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The Effect of Aerobic Exercise Training on Hepatic Glycogen Metabolism in Type 1 Diabetic Rats

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Kinesiology

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THE EFFECT OF AEROBIC EXERCISE TRAINING ON HEPATIC GLYCOGEN
METABOLISM IN TYPE 1 DIABETIC RATS

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by

Michael Murray

Graduate Program in Kinesiology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

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ABSTRACT

The purpose of this investigation was to determine if ten weeks of aerobic exercise training could increase hepatic glycogen storage in rats with Type 1 Diabetes Mellitus (T1DM) and whether elevated hepatic glycogen content is associated with alterations in glycogenic proteins and insulin signaling. Rats were divided into control-sedentary, control-exercised, T1DM-sedentary and T1DM-exercised groups. Animals from each group underwent a euglycemic-hyperinsulinemic clamp at the conclusion of the study. Exercise training consisted of treadmill running at 27m/min, 6% incline for 1hr, five days/week for ten weeks. T1DM rats had lower liver glycogen concentrations than control rats and glycogen was not increased with training. GS, GK and PEPCK protein contents were also increased in the T1DM groups. Insulin-clamp stimulated GSK phosphorylation was not different between treatments. These findings indicate that aerobic exercise training does not increase liver glycogen content in T1DM rats despite increases in glycogenic protein content and normal insulin signaling.

Keywords: type 1 diabetes mellitus, exercise, hepatic glycogen, hepatic insulin signaling

CO-AUTHORSHIP

Dr. Jamie Melling was involved in project design, interpretation of the results and thesis revisions.

DEDICATION

For my parents and sister, I wouldn't be here without you.

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LIST OF ABBREVIATIONS

| | |
|-------------------|---|
| Acetyl-CoA | Acetyl Coenzyme A |
| AMPK | Adenosine Monophosphate Protein Kinase |
| ATP | Adenosine Triphosphate |
| CamK | Calcium Calmodulin-Dependent Protein Kinase |
| CSII | Continuous Subcutaneous Insulin Infusion |
| EGP | Endogenous Glucose Production |
| ERK | Extracellular Regulated Kinase |
| F6P | Fructose-6-Phosphate |
| F6Pase | Fructose 6-Phosphatase |
| FADH ₂ | Flavin Adenine Dinucleotide (reduced form) |
| G1P | Glucose-1-Phosphate |
| G6P | Glucose-6-Phosphate |
| G6Pase | Glucose 6-Phosphatase |
| GK | Glucokinase |
| GKRP | Glucokinase Regulatory Protein |
| GLUT2 | Glucose Transporter 2 |
| GLUT4 | Glucose Transporter 4 |

| | |
|-------------------|---------------------------------------|
| GP | Glycogen Phosphorylase |
| GS | Glycogen Synthase |
| GSK | Glycogen Synthase Kinase |
| HB _{A1c} | Glycosylated Hemoglobin |
| HDL | High Density Lipoprotein |
| HISS | Hepatic Insulin Sensitizing Substance |
| IL | Interleukin |
| IP | Intraperitoneal |
| IR | Insulin Receptor |
| IRS | Insulin Receptor Substrate |
| JNK | c-Jun N-Terminal Kinase |
| K _m | Michaelis Constant |
| LDL | Low Density Lipoprotein |
| LIRKO | Liver Insulin Receptor Knockout |
| MAPK | Mitogen Activated Protein Kinase |
| MODY | Maturity Onset Diabetes of the Young |
| NADH | Nicotinamide Adenine Dinucleotide |
| NFκB | Nuclear Factor Kappa B |

| | |
|------------------|--|
| NPY | Neuropeptide Y |
| PDK | 3-Phosphoinositide Dependent Kinase |
| PEPCK | Phosphoenolpyruvate Carboxykinase |
| PI3K | Phosphatidylinositol-3 Kinase |
| PIP ₂ | Phosphatidylinositol Diphosphate |
| PIP ₃ | Phosphatidylinositol Triphosphate |
| PKA | Protein Kinase A |
| PKC | Protein Kinase C |
| PNS | Parasympathetic Nervous System |
| PP1 | Phosphoprotein Phosphatase 1 |
| RTK | Receptor Tyrosine Kinase |
| SNS | Sympathetic Nervous System |
| SOCS | Suppressor Of Cytokine Signaling |
| Stat | Signal Transducer Activator of Transcription |
| STZ | Streptozotocin |
| T1DM | Type 1 Diabetes Mellitus |
| T2DM | Type 2 Diabetes Mellitus |
| TLR | Toll-Like Receptor |

| | |
|------------|------------------------------|
| UDPG | Uridine Diphosphate-Glucose |
| VLDL | Very Low Density Lipoprotein |
| V_{\max} | Maximal Rate of Catalysis |

CHAPTER 1

1.1 Introduction

Type 1 diabetes mellitus (T1DM) is a disease associated with the autoimmune-mediated destruction of the insulin secreting cells of the endocrine pancreas, the islet β cells, which prevents the natural secretion of insulin in response to elevated blood glucose levels. As the primary role of insulin is to initiate glucose uptake in peripheral tissues, patients suffer from chronic hyperglycemia. This deficit in glucose metabolism manifests a host of diabetes-related complications including cardiovascular disease, neuropathy, nephropathy, retinopathy and insulin resistance^{1,2}. Furthermore, these complications worsen as the patient progresses through life, such that the life expectancy of a patient with T1DM is dramatically reduced from 4 to 19 years depending on age at diagnosis and treatment^{3,4}. Similar to the prevalence of type 2 diabetes mellitus (T2DM), epidemiological data suggests that T1DM onset is increasing worldwide and in both low (low incidence of disease) and high (high incidence of disease) risk populations⁵.

To lower the risk of complications, T1DM patients are required to consistently monitor blood glucose levels and adjust synthetic insulin injections to maintain their blood glucose to near normalized levels. This modernized insulin therapy regime consists of multiple daily injections (referred to as intensive insulin therapy), which is in stark contrast to more conventional insulin therapy regimes that typically consist of a daily single dose of insulin^{1,6}. The Diabetes Control and Complications Trial, a large multi-center and long term study of T1DM pathology, found that this intensive insulin therapy produced lower blood glucose and glycosylated hemoglobin (Hb_{A1C}) over time¹. These changes were in concert with reductions in both retinopathy and neuropathy and improved the low-density lipoprotein profile of patients (an indicator for cardiovascular disease risk)¹. This type of insulin treatment strategy is not without its

challenges, including the incidence of severe hypoglycemia onset being increased 3-fold by intensive treatment versus more conventional insulin therapy ^{1,6}.

In addition to insulin supplementation, regular exercise is often advised as it has been shown to help counteract many of the complications associated with T1DM. For example, regular exercise has been shown to lower blood glucose concentrations and triglycerides, improve bone health, decrease cardiovascular risk and increase insulin sensitivity, as well as increase lean muscle and bone mass and decrease fat mass ⁷⁻⁹. Additionally, improved quality of life and sense of well-being have also been reported in patients with T1DM following an exercise intervention ¹⁰. Despite these known benefits, many patients with T1DM refrain from regular exercise due to fear of experiencing severe hypoglycemia ^{10,11}. The combination of insulin therapy and regular exercise has been shown to improve health markers, but potentiate the risk of hypoglycemia onset due to the potentiating effects of both factors on glucose uptake ¹². As such, patients with T1DM struggle to find a delicate and individualized balance between the dosage of insulin and the amount of exercise required to achieve the health benefits associated with exercise and avoid hypoglycemic episodes.

Patients with T1DM suffer from significant deficits in glucose metabolism, including uptake, storage and release ¹³⁻¹⁷. Following exercise in both healthy and diabetic, glucose uptake into peripheral tissues such as skeletal muscle increases dramatically and glucose production, from hepatic stores or simple ingestion, must increase accordingly. However, hepatic glycogen stores are known to be decreased in T1DM, impairing the liver's ability to naturally release glucose ^{17,18}. Decreased production, combined with increased glucose use and improved insulin sensitivity following exercise make it extremely difficult to maintain glucose homeostasis, leading the T1DM patient to a severe risk of hypoglycemia onset ⁸. The mechanisms underlying lowered levels of hepatic glycogen are not well understood; understanding these mechanisms may provide insight into how we can mitigate post exercise hypoglycemia in T1DM.

1.2 Liver Physiology

The liver is a complex organ which is involved in several processes essential for survival. Located in the upper right section of the abdominal cavity, the liver can be structurally divided into four lobes, the left, right, caudate and quadrate lobes (located under the left and right lobes respectively). The liver tissues consist of two primary cell types, parenchymal cells known as hepatocytes, and non-parenchymal cells including sinusoidal epithelial cells, kupfer cells and stellate cells. The blood supply to the liver comes from two sources, the hepatic artery which supplies oxygenated blood directly from the aorta and the hepatic portal vein which brings deoxygenated blood from the gastrointestinal tract, as well as the spleen and other organs ¹⁹. The liver is able to obtain about half of its required oxygen from the portal vein, while the other half comes from the hepatic artery ¹⁹. Arterial and venous blood mixes within hepatic sinusoids prior to entering the hepatic vein for transport out of the liver. Functionally, the liver can be subdivided into small clusters, or acini, which consist of a central arteriole surrounded by a group of hepatocytes, which, in turn, are surrounded by venules which carry blood away from the liver towards the vena cava ¹⁹. This arrangement creates a strong oxygen gradient (highest centrally, decreasing distally), resulting in additional gradients in the activities of various enzymes. For example, citric acid cycle and electron transport enzyme activities are highest centrally within each acinus, where there is an abundance of oxygen, whereas glycolytic, non-oxygen requiring enzymes, are higher in the periphery ¹⁹.

The liver is highly innervated with both afferent and efferent sympathetic (SNS), parasympathetic (PNS) and sensory neurons, allowing constant communication between the liver and the brain ¹⁹. Afferent fibers are able to communicate directly with the central nervous system, relaying information on blood temperature and pressure, as well as ion and nutrient content (carbohydrate, lipids, proteins, and circulation hormones and growth factor levels) ²⁰⁻²¹. Efferent nervous fibres allow central control over a multitude of functions for which the liver is

responsible. Specifically applicable, the SNS is known to decrease liver glycogen content through adrenergic-mediated release of glucose²². SNS denervation has also been shown to decrease the rate of fatty acid oxidation via carnitine-palmitoyltransferase, which increases circulating triglycerides and very low density lipoproteins (VLDL) as well as decreasing the production of ketone bodies²³⁻²⁵. Conversely, stimulation of the PNS has been shown to increase rates of hepatic glycogen synthesis and increase production of a hepatic insulin sensitizing substance (HISS), a yet unidentified molecule known to increase glucose uptake in skeletal muscle²⁶⁻²⁸. In addition to these metabolic influences, the SNS is known to decrease blood flow to the liver via the hepatic artery without changing the overall distribution of blood within the liver while the PNS has little impact¹⁹.

1.2.1 Liver Functions

Liver functions can be divided into three main categories: nutrient processing, biosynthesis and protection/filtering.

Nutrient Processing:

As the portal vein brings blood directly from the gastrointestinal tract to the liver, it is exposed to all the nutrients absorbed following meal ingestion. One of the fundamental roles of the liver is to maintain a normalized blood glucose concentration (~4 mM)²⁹. Carbohydrates, primarily glucose and fructose, are taken up into hepatocytes where they have several potential fates: direct metabolism into usable energy for the liver itself, storage as glycogen, or entered into the pentose phosphate cycle and converted either into NADPH or ribose-5 phosphate³⁰. Meals containing excess amounts of carbohydrate stimulate lipogenesis resulting in their conversion to both glycerol and fatty acids for storage³⁰. The liver also functions in de novo glucose production via gluconeogenesis^{29,30}. This process uses non-carbohydrate precursors such as lactate and certain amino acids to generate glucose³⁰. This is done by reversing, with a few additional

enzymatic reactions, the process of glycolysis. During states of nutrient deprivation such as fasting or in various disease states such as diabetes where glucose homeostasis is disrupted, this process is critical to maintaining circulating blood glucose levels.

Fatty acids are also absorbed directly from the diet and are metabolized into usable energy for the hepatocytes themselves, or, can be packaged for export³¹. Individual fatty acids combine to form triglycerides which are subsequently organized into lipoproteins (be it high, low or very low density (HDL, LDL, VLDL, respectively)) for export or stored in hepatic vesicles for future use³². During periods of carbohydrate shortage, use of fatty acids increases greatly and as a result, the production of ketone bodies, acetone, acetoacetate and 3-hydroxybutyrate, increase as well³³. The liver is also responsible for the processing of cholesterol, necessary for the production of many important molecules (steroid hormones including cortisol, testosterone and estradiol, lipoproteins, as well as critical components in all cellular membranes). Cholesterol molecules can be used within hepatocytes for the production of bile acids or packaged into lipoproteins for export to peripheral tissues.

The liver also processes excess amino acids, resulting from either dietary sources or normal protein turnover. These amino acids are degraded in the urea cycle for excretion. Non-essential amino acids can also be synthesized within the liver, provided the necessary essential amino acids are present³⁴. Finally, the liver is also a site for the storage of excess vitamins and minerals including vitamins A, D, B12, K and iron³⁵⁻³⁸.

Biosynthesis:

A second important function of the liver is the biosynthesis of compounds necessary for normal body function. In addition to glucose, fat and amino acid synthesis, albumin, a major component of blood plasma, is produced in the liver and binds proteins, fats, hormones and many other constituents, within the blood to maintain the osmotic pressure³⁹. Coagulation factors such

as fibrinogen and prothrombin are also synthesized in the liver, as well as binding proteins, apoferritin and transferrin, which are important for the transport of iron in the blood ^{40,41}. Furthermore, bile salts, compounds necessary for the digestion and absorption of fats from the digestive tract, are also produced and secreted from the liver ⁴².

Protection:

The last major function of the liver is protection and clearance of potentially harmful substances from the body. The gastrointestinal tract, particularly the colon, contains bacteria which is a potential risk to enter circulation. Kupfer cells within the hepatic sinusoids are potent macrophages which scavenge and can clear bacterium from hepatic sinusoids ¹⁹. Similarly, the liver is able to clear toxins, hormones, and other harmful substances from the blood through oxidative reactions that disrupt foreign substances without damaging its own proteins and DNA ^{34,43}. For instance, the liver is heavily involved in the clearance of hormones such as insulin and glucagon and plays an important role in the metabolism of the drugs used to treat any number of conditions ³⁴.

1.3 Glycogen Metabolism

There are two primary stores of carbohydrate in the body, liver and muscle glycogen. While small amounts have been shown to exist in other areas (neurons, the brain etc.) these quantities make up a very small proportion of total energy storage ⁴⁴. Glycogen is a branched polymer of glucose residues and functions to release glucose into circulation when it is unavailable from the diet (i.e. during fasting) ⁴⁴. Muscle glycogen is stored mainly for fueling muscle contraction, while liver glycogen stores serve to maintain blood glucose homeostasis ⁴⁴⁻⁴⁶. Glucose release from the liver is critical in times of increased glucose uptake into peripheral tissues, such as skeletal muscle, during exercise ^{45,46}. The maintenance of normalized

concentrations of glucose in the blood is critical for organs such as the brain which rely heavily on a steady flow of glucose in the blood to function ^{34,44}.

