglucone isomerase [105]. Phosphoglucone isomerase catalyzes the conversion of G6P to G1P, which can then be used as substrate for uridine diphosphate glucose pyrophosphorylase [136]. The product of this catalysis, UDP-Glc, provides the direct substrate for GS polymerization of glucose into α-1-4 glycosidic chains [136]. PP1 activation in
association with the G\textsubscript{M} has the dual effect, inhibiting GYP and activating GS via dephosphorylation [132]. Insulin stimulation also regulates glucose homeostatic gene expression and subsequent protein expression of key regulators of energy metabolism [137, 138]. Insulin’s effects on gene regulation is an area of current study and outside the scope of this review. However, the effects of insulin signaling on regulation of gene transcription and translation must be considered when examining the overall environment of glycogen homeostasis [137]. It is through these combined effects that insulin signaling stimulates glycogen synthesis through AKT regulation of GS activity.

2.8 \textit{In vitro} models for skeletal muscle in glycogen research

There are relatively few \textit{in vitro} models for skeletal muscle suitable for research in glycogen homeostasis. Commonly used cell lines for examination of glucose, glycogen, and insulin signaling are the C2C12 mouse myoblasts, L6 rat myoblasts, and human skeletal muscle cell lines [139]. There are other skeletal muscle cell lines such as BC3H1 mouse myoblasts and MM14 mouse satellite cell lines, each having strengths and weaknesses depending on research purposes. Within glycogen research, each of the common models exhibit differences in genetic makeup, glucose transport, glucose oxidation, and glycogen synthesis regulation. Other factors that can influence treatment responses include differentiation methodology, morphological phenotype, serum concentration, and metabolic challenges. These factors can be a function of cell culture methods, passage count, and treatment concentrations. There can be significant variability in differentiation protocols and measured treatment responses between and within labs depending on these factors. While standard protocols have been established per lab when conducting experiments with these cell lines, nuances in variables such as
differing lot numbers for differentiation serum can necessitate significant deviations from standardized protocols. Considering the potential variability of responses and their effects on cell culture schedules, further research into differentiation can yield more representative and reproducible results in glycogen research.

2.8.1 *In vitro* skeletal muscle cell model comparison

There are critical differences between the common models of *in vitro* skeletal muscle in proliferative, metabolic, and differentiation gene expression [139]. These differences manifest through differential expression and regulation of key metabolic proteins in critical processes. The extrapolation of conclusions using *in vitro* models to whole organism regulation of glycogen regulation must also be cautioned. Skeletal muscle tissue is supported by endocrine communication, vascular, neuronal, and satellite cells, as well as mechanical infrastructure. In contrast, *in vitro* models are isolated cell culture monolayers without the regulatory feedback mechanisms present in an animal. Skeletal muscle tissue exhibits adaptive responses to mechanical demands and endocrine signaling over the life of an animal. Cell culture experiments are conducted with consideration of metabolic conditions in an animal but not representative of the homeostatic regulatory mechanisms which are persistently negotiated *in vivo*.

2.8.2 Primary cultures of human skeletal muscle cells

Glucose and glycogen homeostasis differ in significant ways within C2C12 cells, L6 cells, and human skeletal muscle cell (HSMC) primary cultures. HSMC cells possess the lowest oxidative capacity among the three models *in vitro* [139, 140]. HSMCs accumulate lactate in media at a significantly greater rate over the other models, providing further evidence of a lack of mitochondrial activity within HSMC cultures [139,
These primary cultures also express higher rates of proteins involved with contractile machinery, suggesting a phenotype resembling type I fibers [142]. The metabolic profile in type I fibers have greater capacity for energy production using glycolytic products [143]. Yet in vitro HSMC cultures have the lowest capacity for insulin stimulated glucose uptake and express GLUT1 and GLUT3 in greater abundance rather than GLUT4 [139]. This expression of glucose uptake machinery more closely resembles a type II phenotype with greater mitochondrial oxidative capacity relying more heavily on fatty acid oxidation for energy production rather than glycolytic products [142, 143]. GLUT1 and GLUT3 expression are associated with the modulation of basal glucose transport and are not responsive to insulin stimulation [144, 145]. HSMC cells possess a greater capacity to synthesize and store glycogen in comparison to C2C12 cells with insulin stimulation but not significantly more than L6 cells [139]. The HSMC cells have the greatest expression of GYS1 over L6 and C2C12 cells and have a higher level of GSK3β expression than L6 cells. HSMC cells exhibit the greatest rate of glycogen synthesis over L6 and C2C12 cells without significant difference in total glycogen accumulation [139]. This suggests that GSK3β is regulated differently in HSMCs compared to the rat and mouse models. These expression patterns may be due to monolayer tissue culture of primary cells rather than represent skeletal muscle physiology [139].

### 2.8.3 C2C12 mouse skeletal muscle cells

Mouse C2C12 cells are an immortalized cell line subcloned from the myoblasts of 2 month old mice thigh muscle by David Yaffe and Ora Saxel in 1977 [146]. C2C12 mouse cells exhibit a greater abundance of skeletal muscle protein than the other two
common models. C2C12 also has a different actin and myosin isoform messenger ribonucleic acid (mRNA) expression profile than HSMCs and L6 cells, with increased Myl2 myosin light chain and Acta2 actin alpha 2 [139]. Metabolically, C2C12 cells relied more on glycolytic products as energy substrate than HSMC, similar to the L6 cells [139]. However, lactate production is significantly lower in C2C12 cells when compared to HSMCs [139]. Mechanically, C2C12 cells exhibit the highest contractile response to electrical stimulation, as opposed to the L6 cells which exhibit no response [139]. HSMC cells exhibit an intermediate contractile response, suggesting that C2C12 may be preferred over L6 and HSMC in studies examining contractile features of skeletal muscle in vitro. Despite these advantages over other in vitro cell models, the expression of mRNA for contractile proteins in C2C12 cultures is dramatically reduced compared to the mRNA expression in an animal [139]. Overall, C2C12 cells have greater redistribution of tricarboxylic acid (TCA) cycle intermediates from catabolic products into amino acid biosynthetic pathways over L6 and HSMC cells, making them a good model for TCA and protein synthesis studies [139].

2.8.4 L6 rat skeletal muscle cells

Rat L6 skeletal muscle cells are an immortalized cell line isolated from the thigh of newborn rats by David Yafee in 1968 [147]. L6 cells are commonly utilized in metabolic studies examining insulin stimulation, with insulin eliciting a greater response than HSMC and C2C12 cells in glucose uptake [139]. L6 cells express Slc2a4 mRNA, encoding for GLUT4, in greater abundance than the other models [139]. Expression of As160 (Tbc1d1) and Rac1 mRNA, intermediates in the AKT mediated GLUT4 translocation and membrane fusion, are both decreased over HSMC [139]. However,
these do not appear to affect insulin stimulated glucose uptake. This discrepancy in mRNA expression of distal glucose transport intermediates and the increase in glucose transport response to insulin suggests that the regulation of these intermediates may supersede expression in L6 cells. Expression of Pik3cd mRNA, encoding the PI3Kδ subunit is also increased in the L6 cells, supporting the 1.8-fold response in insulin stimulated glucose uptake compared to the 1.3-fold and 1.2-fold responses in C2C12 and HSMC cells respectively [139]. Expression of Gys1 is lower in L6 cells compared to HSMCs, though there are no significant differences in glycogen accumulation between the two under basal or insulin stimulated conditions [139]. Interestingly, L6 cells express less Gsk3α and Gsk3β when compared to HMSCs, suggesting that GSK3 may have different regulatory patterns across models [139].

L6 cells exhibit the greatest capacity for glucose oxidation among the models and have greater expression of each mitochondrial oxidative phosphorylation enzyme complexes over HSMC [139]. L6 cells express significantly more protein than HSMC and significantly less than C2C12 cells and exhibit greater metabolism of glucose to carbon dioxide than HSMC and C2C12 cells [139]. The lack of skeletal muscle protein and contractile features in the L6 cells likely drives this metabolic tendency and is not represented in rat skeletal muscle. L6 cells have less CamkIIα expression than HSMC and C2C12 cell models, a critical component of calcium sensing indispensable for mechanical demand. This limitation of L6 cells must be taken into consideration when examining mechanistic relationships of signal transduction participants. The discrepancy between the in vitro L6 skeletal muscle model and in vivo rat skeletal
muscle tissue potentially represent a critical divergence in glucose metabolism between the two [139, 148].

2.9 Differentiation of skeletal muscle myoblasts to myotubules

The differentiation of myoblasts into myotubules is critically important when using cellular models of skeletal muscle in vitro. In animals, myogenesis can be considered to occur within two major developmental stages [149]. During embryonic and neonatal development skeletal muscle myofibers are constructed via commitment of pluripotent mesodermal cells to myoblasts [150]. Myofibers consist of fully differentiated myotubules, the number of which are determined during embryonic development [151, 152]. Skeletal muscle tissue develops via myoblast fusion with myotubules, elongating the structure from origin to insertion [149, 152]. Integration of myoblasts into myotubules results in long, polynucleated cells which contain the contractile machinery for voluntary motility function [149]. As myofibers elongate, the peripheral neuronal, vascular, connective, and lymph infrastructure of the skeletal muscle develops via growth factor signaling and paracrine communication to support mechanical processes both metabolically and structurally [153-155]. Post-neonatal development, the role of differentiation in skeletal muscle tissue is limited to satellite cells, which differentiate into myoblasts as a mechanism for repair and growth [156].

The process by which differentiation occurs in vivo differs from induction of differentiation in in vitro cell cultures [156]. Fetal serums containing the cocktail of growth factors and differentiation agents are added to cell media inducing proliferation of myoblasts and fusion to myotubules in cell culture plates [157, 158]. Unlike the physiological environment of an animal, the nuanced endocrine feedback from
developing tissue and peripheral organs and supportive cell types is absent [159].
Another critical difference between differentiating myoblasts in cell culture and animal tissues is the lack of structural features [160]. C2C12 cells and HSMCs tend to form organized skeletal muscle structures in culture, assembling into parallel elongated clusters more closely resembling skeletal muscle. In contrast, L6 cells exhibit a high degree of disorganization in cellular differentiation, lacking the parallel myofiber structures [161]. Each cell model is limited in this regard as there are no origin and insertion points or supportive structures. Differentiation in vivo is guided precisely by paracrine and endocrine feedback as well as structural reorganization as myoblast progenitors are integrated into myotubules. Cells cultured in a monolayer rely on gas diffusion from media rather than vascular perfusion and lack the circulatory components of the cardiovascular and lymph systems [162]. This enables buildup of metabolic products in media such as lactate, which participate in regulatory feedback pathways [139, 163]. Glucose transport is also significantly altered under these conditions as the molar concentration of glucose transported into skeletal muscle tissues is in flux with the rest of systemic glucose homeostasis [164]. Many cell protocols contain glucose concentrations which mimic hyperglycemic conditions, inducing alterations in homeostatic regulation of glucose within the myotubules [165]. Treatments in cell media also pose another limitation in the utility of skeletal muscle cell models. Insulin concentration in an animal is tightly controlled via glucose sensing in pancreas, insulin release, peripheral glucose uptake by adipose, brain, and liver tissues, and insulin degradation via circulating inactivators [166, 167].
In an animal, insulinemia closely follows glycemia and the half-life of circulating insulin is measured in minutes [168]. The fate of insulin in cell culture media is dramatically different without these organismal regulatory mechanisms and persists until it is endocytosed as a ligand for an IR or is degraded in the media [169, 170]. Glucose concentration in cell media does not follow an animal model of ebb and flow with hepatic glucose production from gluconeogenic and dietary sources, renal glucose production from resorption and gluconeogenesis, and disposal of glucose by cells and tissues distal to the skeletal muscle [171]. These factors result in different regulatory patterns of glucose homeostasis in cell culture when compared to animal models. This effect is exemplified in the induced development of insulin resistance in L6 cells with long term exposure to insulin on the order of days in the presence of high glucose media [172, 173]. Insulin resistance within these models exhibits significant deviations to the development of insulin resistance in animals without the mediation of inflammatory signaling, peripheral tissue communication and compensation, and functional outcomes in an animal [174].

Skeletal muscle is voluntarily engaged in an animal, performing concentric and eccentric motility functions and subject to the metabolic fluxes of substrates, products, and intermediates which enable that function [175, 176]. C2C12 and HSMC each are capable of spontaneous and electrically stimulated contraction, in contrast to L6 cells which lack the machinery for this response [139, 177, 178]. However, these in vitro stimulatory conditions are not representative of the electrical and biochemical communication between skeletal muscle and neurons forming a neuromuscular junction [179]. Consideration of these critical differences in biomechanical homeostasis is
imperative when making conclusions based on experimental outcomes generated with
\textit{in vitro} cellular models of skeletal muscle [180].

\section*{2.9.1 Induction of myoblast differentiation \textit{in vitro}}

Cell culture methodologies for myoblast differentiation can vary a significant amount between protocols [181-183]. Laboratories which use these models differ in proliferation time, differentiation time, growth serum and concentration, and differentiation serum and concentration [184-187]. Commonly used growth protocols involve using fetal calf serum (FCS) or fetal bovine serum (FBS) at or around 10% concentration [188]. Differentiation protocols involve reducing serum concentration and can include using a different serum such as horse serum (HS) [189]. Determination of differentiation status can be accomplished via several laboratory assays verifying the presence of mRNA and/or protein specific to differentiating myoblasts or terminally differentiated myotubules [190].

Quantitative reverse transcription PCR can approximate the quantity of mRNA transcripts present at the point of harvest. Commonly measured mRNA transcripts indicative of skeletal muscle cell intermediate states correspond to the cell cycle status during the differentiation process [191]. Myoblast determination protein 1 (Myod1) is expressed during the activation of satellite cells in their transition toward myoblasts [192, 193]. Transcription factor paired box 7 (Pax7) is expressed in quiescent muscle stem satellite cells and is required for the sequential expression of myogenic factor 5 (Myf5) [194, 195]. \textit{Myf5} is expressed during commitment to differentiation into myotubules [196]. Myoblasts will then express myogenin (Myog), suppressing expression of \textit{Pax7}, which enables myoblast fusion into myotubule structures [197].
During incorporation, the fusing myoblast will express myosin heavy chain (Myh2), contractile machinery absent prior to the structural incorporation into the polynucleated myofiber [198, 199]. Complete differentiation into myotubules and expression of terminally differentiated proteins will be indicated by the complimentary lack of expression of the differentiating factors associated with the myoblast phenotype [199]. Western blotting can then be used to confirm expression of these proteins associated with respective mRNA transcripts. Activity assays can also be used to verify the presence of and quantify the activity of catalysts such as creatine kinase, which phosphorylates creatine as an energy buffering mechanism between the transition of ATP consumption and energy production for sustained activation of motor function in myotubules [200]. Other supportive indicators of differentiation include creating a fusion index via immunofluorescence microscopy which can quantify cell morphology, and nuclear staining indicative of myonucleation and myoblast assimilation [193, 201]. Insulin has been examined as a differentiation inducing agent, however it is unnecessary for myoblast fusion into myotubules under low serum conditions [147, 161, 202]. [203]. Spontaneous fusion of myoblasts to myotubules is possible in the absence of serum, however standardized protocols use serum for consistency and reliability [204].