Glycogen synthesis (shown in figure 1.1) is initiated by a single protein, glycogenin, which is able to self-glycosylate, creating an eight residue chain ⁴⁷. This glucosyl chain, in turn, is extended by forming alpha-1,4 glycosidic bonds to the existing end of the chain ⁴⁷. Once thirteen residues are linked linearly, a branch is then created by removing the final four residues and reattaching them via an alpha-1,6 linkage ^{44,47}. Each thirteen-residue chain will have two alpha-1,6 branch points, creating a densely packed granule. Successive “tiers” are created in this fashion until the upper limit of twelve tiers, has been reached ⁴⁴. A full twelve-tier glycogen molecule would contain approximately fifty-five thousand residues; although, analysis of particle size suggests the average is closer to seven tiers ⁴⁶. The glycogen granule is not solely composed of carbohydrate. Known as a glycosome, many proteins involved in the synthesis and degradation of glycogen, as well as an abundance of downstream regulators, also participate in binding to the granule ⁴⁸. While the specific function is unknown, it is believed the function of the glycosome is to provide a scaffold to bring many of the necessary enzymes into close association with each other to allow coordinated and timely control of glycogen metabolism ^{44,48}.

The glycogen concentration is controlled by the relative activities of two enzymes, glycogen synthase (GS) and glycogen phosphorylase (GP). The synthesis of glycogen is mediated by GS which functions to add glucose units to a glycogen chain, while GP is responsible for the breakdown of the glycogen molecule through the removal of glucose units ⁴⁴. GS is expressed in two isoforms, a liver-specific form and a more widely expressed, muscle isoform ^{44,49,50} and can synthesize glycogen through a direct or indirect pathway ^{44,51}. The direct pathway involves the uptake of glucose into either the liver or skeletal muscle and its subsequent phosphorylation into glucose-6-phosphate (G6P) ^{44,52}. G6P is then converted into glucose-1-phosphate (G1P), which is subsequently converted to uridine diphosphate-glucose (UDPG) by UDP-glucose-

pyrophosphorylase. UDPG molecules are assembled into a glycogen chain by GS, adding residues by the formation of α 1-4 bonds, while an additional enzyme, the branching enzyme, removes existing 1-4 bonds and creates the α 1-6 branch points. The indirect pathway exists primarily in the liver, but can also occur in the kidneys, involving the gluconeogenic creation of G6P from three-carbon precursors including lactate, glycerol and alanine^{51,53}. This pathway is less efficient than direct synthesis but allows glycogen to be synthesized from non-carbohydrate sources^{51,53}.

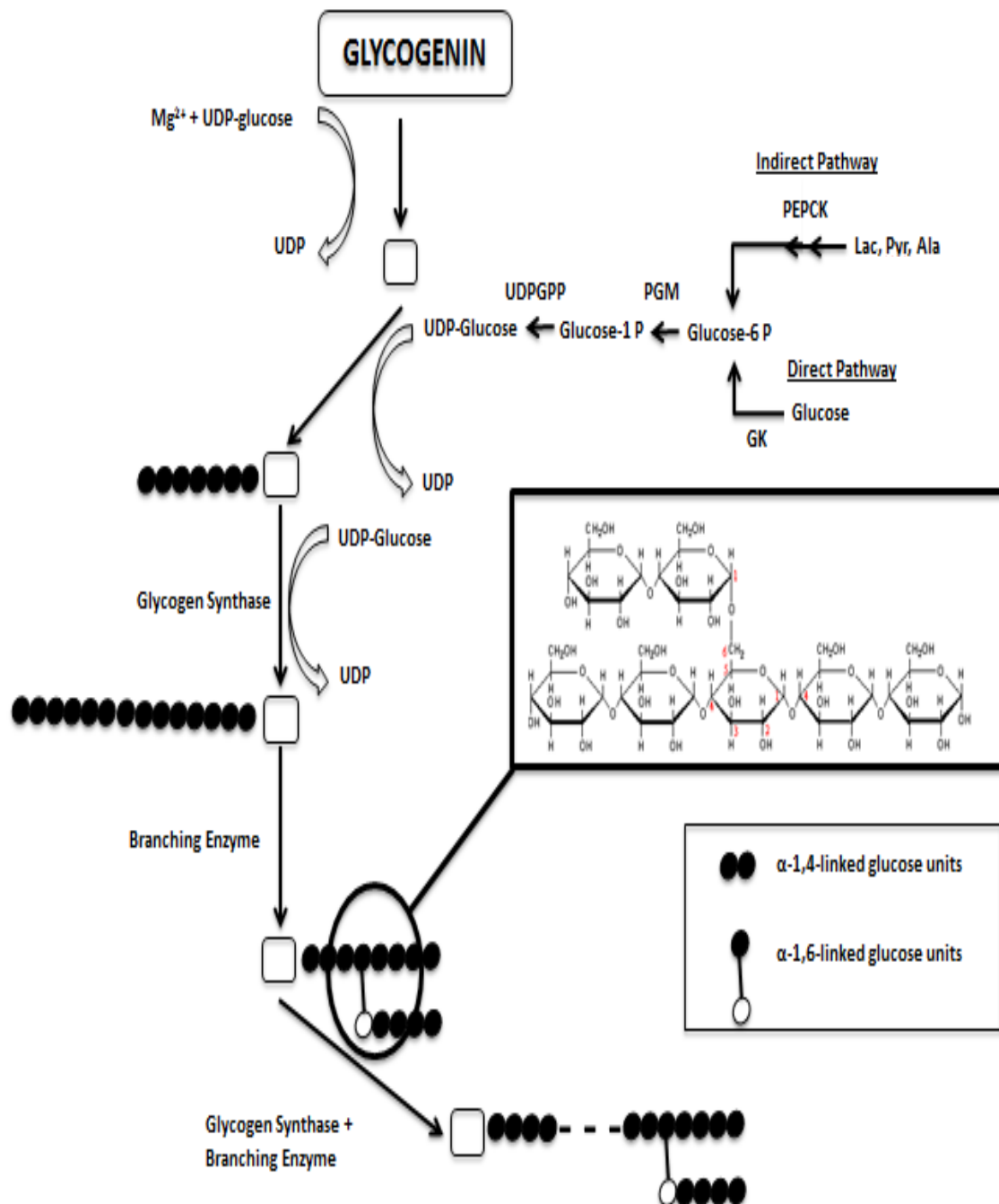


Figure 1.1 Steps in Glycogen Synthesis.

Abbreviations: UDP, uridine diphosphate; UDPG, uridine diphosphate-glucose; UDPGPP, UDPG-pyrophosphorylase; PGM, phosphoglucomutase; GK, glucokinase; PEPCK, phosphoenolpyruvate carboxykinase; Lac, lactate, Pyr, pyruvate; Ala, alanine

Adapted from:

Bollen, M., Keppens, S. & Stalmans, W. Specific features of glycogen metabolism in the liver. *Biochem J* **336**, 19–31 (1998).

1.4 Glucose Processing

Glucose uptake into the liver is mainly accomplished through the glucose transporter 2 (GLUT2) protein⁵⁴. Although GLUT2 is located in several tissues, the highest levels of this protein are expressed in the liver hepatocytes and pancreatic β -cells^{54,55}. It is constitutive to the membrane and allows glucose uptake via facilitated, yet passive, transport. Although GLUT2 has a high capacity for glucose uptake, the affinity for the glucose molecule is low by comparison to other GLUT isoforms^{54,56,57}. This low affinity for glucose is favourable during conditions of lowered blood glucose concentrations, preventing excessive glucose uptake and subsequent hypoglycemia onset. Glucose uptake and subsequent metabolism is critical in the β -cells mechanism for insulin secretion, reinforcing the importance of a selective transporter^{55,58}.

Upon entering the hepatocyte, glucose is phosphorylated into G6P by a liver-specific hexokinase, glucokinase (GK). GK differs from other hexokinase isoforms in that it is not allosterically inhibited by its own product, G6P; as well as having a higher Michaelis constant (K_m) for glucose^{49,51}. Additionally, GK is also controlled through interaction with its own regulatory protein, glucokinase regulatory protein (GKRP), which in combination with glycolytic metabolite fructose-6-phosphate (F6P), acts to bind and inhibit GK activity^{59,60}. More elaborate control mechanisms are also important for modulating GK activity as there is evidence of this step being rate limiting in glycogen synthesis⁶¹. This is important in the liver because it is the site of not only glucose storage, but in times of excess, conversion of glucose into fatty acids⁶². As glucose uptake is dependent upon the concentration gradient between the hepatocyte and the blood, a high rate of glucose phosphorylation to G6P is critical to maintain a low concentration of free glucose within hepatocytes, and subsequent glucose uptake. However, when glycogen stores are full, high glycolytic flux (and subsequently, high F6P) can feedback to GK to slow glucose uptake and prevent conversion to fat⁶²⁻⁶⁴.

There are two primary fates for G6P, glycolysis and glycogen synthesis. Glycolysis utilizes G6P, and, through a series of reactions, converts it into two molecules of pyruvate, two ATP molecules and four reducing equivalents in the form of NADH. Pyruvate, depending on the environment, can be converted to lactate when the overall energy state of the cell is low (a shortage of NAD^+) or into acetyl-coenzyme A (acetyl-CoA), which in turn is metabolized in the citric acid cycle to produce additional NADH for electron transport and oxidative phosphorylation. If directed towards glycogen synthesis, G6P is converted to G1P and then in the glycogenic substrate, UDPG, by phosphoglucomutase and UDPG pyrophosphorylase, respectively⁵¹.

When conditions favour glycogen synthesis, such as following a carbohydrate-containing meal, glucose units will be funneled towards storage. This requires the combined control of two regulatory enzymes, glycogen synthase (GS) and glycogen phosphorylase (GP). Activation of GS is mediated by both allosteric and covalent mechanisms^{44,49}. Glucose and glucose metabolites account for the allosteric modulation while insulin signalling is the main covalent effector⁴⁹. GS is a homodimer and exists in two states, an active *a* form and an inactive *b* form. Phosphorylation occurs through the activity of a variety of protein kinases including protein kinase A (PKA), AKT (also known as protein kinase B (PKB)), glycogen synthase kinase 3 (GSK3), calmodulin dependent protein kinase (CamK), protein kinase C (PKC) and adenosine monophosphate dependent protein kinase (AMPK) among others^{49,65,66}. Phosphorylation of any of GS' six possible sites acts to convert the *a* form to the *b* form, decreasing its maximal rate (V_{\max}) of catalysis⁴⁹. This illustrates a key difference between the muscle and liver isoforms of GS as phosphorylation of the muscle isoform decreases its affinity towards its substrate UDPG, not the maximal rate of catalysis as in the liver isoform⁴⁹. Additionally, G6P is a potent activator of the *b* form of GS, such that maximal activity of G6P through the exposure of the enzyme to supraphysiological conditions results in equivalent rates of glycogen synthesis as seen with the

active *a* form of the enzyme⁴⁹. In addition, intracellular localization is also different between the muscle and liver isoforms of GS⁶⁷. GS translocates from a diffuse distribution in the cytosol towards the cell periphery in hepatocytes; whereas in the muscle, it moves from the nucleus into the cytosol to synthesize glycogen⁶⁷⁻⁶⁹.

1.5 Modulators of Glycogen Synthase Activity

1.5.1 Glucose Induced Glycogen Synthesis

The first mechanism by which elevated levels of intracellular glucose can increase the rate of glycogen synthesis is through interaction with GP (see figure 1.2). The active form of GP acts as a glucose sensor, binding to free glucose which competitively inhibits the enzymes active site and alters GP conformation^{49,70}. Conformational changes in GP have a twofold effect as it increases the rate of GP dephosphorylation (and inactivation) and disrupts the complex that forms between active GP and the primary GS phosphatase, phosphoprotein phosphatase 1 (PP1)^{49,70}. Dissociation of PP1 allows this protein to dephosphorylate and activate GS. In addition, glucose is an important regulator of the GK-GKRP interaction. Increases in glucose concentration have been shown to free GK from this complex to increase the production of G6P, another potent GS activator^{71,72}. As mentioned above, G6P allosterically activates GS *b* by increasing the rate of GS dephosphorylation and directly interacting with the enzyme⁷¹. Finally, as previously discussed, glucose is also a regulator of GS localization, causing it to move to sites of glycogen storage.

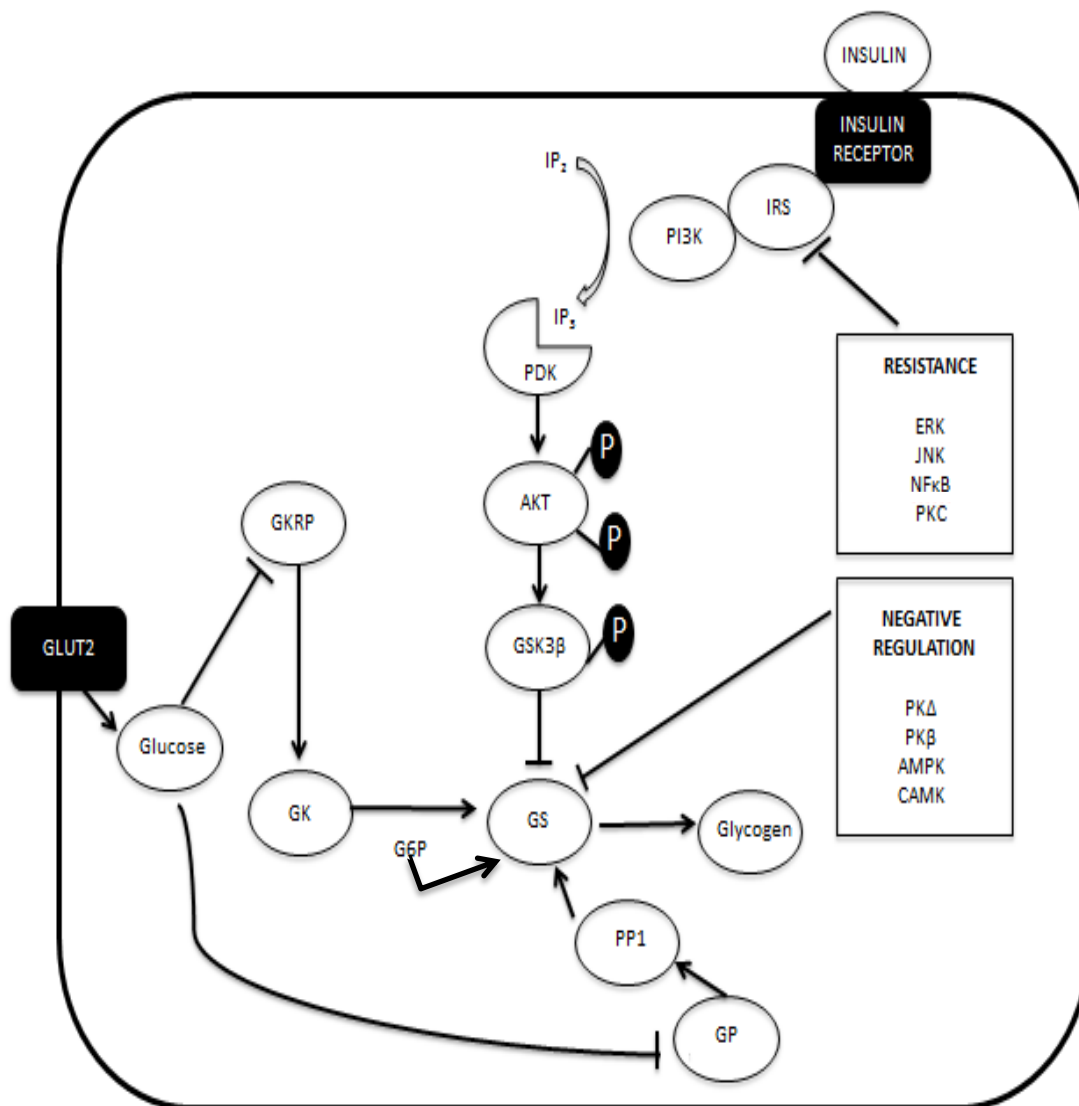


Figure 1.2 Modulators of Glycogen Synthesis. GS activity is altered by a variety of signals including covalent phosphorylation by the insulin signalling (inhibitory) cascade as well as small metabolite allosteric control.