2.10 Cycloheximide research

Cycloheximide (CHX) is a protein synthesis inhibitor used in biological research to examine mechanisms related to the abundance of protein within a system. CHX is a fungicide synthesized by Streptomyces griseus that inhibits translation via stabilization of polyribosome translocation [205]. A diagram of CHX inhibition of protein synthesis
can be found in Figure 1.8. CHX can be introduced into cell culture media and its effects on protein expression can be measured using western blotting via chemiluminescence or fluorophore binding, flow cytometry, and fluorescence microscopy. A common method to determine CHX effects is by utilizing CHX chase protocols. Measuring protein expression after treatment with CHX during a time course experiment can provide a measure of protein stability within a system by inhibiting the synthetic flux. Examination of the abundance of a target protein in this manner can provide a measure of protein half-life and alterations based on other conditions within the cellular system. The main advantage of using this system in the examination of protein stability is that it does not require the use of radiolabeling. However, using CHX in cellular systems comes with many limitations.

CHX induces metabolic effects during cell treatment in addition to the inhibition of protein synthesis. In rat primary hepatocytes and adipocytes, CHX can stimulate glycogenolysis, gluconeogenesis, and ureagenesis secondary to α and β-adrenergic activation, not inhibition of protein synthesis [206]. CHX induces glucose uptake, lipid synthesis and fatty acid esterification from glycolytic products, and glucose oxidation independent of protein synthesis inhibition in epididymal rat fat pads ex vivo [207]. CHX alters glycogen homeostasis in rats after injection and in rat isolated hepatocytes, preventing the conformational shift of GS from b to a, dependent upon protein synthesis [208, 209]. In HEK-293 cells CHX induces phosphorylation of AKT at Ser473 after 3 hours of treatment and at Thr308 after 36 hours of treatment [210]. Substrates of AKT such as GSK3β are also phosphorylated in response to CHX treatment in HEK-293 cells, and protein degradation is altered as a result of this signal transduction [210].
Within skeletal muscle, CHX treatment has been observed to significantly alter glucose homeostasis. CHX treatment induces glucose uptake into skeletal muscle via increased translocation of GLUT4 vesicles to the cell membrane without changes to total GLUT4 expression [211]. This effect may be due to the inhibition of a short half-life of a negative regulator in the AS160 signal transduction cascade. The increase of cytosolic glucose also occurs in ex vivo treatment of insulin resistant rat skeletal muscle, and glycogen also accumulates within 5 hours of CHX treatment [212]. Whether these changes are primary effects of CHX interactions with signaling participants or secondary effects of the inhibition of short half-life of some regulators has yet to be determined. However, the emergent metabolic effects of CHX administration may be a partial function of the protein synthesis inhibition with longer duration of treatment.

2.11 Retinoic acid research in skeletal muscle glycogen homeostasis

Retinoic acid (RA) is the active metabolite of vitamin A and must be acquired through dietary means. RA has two isoforms with biological activity in humans, 9-cis-RA (9CRA) which is the ligand for retinoid X receptors (RXR), and all-trans-RA (ATRA) which is the ligand for retinoic acid receptors (RAR). RA is lipophilic and it is absorbed via nonspecific mechanisms before transiting through the lymph system and entering into general circulation via lipoprotein metabolism. Hepatic stellate cells serve as a reservoir for storing its precursor retinyl esters in normal homeostasis. Retinoids are subsequently transported systemically and intracellularly via specific retinol binding proteins. The regulation of RA within cells is tightly controlled due to its activity in the regulation of gene transcription. RA receptors form heterodimers and homodimers, enabling complex and conditional modulation of gene transcription in combination with
other transcription factors. There is much literature on the effects of the role of RA during embryonic development and the direction of the formation of somatic structures. Less is known about the metabolic role of RA in skeletal muscle and glycogen metabolism.

### 2.11.1 Vitamin A introduction and nutrition

Vitamin A is defined as all-trans-retinol [213]. Evidence for vitamin A’s integral roles in systemic homeostasis have been investigated for more than a century [214]. Throughout this time vitamin A’s critical roles have been observed and demonstrated in energy substrate homeostasis [215], taste sensory organs [216], gastrointestinal epithelium function [217], inflammation [218, 219], immunity [220], the microbiome [221], reproduction [222], embryogenesis and fetal development [223], growth [224], vision [225], and iron status [226]. Vitamin A’s role in these systems is further reviewed by Wiseman et al. [227].

Vitamin A is acquired via the diet as its precursor carotenoids from plants and retinoids from animal tissues [228]. Carotenoid content in plants can generally be correlated with coloration and ripeness, with orange-red vegetables containing the highest amount [229, 230]. Retinoids from animal sources of vitamin A tend to correlate with fat content, with egg yolks and whole milk being exemplary sources [229]. Prolonged dietary deficiency of vitamin A results in changes to the eye, diminishes immune response to infection and susceptibility, and eventually leads to death [231]. Clinical biochemical indicators of vitamin A deficiency include plasma retinol concentrations of less than 0.7 µM, and less than 0.35 µM indicate severe deficiency in average adults [227, 232]. Intakes of vitamin A sufficiency are now measured in retinol
activity equivalent (RAE) for both carotenoids and retinoids [233]. The RAEs for β-carotene are 1:12, and 1:24 for β-cryptoxanthin and α-carotene [233]. The recommended dietary allowance for these nutrients is 900 RAE for males and between 700 and 1300 for females depending upon pregnancy or lactation [233, 234].

2.11.2 Retinoid metabolism in skeletal muscle

Retinoids are metabolites of vitamin A. Retinol must first be converted to retinaldehyde, then to RA before it can proceed to function in a regulatory manner within skeletal muscle. RA translocates into the nucleus where it interacts with the RAR and RXR. A diagram of skeletal muscle retinoid metabolism can be found in Figure 1.9. Once bound to its receptor it can proceed to bind to retinoic acid receptor response element (RARE) to promote gene expression [235]. The study of the action of RA in skeletal muscle is focused within embryogenesis and regenerative medicine via modulation of satellite cell commitment to myoblast morphology. The influence of RA as it relates to glucose homeostasis and glycogen metabolism is less clear. Our lab has demonstrated that RA asserts indirect regulatory influences on intracellular and systemic glucose homeostasis through its action within myotubules [236, 237]. The role of RA has yet to be fully resolved within myotubule glycogen regulation.

2.11.3 Retinoid influence of glycogen in skeletal muscle

The role retinoids play in the regulation of skeletal muscle glycogen metabolism has not been thoroughly investigated. An elevation in hepatic vitamin A content in patients with T2DM was initially reported by Moore in 1937, suggesting a relationship between vitamin A and glucose metabolism [238]. Subsequent studies using human models have revealed alterations of retinoid homeostasis in individuals with T2DM.
Elevated serum vitamin A levels have been documented in individuals with impaired glucose tolerance or T2DM [239-241]. Elevated levels in the retinol binding protein-4 to retinol ratio have also been reported in hyperinsulinemic, glucose intolerant patients [242]. Retinoids have been considered as a T2DM therapy to induce pancreatic β cell proliferation [243]. Retinoids have also been suggested to play an antioxidant role in the progression of obesity induced insulin resistance to T2DM via immunomodulatory effects [244]. However, the exact nature of retinoid interaction with the mechanisms of systemic glucose management remains unclear.

**2.11.4 Retinoic acid effects on differentiating myoblasts**

RA has also been examined to a lesser extent within the context of glycogen homeostasis during differentiation. RA enhances insulin effects in the presence of insulin-like growth factor 1 (IGF1) during L6 myoblast differentiation inducing creatine kinase activity, GS activity, and glucose transport [245]. Dexamethasone as a differentiation factor was also examined by Elsner et al. and attenuated the effects of RA, IGF1, and insulin action on glycogen accumulation and homeostasis. RA induces C2C12 differentiation in the presence of the differentiation inhibiting transforming growth factor-β which inhibits the action of transcription factors Smad2 and Smad3 [246, 247]. RA treatment induces expression of Smad3 which enables differentiation by binding inhibitory CCAAT/enhancer binding protein-β, preventing it from halting differentiation in C2C12 cells [246]. However, a separate study demonstrated that RA asserts inhibitory effects on C2C12 cells as well as HSMCs, and that knockdown of RARβ and RARγ attenuates the inhibitory effects of RA treatment on myoblast differentiation [248]. The effects of RA in skeletal muscles are contingent upon the expression RAR and RXR.
9CRA achieves greater effect in differentiated myotubules through RXR activation in C2C12 cells [249]. All-trans-RA plays a greater role during differentiation by regulating expression of MyoD, Myog, and Myf5 through RAR activity in C2C12 cells [249]. There is much conflicting evidence regarding the effects of RA during and after differentiation of in vitro skeletal muscle models, and a paucity of information regarding RA effects on glucose homeostasis of differentiating myoblasts. Due to the variability of differentiation protocols experimental variability, it is critical to examine the metabolic effects of these treatments during differentiation.

2.11.5 Retinoid influence on glycogen in animal models

Experimentation with animal models has revealed more evidence of the role that retinoids play in glucose homeostasis. The quantity of vitamin A consumed in the diet of rats has been demonstrated to correlate with hepatic glycogen content. Wolf et al. reported no measurable hepatic glycogen content in vitamin A deficient rats, whereas pair-fed rats were able to maintain glycogen content [250]. Injection with glucose similarly resulted in significant impairment of glycogen synthesis in vitamin A deficient rats compared to controls [250]. In 1968, Singh et al. investigated a discrepancy in the reported effects of hypervitaminosis on hepatic glycogen concentration of pair-fed rats [251]. The experiments Singh et al. conducted were further exploration of hypervitaminosis effects within the liver and diaphragm muscle of rats reported from Ray et al. in 1959 [252]. Ray et al. described a decrease in liver glycogen content and depressed glucose usage in the diaphragm with hypervitaminosis concurrent with hyperthyroxinemia [252]. Kumar et al. later reported that hypervitaminosis exhibited a positive correlation with hepatic glycogen in rats [251]. Singh et al. observed a 600%
increase in hepatic glycogen content over the control after 2 days of treatment, and a continual increase with up to 8 days of treatment [251]. In these experiments, Singh et al. also noted that glycogen retention during 20 hours of fasting was between six and ten-fold greater than the control [251]. The mechanism of this relationship was later investigated by Chen et al. [253]. In 2009 Chen et al. reported that the expression and activity of glucokinase in primary hepatocytes and Zucker lean rats is dramatically induced by the synergistic effect of insulin and retinoids containing lipophilic liver extract [253]. This finding is significant as it demonstrates a potential direct role of retinoid modulation of glucose homeostasis in vivo, and identifies that effects are mediated through the activation of RAR in concert with the effects of insulin.

Evidence from metabolic studies has shown an important yet unclear role which retinoids play in skeletal muscle. ATRA administration in mice has been linked to increased energy consumption [254, 255]. Felipe et al. reported that vitamin A supplementation in chow diet reduced weight gain in obesogenic C57BL/6J mice without significant difference in dietary energy consumption [255]. Measurement of uncoupling protein 3 (UCP3) mRNA showed a significant increase over the control with ATRA injection in NMRI mice [255]. A significant increase in UCP3 expression was also seen with vitamin A supplementation in the normal fat group [255].

A later study which further investigated the mitochondrial changes associated with vitamin A supplementation was conducted by Berry et al. in 2009. Berry et al. reported that administration of ATRA to obese mice resulted in significantly increased mRNA expression of UCP3, phosphoenolpyruvate carboxykinase, as well as the mitochondrial fatty acid transporter carnitine palmitoyltransferase B, within the skeletal muscle over
the control [254]. These changes were seen concurrently with a decrease in blood glucose levels without changes to the skeletal muscle mass between the RA supplemented group and control [254]. Histological examination of the gastrocnemius and soleus revealed a dramatic reduction in intramuscular fat, a feature which positively correlates with the development of insulin resistance [254]. Another indicator of modulation of mitochondrial biogenesis was the increased expression over the control of succinate dehydrogenase observed in western blotting and confirmed by immunofluorescence microscopy [254]. This information suggests that RA induces greater mitochondrial oxidation of substrates through multiple pathways which modulate energy homeostasis within skeletal muscle.

2.11.6 Retinoid influence on glucose homeostasis in differentiating L6 myoblasts

Experimentation with rat L6 myoblasts examined the relationship between insulin signaling and ATRA. Sleeman et al. demonstrated that the administration of ATRA in the presence of insulin induces greater glucose uptake as well as greater expression of GLUT4 mRNA in a time and dose dependent manner in L6 cells [256]. Investigation of the further effects of retinoid administration in the media of L6 cells by Matthew Goff revealed that long term exposure with ATRA and insulin increased glucose uptake of L6 cells and increased media acidity, indicating increased glucose metabolism over the control [237]. Experimentation in L6 cells with retinol, ATRA, and retinal, along with LG268 (RXR agonist), TTNPB (RAR agonist), and T1317 (a Liver X Receptor agonist) revealed that the alterations in glucose metabolism observed were mediated through RAR signaling [237]. Interestingly, Goff reported a significant decrease in GLUT4 protein expression levels over controls in the presence of insulin, and a significant
decrease in GLUT4 expression with the presence of ATRA over insulin alone [237]. Goff also detected differences in AKT phosphorylation at Thr308, GSK3β at Ser9, and GS at Ser641 in vivo using Zucker lean rats with streptozotocin induced diabetes [237]. Streptozotocin destroys pancreatic β-cells and prevents insulin secretion, providing a model for insulin dependent type 1 diabetes mellitus. The rats were fed either a vitamin A sufficient diet or a vitamin A deficient diet [237]. Measurement of GS expression levels from the gastrocnemius of the group which received insulin treatment revealed a dramatic increase in the quantity of GS in rats with sufficient vitamin A compared to those which received the vitamin A deficient diet [237]. Measurement of the Gys1 mRNA levels from these tissues was not reported, however measurement of Gys1 mRNA in L6 myotubules treated with both insulin and ATRA showed no increase over control [237].

These data support an active metabolic role for RA in the regulation of glucose and glycogen homeostasis in skeletal muscle, although the mechanism by which these effects occur remains unclear. Few studies have been conducted examining RA effects on glucose homeostasis during myoblast differentiation to myotubules. RA enhances insulin action during differentiation of L6 myoblasts, inducing glycogen accumulation via modulation of GS activity [245]. Limitations in the study of Elsner et al. include the absence of negative controls. Many treatments were tested simultaneously, and the isolated effects of insulin, IGF1, and RA during the experiments reported within this study were not examined. Other work examining RA examines RA effects in satellite cells or RA and insulin effects in fully differentiated myotubules. Due to the variability in
protocols, cell responses, and verification of differentiation status, further investigation of RA and insulin during myoblast differentiation is needed.

2.12 Gaps in current understanding

The regulation of glycogen in skeletal muscle is a robust, flexible system capable of responding to internal and external regulatory stimuli. Increasing the capacity for glucose uptake and glycogen synthesis in skeletal muscle represents an avenue for intervention in addressing hyperglycemia as insulin resistance progresses to diabetes. The effects of retinoid action within skeletal muscle are most studied within the context of embryogenesis, satellite cell differentiation, and regenerative medicine. RA enhances insulin stimulation in fully differentiated myotubules to induce glycogen accumulation and alterations to key regulators of glycogen metabolism.

Current understanding of RA indicates that its primary function is to regulate gene transcription via RARE and heterodimerization with other transcription factors. Posttranslational modification of key regulators of glycogen synthesis in response to insulin stimulation are enhanced with the addition of RA in fully differentiated myotubules [237]. RA also affects insulin signaling in differentiating myoblasts to alter glycogen homeostasis, though the modulatory effects on key regulators in this critical window have not been explored. Among these regulators, GSK3β inhibition of GS exerts the most significant effect in the modulation of glycogen synthesis. The complexity of GSK3β’s regulatory influence is often overlooked and simplified when describing its role within its different systems. The phosphorylation state of Ser9 is most strongly associated with the activity of GSK3β and is often used to represent its kinase activity. Much information regarding the mechanistic relationships within skeletal muscle
has been derived from *in vitro* work using L6 skeletal muscle cells. Considering the critical differences between *in vitro* and *in vivo* metabolism and adaptation and the phenotypical alterations in contractile machinery, conclusions from *in vitro* experiments are inherently limited.

Insulin signaling and RA effects on glycogen homeostasis in differentiating myoblasts can be different from fully differentiated myotubules. This can be due to the differential expression of IRs, insulin signaling transduction participants, and metabolic machinery responsible for glycogen homeostasis. We have observed significant variability in the differentiation timelines of L6 myoblasts using consistent protocols, and the effects of altered differentiation patterns on glycogen homeostasis with RA and insulin treatments is not considered or reported in the literature. Delays in growth to confluence can range from 18 hours to 4 days. We have conducted several experiments examining progressive effects of insulin action in the presence and absence of serum, vehicle contribution, insulin types and dilutions, etc. It has become apparent within our experiments that the differentiation process can greatly influence experimental outcomes. Investigation into the differences in response to treatments at different stages of differentiation may lead to greater experimental reproducibility of data. Investigation may also provide insight into alternate regulatory mechanisms which govern glucose homeostasis in skeletal muscle.

The effects of RA in skeletal muscle have a complex relationship with insulin signal transduction. RA was able to induce a synergistic effect on glucose uptake and homeostasis with insulin after 6 days of treatment in L6 cells [237]. Insulin resistance develops in L6 cells after 6 days of treatment [257]. How does RA change the fate of
glucose which has been transported into the cell? Goff reported that significant changes in glycogen content, insulin signaling, and glucose oxidation occur after 6 days of treatment with RA and insulin [237]. The findings of Goff indicate that RA augments aspects of glucose homeostasis, glycogen metabolism, and the regulatory proteins which negotiate the transition into an insulin resistant state in L6 myotubules [237]. Examining the onset of these transitions in insulin response, glycogen accumulation, and regulators of these metabolic features during differentiation can provide insight into the development of insulin resistance using in vitro models. The timing of these metabolic adaptations may be further used to guide experimentation to establish a more robust model of the development of skeletal muscle insulin resistance in the future.

The purpose of this dissertation is to examine the effects of RA and insulin on glycogen homeostasis in differentiating L6 myoblasts before 6 days of treatment within Chapter III. Chapter IV examines the effects of CHX on the regulators of glycogen synthesis and RA and insulin administration in differentiating L6 myoblasts as well as rat skeletal muscle ex vivo. Chapter V discusses the implications of the research conducted in this dissertation and suggests further experimentation to continue examining regulation of glycogen within skeletal muscle.
References


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Figure 2.1 Molecular structure of the glycogen particle Schematic representation of a glycogen particle. Glycogenin serves as a primer for glycogen synthesis, with glucose moieties attached Tyr194 via $\alpha$-1,4-glycosidic linkages providing the core structure for additional linear glucose $\alpha$-1,4-glycosidic linkages, while branching points are formed by $\alpha$-1,6-glycosidic linkages. This arrangement provides many access points for glycogen phosphorylase to rapidly digest the glycogen particle [1].
Figure 2.2 Cytosolic Glucose Utilized for Glycogen Synthesis in Skeletal Muscle. Schematic representation of cytosolic glucose utilization for glycogen synthesis in skeletal muscle. Abbreviations: G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; GBE, glycogen branching enzyme; GLUT4, glucose transporter type 4; GPI, glucose-6-phosphate isomerase; GS, glycogen synthase; HK, hexokinase; UDP-Glc, uridine diphosphate glucose; UDP-Glc-PP, uridine diphosphate glucose pyrophosphorylase.
Figure 2.3 Simultaneous inhibition of glycogen degradation and stimulation of glycogen synthesis by AKT. AKT activation of PP1 resulting in dephosphorylation of both GS and GYP, inhibition of GSK3β resulting in net glycogen synthesis. Abbreviations: AKT, protein kinase B; GSK3β, glycogen synthase kinase 3 beta; GS, glycogen synthase; PP1, protein phosphatase 1; Ser9, serine 9.
Figure 2.4 Feedback mechanisms in insulin resistance resulting in fatty acid accumulation in skeletal muscle. Lipid deposition resulting from dysregulation of adipose, liver, and skeletal muscle during the development of insulin resistance. Abbreviations: FFA, free fatty acids; NAFLD, non-alcoholic fatty liver disease; ROS, reactive oxygen species; SM, skeletal muscle; TG, triglycerides.
Figure 2.5 AKT dissociation from membrane PI3K complex to regulate cytosolic substrate. AKT is recruited to the membrane complex via PIP3 and is activated by PDK1 and mTORC2. AKT dissociates from the PI3K complex and proceeds to regulate cytosolic substrate via phosphorylation. Abbreviations: AKT, protein kinase B; IRS1, insulin receptor substrate 1; mTORC2, mammalian target of rapamycin complex 2; PDK1, 3-phosphoinositide-dependent protein kinase-1; PI3K, phosphoinositol 3-kinase.
**Table 1.1 Direct regulators of GSK3β at Ser9 in skeletal muscle.** Abbreviations: PKCα, protein kinase C-α; PKCβ1, protein kinase C-β1; PKCβ2, protein kinase C-β2; PKCγ, PKCβ2, protein kinase C-γ; P70S6K, p70 ribosomal protein S6 kinase; P90RSK-1, p70 ribosomal protein S6 kinase; PKB, protein kinase B; AKT, protein kinase B; PKA, protein kinase A; GSK3, glycogen synthase kinase-3; PP1, protein phosphatase 1; GS, glycogen synthase; PP2A, protein phosphatase 2 A; S9A, serine to alanine site specific mutation; cAMP, 3',5'-cyclic adenosine monophosphate; HeLa, Henrietta Lacks cell line.

<table>
<thead>
<tr>
<th>Regulator</th>
<th>Methods</th>
<th>Model</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCα, PKCβ1, PKCβ2, PKCγ</td>
<td>Recombinant PKC and GSK3 isoforms purified, radioassay for activity, confirmed with PP1 and PP1 inhibition</td>
<td>Recombinant</td>
<td>[258]</td>
</tr>
<tr>
<td>P70S6K</td>
<td>GS Peptide-1 phosphorylation C-terminal serine by GSK3β is inhibited by P70S6K and restored in the presence of PP2A in vitro</td>
<td>Purified from rabbit skeletal muscle</td>
<td>[259]</td>
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<tr>
<td>P90RSK-1</td>
<td>HeLa cells expressed either GSK3β or mutant S9A, immunoprecipitated and incubated with tryptic phosphopeptide, phosphorylation observed in vitro when cotransfected with P90RSK-1 dependent upon Ser9</td>
<td>HeLa</td>
<td>[260] [261]</td>
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<tr>
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<tr>
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Figure 2.6 Schematic representation of GS conformation regulation by GSK3β. Phosphorylation at site 3 by GSK3β induces conformation shift from active GS conformation to GS basal conformation resulting in a reduction of GS activity. Phosphorylation of GSK3β at Ser9 induces N-terminus conformation shift from trans to cis resulting in a reduction of GSK3β activity. Abbreviations: GS, glycogen synthase; GS A, glycogen synthase form a; GS B, glycogen synthase form b; GSK3B, glycogen synthase kinase 3 beta; Ser641, serine 641.
Figure 2.7 Site specific regulation of glycogen synthase. Regulators of GS within structurally organized clusters. Abbreviations: AMPK, AMP-activated protein kinase; CaMKII, calcium/calmodulin-dependent protein kinase II; CK1, casein kinase 1; CK2, casein kinase 2; DYRK1A, dual specificity tyrosine-phosphorylation-regulated kinase 1A; DYRK1B, dual specificity tyrosine-phosphorylation-regulated kinase 1B; DYRK2, dual specificity tyrosine-phosphorylation-regulated kinase 2; GSK3B, glycogen synthase kinase 3 beta; GYS1, glycogen synthase 1; p38MAPK, p38 mitogen-activated protein kinases; PASK, PAS domain containing serine/threonine kinase; PhK, phosphorylase kinase; PKA, protein kinase A.
Figure 2.8 Stabilization of polyribosome structure by cycloheximide. CHX binding to the polyribosome structure resulting in stabilization preventing the release of nascent peptides. Abbreviations: CHX, cycloheximide; Au, gold.
Figure 2.9 Retinoic acid regulation and activity in skeletal muscle. RA synthetic pathway from retinyl esters and diagram of RAR/RXR ligand binding, RARE activation, and transcription regulation. Abbreviations: ARAT, acyl-CoA:retinol acyltransferase; LRAT, lecithin retinol acyltransferase; RAR, retinoic acid receptor; RARE, retinoic acid response element; RXR, retinoid X receptor.
Chapter III: Time Dependent Changes in Glycogen Accumulation and Regulators of Glycogen Synthesis in Response to Retinoic Acid and Insulin Treatment in Differentiating L6 Myoblasts
3.1 Introduction

Skeletal muscle transports glucose from the bloodstream in response to insulin signaling. Due to the mass of skeletal muscle, enhancing this process represents the greatest potential to reduce hyperglycemia T2DM [1]. Hyperglycemia is etiologically driven by a lack of skeletal muscle glucose transport in response to insulin signaling. Impairment of the proximal insulin signaling is often observed in hyperglycemia concurrently with a reduction in AKT phosphorylation at Thr308 and Ser473 [2]. There is also a decreased Akt Substrate of 160 kDa (AS160) phosphorylation at sites Ser318, Ser588, and Ser751 resulting in a reduction of GLUT4 translocation and transport of extracellular glucose [2, 3]. GS activity and glycogen synthesis are also reduced in individuals with T2DM [4].

Skeletal muscle is composed primarily of two types of fibers. Type I muscle fibers, or slow-twitch, rely on a greater proportion of energy substrate derived from aerobic oxidation of fatty acids [5]. Type II muscle fibers, or fast-twitch, rely more heavily on anaerobic, glycolytic energy substrate production [5]. Frankenberg et al. reported that the individuals with T2DM have proportionally less type I muscle fibers compared to control [6]. Glycogen organization within T2DM muscle fibers is also organized differently, with a much greater proportion of glycogen-associated proteins localized to cytosolic compartments [6]. No differences in the total GS expression or total glycogen content were observed between T2DM and control individuals [6]. Frankenberg et al. proposed that dysregulation in localization of key glycogen associated proteins and muscle fiber type differences are responsible for the metabolic compromises of skeletal muscle observed in T2DM [6]. This observation highlights the
critical role which subcellular location of enzymatic participants plays in the regulation of glycogen homeostasis beyond phosphorylation of key residues.

The role that RA plays in conjunction with insulin signaling in regulating glycogen within skeletal muscle is unclear. Although many studies have been conducted to determine the role of RA in myoblast differentiation and skeletal muscle regeneration, less is known regarding the effects of RA’s regulatory influence in differentiating myoblasts. RA has a myriad of effects in differentiated myotubules, including alterations in glycogen homeostasis. Our lab has reported that treating fully differentiated L6 myotubules with RA (1 µM) or TTNPB (1 µM) (an RAR specific agonist), but not LG268, increases glycogen content after 6 days of treatment [7]. Increases phosphorylation of GSK3β at Ser9 as well as decreases phosphorylation of GS at Ser641 were also observed after 6 days of treatment with RA or TTNPB [7]. These changes in glycogen and its regulators were observed concurrently with decreases in media pH and media glucose concentration, indicating increased uptake and utilization of glucose for energy production and glycogen synthesis [7]. The effects observed in fully differentiated myotubules after 6 days of treatment with insulin, RA, and TTNPB did not reveal whether onset of the synergistic effects between RA and insulin occurred prior to 6 days of treatment.

Long term treatment with insulin has been demonstrated to induce insulin resistance in L6 cells [8, 9]. The onset of insulin resistance in L6 cells exposed to insulin (50 nM) and glucose (25 mM) occurs within 24 hours [8]. Alterations indicating an insulin resistant state include a decrease in relative expression of the insulin receptor, a decrease in its phosphorylation, a decrease in IRS-1 and its phosphorylation, and
decreased PI3K activity in response to insulin signaling [8]. The previous results generated in our lab show that RA can augment the response to insulin, attenuating the insulin resistance which develops during long-term exposure. Glycogen synthesis is regulated canonically by insulin signaling through IRS-PI3K-AKT signaling.

The aim of this study is to evaluate whether the effects of RA and insulin in differentiating L6 myoblasts to alter glycogen homeostasis occur in a similar fashion to prior myotubule studies and whether the effects are time and dose dependent.

3.2 Materials and Methods

3.2.1 Reagents

Cells were grown and differentiated in Dulbecco’s Modification of Eagle’s Medium 4.5 g/L glucose, L-glutamine & sodium pyruvate, purchased from Mediatech (Manassas, VA, U.S.A.). Penicillin streptomycin and (PS) (10,000 units/mL each) were purchased from Life Technologies (Grand Island, NY, U.S.A.). Fetal Bovine Serum (FBS) purchased from Life Technologies (Grand Island, NY, U.S.A.). Horse Serum (HS) was purchased from Hyclone Laboratories (Logan, UT, U.S.A.). All-trans-retinoic acid (RA) was purchased from Sigma (St. Louis, MO, U.S.A.) and diluted in dimethyl sulfoxide (DMSO). Insulin used in these experiments was Novalin pharmaceutical grade human insulin, generously provided by Dr. Guoxun Chen and diluted in phosphate-buffered saline (PBS) containing sodium chloride (NaCl) (137 mM), potassium chloride (2.7 mM), sodium phosphate (10 mM), and potassium phosphate (1.8 mM). Cells were cultured on NEST 60 mm tissue culture treated polystyrene dishes for protein analysis and NEST 100 mm dishes for glycogen analysis. Cells used for protein quantification were lysed using Tris(hydroxymethyl)aminomethane hydrochloride (Tris HCl) (10 mM)
7.4 pH, NaCl (100 mM), sodium dodecyl sulfate (SDS) (0.1%), sodium deoxycholate (0.5%), Triton X 100 (2%), NP-40 (1%), Glycerol (10%), ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) (1 mM), ethylenediaminetetraacetic acid (EDTA) (1 mM). Unless otherwise stated, protease and phosphatase inhibitors were diluted in ddH2O. The lysis buffer contained the protease inhibitors aprotonin (310 nM), pepstatin A (1 μM) diluted in 10:1 methanol:acetic acid solution, leupeptin (10 μM), phenylmethylsulfonyl fluoride (PMSF) (1 mM) diluted in 2-propanol, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (1 mM), bestatin (6.49 μM), and N-[N-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine (E-64) (20 μM) diluted in 50% ethanol solution. The lysis buffer contained the phosphatase inhibitors sodium orthovanadate (Na₃VO₄) (10 mM) prepared via boiling and ice bath cycles with HCl titration to pH 10 replacing evaporated ddH2O, sodium fluoride (10 mM), β-glycerol phosphate (10 mM), sodium tartrate (10 mM), sodium pyrophosphate (10 mM), sodium molybdate (Na₂MoO₄) (7 mM). Protein was measured using the Pierce bicinchoninic acid (BCA) kit purchased from Pierce (Rockford, IL, U.S.A.). Cell lysates were prepared for western blotting using a loading buffer containing Tris HCl 6.8 pH (75 mM), SDS (2%), bromophenol blue (0.002%), glycerol (10%), dithiothreitol (DTT) (100 mM).