Abbreviations: IRS, insulin receptor substrate; PI3K, phosphatidylinositol phosphate kinase; IP₂ Inositol diphosphate, IP₃, Inositol triphosphate; PDK, phosphoinositide dependent protein kinase; GSK3 β , glycogen synthase kinase 3 β ; GS, glycogen synthase, GKRP, glucokinase regulatory protein; GK, glucokinase; PP1, phosphoprotein phosphatase 1; GP, glycogen phosphorylase; ERK, extracellular regulated kinase; JNK, c-jun n-terminal kinase; NF κ B, nuclear factor kappa B; PKC, protein kinase C; PKA, protein kinase A; AMPK, adenosine monophosphate dependent kinase, CAMK, calmodulin dependent protein kinase

1.5.2 Insulin Signalling

Insulin signalling is responsible for controlling many intracellular processes, including glucose metabolism and is illustrated in figure 2.2⁷³. The insulin receptor (IR) is a ubiquitously expressed, heterodimeric receptor, belonging to the receptor tyrosine kinase (RTK) superfamily. IR activation leads to the stimulation of both canonical arms of the insulin signalling pathway, the metabolic phosphatidylinositol-3 kinase (PI3K) - AKT node; and the mitogenic ras-mitogen activated protein kinase (MAPK) signalling pathways^{74,75}. As the AKT side of this pathway is primarily responsible for regulating glucose metabolism, it will be the focus of the following section. It should be noted however, that despite the apparent divergence of these two cascades, significant cross-talk exists, contributing to the complexity of insulin signalling network⁷³.

Circulating insulin binds to the (extracellular) α subunit of the insulin receptor, causing the autophosphorylation of tyrosine residues in the intracellular β subunit, creating an active docking site for insulin receptor substrate (IRS) proteins⁷⁶. The IRS family contains six members (dubbed IRS 1-6), of which, IRS1 and IRS2 are the most widely expressed in both the liver and skeletal muscle^{73,77,78}. IRS proteins then bind to phosphorylated tyrosine residues on the activated IR^{73,78,79}. IR-IRS interaction promote phosphorylation of one or more of IRS' approximately twenty tyrosine residues, allowing interaction with the p85/p55 regulatory subunit of PI3K⁷⁹. Subsequent activation of the p110 kinase domain of PI3K allows the conversion of phosphatidylinositol diphosphate (PIP₂) to the active second messenger phosphatidylinositol triphosphate (PIP₃). PIP₃ then binds to and activates 3-phosphoinositide dependent kinase (PDK) which phosphorylates 2 residues (a serine and a threonine) to activate AKT⁷³.

AKT is the principle mediator of an insulin signal as it is capable of mediating changes in other kinases, signalling molecules and transcription factors. GSK3 is a key effector on glycogen metabolism and is down regulated by AKT phosphorylation⁷³. This decreases its capacity to

phosphorylate and inactivate GS, favouring glycogen synthesis⁴⁹. GSK3 exists in two isoforms, GSK3 α and GSK3 β . The two forms share high levels of sequence homology within their kinase domains but little throughout the rest of the protein and have largely redundant functions⁸⁰. GSK3 is an important regulator of many intracellular processes with over 40 recognized putative substrates including transcription factors, structural proteins involved in cell motility and trafficking as well as metabolic substrates such as GS⁸⁰. GSK3 is controlled by reversible phosphorylation (as in the insulin cascade outlined above as well as in pathways controlled by G-protein coupled receptors); as well as by intracellular localization (nuclear GSK3 is more active than cytosolic GSK3) and its association with signalling complexes (seen in its role in Wnt- β Catenin signalling)⁸⁰. It should be noted that, unlike traditional positive regulation by phosphorylation, serine phosphorylation, such as that mediated by AKT, of GSK3 decreases its activity toward its substrates⁸⁰.

1.6 Insulin Resistance

Insulin resistance is a condition in which peripheral tissues are unable to properly respond to the insulin hormone. Resistance to insulin has profound consequences not only to glucose metabolism but other metabolic signalling pathways (lipolysis/fatty acid metabolism, transcription, translation, etc.) influenced by the insulin hormone as well. While common to both T1DM and T2DM, the origin of the disease appears to differ. Insulin resistance seems to preclude the development of T2DM as it has been evident in patients 10 to 20 years prior to the onset of clinical hyperglycemia⁸¹. Tissue resistance to insulin causes islet β -cells to secrete progressively higher levels of insulin, which eventually causes β -cells to fail, resulting in overt hyperglycemia and T2DM⁸². On the other hand, T1DM begins with immune-related β -cell death and insulin resistance manifests later in the progression of the disease, impairing the efficacy of administered insulin in controlling blood glucose concentrations⁸³.

Despite etiological differences, a possible mechanism is similar between these two conditions and revolves around decreased insulin-induced, IRS mediated PI3K activation^{73,81}. This effectively decreases overall insulin signalling as it decreases the activity of AKT, the principle effector on both glucose uptake and glycogen synthesis. The exact mechanism behind this has yet to be fully elucidated; however, increased levels of IRS serine phosphorylation are seen in insulin resistant states and have been implicated⁷³. IRS proteins contain over 70 potential serine phosphorylation sites capable of modifying its activity. Interestingly, insulin-responsive kinases including extracellular regulated kinase (ERK), S6 kinase, c-jun N-terminal kinase (JNK) and nuclear factor kappa B kinase (NFκB) are all known to phosphorylate serine residues within IRS proteins, indicating a feedback mechanism to limit an insulin signal⁸⁴⁻⁸⁷. These proteins are also positively controlled by levels of circulating cytokines as well as fatty acids, creating a causative link between fat metabolism/obesity and insulin resistance^{73,81,88,89}. In addition, alterations in insulin receptor phosphorylation, decreased ability for insulin to bind its receptor and depleted intracellular pools of IR have also been cited as potential mechanisms underlying insulin resistance^{87,90}.

1.6.1 Hepatic Insulin Resistance

The IR represents the first step in transducing an insulin signal into intracellular events and as such, plays a key role in insulin resistance⁹¹. Hepatic IR knockout (known as LIRKO) mice demonstrate severe insulin resistance and dysfunctional glucose production and gene expression⁹¹. Recent work utilizing another genetically-induced model of hepatic insulin resistance in which NFκB is constitutively active have shed further light on the matter. Hyperactive NFκB mimics the subacute inflammatory state seen in the fatty livers of patients with T2DM.⁸⁷ Evidence from this model has demonstrated decreased basal and insulin-stimulated IR and IRS2 tyr-phosphorylation, decreased insulin-induced suppression of hepatic glucose production, decreased glycogen storage and impaired GSK-3β phosphorylation⁸⁷. The

gluconeogenic response to feeding was also disrupted such that glucose-6 phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) mRNAs were inappropriately elevated postprandially⁸⁷. NFκB activation also increased mRNA production of pro-inflammatory cytokines interleukin (IL) 1B and IL6 as well as cytokine receptors for IL1, IL6, IL8 and toll-like receptor (TLR) 2⁸⁷. These were shown to feedback to the liver itself as mRNAs for Stat3 and SOCS1, 2 and 3 were elevated⁸⁷. Despite hepatic-specific NFκB activation, systemic effects in skeletal muscle were also observed as IRS1 phosphorylation, glucose uptake and glycogen synthesis were all impaired, lending support to the idea that insulin resistance could potentially begin in the liver and travel systemically^{87,92}.

T1DM is associated with both peripheral and hepatic insulin resistance. Early work from DeFronzo et al demonstrated that, during hyperinsulinemia, patients with T1DM had higher levels of endogenous glucose production than healthy control subjects, indicative of the liver's inability to respond to insulin⁹³. These results have been confirmed more recently by several groups reporting that T1DM patients with long term continuous subcutaneous insulin therapy (CSII) had deficits in suppression of endogenous glucose production (EGP). Furthermore, following overnight normalization of blood glucose concentrations, patients with T1DM required significantly more insulin to suppress hepatic glucose production^{94,95}. Despite a lack of mechanistic studies in T1DM, potential links can be drawn between recent works. Insulin resistance in T2DM has long been associated with obesity, specifically alterations in lipid partitioning such that abnormal amounts of lipid accumulate in the liver and skeletal muscle. A mouse model lacking adipose tissue was created and mimicked this response by causing significant fat accumulation in the liver and skeletal muscle; and insulin resistance⁹⁶. Despite lack of obesity, recent work using liver biopsies from T1DM patients has demonstrated that over 50% demonstrate steatosis, 20% had non-alcoholic fatty liver disease and over 70% had fibrotic

scarring⁹⁷. This is the first study to date demonstrating this in T1DM and may provide insight into the origin of insulin resistance in this condition.

1.7 Diabetes and Glycogen Storage

While patients with T1DM are likely to experience both fed and fasting hyperglycemia, they are also susceptible to the development of insulin- and exercise-mediated hypoglycemia. Despite the seemingly opposite nature of these conditions, it has been suggested that deficits in hepatic glycogen storage contribute to both¹⁷. Early work by Hwang et al, demonstrated using C13 magnetic resonance spectroscopy that, following ingestion of isocaloric meals (breakfast, lunch and dinner), patients with T1DM stored less liver glycogen after each individual meal (measured 4hrs later) as well as accumulated less total glycogen (approximately 70% less) over the course of the day¹⁷. This same study also demonstrated that T1DM subjects synthesized only 41% of their daily liver glycogen from the direct pathway; whereas healthy control subjects synthesized 65% from direct sources¹⁷. The authors conclude that alterations in the insulin:glucagon ratio (T1DM subjects were hypoinsulinemic and hyperglucagonemic following each meal relative to the controls) likely contribute to this deficit but acknowledge that hepatic alterations in T1DM are also likely. Decrements in glycogen storage and direct:indirect pathway flux is a strong candidate for postprandial hyperglycemia as increased gluconeogenic activity and glucose release, combined with impaired glucose uptake/storage leaves absorbed glucose nowhere to go. Later work from Kishore and colleagues connected these findings to impairments in hypoglycemic recovery¹⁸. They found that, during a hypoglycemic clamp in which patients blood glucose was held constant at ~3.33mM by insulin infusion, patients with T1DM were unable to increase the rate of endogenous glucose production (total of hepatic glycogenolysis and gluconeogenesis) due to the complete inability to utilize hepatic glycogen¹⁸. It was concluded that a 30% decrement in baseline hepatic glycogen relative to control subjects, in addition to defects in hormonal counterregulation (lower epinephrine, norepinephrine and glucagon were

observed during hypoglycemia) were the cause of impaired response to hypoglycemia in T1DM¹⁸.

While patients with T1DM in the above studies were insulin treated, there was still some doubt as to whether intensive insulin treatment could normalize hepatic glycogen content. Consecutive works from Bischoff et al set out to answer this question^{98,99}. The first of the two experiments examined if short-term intensive insulin therapy could normalize the alterations in hepatic glycogen metabolism seen in T1DM. Untreated subjects with T1DM showed lower rates of both glycogen synthesis and breakdown (74% and 41% respectively, relative to healthy subjects), both of which were significantly improved with intensive insulin treatment⁹⁸. The second experiment utilized similar short-term insulin treatment in addition to a full week of treatment prior to the study during which T1DM patients were normoglycemic. Rates of hepatic glycogen synthesis were normalized to the level of control subjects; however, the proportion of direct-to-indirect synthesis was still favouring indirect synthesis in the patients with T1DM⁹⁹. This finding in patients with long term T1DM is in opposition to more recent work in an STZ-induced model of rodent diabetes. Soares et al (2012) found that, when insulin was administered 9 days post Streptozotocin (STZ), by day 18 (post STZ) the rates of direct pathway glycogen synthesis were normalized in the rats with T1DM¹⁰⁰. This suggests that, if intensive insulin therapy is started early following the onset of T1D, the hepatic defects seen in long standing T1D may in fact be reversible.

Insulin signalling and glycogen storage in T1DM is associated with deficits in the expression and activation of many proteins involved in hepatic glycogen synthesis. Libal-Weksler et al (2001) recently demonstrated that STZ-induced diabetic animals had lower total and activated GS activity relative to healthy animals and that insulin stimulation was unable to increase GS activity *in vitro*¹⁰¹. Despite this, they found that the total amount of GS protein was equivalent between the two conditions and that insulin stimulation increased GS protein content

in both groups¹⁰¹. This is interesting as it suggests that insulin's effects on protein production are functional in this model of T1DM, but its ability to increase enzyme activity is hindered. These results were verified in the same study using two dietary models of insulin resistance, high fat and high simple sugar diets. Both induced decreases in GS activity¹⁰¹. This is of particular interest as hyperglycemia is also a known characteristic of T1DM and, despite the presence of insulin secretion, glucose was also able to decrease GS activity¹⁰¹.

Work from Gannon and Nuttall, however, have shown that GS activity is increased in T1DM as a function of duration of the disease¹⁰². Specific activity and GS mRNA were increased in T1DM after both 3 and 8 days of the disease¹⁰². This occurred despite only small changes in total GS content and was unchanged with feeding or fasting¹⁰². It was concluded that T1DM increases the efficiency of the enzyme as enzyme mass and mRNA could not explain the change in activity. Furthermore, by day 8, protein mass and mRNA were lower than that seen in controls animals¹⁰². Enzyme activity was also equivalent in the fed and fasted state, yet mRNA and protein contents differed, hence, changes in enzyme phosphorylation and activation by G6P were cited as to why enzyme activity was increased. This work is not the only to contradict Libal-Weksler and colleagues. Increases in GS activity in T1DM have been reported by several groups as well as in differing diabetic models including STZ and alloxan-induced diabetes¹⁰³⁻¹⁰⁷.

Despite apparent increases in GS activity, T1DM animals in the aforementioned works had significantly decreased hepatic glycogen levels and no change in GS content at both 3 and 8 days¹⁰². This is interesting as increases in GS protein content by adenoviral overexpression have been shown to increase hepatic glycogen storage¹⁰⁸. As simple overexpression of GS is clearly unable to account for all changes accompanying the hepatic proteome in T1DM, direct comparisons are unfounded; however, it does raise interest as to what else might be causing the deficit in glycogen storage.

Differences in the above studies are likely due to differences in T1DM model as well as disease duration. In vitro versus in vivo activity analyses yield seemingly conflicting results as cell culture of diabetic hepatocytes suggests decreases in GS activity while hepatocytes from whole-animal livers suggest an increase^{101,102}. Reasons for these discrepancies are unclear but may be due to differences in the hormonal and neural input to an intact liver, causing alterations in enzyme activity not mimicked by culture media. Additionally, studies in T1DM animals without insulin treatment remove a key hormonal stimulus for GS activation that has been reported to be impaired in vitro¹⁰¹. Additionally, the duration of diabetes, as well as the severity, has also been shown to change GS activity¹⁰². Durations up to 8 days as used by Gannon and Nuttall demonstrate increased GS activity, however Rao and colleagues found, in insulin treated animals after three weeks of diabetes, that GS activity and expression were unchanged from control levels¹⁰⁹. To date, no studies on long term diabetes and GS activity or expression have been conducted but if the trend of decreased activity from 8 to 21 days continues, it is possible that GS activity would be decreased relative to control levels and may be a contributing factor to decreased levels of hepatic glycogen.