Polyacrylamide gel electrophoresis (PAGE) was conducted using 10% resolving gels constituted from Tris HCl 8.8 pH (370.1 mM), 30% acrylamide 29:1 bis-acrylamide (10%), ammonium persulfate (APS) (0.0001%), SDS (0.0001%), tetramethylethlenediamine (TEMED) (5.6 mM) and 4% stacking gels constituted from Tris HCl pH 6.8 (123.5 mM), 30% acrylamide 29:1 bis-acrylamide (4%), APS (0.0001%), SDS (0.0001%), TEMED (17 mM). Electrophoresis was conducted in buffer containing
Tris HCl pH 8.3 (25 mM), glycine (190 mM), and SDS (0.1%). Protein was transferred to Immobilon®-PSQ polyvinylidene fluoride (PVDF) membrane 0.45 µm pore size which was purchased from EMD Millipore (Billerica, MA, U.S.A.). Protein transfer was conducted in buffer which contained Tris pH 8.3 (25 mM), glycine (190 mM), and methanol (20%). Membranes were blocked in a buffer containing Tween®20 (0.1%) (TBST), Tris HCl pH 7.6 (20 mM), NaCl (150 mM), nonfat milk protein (5%), and BSA (2.5%). Primary antibodies purchased from Cell Signaling Technologies (CST) (Danvers, MA, U.S.A.) were GS #3886, pGS (Ser641) #3891, GSK3β #9315, pGSK3β (Ser9) #9336, each diluted 1:1,000 in TBST. The secondary antibody purchased from CST (Danvers, MA, U.S.A.) was anti-Rabbit #7074P2 diluted 1:4000 in TBST. Horseradish peroxidase (HRP) primary conjugated β-actin #SC-47778 antibody was purchased from Santa Cruz Biotechnology, diluted 1:20,000 in TBST containing 5% nonfat milk protein. Pierce enhanced chemiluminescence (ECL) for HRP kit #32106 was used for chemiluminescence reaction. Luminescence signal was detected using Bio-Rad ChemiDoc XRS+ Image Capture. Quantification of optical density was performed using Bio-Rad Image Lab and Bio-Rad Analysis Software.

3.2.2 Cell culture

L6 Rat myoblasts were grown to 100% confluence in DMEM 4.5 g/L glucose, L-glutamine & sodium pyruvate (110 mg/L) containing 10% FBS, 1% PS. Post-confluence, media was replaced with DMEM containing HS (2%) and PS (1%) to initiate differentiation two days prior to treatment. DMEM cell culture media with HS (2%) and PS (1%) was replaced every two days thereafter containing treatment or vehicle control. Cells continued to differentiate under control and treatment conditions in 2% HS during
treatment. Treatments consisted of DMSO for the vehicle control for RA treated experiments and PBS for insulin treatments, Novalin human insulin (10 nM or 50 nM), RA (0.5 µM or 1 µM), or a combination of insulin or RA concentrations. Cells were harvested after 2 days and 6 days for time course analysis of treatment effects. Cells were cultured on 100 mm plates for glycogen analysis and 60 mm plates for protein analysis.

3.2.3 Glycogen assay

Cell culture plates were placed on ice and media was removed with a vacuum pump. Cells were washed with 5 mL of ice-cold PBS and removed with a vacuum pump. Cells were scraped with 1 mL of ice-cold PBS and pipetted into 1.5 mL microcentrifuge tubes in an ice-cold aluminum block. Cells were centrifugated at 1000 RPM for 5 minutes at 4°C to form a soft pellet. The supernatant was removed, and the cell pellet was flash-frozen in liquid nitrogen, then placed into -80°C freezer for storage. Cells were thawed on ice for assay. Cells were lysed with 300 µL of potassium hydroxide (KOH) (30%) at 100°C for 1 hour. Ethanol (200 proof) was added to cell lysate for a final concentration of 70% and cooled to -20°C for 1 hour for glycogen precipitation. Precipitated cell lysate was then centrifugated at 16,000 RPM for 15 minutes at 4°C to form a pellet. Supernatant was removed and the pellet was resuspended in HCl (3.0 M), then heated to 100°C for 1 hour for glycogen hydrolysis. Hydrolysate was cooled to room temperature and centrifugated briefly for volume normalization. Hydrolysate was then measured using the StanBio Glucose Liquicolor kit according to manufacturer protocol.
### 3.2.4 Western blot

Cells were placed on ice and media was removed with a vacuum pump. Cells were washed with 2 mL of ice-cold PBS which was immediately vacuumed out. Cells were scraped in 300 µL of ice-cold Tris-Triton lysis buffer and pipetted into 1.5 mL microcentrifuge tubes. Cells were agitated for 30 minutes at 4°C for lysis via microcentrifuge tube adaptor with a 4-E's vortex at low RPM. Cell lysate was then centrifuged at 16,000 RPM for 15 minutes at 4°C to form a pellet. Supernatant was transferred to fresh microcentrifuge tubes and the pellet was discarded. Cell lysate was placed in -80°C for storage. Protein concentration was measured using Pierce BCA kit and measured with the Glomax Multi Detection System at 560 nm absorbance wavelength. Protein concentration for each sample was normalized in loading buffer which consisted of Tris HCL 6.8 pH (75 mM), SDS (2%) glycerol (10%), bromophenol blue (0.002%). and 100 mM dithiothreitol (DTT). Prepared samples were heated to 100°C for 5 minutes for denaturation. Prepared samples were separated on PAGE gels using BioRad MiniProtean electrophoresis system. Samples were separated using voltage at 80 volts until samples had migrated past the 4% stacking gel and then at 120 volts for the remainder of protein migration. Protein was transferred to PVDF membranes prepared in methanol and transfer was conducted using BioRad MiniProtean transfer cassette system. Protein transfer was conducted with voltage at 50 volts for 2 hours at 4°C. Blocking solution was TBST with 5% nonfat milk, filtered to remove undissolved solids. Membranes were placed in blocking solution for 1 hour and gently agitated via orbital shaker at room temperature. Blocked membranes were incubated overnight in primary antibody at 4°C. Incubated membranes were then
washed with TBST for 5 minutes, 3 times at room temperature. Washed membranes were then incubated in secondary anti-rabbit antibody diluted 1:4000 for 1 hour at room temperature. The membranes were then washed with TBST for 5 minutes, 3 times at room temperature. Membranes were then treated with Pierce ECL prepared according to manufacturer protocol. Luminescence was detected using Bio-Rad ChemiDoc system and quantification was conducted using BioRad Image Lab software.

3.2.5 Statistical analysis

One-way ANOVA with Fisher’s LSD post-hoc was conducted using Graphpad Prism software version 9.5.1. The statistical significance of \( p \leq 0.05 \) is represented as *; \( p \leq 0.005 \) is represented as **; \( p \leq 0.0005 \) is represented as ***; \( p \leq 0.0005 \) is represented as ****; Data are presented as ±S.E.M. (n=4).

3.3 Results

3.3.1 Changes in glycogen accumulation and expression levels of regulators of glycogen synthesis in differentiating L6 myoblasts treated with insulin (10 nM) or insulin (50 nM)

Differentiating L6 myoblasts were grown to confluence and treated with control (vehicle), insulin (10 nM) or insulin (50 nM) as described in the methods. An n=1 dose response pilot study was conducted to measure insulin stimulated alterations to glycogen homeostasis. Insulin treatment stimulated glycogen accumulation in a time and dose dependent manner (Figure 3.1A). All Chapter III figures can be found in Chapter III Appendix. To observe the effects of serum on glycogen accumulation, a group was given a serum-free media after being grown to confluence for two days. The group which received the 2% HS differentiation media for two days exhibited an 8-fold
increase in total glucose released over its corresponding serum-free control, the
greatest difference within the treatment schedule (Figure 3.1A). Differentiating L6
myoblasts treated with vehicle, insulin (10 nM), or insulin (50 nM) for either two or six
days were controlled to total protein and similar induction was observed at day 2 and
day 6 (Figure 3.1B). Insulin treatment resulted in a decrease of GS expression after 2
days in a dose dependent manner (Figure 3.1C). After 6 days of insulin treatment GS
expression was reduced in both insulin treated groups, and insulin (10 nM) led to a
greater reduction than insulin (50 nM) (Figure 3.1D). Neither dose of insulin affected
pGS (Ser641) expression after 2 days of insulin treatment (Figure 3.1E). Insulin (10 nM)
induced a greater increase of pGS (Ser641) than insulin (50 nM) after 6 days of
treatment (Figure 3.1F). Expression of GSK3β exhibited a dose dependent response to
insulin treatment after 2 days (Figure 3.1G). After 6 days of treatment, expression of
GSK3β was elevated similarly in both groups treated with insulin (Figure 3.1H).
Phosphorylation of GSK3β at Ser9 was sharply induced in a dose dependent response
to insulin treatment after 2 days (Figure 3.1I). Expression of pGSK3β (Ser9) was sharply
increased after 6 days of insulin treatment with no additional effects with increased
insulin dose. (Figure 3.1J).

3.3.2 Glycogen accumulation and expression levels of regulators of glycogen
synthesis in differentiating L6 myoblasts treated with RA (0.5 µM or 1 µM) and
insulin (10 nM or 50 nM)

Differentiating L6 myoblasts were grown to confluence and treated with control
(vehicle), insulin (10 nM), insulin (50 nM), RA (0.5 µM), RA (1 µM), or a combination of
treatments as described in the methods. RA enhanced insulin induction of glycogen
accumulation with the greatest response at insulin (50 nM) + RA (0.5 µM) upon 2 days’ treatment (Figure 3.2A, C). After 6 days of treatment, RA (1 µM) + insulin (50 nM) induced the greatest increase in glycogen accumulation (Figure 3.2B, D). Expression of GS was reduced in all groups except RA (0.5 µM) at 2 days’ treatment (Figure 3.2E). After 6 days of treatments, GS expression was dramatically reduced in groups treated with RA (0.5 µM) and RA (1 µM) + insulin (50 nM) (Figure 3.2F). RA enhanced insulin’s effect on pGS (Ser641) expression after 2 days resulting in a dramatic reduction over insulin treatment alone (Figure 3.2G). After 6 days of treatment, the relative expression of pGS (Ser641) was induced with insulin (10 nM) treatment but was stable in other treatment groups (Figure 3.2H). GSK3β expression remained stable after 2 days of treatment, was induced by day 6 with RA (1 µM) + insulin (50 nM) treatment (Figure 3.2I, J). pGSK3β (Ser9) expression was induced in a dose dependent manner with insulin treatment without additional effects from RA (Figure 3.2K). After 6 days of treatment, the relative expression of pGSK3β (Ser9) was increased in all groups which received insulin treatment without additional effects from RA (Figure 3.2L).

3.3.3 Glycogen accumulation and expression levels of regulators of glycogen synthesis in differentiating L6 myoblasts treated with RA (0.5 µM) and insulin (10 nM)

Based on data from pilot experiments and previously generated data, treatment doses of RA (0.5 µM) and insulin (10 nM) were chosen for the time course experiment. RA enhanced insulin induction of glycogen accumulation after 2 days of treatment (Figure 3.3A, C). After 6 days of treatment, RA effects on insulin stimulated glycogen accumulation were attenuated (Figure 3.3B, D). Insulin treatment induced AKT
expression after 2 days of treatment with marginal enhancement from RA (Figure 3.3E). After 6 days of treatment the induction of AKT expression was normalized to control without effects from either RA or insulin treatment (Figure 3.3F). Insulin induced $p\text{AKT}$ (Ser473) expression after 2 days with no additional effect from RA (Figure 3.3G). After 6 days of treatment, RA (0.5 µM) marginally enhanced insulin (10 nM) stimulated expression of $p\text{AKT}$ (Ser473) (Figure 3.3H). The expression of GS remained unchanged with treatment throughout the time course (Figure 3.3I, J). There were no changes in $p\text{GS}$ (Ser641) expression after 2 days of treatment (Figure 3.2K). However, insulin induced $p\text{GS}$ (Ser641) expression after 6 days and was marginally enhanced by RA (Figure 3.3L). Similar to GS, GSK3β expression remained unchanged with treatment throughout the time course (Figure 3.3M, N). Insulin induced $p\text{GSK3β}$ (Ser9) expression after 2 days of treatment without additional effect from RA (Figure 3.3O). After 6 days, insulin stimulated $p\text{GSK3β}$ (Ser9) expression was marginally enhanced with RA (Figure 3.3P).

3.4 Discussion

In the present study, glycogen accumulation is significantly increased as a response to insulin and RA treatment concurrently with the induction of $p\text{AKT}$ (Ser473), $p\text{GSK3β}$ (Ser9), and $p\text{GS}$ (Ser641) in differentiating L6 myoblasts. Both 2 days and 6 days of treatment with insulin can be considered long-term exposure, as insulin is released post-prandially in response to bolus feeding and plasma concentrations closely trail blood glucose [10]. In normal physiological conditions, insulin secretion is biphasic, tightly correlated with blood glucose, rising and falling sensitively in response to glucose uptake [11]. In response to a bolus of glucose, insulin secretion initially rises sharply,
dips, and then is secreted more gradually trailing the return of blood glucose to post-absorptive glycemic conditions [11]. Insulin secretion in rats will fluctuate between 200 picomol/L and 600 picomol/L in response to a glucose bolus [12]. The half-life of insulin within rat serum *in vivo* is 0.18-1.67 minutes [12]. The exposure of skeletal muscle cells to insulin 10 nM represents a 50-fold increase over physiological basal concentration and 17-fold increase over physiological bolus concentration of circulating insulin levels in rats [12].