In addition to GS, other proteins involved in hepatic glycogen synthesis have been shown to be altered in T1DM. GLUT2 protein and mRNA content appears to be stable in T1DM patients and is largely unchanged with insulin administration¹¹⁰. Additionally, although feeding and fasting have been known to alter GLUT2 mRNA, there is little change in protein content^{57,110}. Despite these findings, Libal-Weksler et al have shown that, in cultured hepatocytes, both high sugar and high fat feeding decreased GLUT2 protein content, which, consistent with additional works, was not improved by acute insulin administration^{49,57}. This is an interesting finding as glucose has also been shown to increase GLUT2 mRNA content in hepatocytes which suggests a post-transcriptional control mechanism is dominant in GLUT2 expression¹¹¹.

Downstream of GLUT2, GK is an important regulator of glycogen synthesis in the liver. Adenoviral overexpression of GK in hepatocytes has been shown to significantly increase glycogen storage, independent of GS content or activity^{61,108,112}. Insulin is the primary factor responsible for increasing GK content in hepatocytes, and accordingly, insulin deficient STZ-induced T1DM animals have significantly lower levels of GK; and humans deficient for GK develop maturity onset diabetes of the young (MODY) type 2^{51,113–115}. Furthermore, obese T2DM patients also have decreased levels of GK, indicating once again GK's importance in whole body glucose homeostasis¹¹⁶. Insulin administration to T1DM subjects has been shown to normalize both GK content and activity, and overexpression in diabetic animals also restores both blood glucose concentration and glycogen content, as well as the animals ability to avoid hypoglycemia during a fast^{100,115}.

In addition to alterations in direct pathway glycogen storage, T1DM is also known to generate significant amounts of glycogen by the indirect pathway. Proteins involved in the gluconeogenic production of G6P are also altered in T1DM. Soares and colleagues noted, in a short-term insulin treated, STZ-induced diabetic model, that T1DM animals (with or without insulin) demonstrated significant increases in the mRNA content of gluconeogenic proteins PEPCK, fructose-1,6-bisphosphatase (F6Pase) and G6Pase¹⁰⁰.

1.8 Exercise and Glycogen

Physical exercise is a metabolic stressor well known to improve insulin sensitivity as well as alter substrate utilization, increase lean muscle mass and decrease fat mass^{117,118}. Exercise also produces alterations in key metabolic enzyme concentrations and activities, as well as cardiac and respiratory efficiency, allowing better performance in subsequent exercise bouts. Muscle glycogen is used in an intensity and duration dependent manner to fuel muscle

contractions; and loss of glycogen is associated with exhaustion and withdrawal from exercise ¹¹⁹. During and following an exercise bout, muscle glucose uptake is increased in an insulin-independent fashion which functions to both provide glucose for glycolysis during exercise, and substrate for glycogen resynthesis following exercise ^{119,120}. Following long term training programs, increases in oxidative enzyme capacity generate a glycogen-sparing effect such that a greater proportion of energy can be obtained from fatty acids at increasingly higher intensities. Additionally, exercise training also increases the overall storage capacity for muscle glycogen ¹²¹. With respect to function in blood glucose maintenance, hepatic glycogen storage and use with both acute and prolonged training will be the focus of the following section.

1.8.1 Acute Exercise

Stored hepatic glycogen is utilized for the maintenance of blood glucose concentrations in times of increased use, not for fueling muscle contractions. Earlier studies from Ivey and colleagues demonstrated significant glycogen use in skeletal muscle but not the liver during 5 minutes of intense treadmill exercise ¹²². Exercise of this short duration is unlikely to be long enough for blood glucose to become a significant contributor to exercise energy production, leaving hepatic glycogen untouched. However, following the bout, liver glycogen was depleted over the following four hours, while significant glycogen accumulated in muscle ¹²². Studies utilizing prolonged low-intensity or high-intensity exercise to exhaustion, however, demonstrate complete depletion of hepatic glycogen ¹²³⁻¹²⁷. Following an acute exercise bout, very little hepatic glycogen is resynthesized in the absence of food intake, and by 24 hours post-exercise, glycogen concentrations are lower than those seen following 36 hours of fasting ¹²⁴. In a follow up study, animals were followed for 24 hours and again, during the first four hours following either continuous or intermittent exercise to exhaustion, no hepatic glycogen was stored when food was restricted ¹²⁷. Despite this, skeletal muscle glycogen concentrations increased in this time frame to approximately 50% of resting levels ¹²⁷. When food was provided ad libitum for

the following 20 hours, liver glycogen returned to the level of an exercised rat, but not to the level of a non-exercised control rat ¹²⁷. In this same time frame however, muscle glycogen was seen to reach approximately 190% of pre-exercise resting values, suggestive of preferential resynthesis in muscle over the liver ^{126,127}.

1.8.2 Exercise Training

Exercise training has been established to increase hepatic glycogen storage ^{128,129}. Baldwin and coworkers demonstrated increased resting hepatic glycogen levels in rats following 14 weeks of swimming, 6 hours per day, 5 days a week ¹²⁹. This improvement was also associated with a smaller decrease in liver glycogen following a 45 min progressive treadmill test ¹²⁹. In contrast to the extreme endurance activity used in those studies, only 6 weeks of treadmill running, 6 weeks of weighted jumps (4 sets of 10, 5 days per week) and 12 weeks of ladder climbing (4-8 climbs, 3 days per week) have all be shown to increase liver glycogen stores ^{130,131}. Additionally, exercise training supplemented with either a diet high in fructose or whey protein, have also been shown to further increase liver glycogen ¹³²⁻¹³⁴. In addition to alterations in hepatic glycogen content, exercise training has also been shown to increase GS activity, in conjunction with increases in liver size, further enhancing the capacity of the liver to store glycogen ¹²⁸. Contrary to these findings, work from James and colleagues found that 10 weeks of treadmill running at a light, moderate or high intensity did not alter glycogen content or GS activity in the liver ¹³⁵.

1.9 Summary

Patients with T1DM suffer from hepatic glycogen deficits, which alter their ability to increase glucose release during a hypoglycemic episode. Additionally, hepatic insulin resistance is present and may contribute to the lack of hepatic glycogen stores. Exercise training has been shown to improve a host of T1DM-related complications, however many patients refrain from

exercise due to fear of hypoglycemia. Training studies have demonstrated that exercise might act as a means of increasing hepatic glycogen storage and would aid in hypoglycemia prevention and recovery. Further study is required to elucidate molecular mechanisms involved in both the glycogen deficit and hepatic-insulin resistance noted in patients with T1DM and to examine the effects of exercise training on these parameters.

1.11 Reference List

1. Group, T. D. C. and C. T. R. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* **329**, 977–986 (1993).
2. Chiang, J. L., Kirkman, M. S., Laffel, L. M. B. & Peters, A. L. Type 1 diabetes through the life span: a position statement of the american diabetes association. *Diabetes Care* **37**, 2034–2054 (2014).
3. Miller, R. G., Secrest, A. M., Sharma, R. K., Songer, T. J. & Orchard, T. J. Improvements in the life expectancy of type 1 diabetes: the Pittsburgh epidemiology of diabetes complications study cohort. *Diabetes* **61**, 2987–2992 (2012).
4. Narayan, K. M. ., Boyle, J. P., Thompson, T. J., Sorensen, S. W. & Williamson, D. F. Lifetime risk for diabetes mellitus in the United States. *JAMA* **290**, 4–10 (2003).
5. Onkamo, P., Väänänen, S., Karvonen, M. & Tuomilehto, J. Worldwide increase in incidence of type 1 diabetes--the analysis of the data on published incidence trends. *Diabetologia* **42**, 1395–1403 (1999).
6. Verrotti, A., Chiarelli, F., Blasetti, A. & Bruni, E. Severe hypoglycemia in insulin-dependent diabetic children treated by multiple injection insulin regimen. *Acta diabetol* **33**, 53–57 (1996).
7. Maggio, A. B. R. *et al.* Physical activity increases bone mineral density in children with type 1 diabetes. *Med Sci Sport Ex* **44**, 1206–1211 (2012).
8. Riddell, M. C. & Perkins, B. A. Type 1 diabetes and vigorous exercise: Applications of exercise physiology to patient management. *J Diabetes* **30**, 63–71 (2006).
9. Reaven, G. M. & Chang, F. Effect of exercise-training on the metabolic manifestations of streptozotocin-induced diabetes in the rat. *Diabetologia* **21**, 415–417 (1981).
10. Kavookjian, J., Elswick, B. M. & Whetsel, T. Interventions for being active among individuals with diabetes: a systematic review of the literature. *Diabetes Educ.* **33**, 962–988 (2007)
11. Brazeau, A. S., Rabasa-Lhoret, R., Strychar, I. & Mircescu, H. Barriers to physical activity among patients With type 1 diabetes. *Diabetes Care* **31**, 2108–2109 (2008).
12. McMahan, S. K. *et al.* Glucose requirements to maintain euglycemia after moderate-intensity afternoon exercise in adolescents with type 1 diabetes are increased in a biphasic manner. *J Clin Endocrinol Metab* **92**, 963–968 (2007).
13. Marette, A. *et al.* Glucose rapidly decreases plasma membrane GLUT4 content in rat skeletal muscle. *Endocrine* **10**, 13–18 (1999).

14. Fisher, J. S. *et al.* Glucose transport rate and glycogen synthase activity both limit skeletal muscle glycogen accumulation. *Am J Physiol Endocrinol Metab* **282**, 1214–1221 (2002).
15. Hall, K. E. *et al.* The role of resistance and aerobic exercise training on insulin sensitivity measures in STZ-induced type 1 diabetic rodents. *Metabolism* 1–11 (2013).
doi:10.1016/j.metabol.2013.05.012
16. Chen, V. & Ianuzzo, C. D. Dosage effect of streptozotocin on rat tissue enzyme activities and glycogen concentration. *Can J Physiol Pharm* **60**, 1251–1256 (1982).
17. Hwang, J. H. *et al.* Impaired net hepatic glycogen synthesis in insulin-dependent diabetic subjects during mixed meal ingestion. A ¹³C nuclear magnetic resonance spectroscopy study. *J Clin Invest* **95**, 783–787 (1995).
18. Kishore, P. *et al.* Role of hepatic glycogen breakdown in defective counterregulation of hypoglycemia in intensively treated type 1 diabetes. *Diabetes* **55**, 659–666 (2006).
19. Lutt, W. W. *Hepatic Circulation Physiology and Pathophysiology*. (Claypool Life Sciences, 2009).
20. Yi, C.-X., la Fleur, S. E., Fliers, E. & Kalsbeek, A. The role of the autonomic nervous liver innervation in the control of energy metabolism. *Biochim. Biophys. Acta - Mol. Basis Dis.* **1802**, 416–431 (2010).
21. Spanswick, D., Smith, M. A., Mirshamsi, S., Routh, V. H. & Ashford, M. L. J. Insulin activates ATP- sensitive K⁺ channels in hypothalamic neurons of lean , but not obese rats. *Nat Neurosci* **3**, 757–758 (2000).
22. Shimazu, T. & Fukuda, A. Increased activities of glycogenolytic enzymes in liver after splanchni- nerve stimulation. *Science*. **150**, 1607–1608 (1965).
23. Carreno, F. R. & Seelaender, M. C. L. Liver denervation affects hepatocyte mitochondrial fatty acid transport capacity. *Cell Biochem Funct* **22**, 9–17 (2004).
24. Tavares, F. L. & Seelaender, M. C. L. Hepatic denervation impairs the assembly and secretion of VLDL-TAG. *Cell Biochem Funct* **26**, 557–565 (2008).
25. Yamamoto, T., Iwai, M., Kimura, S. & Shimazu, T. The mechanism of action of hepatic sympathetic nerves on ketone-body output from perfused rat liver The effect of the interaction of noradrenaline with ATP on the release of / 3-hydroxybutyrate. *Eur J Biochem* **234**, 466–471 (1995).
26. Shimazu, T. Glycogen synthetase activity in the liver: regulation by the autonomic nerves. *Science*. **156**, 1256–1257 (1967).
27. Lutt, W. W. A ew paradigm for diabetes and obesity : the hepatic insulin sensitizing substance (HISS) Hypothesis. *J Pharmacol Sci* **95**, 9–17 (2004).

28. Shimazu, T. Regulation of glycogen metabolism in the liver by the autonomic nervous system V. Activation of glycogen synthetase by vagal stimulation. *Biochim. Biophys. Acta* **252**, 28–38 (1971).
29. Nordlie, R. C., Foster, J. D. & Lange, A. J. Regulation of glucose production by the liver. *Ann Rev Nutr* **19**, 379–406 (1999).
30. Dashty, M. A quick look at biochemistry: carbohydrate metabolism. *Clin Biochem* **46**, 1339–1352 (2013).
31. Neuschwander-Tetri, B. A. Carbohydrate intake and nonalcoholic fatty liver disease. *Curr Opin Clin Nutr Metab Care* **16**, 446–452 (2013).
32. Sundaram, M. & Yao, Z. Intrahepatic role of exchangeable apolipoproteins in lipoprotein assembly and secretion. *Atheroscler Thromb Vasc Bio* **32**, 1073–1038 (2012).
33. McGarry, J. D. & Foster, D. W. Regulation of hepatic fatty acid oxidation and ketone body production. *Ann Rev Biochem* **49**, 395–420 (1980).
34. Sherwood, L. *Human Physiology From Cells to Systems*. (Thompson, Brooks/Cole, 2007).
35. Pongron, G., Kennan, A. L. & Deluca, H. F. ‘Activation’ of vitamin D by the liver. *J Clin Invest* **48**, 2032–2037 (1969).
36. Swendseid, M. E., Bethell, F. H. & Ackerman, W. W. The intracellular distribution of vitamin B12 and folic acid in mouse liver. *J Biol Chem* **190**, 791–798 (1951).
37. Young, S., Roberts, S. & Bomford, A. Intracellular processing of transferrin and iron by isolated rat hepatocytes. *Biochem. J* **232**, 819–823 (1985).
38. Blomhoff, R. Transport and Metabolism of Vitamin A. *Nutr Rev* **52**, S13–S23 (2009).
39. Moshage, H. J., Janssen, J. A. M., Franssen, J. H., Hafkenscheid, J. C. M. & Yap, S. H. Study of the molecular mechanism of decreased liver synthesis of albumin in inflammation. *J Clin Invest* **79**, 1635–1641 (1987).
40. Mann, K. G. Biochemistry and physiology of blood coagulation. *Thromb Haemost* **82**, 165–174 (1999).
41. Huebers, H. A. & Finch, C. A. The physiology of transferrin and transferrin receptors. *Physi* **67**, 520–582 (1987).
42. Hofmann, A. F. Bile acids: the good, the bad, and the ugly. *Physiology* **14**, 24–29 (1999).
43. Bouwens, L., De Bleser, P., Vanderkerken, K., Geerts, B. & Wisse, E. Liver cell heterogeneity: functions of non-parenchymal cells. *Enzyme* **46**, 155–168 (1992).
44. Roach, P. J., Depaoli-Roach, A. A., Hurley, T. D. & Tagliabracci, V. S. Glycogen and its metabolism: some new developments and old themes. *Biochem J* **441**, 763–787 (2012).

45. Greenberg, C. C., Jurczak, M. J., Danos, A. M. & Brady, M. J. Glycogen branches out: new perspectives on the role of glycogen metabolism in the integration of metabolic pathways. *Am J Physiol Endocrinol Metab* **291**, E1–8 (2006).
46. Shearer, J. & Graham, T. E. Novel aspects of skeletal muscle glycogen and its regulation during rest and exercise. *Exerc Sport Sci Rev* **32**, 120–126 (2004).
47. Lomako, J., Lomako, W. M. & Whelan, W. J. Glycogenin: the primer for mammalian and yeast glycogen synthesis. *Biochim. Biophys. Acta* **1673**, 45–55 (2004).
48. Rybicka, K. K. Glycosomes — the organelles of glycogen metabolism. *Tissue Cell* **28**, 253–265 (1996).
49. Bollen, M., Keppens, S. & Stalmans, W. Specific features of glycogen metabolism in the liver. *Biochem J* **336**, 19–31 (1998).
50. Gomis, R. R., Cid, E., García-Rocha, M., Ferrer, J. C. & Guinovart, J. J. Liver glycogen synthase but not the muscle isoform differentiates between glucose 6-phosphate produced by glucokinase or hexokinase. *J Biol Chem* **277**, 23246–23252 (2002).
51. Agius, L. Glucokinase and the molecular aspects of liver glycogen metabolism. *Biochem J* **414**, 1–18 (2008).
52. Thorens, B. & Mueckler, M. Glucose transporters in the 21st century. *Am J Physiol Endocrinol Metab* **298**, E141–145 (2010).
53. Radziuk, J. & Pye, S. Hepatic glucose uptake , gluconeogenesis and the regulation of glycogen synthesis. *Diabetes Metab Res Rev* **17**, 250–272 (2001).
54. Thorens, B. Molecular and cellular physiology of GLUT2: a high km facilitated diffusion glucose transporter. *Int Rev Cytol* **137**, 209–238 (1992).
55. Thorens, B., Gerard, N. & Deriaz, N. GLUT2 surface expression and intracellular transport via the constitutive pathway in pancreatic beta cells and insulinoma: evidence for a block in trans-Golgi network exit by brefeldin A. *J Cell Biol* **123**, 1687–1694 (1993).
56. Huang, S. & Czech, M. P. The GLUT4 glucose transporter. *Cell metab* **5**, 237–252 (2007).
57. Kahn, B. B. Facilitative glucose transporters: regulatory mechanisms and dysregulation in diabetes. *J Clin Invest* **89**, 1367–1374 (1992).
58. Unger, R. H. Diabetic hyperglycemia: link to impaired glucose transport in pancreatic beta cells. *Science (80-)*. **251**, 1200–1205 (1991).
59. Van Schaftingen, E., Detheux, M. & Da Cunha, M. V. Short-term control of glucokinase activity: role of a regulatory protein. *FASEB J* **8**, 414–419 (1994).

60. Niculescu, L., Da Cunha, M. V. & Van Schaftingen, E. Investigation of the mechanism by which fructose, hexitols and their compounds regulate the translocation of glucokinase in rat hepatocytes. *Biochem J* **321**, 239–246 (1997).
61. Ferre, T., Riu, E., Bosch, F. & Valera, A. Evidence from transgenic mice that glucokinase is rate limiting for glucose utilization in the liver. *FASEB J* **10**, 1213–1218 (1996).
62. Foufelle, F. & Ferré, P. New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c. *Biochem J* **366**, 377–391 (2002).
63. Prip-Buus, C. *et al.* Induction of fatty-acid-synthase gene expression by glucose in primary culture of rat hepatocytes Dependency upon glucokinase activity. *Eur J Biochem* **230**, 309–315 (1995).
64. Van Dijk, T. H. *et al.* Acute inhibition of hepatic glucose-6-phosphatase does not affect gluconeogenesis but directs gluconeogenic flux toward glycogen in fasted rats. A pharmacological study with the chlorogenic acid derivative S4048. *J Biol Chem* **276**, 25727–25735 (2001).
65. Villar-Palasi, C. & Guinovart, J. J. The role of glucose 6-phosphate in the control of glycogen synthase. *FASEB J* **11**, 544–558 (1997).
66. Imazu, M., Strickland, W. G., Chrisman, T. D. & Exton, J. H. Phosphorylation and inactivation of liver glycogen synthase by liver protein kinases. *J Biol Chem* **259**, 1813–1821 (1984).
67. Ros, S., García-Rocha, M., Domínguez, J., Ferrer, J. C. & Guinovart, J. J. Control of liver glycogen synthase activity and intracellular distribution by phosphorylation. *J Biol Chem* **284**, 6370–6378 (2009).
68. Cid, E., Gomis, R. R., Geremia, R. a, Guinovart, J. J. & Ferrer, J. C. Identification of two essential glutamic acid residues in glycogen synthase. *J Biol Chem* **275**, 33614–33621 (2000).
69. Fernandez-Novell, J. M., Bellido, D., Vilaro, S. & Guinovart, J. J. Glucose induces the translocation of glycogen synthase to the cell cortex in rat hepatocytes. **231**, 227–231 (1997).
70. Aiston, S., Coghlan, M. P. & Agius, L. Inactivation of phosphorylase is a major component of the mechanism by which insulin stimulates hepatic glycogen synthesis. *Eur J Biochem* **270**, 2773–2781 (2003).
71. Cadefau, J., Bollen, M. & Stalmans, W. Glucose-induced glycogenesis in the liver involves the glucose-6-phosphate-dependent dephosphorylation of glycogen synthase. *Biochem J* **322**, 745–750 (1997).

72. Fernandez-Novell, J. M., Arino, J., Vilaro, S., Bellido, D. & Guinovart, J. J. Role of glucose 6-phosphate in the translocation of glycogen synthase in rat hepatocytes. *Biochem J* **288**, 497–501 (1992).
73. Taniguchi, C. M., Emanuelli, B. & Kahn, C. R. Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* **7**, 85–96 (2006).
74. Cheatham, B. *et al.* Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. *Mol Cell Biol* **14**, 4902–4911 (1994).
75. Pouyssegur, J., Vomat, V. & Lenormand, P. Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling. *Biochem Pharmacol* **64**, 755–763 (2002).
76. Goren, H. J., White, M. F. & Kahn, C. R. Separate domains of the insulin receptor contain sites of autophosphorylation and tyrosine kinase activity. *Biochemistry* **26**, 2374–2382 (1987).
77. Sun, X. J. *et al.* Role of IRS-2 in insulin and cytokine signalling. *Nature* **377**, 173–177 (1995).
78. Sun, X. J. *et al.* Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature* **352**, 73–77 (1991).
79. Virkamäki, A., Ueki, K. & Kahn, C. R. Protein – protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. *J Clin Invest* **103**, 931–943 (1999).
80. Jope, R. S. & Johnson, G. V. W. The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci* **29**, 95–102 (2004).
81. Perseghin, G., Petersen, K. & Shulman, G. I. Cellular mechanism of insulin resistance: potential links with inflammation. *Int. J. Obes.* **27**, 6–11 (2003).
82. Fonseca, V. A. Defining and characterizing the progression of type 2 diabetes. *Diabetes Care* **32**, 151–156 (2009).
83. Greenbaum, C. J. Insulin resistance in type 1 diabetes. *Diabetes Metab Res Rev* **18**, 192–200 (2002).
84. Swantek, J. L., Cobb, M. H. & Geppert, T. D. Jun N-Terminal Kinase / Stress-Activated Protein Kinase (JNK / SAPK) Is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF- α) translation : glucocorticoids inhibit TNF- α translation by blocking JNK / SAPK. *Mol Cell Biol* **17**, 6274–6282 (1997).
85. Bouzakri, K. *et al.* Reduced activation of phosphatidylinositol-3 kinase and increased serine 636 phosphorylation of insulin receptor substrate-1 in primary culture of skeletal muscle cells from patients with type 2 diabetes. *Diabetes* **52**, 19–23 (2003).

86. Harrington, L. S. *et al.* The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. *J Cell Biol* **166**, 213–223 (2004).
87. Cai, D. *et al.* Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med* **11**, 183–190 (2005).
88. Mlinar, B., Marc, J., Janez, A. & Pfeifer, M. Molecular mechanisms of insulin resistance and associated diseases. *Clin. Chim. Acta* **375**, 20–35 (2007).
89. Perseghin, G. *et al.* Insulin resistance, intramyocellular lipid content, and plasma adiponectin in patients with type 1 diabetes. *Am J Physiol Endocrinol Metab* **285**, E1174–E1181 (2003).
90. Caro, J. F. *et al.* Studies on the mechanism of insulin resistance in the liver from humans with noninsulin-dependent diabetes: Insulin action and binding in isolated hepatocytes, insulin receptor structure and kinase activity. *J Clin Invest* **78**, 249–258 (1986).
91. Michael, M. D. *et al.* Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Mol Cell* **6**, 87–97 (2000).
92. Kraegen, E. W. *et al.* Development of muscle insulin resistance after liver insulin resistance in high-fat-fed rats. *Diabetes* **40**, 1397–1403 (2010).
93. DeFronzo, R. A., Simonson, D. & Ferrannini, E. Hepatic and peripheral insulin resistance: a common feature of type 2 (non-insulin-dependent) and type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* **23**, 313–319 (1982).
94. Donga, E., van Dijk, M., Hoogma, R. P. L. ., Corssmit, E. P. M. & Romijn, J. A. Insulin resistance in multiple tissues in patients with type 1 diabetes mellitus on long-term continuous subcutaneous insulin infusion therapy. *Diabetes Metab Res Rev* **29**, 33–38 (2013).
95. Bergman, B. C. *et al.* Features of hepatic and skeletal muscle insulin resistance unique to type 1 diabetes. *J Clin Endocrinol Metab* **97**, 1663–1672 (2012).
96. Kim, J. K., Gavrilova, O., Chen, Y., Reitman, M. L. & Shulman, G. I. Mechanism of insulin resistance in A-ZIP/F-1 fatless mice. *J Bio* **275**, 8456–8460 (2000).
97. Harman, D. J. *et al.* Prevalence and natural history of histologically proven chronic liver disease in a longitudinal cohort of patients with type 1 diabetes. *Hepatology* **60**, 158–168 (2014).
98. Bischof, M. G. *et al.* Effects of short-term improvement of insulin treatment and glycemia on hepatic glycogen metabolism in type 1 diabetes. *Diabetes* **50**, 392–8 (2001).
99. Bischof, M. G. *et al.* Hepatic glycogen metabolism in type 1 diabetes after long-term near normoglycemia. *Diabetes* **51**, 49–54 (2002).

100. Soares, A. F. *et al.* Restoration of direct pathway glycogen synthesis flux in the STZ-diabetes rat model by insulin administration. *Am J Physiol Endocrinol Metab* **303**, E875–885 (2012).
101. Libal-Weksler, Y., Gotlibovitz, O., Stark, a H. & Madar, Z. Diet and diabetic state modify glycogen synthase activity and expression in rat hepatocytes. *J Nutr Biochem* **12**, 458–464 (2001).
102. Gannon, M. C. & Nuttall, F. Q. Effect of feeding, fasting, and diabetes on liver glycogen synthase activity, protein, and mRNA in rats. *Diabetologia* **40**, 758–63 (1997).
103. Gold, A. H. The effect of diabetes and insulin on liver glycogen synthetase activation. *J Biol Chem* **245**, 903–905 (1970).
104. Bahnaks, B. R. & Gold, A. H. Effects of alloxan diabetes on the turnover of rat liver glycogen synthase. *J Biol Chem* **257**, 8775–8780 (1982).
105. Hornbrook, K. R. Synthesis of liver glycogen in starved alloxan diabetic rats. *Diabetes* **19**, 916–923 (1970).
106. Whitton, P. D. & Hems, D. A. Glycogen synthesis in the perfused liver of streptozotocin-diabetic rats. *Biochem J* **150**, 153–165 (1975).
107. Miller, T. B., Vicalvi, J. J. & Garnache, A. K. Alteration of hepatic glycogen synthase phosphatase activity by insulin deficiency. *Am J Physiol* **240**, 539–543 (1981).
108. Gomis, R. R., Ferrer, J. C. & Guinovart, J. J. Shared control of hepatic glycogen synthesis by glycogen synthase and glucokinase. *Biochem J* **351**, 811–816 (2000).
109. Rao, P. V., Pugazhenti, S. & Khandelwal, R. L. The effects of streptozotocin-induced diabetes and insulin supplementation on expression of the glycogen phosphorylase gene in rat liver. *J Biol Chem* **270**, 24955–24960 (1995).
110. Thorens, B., Flier, J. S., Lodish, H. F. & Kahn, B. B. Differential regulation of two glucose transporters in rat liver by fasting and refeeding and by diabetes and insulin treatment. *Diabetes* **39**, 712–719 (1990).
111. Postic, C. *et al.* Evidence for a transient inhibitory effect of insulin on GLUT2 expression in the liver : studies in vivo and in vitro. *Biochem J* **293**, 119–124 (1993).
112. Gomis, R. R. *et al.* Glucose 6-phosphate produced by gluconeogenesis and by glucokinase is equally effective in activating hepatic glycogen synthase. *J Biol Chem* **278**, 9740–9746 (2003).
113. Sharma, C., Manjeshwar, R. & Weinhouse, S. Hormonal and dietary regulation of hepatic glucokinase. *Adv Enz Reg* **2**, 189–200 (1964).
114. Magnuson, M. A. Tissue-Specific Regulation of Glucokinase Gene. *J Cell Biochem* **48**, 115–121 (1992).

115. Morral, N. *et al.* Adenovirus-mediated expression of glucokinase in the liver as an adjuvant treatment for type 1 diabetes. *Hum Gene Ther* **13**, 1561–1570 (2002).
116. Caro, J. F. *et al.* Liver glucokinase: decreased activity in patients with type II diabetes. *Horm Metab Res* **27**, 19–22 (1995).
117. Goodyear, L. & Kahn, B. Exercise, glucose transport, and insulin sensitivity. *Annu Rev Med* **49**, 235–261 (1998).
118. Gundersen, K. Excitation-transcription coupling in skeletal muscle: the molecular pathways of exercise. *Biol Rev* **86**, 564–600 (2011).
119. Jensen, T. E. & Richter, E. A. Regulation of glucose and glycogen metabolism during and after exercise. *J Physiol* **5**, 1069–1076 (2012).
120. Richter, E. a & Hargreaves, M. Exercise, GLUT4 and skeletal muscle glucose uptake. *Physiol Rev* **93**, 993–1017 (2013).
121. Spina, R. J. *et al.* Mitochondrial enzymes increase in muscle in response to 7-10 days of cycle exercise. *J Appl Physiol* **80**, 2250–2254 (1996).
122. Ivey, P. A. & Gaesser, G. A. Postexercise muscle and liver glycogen metabolism in male and female rats. *J Appl Physiol* **62**, 1250–1254 (1987).
123. Baldwin, K. M., Reitman, J. S., Terjung, R. L., Winder, W. W. & Holloszy, J. Substrate depletion in different types of muscle and liver during prolonged running. *Am J Physiol* **225**, 1045–1050 (1973).
124. Brooks, G. A., Brauner, K. E. & Cassens, R. G. Glycogen synthesis and metabolism of lactic acid after exercise. *Am J Physiol* **224**, 1162–1166 (1973).
125. Conlee, R. K., Hickson, R. C., Winder, W. W., Hagberg, J. M. & Holloszy, J. O. Regulation of glycogen resynthesis in muscles of rats following exercise. *Am J Physiol* **235**, 145–150 (1978).
126. Fell, R. D., McLane, J. A., Winder, W. W. & Holloszy, J. O. Preferential resynthesis of muscle glycogen in fasting rats after exhausting exercise. *Am J Physiol* **238**, 328–332 (1980).
127. Gaesser, G. A. & Brooks, G. A. Glycogen repletion following continuous and intermittent exercise to exhaustion. *J Appl Physiol Resp Env. Ex Physiol* **49**, 722–728 (1980).
128. Galbo, H., Saugmann, P. & Richter, E. a. Increased hepatic glycogen synthetase and decreased phosphorylase in trained rats. *Acta Physiol Scand* **107**, 269–272 (1979).
129. Baldwin, K. M., Fitts, R. H., Booth, F. W., Winder, W. W. & Holloszy, J. O. Depletion of muscle and liver glycogen during exercise Protective effect of training. *Pflugers Arch* **354**, 203–212 (1975).