Previous investigations of the effects of RA on L6 myotubules have revealed that treatment of L6 cells with RA induces changes in glucose uptake [13]. Sleeman et al. reported in 1995 that RA (1 µM) enhanced insulin stimulated uptake of the uptake of 2-deoxyglucose after 8 hours of treatment [13]. Uptake of 2-deoxyglucose also peaked after 24 hours of treatment with insulin (1 µM) and RA (1 µM) [13]. The effects of RA and insulin on differentiation were not quantified during this study [13]. We observed that RA enhanced insulin stimulated glycogen synthesis after 2 days of treatment (Figure 3.3C) but did not enhance insulin effects after 6 days (Figure 3.3D). RA administration in conjunction with insulin treatment during differentiation may influence the myoblast differentiation process. The divergence of differentiation induced by RA on insulin signaling may be responsible for the altered pattern in GS regulation by GSK3β when compared to terminally differentiated myotubules (Figure2.3L, P) [7]. Prior to this study, Sargeant et al. reported via pulse-chase that treatment with insulin (100 nM) increased the half-life of GLUT4 in murine adipocytes from 50 hours to 65.5, and that GLUT4 expression was initially elevated over control, but had significantly decreased after 72 hours of insulin treatment [14]. Although conducted in adipocytes, this response
indicates that insulin sensitivity and glucose homeostasis maintain acutely adaptable equilibriums with insulin sensitive tissues. Adipocyte GLUT4 translocation and glucose uptake in response to insulin stimulation exhibit common regulation with skeletal muscle via PI3K signaling and have been demonstrated in vitro and in vivo to be dependent upon the syntax 4-binding protein Munc18c [15-17]. A similar desensitization response has been observed in skeletal muscle cells [18]. After two days of treatment, insulin alone did not induce additional glycogen accumulation over control without RA effects during myoblast differentiation (Figure 3.3A, C). Chronic insulin stimulation of L6 cells alters proximal insulin signaling transduction, resulting in reduced phosphorylation of IRS-1, reduced activity of PI3K, and reduced AKT phosphorylation [18]. We observed a similar phenomenon with insulin stimulation inducing pAKT (Ser473) after 2 days but effects were attenuated after 6 days without RA enhancement (Figure 3.3G, H). The difference in AKT expression between day 2 and day 6 may be partly responsible for this observation, as well the metabolic environment during differentiation (Figure 3.3E, F). Similar alterations to proximal insulin signaling are observed in the T2DM phenotype, and this method of inducing insulin resistance in L6 cells can provide insight for the mechanistic study of insulin signaling in T2DM [18, 19]. The alterations of insulin signal transduction concurrent with the development of insulin resistance ultimately affect distal insulin signaling to include GLUT4 synthesis, degradation, translocation, and activity [17, 20]. The compromise or downregulation of glucose uptake in the insulin resistant state is responsible for the hyperglycemia presented in T2DM [21]. The propensity for the development of insulin resistance can also be a result of altered activity, regulation, and expression of the IR [22], IRS [23], PI3K [24], AKT [25], AS160
Our observations indicate that pAKT (Ser473) expression mirrors pGSK3β expression at 2 (Figure 3.3G, O) and 6 days (Figure 3.3H, P) of treatment, suggesting that GSK3β activity may be correlated with insulin sensitivity. The onset of insulin resistance and pathogenesis of T2DM in humans emerges through altered insulin signal transduction [29, 30]. GS phosphorylation at site 3a by GSK3β follows the canonical model at 2 days of treatment (Figure 3.2K) exhibiting a trend of reduction concurrent with increased pGSK3β (Ser9) expression (Figure 3.3G) in differentiating myoblasts. However, after 6 days of treatment GS is desensitized to regulation by GSK3β in differentiating myoblasts (Figure 3.3L, P).

It was first reported in 1965 that muscle glycogen concentration acts as a negative feedback mechanism for GS activity [31]. This regulatory feature of glycogen acting as a GS inhibitor was further explored within the context of T2DM [32]. Jensen et al. reported that lowering the concentration of skeletal muscle glycogen through fasting and refeeding of Wistar rats resulted in significantly increased glucose uptake under basal and insulin stimulated conditions [32]. Notably, the team found that increasing glycogen concentration by refeeding did not interfere with insulin stimulated glucose uptake [32]. This suggests that normal depletion and repletion of glycogen, including elevated glycogen concentrations through supercompensation, are subject to different regulatory mechanisms than skeletal muscle which is chronically exposed to elevated glucose and insulin levels [33]. Due to the capacity of skeletal muscle glycogen synthesis to serve as a major site of blood glucose disposal, dysregulation of this system may be partially responsible for the pathogenesis for T2DM. A later investigation [26], GLUT4 [27], and other participants in insulin stimulated glucose uptake [28].
of this inverse correlation revealed that the inhibition of the activity of GS occurs separately from proximal insulin signaling [34]. Our observation of the desensitization of GS (Figure 3.3L) regulation by GSK3β (Figure 3.3P) in the presence of increased glycogen (Figure 3.3D) provides evidence for alternate mechanisms of glycogen regulation with differentiating myoblasts isolated from skeletal muscle from rats fed low, normal, and high glucose diets exhibited no significant difference in tyrosine phosphorylation of the IR in response to insulin [34]. Similarly, IRS-1 stimulated PI3K activity, and subsequent phosphorylation of AKT (Ser473), and the resulting phosphorylation of GSK3β (Ser9) were also preserved in response to insulin signaling across each diet [34]. Insulin stimulated glucose uptake was also not significantly different between different diet groups and GLUT4 expression was not significantly different between the normal and high glucose diet groups [34]. However, in the group fed the high glucose diet GS phosphorylation of site 3 was significantly increased, GS activity was significantly decreased, and glycogen concentration was 2 fold higher than the group fed normal diet and 3.5 fold higher than the group fed the low glucose diet [34]. We have observed a similar effect of elevated pGS (Ser641) in differentiating L6 myoblasts, concurrent with elevated glycogen accumulation and continuity within proximal insulin signaling (Figure 3.3L). The activity of GS is ultimately regulated structurally, and each combination of the hundreds of allosteric interactions and posttranslational modifications can potentially modulate GS activity in subtle and overt ways [35, 36].

The action of RA to attenuate insulin resistance in differentiating L6 myoblasts may proceed through a myriad of regulatory pathways. Insulin sensitization has been
achieved in mice via inhibition of negative feedback genes involved in insulin signaling [37]. RA has been demonstrated to modulate expression of genes which regulate energy substrate production from lipids and glucose as they relate to normal physiology and insulin resistance [38-40]. After two days of treatment with RA (0.5 µM) + insulin (10 nM), we observed that differentiating L6 myoblasts accumulated more glycogen than treatment with insulin alone (Figure 3.3A). This indicates that RA synergizes with insulin to alter either glycogen synthesis or glycogen degradation to enable skeletal muscle to have a greater capacity for glycogen storage. After 6 days, however, this effect had diminished, and RA did not enable glycogen accumulation greater than treatment with insulin alone (Figure 3.3B). The synthesis of skeletal muscle glycogen by GS is a critical homeostatic regulator of systemic glucose homeostasis. The loss of sensitivity to insulin stimulation is seen within the T2DM phenotype, with decreased skeletal muscle glycogen, decreased glucose transport resulting in hyperglycemia, and insulin insensitivity [41]. The transition from insulin resistant skeletal muscle, in which excess glycogen accumulation concurrent with GS activity inhibition without compromise of proximal insulin signaling and glucose transport, to T2DM is unclear. It is evident that glycogen synthesis plays a dominant role in this long-term transition to the T2DM phenotype. Understanding the mechanistic relationships by which this occurs can provide opportunities for interventions which delay or prevent insulin desensitization from occurring.

3.5 Conclusion

RA enhanced insulin stimulated glycogen accumulation after 2 days of treatment in differentiating L6 myoblasts but was diminished after 6 days. RA marginally enhanced
insulin’s effect on AKT expression after 2 days. Insulin stimulation of pAKT (Ser473), pGS (Ser641), and pGSK3β (Ser9) was marginally enhanced by RA after 6 days of treatment. Glycogen accumulation was increased along with pGS (Ser641) after 6 days of treatment with insulin. This phenomenon suggests that the effects of RA on insulin signaling during differentiation result in altered regulation of glycogen homeostasis in L6 cells.
References


18. Hanbing, L., N. Jing, and P. Yunxue. Astragaloside IV improved insulin resistance in L6 myotubes induced by high glucose and insulin. in 2011


Appendix

Figure 3.1 Increased glycogen accumulation in differentiating L6 myoblasts in response to insulin dose at 2 days and 6 days. Pilot study to determine myoblast response to Novalin human insulin. A. Total glucose µg released by differentiating L6 myoblasts after respective treatments as described in the methods section. B. Relative glycogen accumulation expressed as the ratio of total glucose to total protein normalized to the vehicle control after 2 days of treatment, quantified via BCA assay as described in the methods section. C-J. Representative blots and relative expression of β-actin, GS, pGS (Ser641), GSK3β, and pGSK3β (Ser9) after 2 and 6 days of treatment with vehicle control, insulin (10 nM), or insulin (50 nM). Data are presented as the ratio of the test protein over its respective control. (n=1).
Figure 3.1 Continued
Figure 3.1 Continued
Figure 3.2 Increased glycogen accumulation in differentiating L6 myoblasts in response to insulin and RA treatments at 2 days and 6 days. Pilot study to determine myoblast response to ATRA and Novalin human insulin. A-B. Total glucose µg released by differentiating L6 myoblasts after respective treatments as described in the methods section. C-D. Relative glycogen accumulation expressed as the ratio of total glucose controlled to total protein quantified via BCA assay as described in the methods section. E-L. Representative blots and relative expression of β-actin, GS, pGS (Ser641), GSK3β, and pGSK3β (Ser9) after 2 and 6 days of treatment with vehicle control, RA (0.5 µM), RA (1 µM), insulin (10 nM), insulin (50 nM), RA (0.5 µM) + insulin (10 nM), RA (0.5 µM) + insulin (50 nM), RA (1 µM) + insulin (10 nM), or RA (1 µM) + insulin (50 nM). Data are presented as the ratio of the test protein over its respective control. (n=1)
Figure 3.2 Continued
Figure 3.2 Continued
Figure 3.3 Changes in glycogen accumulation and relative expression of glycogen regulators in response to 2 and 6 days of treatment with RA and insulin. A-B. Total glucose µg released by differentiating L6 myoblasts after respective treatments as described in the methods section. C-D. Relative glycogen accumulation expressed as the ratio of total glucose to total protein quantified via BCA assay as described in the methods section. E-O. Representative blots and relative expression of β-actin, AKT, pAKT (Ser473), GS, pGS (Ser641), GSK3β, and pGSK3β (Ser9) after 2 and 6 days of treatment with vehicle control, RA (0.5 µM), insulin (10 nM), or RA (0.5 µM) + insulin (10 nM). Data are presented as the ratio of the test protein over its respective control. Statistical significance of p≤.05 is represented as *; p≤.005 is represented as **; p≤.0005 is represented as ***; p≤.0005 is represented as ****; One-way ANOVA followed by Fisher’s LSD post-hoc were conducted using Graphpad Prism software version 9.5.1. Data are presented as ±S.E.M. (n=4).
Figure 3.3 Continued
Figure 3.3 Continued
Chapter IV: Alterations of Glycogen and Its Regulators by Cycloheximide, Retinoic Acid, and Insulin in Rat Skeletal Muscle and Differentiating L6 Myoblasts
4.1 Introduction

Skeletal muscle glycogen synthesis is responsible for 70-90% of post-prandial blood glucose disposal in normal physiology [1]. Glycogen synthesis within skeletal muscle is primarily accomplished by GS in response to insulin signaling [2]. The synthesis of glycogen by GS within skeletal muscle provides the greatest non-oxidative mechanism for handling glucose challenges [3]. Glycogen content within skeletal muscle adjusts dynamically to metabolic demand via super compensation, potentially doubling its concentration within skeletal muscle after endurance exercise [4]. However, glycogen concentration within skeletal muscles is relatively stable regardless of carbohydrate intake in the absence of endurance training [5]. Glycogen concentration is elevated under chronic overfeeding conditions, and excess blood glucose is metabolically managed via *de novo* lipid synthesis. This leads to excess lipid accumulation within skeletal muscle [6]. Chronically elevated glycogen content within skeletal muscle exerts a negative feedback effect on glycogen synthesis through inhibition of GS [6-8].

Insulin resistance within the context of skeletal muscle can be viewed as the progressive decompensation of internal signaling components leading to decreasing capacity to transport blood glucose into skeletal muscle [9]. Chronic overfeeding leading to energy abundance within the cell leads to elevated glycogen levels, impairing GS and glycogen synthesis [10]. Early in the pathophysiology, insulin stimulation continues to induce glucose transport via into the cell [11]. The overabundance of glucose in the skeletal muscle coupled with impaired glycogen synthesis from glycogen-induced negative feedback redirects the cytosolic glucose into other biosynthetic and anaplerotic
pathways [12]. This ultimately results in greater lipid synthesis, mitochondrial damage from reactive oxygen species production, and progressive impairment of insulin signaling participants [12]. A greater concentration of insulin is required to induce glucose transport into skeletal muscle as this system is decompensated and is clinically observed as hyperinsulinemia [13]. Further decompensation of the insulin signaling components occurs as mitochondrial damage, lipid accumulation, and insulin signaling components become modified transitions the internal homeostatic conditions to the T2DM phenotype [14]. With T2DM, the impaired regulatory apparatus presents as chronically depressed glycogen concentration, inability to transport glucose in response to insulin stimulation, and aberrant regulation of genetic regulation of metabolic components.

We have previously demonstrated that RA enables glycogen synthesis in differentiating L6 myoblasts after two days of treatment. We observed that GS was also inhibited via Ser641 phosphorylation concurrent with GSK3β phosphorylation at Ser9. During acute insulin stimulation GS is dephosphorylated by PP1 and GSK3β is phosphorylated by AKT [15]. This enables GS activity to increase, free from inhibitory conformation induced by phosphorylation, and glycogen to be synthesized. The effects we have previously observed may be explained by glycogen exerting inhibitory influence over GS, although the mechanism by which that occurs remains elusive [16]. Glycogen concentration was maintained at a higher concentration with the presence of RA along with long-term exposure to supraphysiological concentrations of insulin in differentiating L6 myoblasts. The synthesis and degradation of skeletal muscle glycogen
is a flexible, dynamic system which has evolved to meet a wide variety of metabolic circumstances for survival (Prebil, Jensen, Zorec, & Kreft, 2011).

Phosphorylation of GS at Ser641 reduces its activity, and the regulation of GS occurs through many pathways which may yet be defined. The stability of GS may play a role in the maintenance of glycogen concentration in insulin resistant conditions. The stability of GS can be modulated via changes to its synthesis or degradation. Whether or not this effect occurs through the altered nuclear regulation of gene expression and translation, or metabolic feedback modulation of degradation has yet to be resolved. Our lab has previously demonstrated that the addition of RA to insulin treatment can induce significant expression in total GS after 6 days post differentiation [17]. One of the questions left unanswered from the increase in GS observed was whether or not this was due to increased synthesis or decreased degradation [17]. To test this, the protein synthesis inhibitor cycloheximide (CHX) was used to examine total protein expression. Inhibiting the synthesis of GS may indicate whether the increase Goff observed was due to increased stability.

CHX a fungicide produced by Streptomyces griseus [18], is a commonly used protein synthesis inhibitor to study mechanisms of protein synthesis and degradation [19]. CHX inhibits protein synthesis by binding to the E-site within the 60 S subunit of the ribosome [20, 21]. It competes with aminoacyl tRNA for entering to the same site which prevent the release of nascent peptides after their synthesis [22]. This effect slows down total protein synthesis rates and stabilizes the polyribosome structure. The mechanism by which CHX inhibits protein synthesis enables rescue, with the rate of inhibition proportional to the presence of CHX in a dynamic manner [21]. Total inhibition
of protein synthesis is not achieved with CHX, and synthesis of nascent peptides proceeds at 0.1-0.3 amino acids per second even at high doses [21].