130. Cunha, T. S., Tanno, A. P., Costa Sampaio Moura, M. J. & Marcondes, F. K. Influence of high-intensity exercise training and anabolic androgenic steroid treatment on rat tissue glycogen content. *Life Sci.* **77**, 1030–1043 (2005).
131. Prestes, J. *et al.* Resistance training and glycogen content in ovariectomized rats. *Int J Sport. Med* **33**, 550–554 (2012).
132. Murakami, T. *et al.* Enlargement of glycogen store in rat liver and muscle by fructose-diet intake and exercise training. *J Appl Physiol* **82**, 772–775 (1997).
133. Morifuji, M., Sakai, K., Sanbongi, C. & Sugiura, K. Dietary whey protein increases liver and skeletal muscle glycogen levels in exercise-trained rats. *Br J Nutr* **93**, 439–445 (2007).
134. Morifuji, M., Sakai, K. & Sugiura, K. Dietary whey protein modulates liver glycogen and glycoregulatory enzyme activities in exercise-trained rats. *Exp Biol Med* **230**, 23–30 (2005).
135. James, D. E. & Kraegen, E. W. The effect of exercise training on glycogen, glycogen synthase and phosphorylase in muscle and liver. *Eur J Appl Physio* **52**, 276–281 (1984).

CHAPTER 2

2.1 Introduction

Type 1 diabetes mellitus (T1DM) is characterized by the autoimmune destruction of pancreatic islet β -cells, resulting in inadequate insulin secretion and deficits in blood glucose control. As a result, many T1DM patients suffer from a variety of complications including cardiovascular disease, retinopathy, neuropathy and nephropathy; as well as insulin resistance^{1,2}. While it has been shown that strict control of blood glucose concentrations (4-7mM) through intensive insulin therapy is effective in preventing many of these complications, many patients refrain from strict control and maintain more moderate levels of glycemia (8-11mM, known as conventional insulin therapy) to prevent insulin overcorrection and subsequent hypoglycemia¹. In addition to intensive insulin therapy, physical activity has been shown to be effective in relieving many T1DM-related complications. However, patients refrain due to fear of exercise induced hypoglycemia³⁻⁵.

It is believed that a deficit in hepatic glycogen stores may, in part, be responsible for the inability of patients' with T1DM to respond to a hypoglycemic episode⁶. Hepatic glycogen, unlike that in muscle, is stored for the maintenance of blood glucose concentrations in times when glucose homeostasis is challenged, such as during a fast or following exercise. Early work from Hwang et al. (1995) demonstrated that, following a meal, patients with T1DM synthesized significantly less hepatic glycogen than healthy subjects. Furthermore, a larger proportion of that glycogen came from indirect synthesis⁶. It was concluded that this was largely due to disruptions in the insulin:glucagon ratio following a meal⁶. Glycogen deficits are also seen to persist following short term intensive insulin therapy, however, following a week of normoglycemia, hepatic glycogen levels have been shown to return to those of a healthy subject^{7,8}. Lack of hepatic glycogen stores can be connected to impairments in hypoglycemic recovery. Under a

hypoglycemic clamp, rats with T1DM are completely unable to mobilize hepatic glycogen, suggesting that inadequate hepatic release of glucose during hypoglycemia was a result of insufficient glycogen at the start of the clamp⁹.

Hepatocyte glucose uptake, unlike that seen in muscle, is mediated by the glucose transporter 2 (GLUT2), an insulin insensitive isoform¹⁰⁻¹². Glucose is phosphorylated by glucokinase (GK), a high affinity isoform of hexokinase, into glucose-6-phosphate (G6P)^{10,13}. G6P is then converted to glucose-1-phosphate (G1P), uridine diphosphate-glucose (UDPG) and ultimately converted into glycogen by glycogen synthase (GS)¹⁰. All three of these proteins have been shown to modulate hepatic glycogen concentrations and aberrant expression or activity may underlie defective glycogen synthesis¹⁴⁻¹⁷. These proteins have also been demonstrated to be disrupted in T1DM, although conflicting results have been shown due to differences in diabetic model, as well as disease duration¹⁸⁻²². In addition to alterations in glycogen synthesizing proteins, hepatic insulin resistance may also have a role in impaired glycogen synthesis. Work from Defranzo et al originally demonstrated hepatic insulin resistance in T1DM as demonstrated by reduced suppression of hepatic glucose production by insulin; a finding which has recently been supported by others^{23,24}. Although no mechanistic studies exist in patients with T1DM, a rodent model of hepatic insulin resistance, in which hepatic nuclear factor kappa B (NFκB) is constitutively active, exhibits disruptions in insulin signaling and glycogen storage. More specifically, the activation of insulin receptor substrates (IRS) and glycogen synthase kinase (GSK)-3β were shown to be reduced²⁵.

Exercise training has been shown to mitigate many of the complications associated with T1DM and, in healthy populations, also alters hepatic glycogen metabolism. Recent studies have demonstrated the efficacy of various exercise programs, including aerobic and resistance exercise, in increasing hepatic glycogen stores as well as glycogen synthase (GS) activity²⁶⁻²⁹. However, limited work has been completed in T1DM models³⁰. Following high intensity exercise, patients with T1DM, despite having lower basal hepatic glycogen contents, have similar

rates of glycogenolysis, and higher rates of gluconeogenesis compared to healthy control subjects³¹. Similar rates of glycogenolysis suggest that that if basal glycogen levels had been higher, any drop in glycemia following exercise might have been shorter in duration and/or magnitude, or preventable altogether.

The purpose of the current investigation was to determine if a 10-week aerobic exercise training program could improve hepatic glycogen storage in rats with T1DM and whether improved glycogen storage was associated with changes in expression of glycogen-synthesizing proteins. Furthermore, a secondary aim of the study was to determine if aerobic training was able to alter the insulin signaling pathways mediating glycogen storage in the liver. It was hypothesized that exercise training would increase hepatic glycogen storage as well as glycogenic protein expression, and that this would be associated with improvements in hepatic insulin signaling.

2.2 Methods

Ethics approval

In accordance with the guidelines of the Canadian Council on Animal Care, this study was approved by the University Council of Animal Care and Research Ethics boards of the University of Western Ontario (Appendix B1).

Animals

Eight week old, male Sprague-Dawley rats were obtained from Charles River Laboratories (St. Constant, QC, Canada). Animals were housed two per cage in standard rat cages and kept on a 12-hour light/dark cycle at constant temperature and humidity (20±1°C, 50% respectively). Food (protein=26%, carbohydrate=60%, fat=14%; enriched with vitamins and minerals; 164 PROLAB RMH 3000, Brentwood, MO, USA) and water were given *ad libitum* for the duration of the study. Rats were divided into four experimental groups: non-T1DM sedentary

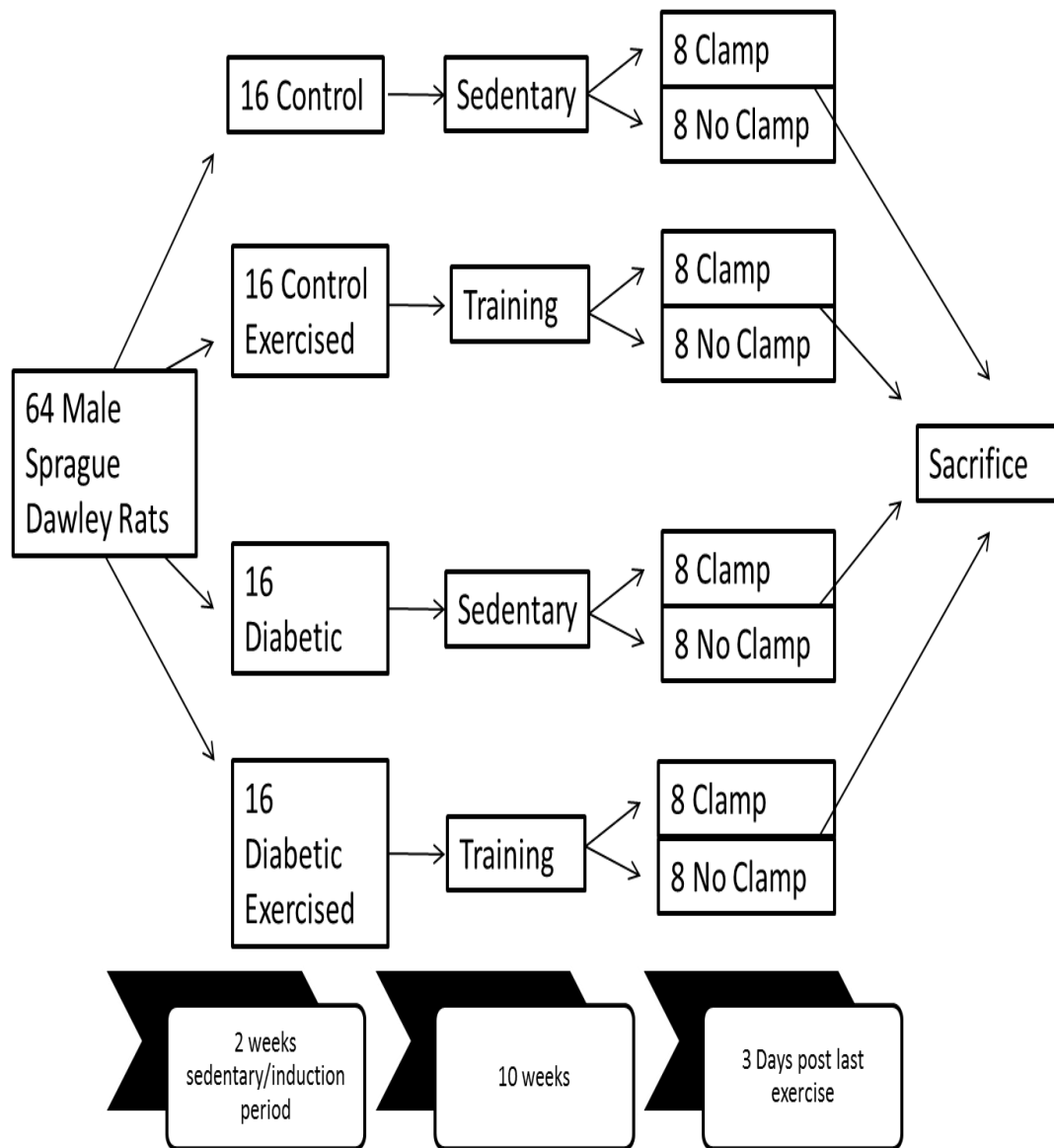
(C, n=16), non-T1DM exercised (CX, n=16), T1DM sedentary (D, n=16), and T1DM exercised (DX, n=16). (See Figure 2.1)

Experimental Procedures

Diabetes Induction

T1DM was induced using multiple low dose streptozotocin (STZ) injections (Appendix A1) to replicate conditions seen in T1DM patients³². On five consecutive days, 20mg/kg of STZ, dissolved in citrate buffer (0.1M, pH 4.5), was given intraperitoneally (IP). T1DM was then confirmed by two consecutive non-fasted blood glucose readings of >18mM. If, after five injections, T1DM was not confirmed, additional injections were given until confirmation was obtained. Blood glucose was then maintained in the range of 9-15mM using insulin pellets, to represent that under conventional insulin treatment³³. Exogenous insulin pellets (1 pellet; 2U insulin/day; Linplant, Linshin Canada, Inc., Toronto, Ontario, Canada) were subcutaneously implanted in the abdominal region (Appendix A2).

Figure 2.1 Study Design



Exercise Protocol

One week prior to the start of exercise training, animals were familiarized with the treadmill to allow rats to become accustomed to both the treadmill and to running itself. Familiarization consisted of 15min of progressive running up to 30m/min over two days. Following the familiarization period, the exercise training consisted of 1hr of treadmill running, 5 days per week, at 27m/min on a 6% gradient for 10 weeks. This intensity of exercise has previously been shown to elicit 70-80% of the animals' VO_{2max} ³⁴.

Experimental Measures

Euglycemic Hyperinsulinemic Clamp

Eight animals from each group were utilized for the euglycemic-hyperinsulinemic clamp. Clamp experiments were initiated three days following the final bout of exercise training. Prior to clamp studies, animals were fasted for 12hrs. Anesthetization consisted of inhaled isoflurane gas (4%) as well as an IP injection of urethane (25mg/kg) and α -chloralose (4mg/kg). Isoflurane gas was removed after approximately 20min and urethane α -chloralose maintained anesthetization during the clamp. A catheter was inserted into the right jugular vein to facilitate infusions of anesthetic, insulin (10mU/kg/min; 0.4 μ IU/mL; Eli Lilly, Toronto, ON, CAN) and glucose (20 mg/kg/min, 0.2 g/mL; EMD Millipore, Darmstadt, HE, Germany). Body temperature was maintained at 37°C with a heating pad and was assessed by a rectal thermometer.

Clamp procedures were originally described by DeFronzo et al. (1979)³⁵. Rats were stabilized for 1hr prior to initiation of clamp experiments. Insulin (10mU/kg/min) was infused using an infusion pump and blood glucose was maintained at basal blood glucose concentration using variable infusions based on glucose measures (FreeStyle Lite, Abbot Diabetes Care, Alameda, CA) every 5min for the first 20min and every 10min thereafter.

Tissue Collection

Animals were euthanized by exsanguination immediately following the cessation of the insulin clamp, where liver and muscle samples were quickly extracted. To remove the effects of insulin clamp on tissue analysis, eight of the sixteen did not undergo the clamp procedure and were sacrificed (isoflurane anesthesia followed immediately by exsanguination) three days following their last bout of exercise for extraction of liver and muscle tissues samples. Upon extraction, all liver and muscle tissues were immediately flash frozen in liquid nitrogen and stored at -70°C for further analysis.

Glycogen Quantification

Liver and muscle glycogen content was determined spectrophotometrically as described by Lo et al. (1970)³⁶. Briefly, liver samples were homogenized in 30% KOH, saturated with Na_2SO_4 and boiled for 30min. Glycogen was then precipitated in 95% ethanol and centrifuged at 3000rpm for 25min. Supernatants were discarded and the glycogen pellet was resuspended in 3ml of water and split into three, 1ml aliquots for triplicate analysis. 1ml of 5% phenol and 5ml concentrated sulfuric acid (96-98%) were added sequentially, allowed to stand for 10min, agitated, and incubated for 20min at $25-30^{\circ}\text{C}$. The colour reaction was then analyzed using a spectrophotometer at a wavelength of 490nm.