Previous investigations have indicated that CHX exerts metabolic influences beyond protein synthesis inhibition. In hepatocytes CHX can interfere with GS regulation [23], activate adrenergic signaling [24], and increase gluconeogenesis and increase ureagenesis [25]. In adipocytes CHX treatment increases glucose uptake, oxidation, and incorporation into fatty acids [26]. In the skeletal muscle CHX treatment has been demonstrated to increase glycogen accumulation [27]. However, much of the literature on the effects of CHX on glucose homeostasis involves high concentrations of CHX and insulin and/or short duration, Additionally, the effects of CHX on alterations of glycogen homeostasis induced by combination of RA and insulin within skeletal muscle and differentiating myoblasts have not been reported.

In the present study, we examine the effects of CHX at 3.5 µM and 10 µM on glycogen homeostasis over a 48-hour time course in differentiating L6 myoblasts. The modulatory effect of CHX on glycogen accumulation on RA and insulin stimulation at 48 hours is also examined. The effects of CHX on glycogen and its regulators are examined in rat skeletal muscle ex vivo after 3 hours of treatment.

4.2 Materials and Methods

4.2.1 Reagents

Cells were grown and differentiated in Dulbecco’s Modification of Eagle’s Medium 4.5 g/L glucose, L-glutamine & sodium pyruvate, purchased from Mediatech (Manassas, VA, U.S.A.). Penicillin streptomycin and (PS) (10,000 units/mL each) were purchased from Life Technologies (Grand Island, NY, U.S.A.). Fetal Bovine Serum
(FBS) purchased from Life Technologies (Grand Island, NY, U.S.A.). Horse Serum (HS) was purchased from HyClone Laboratories (Logan, UT, U.S.A.). Cycloheximide catalog number J66004.XF was purchased from Thermo Fisher. All-trans retinoic acid (RA) was purchased from Sigma (St. Louis, MO, U.S.A.) and diluted in dimethyl sulfoxide (DMSO). Insulin used in these experiments was Novalin pharmaceutical grade human insulin, generously provided by Dr. Guoxun Chen and diluted in phosphate-buffered saline (PBS) containing sodium chloride (NaCl) (137 mM), potassium chloride (2.7 mM), sodium phosphate (10 mM), and potassium phosphate (1.8 mM). Cells were cultured on NEST 60 mm tissue culture treated polystyrene dishes for protein analysis and NEST 100 mm dishes for glycogen analysis. Cells used for protein quantification were lysed using Tris(hydroxymethyl)aminomethane hydrochloride (Tris HCl) (10 mM) 7.4 pH, NaCl (100 mM), sodium dodecyl sulfate (SDS) (0.1%), sodium deoxycholate (0.5%), Triton X 100 (2%), NP-40 (1%), Glycerol (10%), ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) (1 mM), ethylenediaminetetraacetic acid (EDTA) (1 mM). Unless otherwise stated, protease and phosphatase inhibitors were diluted in ddH2O. The lysis buffer contained the protease inhibitors aprotonin (310 nM), pepstatin A (1 μM) diluted in 10:1 methanol:acetic acid solution, leupeptin (10 μM), phenylmethylsulfonyl fluoride (PMSF) (1 mM) diluted in 2-propanol, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (1 mM), bestatin (6.49 μM), and N-[N-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine (E-64) (20 μM) diluted in 50% ethanol solution. The lysis buffer contained the phosphatase inhibitors sodium orthovanadate (Na3VO4) (10 mM) prepared via boiling and ice bath cycles with HCl titration to pH 10 replacing evaporated ddH2O, sodium fluoride (10 mM), β-glycerol
phosphate (10 mM), sodium tartate (10 mM), sodium pyrophosphate (10 mM), sodium molybdate (Na$_2$MoO$_4$) (7 mM). Protein was measured using the Pierce bicinchoninic acid (BCA) kit purchased from Pierce (Rockford, IL, U.S.A.). Cell lysates were prepared for western blotting using a loading buffer containing Tris HCl 6.8 pH (75 mM), SDS (2%), bromophenol blue (0.002%), glycerol (10%), dithiothreitol (DTT) (100 mM).

Polyacrylamide gel electrophoresis (PAGE) was conducted using 10% resolving gels constituted from Tris HCl 8.8 pH (370.1 mM), 30% acrylamide 29:1 bis-acrylamide (10%), ammonium persulfate (APS) (0.0001%), SDS (0.0001%), tetramethylethylenediamine (TEMED) (5.6 mM) and 4% stacking gels constituted from Tris HCl pH 6.8 (123.5 mM), 30% acrylamide 29:1 bis-acrylamide (4%), APS (0.0001%), SDS (0.0001%), TEMED (17 mM). Electrophoresis was conducted in buffer containing Tris HCl pH 8.3 (25 mM), glycine (190 mM), and SDS (0.1%). Protein was transferred to Immobilon®-PSQ polyvinylidene fluoride (PVDF) membrane 0.45 µm pore size which was purchased from EMD Millipore (Billerica, MA, U.S.A.). Protein transfer was conducted in buffer which contained Tris pH 8.3 (25 mM), glycine (190 mM), and methanol (20%). Membranes were blocked in a buffer containing Tween®20 (0.1%) (TBST), Tris HCl pH 7.6 (20 mM), NaCl (150 mM), nonfat milk protein (5%), and BSA (2.5%). Primary antibodies purchased from Cell Signaling Technologies (CST) (Danvers, MA, U.S.A.) were GS #3886, pGS (Ser641) #3891, GSK3β #9315, pGSK3β (Ser9) #9336, each diluted 1:1,000 in TBST. The secondary antibody purchased from CST (Danvers, MA, U.S.A.) was Anti-Rabbit #7074P2 diluted 1:4000 in TBST. Horseradish peroxidase (HRP) primary conjugated β-actin #SC-47778 antibody was purchased from Santa Cruz Biotechnology, diluted 1:20,000 in TBST containing 5%
nonfat milk protein. Pierce enhanced chemiluminescence (ECL) for HRP kit #32106 was used for chemiluminescence reaction. Luminescence signal was detected using Bio-Rad ChemiDoc XRS+ Image Capture. Quantification of optical density was performed using Bio-Rad Image Lab and Bio-Rad Analysis Software.

4.2.2 Cell culture

L6 Rat myoblasts were grown to 100% confluence in DMEM 4.5 g/L glucose, L-glutamine & sodium pyruvate (110 mg/L) containing 10% FBS, 1% PS. Post-confluence, media was replaced with DMEM containing HS (2%) and PS (1%) to initiate differentiation two days prior to treatment. DMEM cell culture media with HS (2%) and PS (1%) was replaced every two days thereafter containing treatment or vehicle control. Cells continued to differentiate under control and treatment conditions in 2% HS during treatment. Treatments consisted of DMSO for vehicle control for RA and CHX treated experiments and PBS for insulin treatments, CHX (3.5 µM or 10 µM), Novalin human insulin (10 nM), RA (0.5 µM), or a combination of (10 nM) + RA (0.5 µM) and CHX (10 µM). Cells were harvested after 0, 6, 12, 24, and 48 hours for time course analysis of CHX treatment effects and after 48 hours to assess CHX effect on RA and insulin treatments. Cells were cultured on 100 mm plates for glycogen analysis and 60 mm plates for protein analysis.

4.2.3 Ex vivo rat skeletal muscle

Heterozygous Zucker Fatty rats were sacrificed at 21 days after birth via primary CO₂ asphyxiation and secondary cervical dislocation. Rats were immediately dissected, and the gastrocnemius was excised from each rat. Skeletal muscle was then separated into portions and weighed. Skeletal muscle was washed in 37°C HBBS buffer pre-
equilibrated at 5% CO\textsubscript{2}. Treatment of the skeletal muscle was administered in serum and antibiotic free high glucose 4.5 g/L DMEM with L-glutamine & sodium pyruvate (110 mg/L). Treatment groups for the skeletal muscle contained DMSO vehicle control or cycloheximide (10 µM) for 3 hours. Glycogen and protein analysis were conducted using skeletal muscle samples taken from the same portion of excised tissue. Samples were incubated in 5% CO\textsubscript{2} pre-equilibrated treatment media at 37°C for 3 hours. After treatment, samples were flash-frozen in liquid nitrogen and placed in -80°C for storage.

4.2.4 Glycogen assay

Cell culture plates were placed on ice and media was removed with a vacuum pump. Cells were washed with 5 mL of ice-cold PBS and removed with a vacuum pump. Cells were scraped with 1 mL of ice-cold PBS and pipetted into 1.5 mL microcentrifuge tubes in an ice-cold aluminum block. Cells were centrifugated at 1000 RPM for 5 minutes at 4°C to form a soft pellet. The supernatant was removed, and the cell pellet was flash-frozen in liquid nitrogen, then placed into -80°C freezer for storage. Cells were thawed on ice for assay. Cells were lysed with 300 µL of potassium hydroxide (KOH) (30%) at 100°C for 1 hour. Ethanol (200 proof) was added to cell lysate for a final concentration of 70% and cooled to -20°C for 1 hour for glycogen precipitation. Precipitated cell lysate was then centrifugated at 16,000 RPM for 15 minutes at 4°C to form a pellet. Supernatant was removed and the pellet was resuspended in HCl (3.0 M), then heated to 100°C for 1 hour for glycogen hydrolysis. Hydrolysate was cooled to room temperature and centrifugated briefly for volume normalization. Hydrolysate was then measured using the StanBio Glucose Liquicolor kit according to manufacturer protocol.
Skeletal muscle for glycogen analysis was thawed on ice and dissolved in 1 mL 30% KOH at 100°C. Glycogen was precipitated from tissue lysate at a final concentration of 70% ethanol at -20°C for 1 hour. Precipitated glycogen was centrifuged at 7500 RPM in 15 mL centrifuge tubes at 4°C to form a pellet, supernatant was discarded. The pellet was resuspended in 3.0 M HCl and heated to 100°C for 1 hour for glycogen hydrolysis. Hydrolysate was cooled to room temperature, centrifugated to normalize volume, and released glucose was then measured using the StanBio Glucose Liquicolor kit according to manufacturer protocol.

4.2.5 Western blot

Cells were placed on ice and media was removed with a vacuum pump. Cells were washed with 2 mL of ice-cold PBS which was immediately vacuumed out. Cells were scraped in 300 μL of ice-cold Tris-Triton lysis buffer and pipetted into 1.5 mL microcentrifuge tubes. Cells were agitated for 30 minutes at 4°C for lysis via microcentrifuge tube adaptor with a 4-E’s vortex at low RPM. Cell lysate was then centrifuged at 16,000 RPM for 15 minutes at 4°C to form a pellet. Supernatant was transferred to fresh microcentrifuge tubes and the pellet was discarded. Cell lysate was placed in -80°C for storage. Skeletal muscle tissue samples were prepared for western blotting via mechanical tissue disruption. Skeletal muscle was thawed from -80°C storage on ice and placed into 15 mL tubes containing Tris Triton lysis buffer and inhibitors described previously. Tissues were homogenized using a Polytron tissue disruptor in lysis buffer on ice. Homogenized tissue was transferred to 1.5 mL microcentrifuge tubes then agitated via microcentrifuge tube adaptor with a 4-E’s vortex at low RPM for 4 hours at 4°C. Tissue lysates were centrifugated at 16,000 RPM for 15
minutes to form a pellet. Supernatant was transferred fresh 1.5 mL microcentrifuge tubes and the pellet was discarded. Tissue lysates were stored at -80°C for assay. Samples were thawed on ice and protein concentration was measured using Pierce BCA kit and GloMax Multi Detection System at 560 nm absorbance wavelength. Protein concentration for each sample was normalized in loading buffer which consisted of Tris HCL 6.8 pH (75 mM), SDS (2%) glycerol (10%), bromophenol blue (0.002%). and 100 mM dithiothreitol (DTT). Prepared samples were heated to 100°C for 5 minutes for denaturation. Prepared samples were separated on PAGE gels using BioRad MiniProtean electrophoresis system. Samples were separated with voltage at 80 volts until samples had migrated past the 4% stacking gel and then at 120 volts for the remainder of protein migration. Protein was transferred to PVDF membranes prepared in methanol and transfer was conducted using BioRad MiniProtean transfer cassette system. Protein transfer was conducted with voltage at 50 volts for 2 hours at 4°C. Blocking solution was prepared consisting of TBST and 5% by weight nonfat milk powder, filtered to separate undissolved solids. Membranes were placed in blocking solution for 1 hour and gently agitated via orbital shaker at room temperature. Blocked membranes were incubated overnight in primary antibody at 4°C. Incubated membranes were then washed with TBST for 5 minutes, 3 times at room temperature. Washed membranes were then incubated in secondary anti-rabbit antibody diluted 1:4000 for 1 hour at room temperature. Secondary-incubated membranes were then washed with TBST for 5 minutes, 3 times at room temperature. Membranes were then treated with Pierce ECL prepared according to manufacturer protocol. Luminescence
was detected using Bio-Rad ChemiDoc system and quantification was conducted using BioRad Image Lab software

4.2.6 Half-life calculation based on relative expression

Half-lives of AKT, GS, and GSK3β total proteins were calculated using relative expression based on CHX treatment dose and time-course. Reported half-lives of AKT and GSK3β were used to compare the half-life estimate calculated from our experimental results. The average of the groups within respective treatment and time-point were plotted using a nonlinear regression (curve fit). The slope and intercept were derived from the x values representing the time-point harvest, and with y values representing average ratio of total protein by the loading control. The value corresponding to the 50% relative expression at the target hour in cells treated with CHX (10 μM and 3.5 μM) was selected to compare against the reported half-lives of AKT and GSK3β. Treatment with CHX (10 μM) generated predictive curves and half-lives for proteins, which were within ≤6% of reported AKT and GSK3β half-lives and this dose was used to estimate the half-life of GS.

4.2.7 Statistical analysis

All statistical analysis was conducted using Graphpad Prism software version 9.5.1. Ordinary one-way ANOVA with Fisher’s LSD post-hoc for multiple comparisons were conducted. For skeletal muscle comparisons, unpaired parametric two-tailed t tests were conducted. For half-life calculation, the R² from nonlinear regression analysis was used to determine the predictive power for interpolation of the average ratios within treatment groups and timepoints. Unweighted, least squares fitting of a line was plotted without comparisons, using groups within treatments as replicate values. The statistical
significance of $p \leq 0.05$ is represented as *; $p \leq 0.005$ is represented as **; $p \leq 0.0005$ is represented as ***; $p \leq 0.0005$ is represented as ****; Data are presented as ±S.E.M. (n ≥ 4).