Immuno Blot Analysis

Liver samples were homogenized at a 1:10 (weight : volume) ratio in homogenizing buffer containing 100mM tris, 0.1mM EDTA, 0.1mM EGTA, 1% triton-X 100, 1% phosphatase inhibitor, 1% protease inhibitor, pH 7.5. Loading volumes were determined using the Bradford protein assay.

Proteins were run on polyacrylamide gels (10% separating, 4% stacking) at a constant voltage (125V) for approximately 90min in running buffer containing 25mM tris, 200mM L-

glycine and 0.1% SDS, pH 8.3. Proteins were then transferred to nitrocellulose membranes, blocked for 4 hours at 4°C in 10% non-fat dry milk, 1% BSA and probed with primary antibodies (anti-GS, anti-IR and anti-pGSK-3 β were from Cell Signaling; anti-GLK, anti-GLUT2 and anti-PEPCK from abcam and anti-GSK-3 β was from Santa Cruz) overnight. Membranes were then washed and incubated for 1 hour in horseradish-peroxidase conjugated secondary antibodies and detected using luminol-based chemiluminescence in a chemidoc imager (BioRad). Optical densities were quantified in BioRad Quantity One software. Total GS protein content was shown as the additive total of the two bands detected.

Statistical Analysis

Basic characteristics, glycogen contents and western blot group differences were tested using a two-way ANOVA and tukey's post hoc test. Group differences for the euglycemic-hyperinsulinemic clamp were assessed using a repeated measures two-way ANOVA. Significance was declared with α set to 0.05.

2.3 Results

Animal Characteristics

Animal weights and weekly blood glucose values are shown from weeks 1 and 10 in table 2.1. There were main effects of diabetes on blood glucose levels such that T1DM animals had higher blood glucose concentrations at both weeks 1 and 10 ($p < 0.05$). No effects of exercise were seen at either week 1 or 10 ($p > 0.05$). Body weights were significantly lower in animals with T1DM at both weeks 1 and 10 ($p < 0.05$) and exercised animals also weighed significantly less than unexercised counterparts at these time points ($p < 0.05$). These data have also been reported elsewhere^{37,38}.

Liver Glycogen

There was a main effect for T1DM on the hepatic glycogen content ($p < 0.05$; figure 2.2) such that rats with T1DM had lower liver glycogen contents than those without T1DM. 10 weeks of aerobic exercise training had no effect on liver glycogen levels ($p > 0.05$; figure 2.2).

Protein Expression

To determine if aberrant expression of glycogenic proteins could underlie discrepancies in hepatic glycogen storage in T1DM, GLUT2, GK and GS levels were quantified in the liver. Neither T1DM nor exercise training significantly altered GLUT2 expression ($p > 0.05$; figure 2.3). GK was significantly increased in rats with T1DM ($p < 0.05$; figure 2.4), while exercise training had no effect ($p > 0.05$; figure 2.4). There was a significant interaction between T1DM and exercise ($p < 0.05$; figure 2.5) with respect to total GS content. Pairwise comparisons indicate that DX is significantly greater than D ($p < 0.05$), no other differences were found. Finally, PEPCCK protein content was significantly elevated by T1DM ($p < 0.05$, figure 2.6). No main effects were found with exercise training ($p > 0.05$, figure 2.6).

Euglycemic-hyperinsulinemic Clamp and Hepatic Insulin Signaling

Glucose infusion rates are shown in figure 2.7. Individual glucose infusion rates did not change significantly for any group over the course of the clamp ($p > 0.05$). There was a significant main effect of groups on glucose infusion rate ($p < 0.05$). Pairwise comparisons indicate that D is significantly lower than C, CX and DX ($p < 0.05$), C and DX are not significantly different from each other ($P > 0.05$) and CX is significantly higher than C, D and DX ($p < 0.05$).

Prior to insulin stimulation, IR protein content was significantly elevated in rats with T1DM compared to non-T1DM rats ($p > 0.05$; figure 2.8). Exercise training had no effect on IR protein content ($p > 0.05$; figure 2.8). To examine if an insulin signal was being propagated down

the insulin signaling cascade, phosphorylated GSK was examined following the insulin clamp. There were no effects of either T1DM or exercise training on the phosphorylation of GSK following insulin stimulation ($p>0.05$; figure 2.9).

Following the insulin clamp, liver and soleus glycogen contents were measured to determine which tissues were responsible for glucose uptake. No effects of T1DM or exercise training were observed for liver glycogen content ($p>0.05$; figure 2.10). Soleus glycogen was found to be significantly increased by exercise training ($p<0.05$; figure 2.11), and no effects of T1DM were observed ($p>0.05$; figure 2.11).

Table 2.1 Animal Characteristics. Animal blood glucose concentration and body weight from weeks 1 and 10. Data are resented as mean \pm SE. Asterisk (*) indicates a main effect for diabetes ($p < 0.05$). Hash (#) indicates a main effect of exercise ($p < 0.05$). BG, blood glucose.

| Week | 1 | | 10 | | |
|-------------|--------------|-----------------|-----------------|-----------------|------------------|
| | Group | BG (mM) | Weight (g) | BG (mM) | Weight (g) |
| C | | 4.6 \pm 0.1 | 385 \pm 6.4 | 4.2 \pm 0.1 | 550 \pm 11.7 |
| CX | | 4.7 \pm 0.1 | 360 \pm 4.8# | 4.2 \pm 0.1 | 526 \pm 8.9# |
| D | | 10.2 \pm 1.2* | 304 \pm 5.4* | 16.2 \pm 0.8* | 460 \pm 7.9* |
| DX | | 9.4 \pm 1.2* | 292 \pm 7.9*# | 16.9 \pm 0.5* | 415 \pm 12.1*# |

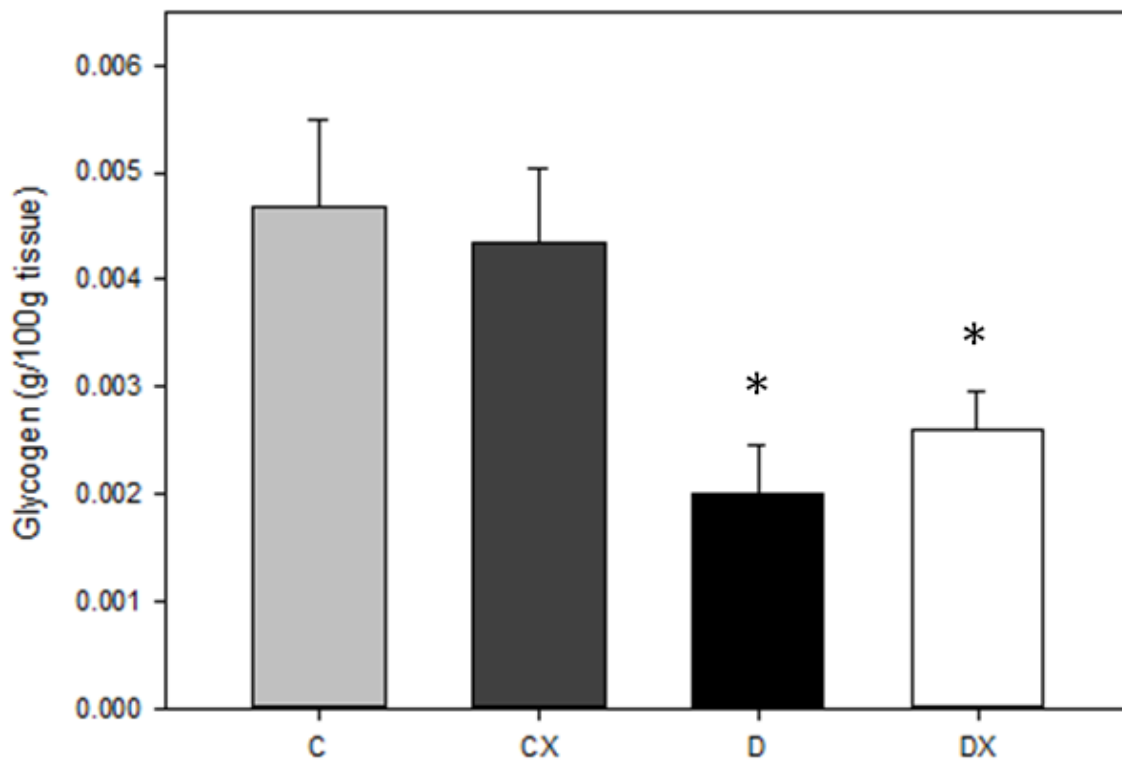


Figure 2.2 Hepatic Glycogen Content. Glycogen content presented as mean \pm SE. An asterisk (*) indicates a significant main effect of diabetes ($p < 0.05$), no effects of exercise were found ($p > 0.05$). $n = 8, 8, 8, 6$ for C, CX, D and DX respectively.

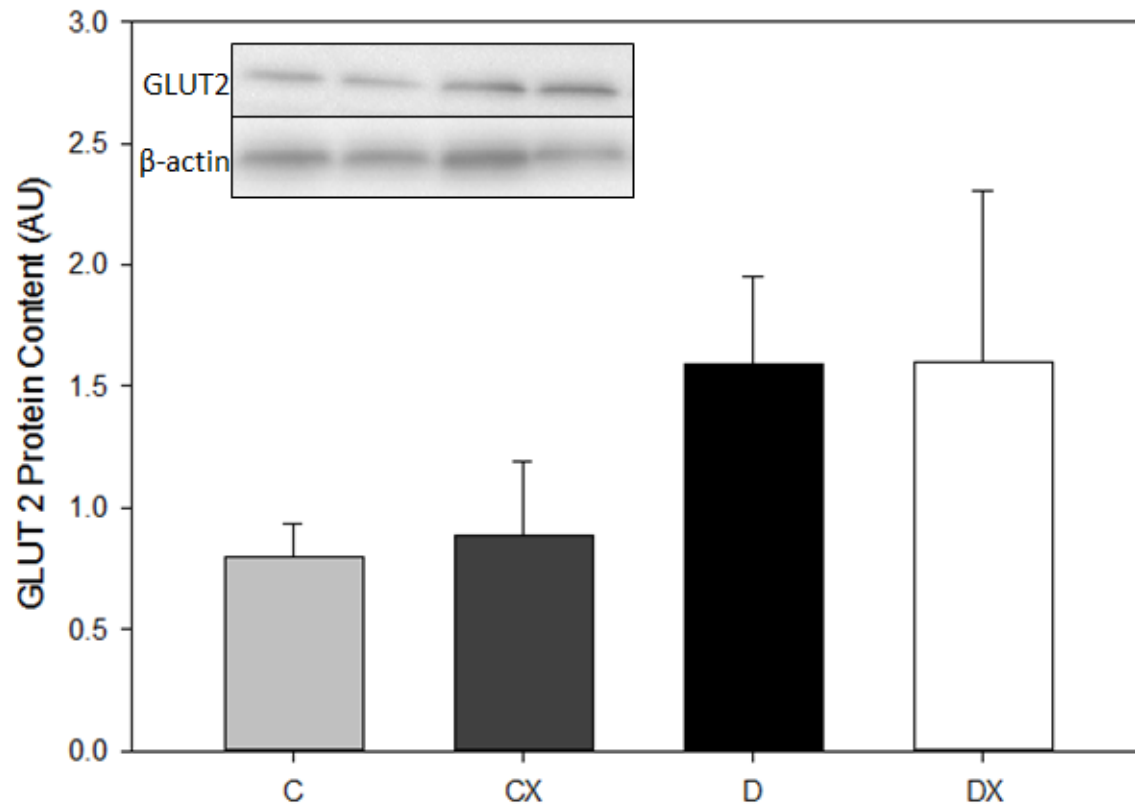


Figure 2.3 Hepatic GLUT2 Protein Content. Hepatic GLUT2 transporter was quantified using western blot analysis, normalized to the expression of β -actin and presented as mean \pm SE. Representative blots are shown in the same order as the graph. No effects were found for either diabetes or exercise ($p > 0.05$). $n = 5$ for every group.

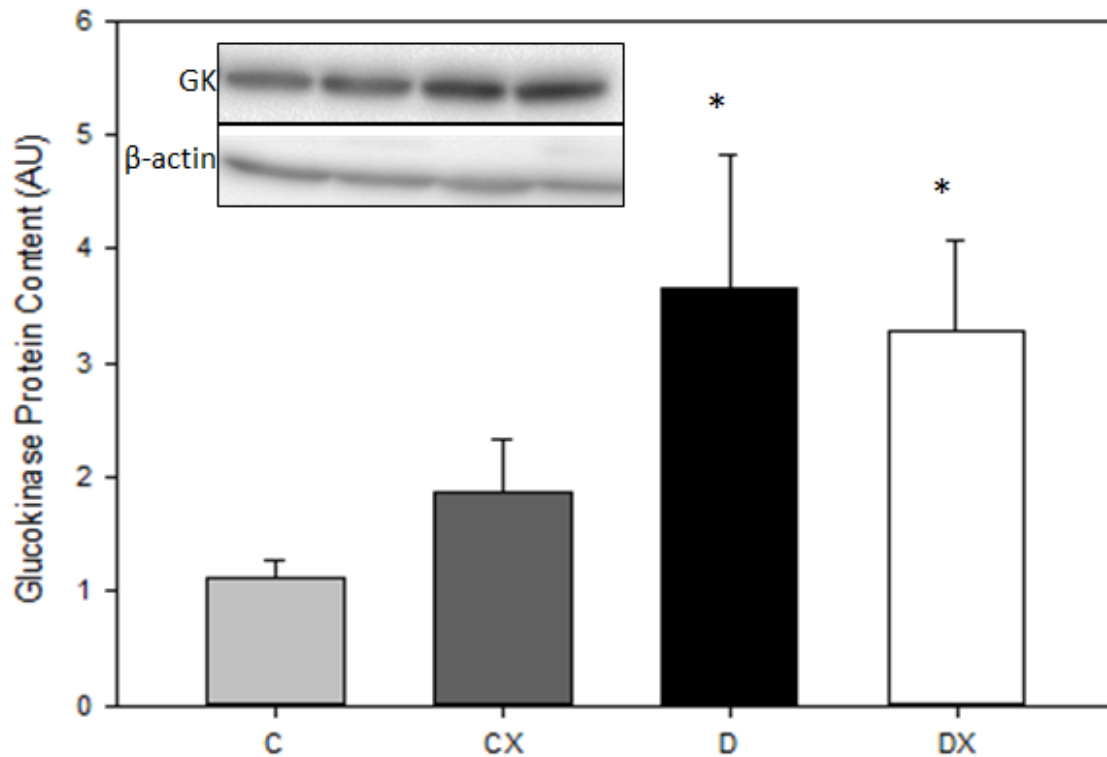


Figure 2.4 Hepatic Glucokinase Protein Content. Glucokinase protein level was quantified using western blot analysis, normalized to the expression of β -actin and presented as mean \pm SE. Representative blots are shown in the same order as on the graph. An asterisk (*) indicates a significant main effect of diabetes ($p < 0.05$), no effects of exercise were found ($p > 0.05$). $n = 8$ for C, CX and D, $n = 7$ for DX.