4.3 Results

4.3.1 Changes in regulators of glycogen synthesis in differentiating L6 myoblasts treated with CHX (3.5 μM), or CHX (10 μM) during a 48-hour time-course

L6 myoblasts were grown to confluence and treated with differentiation media for two days. Differentiating L6 myoblasts were then harvested after 0, 6, 12, 24, and 48 hours of treatment with DMEM containing control (vehicle), CHX (3.5 μM), or CHX (10 μM). Representative western blots for each treatment and time point are arranged according to treatment within harvest times (Figure 4.1A). All Chapter IV figures can be found in Chapter IV Appendix. A plate was harvested after 2 days of differentiation without receiving treatment to serve as a baseline for comparisons of relative expression of target proteins (Figure 4.1A). Comparison between (3.5 μM) and CHX (10 μM) doses revealed no significant differences in relative expression of AKT, GSK3β, or GS within timepoints (Figure 4.1B-D). AKT expression was significantly decreased after 48 hours of treatment with CHX (3.5 μM) and CHX (10 μM) (Figure 4.1E, G). CHX (3.5 μM) induced pAKT (Ser473) expression after 24 hours (Figure 4.1F). CHX (10 μM) induced pAKT (Ser473) expression after 6 and 48 hours (Figure 4.1H). Treatment with neither CHX (3.5 μM) nor CHX (10 μM) resulted in changes to GS expression within timepoints (Figure 4.1I, K). There was a significant induction of pGS (Ser641) expression in groups treated with CHX (3.5 μM) or CHX (10 μM) after 48 hours (Figure
4.1J, L). There were no significant differences in GSK3β expression within timepoints after treatment with either CHX (3.5 μM) or CHX (10 μM) (Figure 4.1M, O). CHX (3.5 μM) induced expression of pGSK3β (Ser9) after 6, 24, and 48 hours (Figure 4.1N). CHX (10 μM) induced expression of pGSK3β (Ser9) after 24 and 48 hours (Figure 4.1P).

4.3.2 Calculated GS half-life using CHX (10 μM) protein synthesis inhibition over a 48-hour time-course

The decreases in relative expression of total proteins were used to calculate the half-life of GS. Using the known half-lives of AKT and GSK3β as a positive control, the half-life of GS was calculated using the same method. Half-lives of proteins were calculated via linear interpolation using the average ratios of total target protein by β-actin for each time point. Slope and intercept from this dataset were used to calculate the time-point in which the relative expression of the target protein degraded to 50% of the value of its peak expression within the dataset. The relative expression of total AKT declined from its peak expression at 6 hours with a predictive R² value of 0.9188, whereas the control maintained a relatively consistent expression throughout the time-course (Figure 4.2A). The relative expression of total GSK3β declined with a predictive R² value of 0.8816 (Figure 4.2B). The relative expression of total GS declined from its peak expression at 6 hours with a predictive R² value of 0.8480. (Figure 4.2C). A summary of the calculated half-lives of AKT, GSK3β, and GS were compared against known half-lives of AKT and GSK3β. Data from the CHX (3.5 μM) treatment group calculated with the same methods did not yield predictive values which correspond to known half-lives. Using the relative expressions from the groups treated with CHX (10 μM) yielded predicted half-life values of 34 hours for AKT and 49 hours for GSK3β.
Using this method, the estimated half-life for AKT is within 6% of its value of 36 hours recorded by Basso et al. [28]. Similarly, within the CHX (10 μM) treatment group, our predicted value for the half-life of GSK3β was 49 hours using this method, within 2% of the half-life of 48 hours recorded by Hongisto et al. [29]. Based on these data, we estimate the half-life of GS to be within 37-41 hours.

### 4.3.3 Glycogen accumulation and expression levels of regulators of glycogen synthesis in differentiating L6 myoblasts treated with RA (0.5 μM) and insulin (10 nM)

L6 myoblasts were grown to confluence and treated with differentiation media for two days. Differentiating L6 myoblasts were then harvested after 48 hours of treatment with DMEM containing DMSO (vehicle control), CHX (10 μM), RA (0.5 μM) + insulin (10 nM), or CHX (10 μM) + RA (0.5 μM) + insulin (10 nM). Representative western blots for each tested protein are arranged according to treatment (Figure 4.3A). Treatment with RA (0.5 μM) + insulin (10 nM) induced significant glycogen accumulation after 48 hours of treatment, however the effects of CHX (10 μM) attenuated the effects of RA (0.5 μM) + insulin (10 nM) (Figure 4.3 B, C). CHX (10 μM) inhibited AKT expression in the presence and absence of RA (0.5 μM) + insulin (10 nM) (Figure 4.3D). Treatment with RA (0.5 μM) + insulin (10 nM) induced pAKT (Ser473) expression and CHX (10 μM) treatment attenuated this effect (Figure 4.3E). There were no significant differences in GS expression between treatment groups (Figure 4.3F). CHX (10 μM) induced expression of pGS (Ser641) in the presence and absence of RA (0.5 μM) + insulin (10 nM) (Figure 4.3G). GSK3β expression was significantly inhibited by CHX (10 μM) in the presence and absence of RA (0.5 μM) + insulin (10 nM) (Figure 4.3H). RA (0.5 μM) +
insulin (10 nM) induction of $\text{pGSK3}\beta$ (Ser9) expression was enhanced by CHX (10 µM) (Figure 4.3i).

### 4.3.4 Glycogen accumulation and expression levels of regulators of glycogen synthesis in heterozygous Zucker fatty rat skeletal muscle

Gastrocnemius muscle was excised from heterozygous Zucker fatty rats and incubated with serum free DMEM containing vehicle control or CHX (10 µM) for 3 hours. Treatment with CHX (10 µM) induced glycogen accumulation over tissues that received the vehicle (Figure 4.4A, B). There were no significant differences in AKT, GS, or GSK3β expression with CHX (10 µM) treatment (Figure 4.4C, E, and G). Expression of $\text{pAKT}$, $\text{pGS}$ (Ser641), and $\text{pGSK3}\beta$ (Ser9) did not significantly change over the controls (Figure 4.4D, F, and H).

### 4.4 Discussion

In the present study we observed significant changes by CHX to the regulation of GS as well as glycogen homeostasis in both rat skeletal muscle as well as L6 cells. A significant increase in glycogen accumulation was detected within 3 hours in rat skeletal muscle in response to CHX (10 µM) (Figure 4.4A, B). Interestingly, we did differences in the relative expression of $\text{pAKT}$ (Ser473), $\text{pGS}$ (Ser641), or $\text{pGSK3}\beta$ (Ser9) in the rat skeletal muscle, despite a significant increase in glycogen (Figure 4.4C-H). Using differentiating L6 myoblasts, we observed significant induction of the relative expression of $\text{pAKT}$ (Ser473) after 6 hours of treatment with CHX (10 µM) (Figure 4.2H). The increased phosphorylation of AKT at Ser473 as a result of 3 hours of treatment with CHX concentrations ranging from 0.1 µM to 300 µM has been previously reported to be PI3K/PDK1 dependent in HEK-293 cells [20]. We also observed the induction of GSK3β
phosphorylation at Ser9 consistently throughout the CHX time course in differentiating L6 myoblasts (Figure 4.1N, P). Canonically, the phosphorylation of GSK3β at Ser9 inhibits its kinase activity, accelerating GS activity and glycogen synthesis. Interestingly, we observed that the phosphorylation of GS at Ser641 was relatively stable up to 24 hours, suggesting that a metabolic shift which decouples phosphorylation of GS at Ser641 by GSK3β might occur between 24 and 48 hours (Figure 4.1J, L). The phosphorylation of GSK3β at Ser9 was significantly elevated over the control at both 24 and 48 hours, indicating a reduction in GSK3β's interaction with GS (Figure 4.1N, P). CHX’s induction of GS phosphorylation at Ser641 in L6 myoblasts is dramatic and dose dependent after 24 hours, indicating that the GS regulation by site 3 phosphorylation is critically altered by something other than GSK3β (Figure 4.1J, L). We reported a similar decoupling of GS activity from GSK3β regulation after 48 hours of treatment with insulin (10 nM) and RA (0.5 µM) in differentiating L6 myoblasts here as well as in Chapter 3. However, glycogen remained elevated over control in groups treated with insulin (10 nM) and RA (0.5 µM) after 48 hours, whereas treatment with CHX (10 µM) eliminated the stimulatory effects insulin (10 nM) and RA (0.5 µM) on glycogen synthesis (Figure 4.3B, C). These two opposite states of glycogen concentration must be negotiated through different mechanisms in differentiating L6 myoblasts as GS phosphorylation at Ser641 remains elevated over control in both insulin (10 nM) and RA (0.5 µM) groups as well as CHX (10 µM) (Figure 4.3B, C, and G). The effects of CHX appear to be similar to insulin stimulation early in treatment via activation of the AKT-PI3K-GSK3β signaling pathway (Figure 4.1 E-P) but induce overriding regulatory mechanisms on glycogen synthesis after 24 hours (Figure 4.3D-I). Although the effects of CHX
treatment in ex vivo skeletal muscle did not achieve statistically significant differences in
the regulators of glycogen synthesis, the trends of increased GS phosphorylation at
Ser641 and GSK3β phosphorylation at Ser9 were in line with the observations on L6
differentiating myoblasts at 48 hours (Figure 4.4F, H).

Interpretation of these results within the context of other studies examining the
effects of CHX in L6 cells, rat skeletal muscle, and other systems raises many
questions. Insulin signaling increases glucose transport into skeletal muscle via
increasing activity, translocation, and increased synthesis of GLUT4 [30]. CHX (500 µM)
treatment for 6 hours increased glucose uptake in L6 myotubules while without
significantly increasing GLUT4 expression, suggesting that the elevated glucose may
have been a result of increased GLUT4 translocation and activity rather than expression
[31]. The increase in glycogen accumulation we observed in ex vivo rat skeletal muscle
(Figure 4.4 A, B) has also been reported after 5 hours of treatment with CHX (500 µM),
along with increased glucose transport in animals which had been induced insulin
resistance [27]. However, the methodological approaches used by the research group
to induce insulin resistance in the rats prior to dissection limit the conclusions which can
be drawn from the study [27]. The rats used were under significant metabolic stress,
having a catheter surgically implanted four days prior to sacrifice as well as having a
solution of 50% glucose infused for 6-24 hours to induce insulin resistance [27]. The ex
vivo treatment of the skeletal muscle also consisted of insulin (100 nM) for 30 minutes,
and CHX (500 µM) for 5 hours in 36 mM glucose media [27]. Insulin in that dose is 500-
fold over basal levels of circulating insulin in wild-type rats [32]. CHX is lethal to rats at 2
mg/kg, or 7.11 nM, and a 500 µM dose represents a 70,337.5 fold increase over a lethal
concentration in vivo [33]. The glucose concentration within the treatment media during the ex vivo experiments was 36 mM, or a 7.72-fold increase over basal circulation of glucose in wild-type rats [34]. The metabolic challenges administered to these rats in vivo and to their tissue ex vivo obscure physiologically relevant conclusions which may be derived from the study.

Other studies examining the effects of CHX on glucose homeostasis have also demonstrated that CHX treatment can dramatically alter the metabolic landscape in addition to direct effects on protein synthesis. CHX induces glycogenolysis, gluconeogenesis and ureagenesis in isolated primary rat hepatocytes through activation of α1-adrenergic signaling [24]. A later study reported that GS activity in rat hepatocytes treated with CHX was dependent upon gluconeogenic precursor dihydroxyacetone only after being primed with L-glutamine and L-leucine, indicating that protein synthesis plays a vital role in the transition of GS from its less active b conformation to its more active a conformation [25]. The transition of GS to its more active a state as a result of dephosphorylation is accomplished by PP1 [35]. The inhibition of PP1 translation may potentially interfere with the dephosphorylation of GS due to the reduced quantity of PP1. Evidence that protein synthesis was a contingent factor for hepatic glycogen synthesis was also demonstrated in rats which received an intramuscular injection with 5 mg/kg body weight CHX over 4 hours [23]. In rat epididymal fat pads, CHX treatment induced de novo lipogenesis, glucose uptake, carbon dioxide production, and a nonsignificant increase in glycogen synthesis [26].

We have demonstrated that CHX treatment alters glycogen synthesis in a time dependent manner using differentiating L6 myoblasts and rat skeletal muscle (Figures
Phosphorylation of GSK3β at Ser9 (Figure 4.1N, P) has previously been examined with CHX, S9A mutation, and inhibition with lithium chloride (LiCl) in L6 myotubules to determine the role of phosphorylation on GSK3β Ser9 in glycogen accumulation [36]. Cytosolic glucose availability, and subsequent UDP-Glc production, as a function of glucose transport was found to be the primary rate-limiting step in glycogen synthesis rather than GSK3β activity [36]. GSK3β inhibition was achieved using the pharmacological inhibitors lithium chloride, SB-415286, and SB-216763 [36]. Inhibition of GSK3β alone did not increase glycogen accumulation in L6 cells without an accompanying increase in glucose transport [36]. The increase of phosphorylation of AKT at Ser473 and Thr308 after 36 hours in human embryonic kidney-293 (HEK-293) cells was observed within 3 hours, suggesting common regulators induced by CHX also exist in differentiating L6 myoblasts [20]. CHX-mediated AKT phosphorylation was determined to proceed in a PI3K/PDK1 dependent manner [20]. Phosphorylation of GSK3β and p70S6K by AKT has been observed with cycloheximide treatment but not with NSC119889, a protein synthesis inhibitor which prevents mRNA-ribosome interaction and inhibits initiation of protein synthesis [20, 37]. Both protein synthesis inhibitors induced the phosphorylation of AKT at Ser473, however only CHX treatment resulted in increased GSK3β phosphorylation at Ser9 after 6 hours in HEK-293 cells [20]. The activity of GSK3β negatively regulates the expression of GS in L6 myotubules, exerting its influence at the level of mRNA translation in a CHX sensitive manner [36]. This effect supports our observation of the upward trend in GS expression early in the time course, with gradual degradation through to 48 hours (Figure 4.1I, K). Expression of total protein content of AKT, GS, and GSK3β decreased over 48 hours CHX
treatment, and the dose response demonstrated no significant difference in total protein expression between CHX (3.5 µM) and CHX (10 µM) when compared to each other (Figure 4.1B-D).

Feedback inhibition of GS by glycogen content induced by long-term stimulation of L6 cells with RA and insulin may retain flexibility. In contrast, the inhibitory effects induced by CHX appear to take priority over GSK3β’s regulatory influence which would enable the synthesis of glycogen in the absence of glycogen-concentration induced feedback inhibition. Known regulators of GS site 3a within regulatory cluster 2 of GS which directly phosphorylate Ser641, include PAS domain-containing serine/threonine-protein kinase (PASK), dual-specificity tyrosine phosphorylation-regulated kinase (DYRK)1A, DYRK1B, DYRK2, and GSK3β [38, 39]. It remains to be determined whether CHX has any effects on other kinases that modulate GS phosphorylation at Ser641. PASK participates in energy sensing and other indicators of cellular status such as reactive oxygen species (ROS), hypoxia responses, and intracellular calcium levels [40]. The action of DYRK2 in the regulation of GS is not sensitive to glycogen concentration, but recombinant human PASK did exhibit sensitivity to glycogen concentration and the phosphorylation of site 3a of GS [39]. The mechanism by which CHX inhibits glycogen synthesis may be dependent upon its effects as a protein synthesis inhibitor, affecting proteins with a short half-life. CHX may also proceed through other unknown mechanisms within the metabolism. Determining the route through which CHX modulates glycogen homeostasis can reveal a more complete model in differentiating L6 myoblasts.
4.5 Conclusion

CHX (10 µM) induces changes in phosphorylation of AKT and GSK3β which mimic the effects of insulin stimulation within 48 hours in differentiating L6 myoblasts. Between 24 and 48 hours of CHX treatment a dramatic shift in the regulation of GS occurs, resulting in its inhibition apart from feedback regulation by glycogen and regulation by GSK3β. In rat skeletal muscle, glycogen accumulation is increased after 3 hours of treatment without significant changes in key regulators of glycogen synthesis. After 48 hours of treatment in differentiating L6 myoblasts, glycogen accumulation is critically inhibited below basal levels via GS phosphorylation at Ser641 apart from insulin signaling regulators. Investigation of known regulators of GS at Ser641 may yield new important relationships between the energy sensing apparatus and protein synthesis, or previously unknown mechanisms of GS regulation.
References


Appendix

Figure 4.1 Changes in relative expression of glycogen regulatory proteins after treatment with CHX over 48 hours. L6 myoblasts were grown to confluence and treated as described in the methods section. A. Representative western blots and relative expression of β-actin, AKT, pAKT (Ser473), GS, pGS (Ser641), GSK3β, and pGSK3β (Ser9) depicting treatment conditions vehicle control, CHX (3.5 μM), and CHX (10 μM) harvested at 0, 6, 12, 24, and 48 hours. B-D. Two-way ANOVA analysis comparing either CHX (3.5 μM), or CHX (10 μM) treatments against negative control within time-points. E-P. Two-way ANOVA analysis of individual treatments against respective control within time-points. Data are presented as the ratio of the optical density of the test protein over its respective control. Phosphorylated protein expressions are compared to their respective total protein expressions, and total protein expressions are compared to their respective β-actin expression. Two-way ANOVA was conducted using Graphpad Prism software. Statistical significance of p≤.05 is represented as *; p≤.005 is represented as **; p≤.0005 is represented as ***; p≤.0005 is represented as ****; Data are presented as ±S.E.M. (n=4).
Figure 4.1 Continued
Figure 4.1 Continued
Figure 4.2 Estimation of the half-life of GS using linear interpolation from 48-hour time-course treatment with CHX. L6 myoblasts were grown to confluence and treated as described in the methods section. A-C. Plots of average ratios of relative expression of total AKT, GSK3β, and GS over β-actin in groups treated with vehicle control and CHX (10 µM). Unweighted, least squares fitting of a line was used in linear regression analysis of the trend of relative expression across time points. R² values of the CHX (10 µM) dataset are listed indicating the dependence of half-life calculation upon the time treated. Slope and intercept were calculated from each dataset and used to calculate the time point after which each respective protein would arrive at 50% from its peak expression within the dataset. Only data from the CHX (10 µM) treatment groups is graphically represented. Differences in predictive potential of the different doses are dramatic, with CHX (10 µM) presenting more potential to provide estimations of protein half-life. Data are presented as ±S.E.M. (n=4).
Figure 4.3 Changes in relative expression of glycogen regulators in response to 48 hours of treatment with CHX, RA, and insulin. L6 myoblasts were grown to confluence and differentiated for 2 days prior to treatment. Differentiating L6 myoblasts were given treatment media for 48 hours before harvest as described in the materials and methods section. Incubation in treatment media consisted of DMEM, HS (2%), and respective treatments diluted 1:1000 in DMSO for CHX and RA and PBS for insulin, equivalent vehicles were provided to each treatment group. Protein was quantified via BCA and protein concentrations were normalized during preparation for western blotting. A. Representative blots and relative expression of β-actin, AKT, pAKT (Ser473), GS, pGS (Ser641), GSK3β, and pGSK3β (Ser9) after 48 hours of treatment with vehicle control, CHX (10 μM), RA (0.5 μM) + insulin (10 nM), or CHX (10 μM) + RA (0.5 μM) + insulin(10 nM). B-C. Glycogen accumulation is represented as the total glucose released as described in the methods section controlled to total protein from BCA measurement in panel A and normalized to the control in panel B. D-G. Data are presented as the average ratios of the test protein over respective controls One-way ANOVA with Fisher’s LSD post-hoc were conducted using Graphpad Prism software version 9.5.1. The statistical significance of p≤.05 is represented as *; p≤.005 is represented as **; p≤.0005 is represented as ***; p≤.0005 is represented as ****; Data are presented as ±S.E.M. (n=4).
Figure 4.3 Continued
Figure 4.4 Changes in glycogen and relative expression of glycogen synthetic proteins in excised rat skeletal muscle after 3 hours of treatment with CHX (10 µM). Skeletal muscle was harvested from rats as described in the methods section. A-B. Total glucose µg released by rat skeletal muscle after respective treatment with either vehicle (DMSO) or CHX (10 µM) for 3 hours. Glycogen analysis was conducted as described in the materials and methods section. Control and treatment tissue groups were harvested from the same excised gastrocnemius of each rat. Excision of the tissue was followed immediately with bisection for control and treatment groups, weighed, and washed with HBBS before being placed in pre-warmed and CO2 equilibrated 12 well culture plates containing individual wells holding 3 mL of either vehicle or treatment media for each sample. A. Total glucose released from the samples is controlled to tissue mass. B. Relative glycogen accumulation expressed as the ratio of total glucose released to tissue mass, as well as fold-induction normalized to control. C-H. Representative blots and relative expression of β-actin, AKT, pAKT (Ser473), GS, pGS (Ser641), GSK3β, and pGSK3β (Ser9) after 3 hours of treatment with either vehicle (DMSO) or CHX (10 µM). Data are presented as the average of the ratios within groups of the test protein over its respective control. Phosphorylated proteins were controlled to their respective total proteins, and total proteins were controlled to β-actin. Tissues were harvested from the gastrocnemius and immediately flash frozen in liquid nitrogen and placed into -80 for later analysis. Tissue was homogenized as described in the methods section, total protein was quantified via BCA, and samples were prepared for western blotting as described in the methods section. Unpaired t tests were conducted using Graphpad Prism software. The statistical significance of p≤.05 is represented as *; Data are presented as ±S.E.M. (n=7).
Chapter V: Conclusion and Future Direction
5.1 Conclusion

In this dissertation, we have demonstrated that the regulation of GS at Ser641 can be decoupled from regulation by GSK3β in long-term treatment with RA + insulin and CHX in L6 cells. Specifically, we used differentiating L6 myoblasts to assess dose response of insulin, RA, and CHX and then conducted time course experiments to examine the long-term effects of these treatments on glycogen homeostasis during differentiation. The research presented in this dissertation provides evidence to address two questions: (1) Do the previously observed effects of RA and insulin occur before 6 days of treatment in L6 cells? (2) Does the stability of GS play a role in the increased glycogen accumulation we observed? These questions are framed into hypotheses and tested in Chapter II and Chapter III and can provide insight into the regulation of GS within skeletal muscle.

The focus of Chapter II was to examine the previously reported synergistic effects of RA and insulin treatment in L6 myoblasts with a time course to determine whether the effect on glycogen accumulation was time dependent. In order to test this, we first conducted two pilot experiments, the first testing insulin response and the second testing RA and insulin + RA response in the differentiating L6 myoblasts before proceeding. We determined that the doses of insulin (10 nM) and RA (0.5 µM) provided a sufficiently robust response over the controls to test our hypothesis. The findings of that experiment indicated that (1) treatment with RA enhances insulin stimulated glycogen accumulation after 2 days during myoblast differentiation. (2) Treatment with RA + insulin significantly induced the expression of total AKT after 2 days and its relative phosphorylation at Ser473 after 2 and 6 days over the controls. However, the
effect of RA combined with insulin did not supersede the effect of insulin alone in its alterations to AKT. (3) The levels of phosphorylated GSK3β were not changed in response to both insulin and RA + insulin across time-points. (4) In opposition to the canonical model of insulin signaling, phosphorylation of GS at Ser641 was significantly induced after 6 days of treatment in differentiating L6 myoblasts. While our results of increased activity of AKT and decreased activity of GSK3β are more closely aligned with the canonical model of insulin signaling, our finding suggests a metabolic shift between 2 and 6 days in terms of the GS inhibition apart from its regulation by GSK3β.

The initial aim of the study was to examine the onset of the effects of RA + insulin previously reported, however, we consistently observed through all the long-term experiments conducted that GS phosphorylation increased with duration rather than decreased. It is possible that glycogen was responsible for providing negative feedback to GS, and that RA could overcome this effect when combined with insulin during differentiation in L6 cells. Neither of the treatments induced a significant difference in the phosphorylation of GS at Ser641 after two days, so it is possible that GS was still sensitive to regulation by GSK3β. After 6 days, GSK3β’s capacity to inhibit the activity of GS might have been overridden by another regulator which can directly phosphorylate GS at site 3a.

In Chapter III, we demonstrate that the regulation of glycogen accumulation in differentiating L6 myoblasts depends on the activity of GS and its phosphorylation at Ser641 to a greater extent than total expression using CHX. We began with a dose response to two doses of CHX, 3.5 µM, and 10 µM, and did not observe significant differences between the two doses in total protein expression. We tested this within a
48-hour time course, testing at 6, 12, 24, and 48 hours to examine progressive effects on total protein expression as well as regulatory influences on glycogen regulators in the insulin signaling pathway. The findings within this chapter indicate that (1) CHX treatment can mimic insulin signaling up to 24 hours in its regulation of AKT and GSK3β, but induces a dramatic, dose-dependent shift between 24 and 48 hours in the regulation of GS and expression of total AKT. (2) After 24 hours, AKT expression is steeply diminished compared to the control in a dose dependent manner. GS Ser641 phosphorylation significantly increases between 24 and 48 hours. (3) The half-life of GS is estimated most likely to be between 37-41 hours. (4) CHX decouples GS regulation from GSK3β after 24 hours, and CHX inhibits glycogen synthesis via regulatory mechanisms which supersede the stimulatory effects of insulin and RA signaling. (5) Glycogen accumulation is induced in rat skeletal muscle after 3 hours of CHX treatment, without significant changes to the relative expression of glycogen regulators.

5.2 Future directions

The findings of this dissertation indicate that the regulation of glycogen synthesis by GS dephosphorylation at site 3a by GSK3β does not necessarily follow the canonical model of insulin signaling in differentiating L6 myoblasts. This may be due to the developing machinery of skeletal muscles, including IR expression, differential calcium regulation, the presence of contractile proteins, GLUT4 modulation, differential degradation regulation of glycogen, as well as other unknown mechanisms. The conclusions which can be drawn from differentiating L6 myoblasts and rat skeletal muscle ex vivo are limited, yet the observations raise many more questions regarding glycogen metabolism in skeletal muscle. Creating a more definitive roadmap of which
regulators act upon GS and under what conditions they do so can yield mechanistic insight into glycogen homeostasis in normal physiology and pathophysiology. Potential experiments leading from the data presented in this dissertation are outlined in Figure 5.1. All figures in Chapter IV can be found in Chapter V Appendix.

5.2.1 Other regulators of GSK3β

Skeletal muscle GSK3β can be directly regulated via Ser9 phosphorylation within several pathways and are outlined in Table 1.1 located in Chapter I Appendix. However, Ser9 is not the only regulatory mechanism which affects GSK3β activity. A total of 36 posttranslational modification sites have been discovered through high throughput and low throughput methods, most having unknown effects and regulators (Figure 5.2). Exploration of these regulatory outcomes using site specific mutations combined with activity assays, western blotting, and downstream targets can yield valuable information regarding GSK3β’s role in skeletal muscle glycogen homeostasis. Changes to GSK3β activity and regulation may result in alterations to the activity and phosphorylation of GS. There are 9 sites within 4 clusters which modulate GS in response to internal and external signaling. GSK3β directly regulates GS at site 3 via phosphorylation, and potentially can affect glycogen homeostasis through its interactions within other regulatory pathways of GS.

5.2.2 Regulation of skeletal muscle glycogen homeostasis in vivo

The effects we observed in glycogen regulation in differentiating myoblasts are limited by many aspects of in vitro experimentation. Glycogen homeostasis during satellite cell differentiation and integration to skeletal muscle tissue may differ significantly from in vitro models due to peripheral regulators of that process.
Phenotypical characterization and studies of primary satellite cell cultures from experimental animals can provide translation of our results to physiological regulation. Examination of growth factor responses on glycogen homeostasis in primary cell cultures as well as within animal tissues may show significant differences from the L6 myoblast cells.

### 5.2.3 The interplay of RA, insulin, and CHX in skeletal muscle glycogen homeostasis

Insulin regulation of glycogen homeostasis in skeletal muscle has been extensively studied due to its relevance in T2DM. The effects of RA on insulin regulation of both mitogenic and metabolic outcomes has yet to be clearly defined. The regulation of metabolic gene expression and fine tuning by insulin stimulation can be altered via RAREs within target genes by RA. CHX also has multiple metabolic effects both dependent and independent of its inhibition of protein synthesis. Identifying transcription factors which dimerize with RAR and RXR and how that alters gene expression can provide insight into the complex interplay of RA and insulin. Further examination of the effects of CHX and how it mechanistically alters signaling pathways may yield additional uses for CHX for research. Performing experiments in different skeletal muscle and *in vitro* cell models at different stages of differentiation, accompanied by analyses of gene expression with quantitative PCR, protein expression with western blotting, and interactions via mass spectrometry are warranted.

### 5.3 Summary

The observations made from the experiments conducted for this dissertation indicate that there are still many unknown mechanisms in glycogen regulation in
myoblasts and skeletal muscle. The canonical model of glycogen metabolism, while elegant, does not provide explanation for the phenomena we have shown in the dissertation. Due to glycogen’s central role in systemic glucose homeostasis and pathophysiology of T2DM, a greater understanding of these mechanistic relationships may provide more robust models to guide future clinical interventions in insulin resistance and T2DM.
References

Appendix

Figure 5.1 Suggested experiments for future exploration. Directions which this research could proceed based upon the experimental data within this dissertation.
Figure 5.2 Posttranslational modification sites of GSK3β discovered using high-throughput methods. Phosphorylation, ubiquitylation, and sumoylation sites discovered using high throughput methods obtained from the PhosphoSitePlus database [1].
Vita

Aaron Lee Armstrong was born in Nashville, TN on April 13, 1987. He obtained his high school diploma through the education system of Macon County, TN in 2005. Aaron joined the U.S. Army in 2006 and was deployed to Iraq in 2009-2010 in support of Operation Iraqi Freedom. He received his B.S. in Hospitality Management and Dietetics in 2015 and completed a dietetic internship at Western Kentucky University in 2016. After becoming a registered dietitian in 2016 he decided to pursue metabolic research due to his interest in physiology. He was accepted into the Cellular and Molecular Nutrition concentration of the Nutrition Sciences program at the University of Tennessee as a student of Dr. Guoxun Chen in Fall 2016. In December of 2022 he was accepted as a student of Dr. Jiangang Chen after Dr. Guoxun Chen’s retirement. During his time as a graduate student, he provided laboratory and research training for several students and served on the Graduate Student Advisory Board. He married Tessa Colette Helmink on March 13, 2013. On May 4, 2021, Tessa and he welcomed their beautiful daughter Eleanor Armstrong into the world. Aaron defended his dissertation on May 2, 2023.