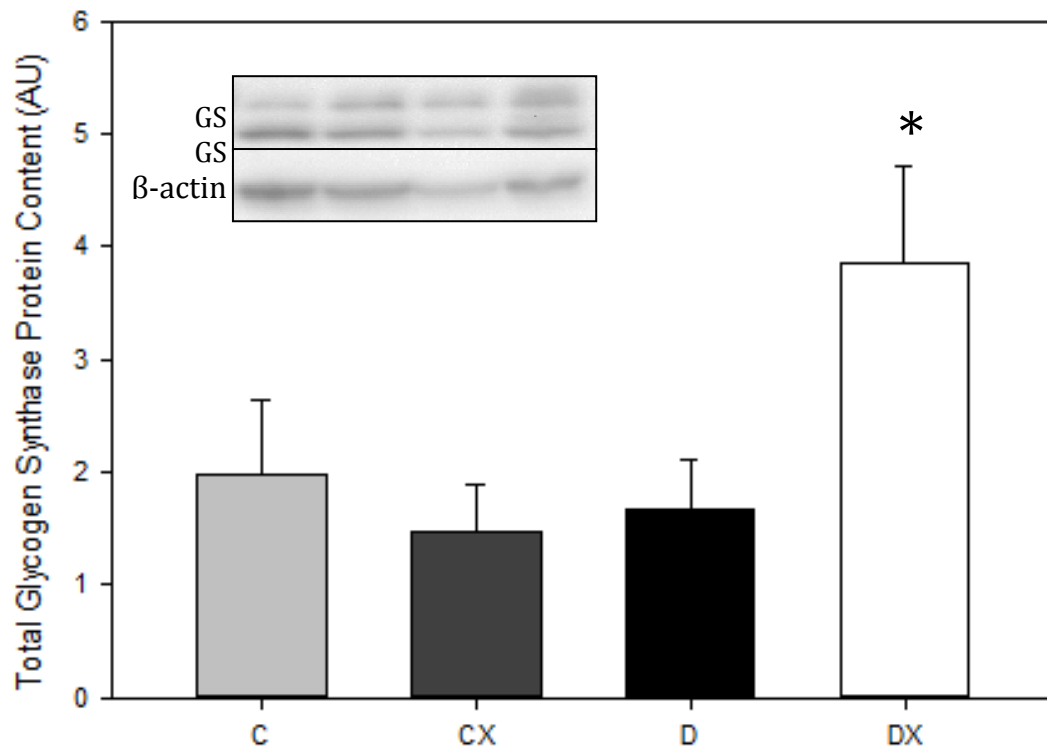


Figure 2.5 Total Hepatic Glycogen Synthase Protein Content. GS protein content was quantified using western blot analysis, normalized to the expression of β -actin and presented as mean \pm SE. Bands represent the muscle (top) and liver (bottom) isoform of the GS protein. Bands were added together for quantification of total content. Representative blots are shown in the same order as on the graph. A significant interaction was found between diabetes and exercise ($p < 0.05$). Pairwise comparison indicates that DX is significantly greater than D (shown by an asterisk (*) $p < 0.05$). $n = 5$ for all groups.

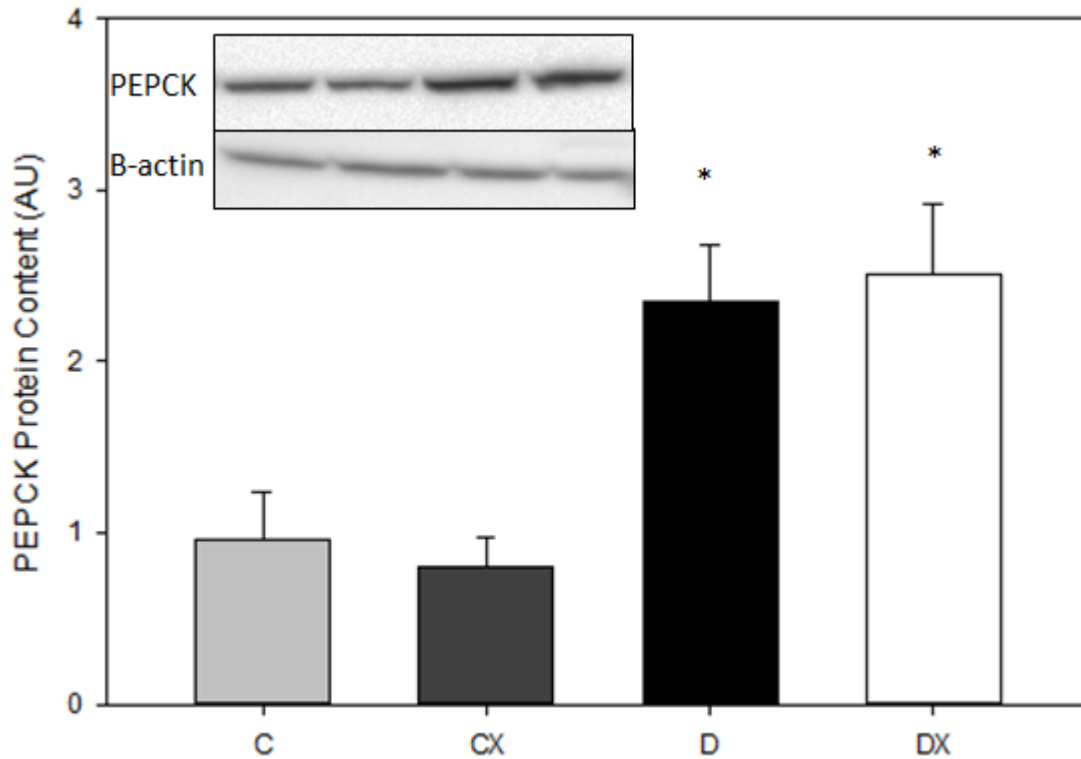


Figure 2.6 Hepatic PEPCK Protein Content. PEPCK protein level was quantified using western blot analysis, normalized to the expression of β -actin and presented as mean \pm SE. Representative blots are shown in the same order as on the graph. An asterisk (*) indicates a significant main effect of diabetes ($p < 0.05$), no effects of exercise were found ($p > 0.05$). $n = 7$ for all groups

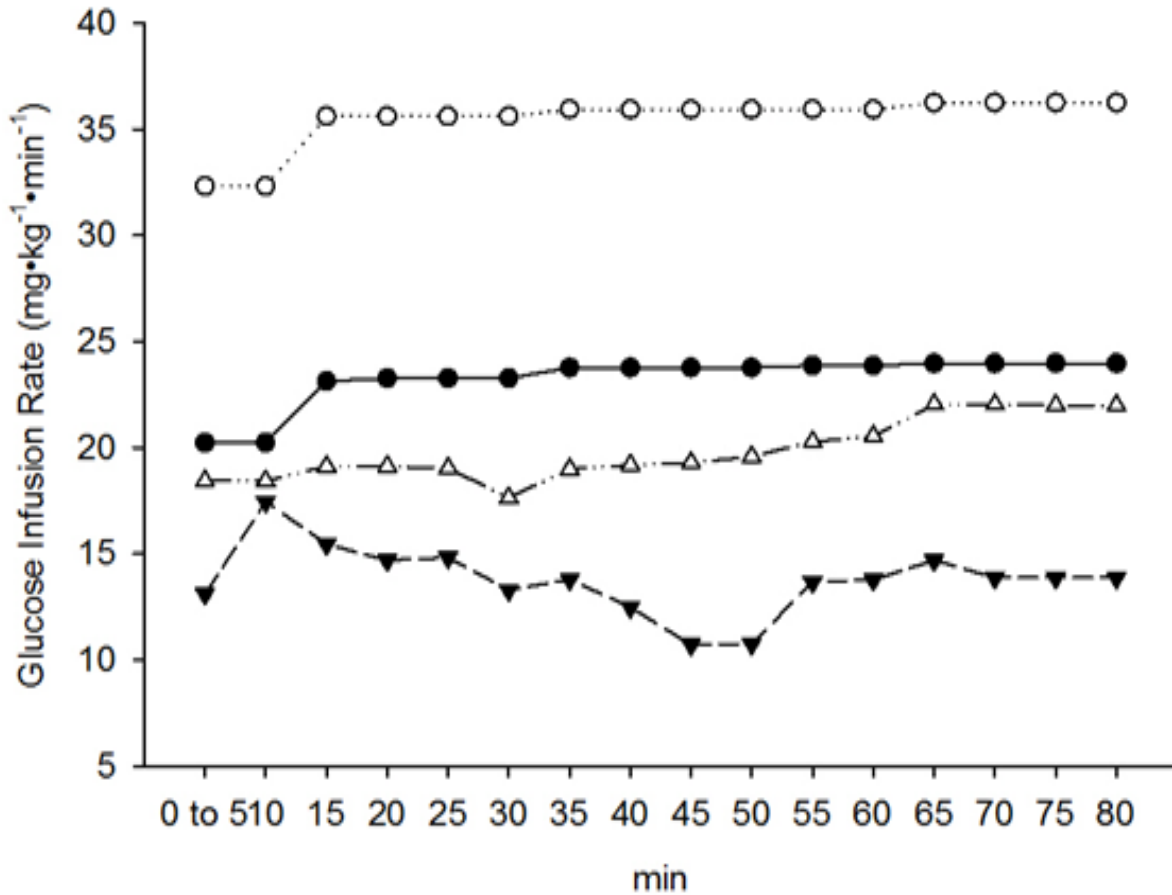


Figure 2.7 Glucose Infusion Rate during a Euglycemic-Hyperinsulinemic Clamp. During the clamp, animal blood glucose was maintained at their basal level ($\sim 4\text{mM}$ for C and CX, $\sim 15\text{mM}$ for D and DX) using continuous infusion. Insulin was infused at a rate of $10\mu\text{u/kg/min}$. Full circles (●) represent C, open circles (○) are CX, full triangles (▲) are D and open triangles (△) are DX. Data are presented as mean \pm SE. There was a significant effect of group ($p < 0.05$). No effect of time was observed ($p > 0.05$). $n = 7, 6, 9, 9$ for C, CX, D and DX respectively.

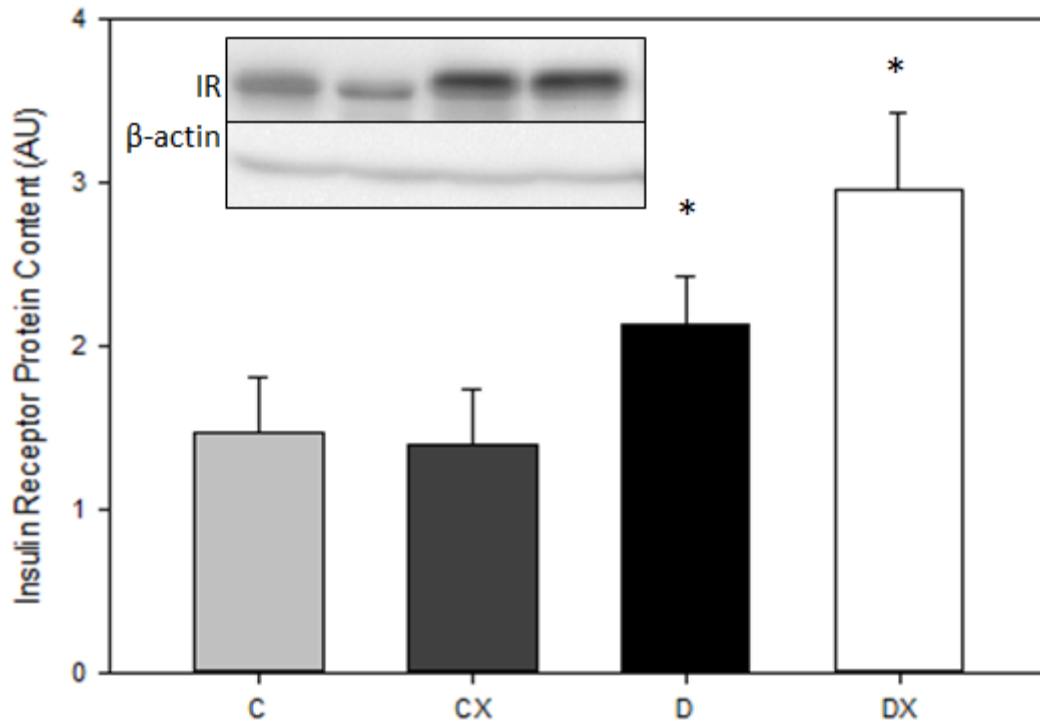


Figure 2.8 Hepatic Insulin Receptor Protein Content. Insulin receptor protein level was quantified using western blot analysis, normalized to the expression of β -actin and presented as mean \pm SE. Representative blots are shown below in the same order as the graph. An asterisk (*) indicates a significant main effect of diabetes ($p < 0.05$), no effects of exercise were found ($p > 0.05$). $n = 5$ for all groups.

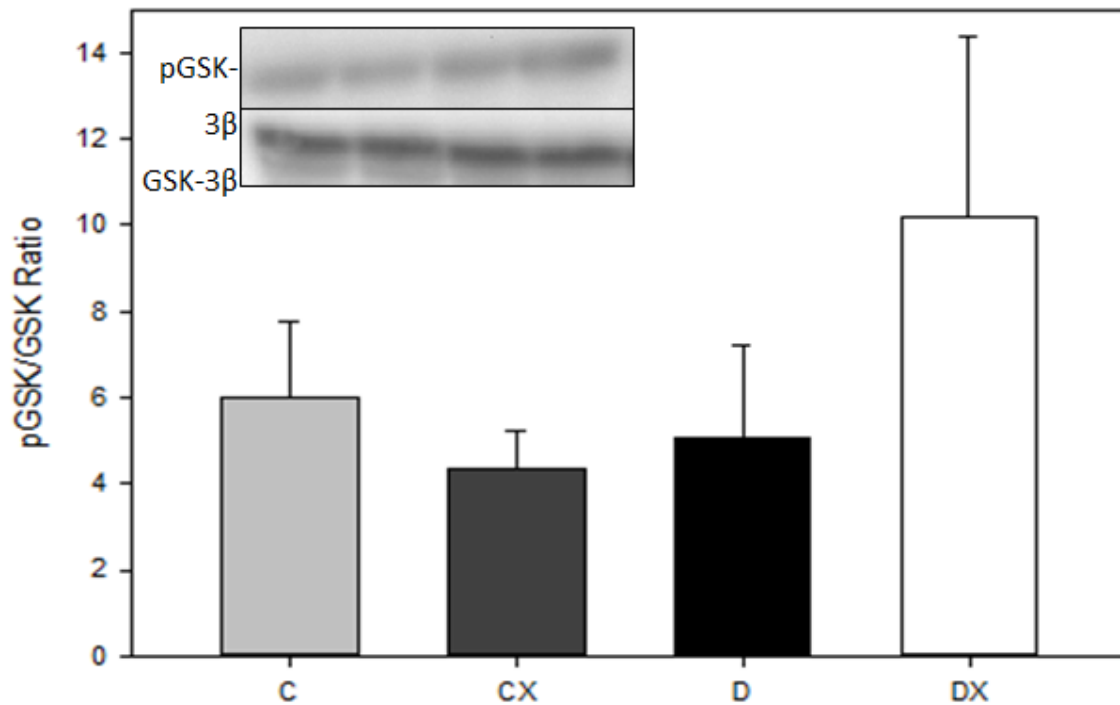


Figure 2.9 Insulin-Induced Hepatic Glycogen Synthase Kinase 3 β Phosphorylation.

Western blot analysis of GSK-3 β phosphorylation was done on animals having undergone the insulin clamp protocol to examine hepatic insulin signaling. Phospho-GSK-3 β was normalized to the total amount of GSK-3 β and presented as mean \pm SE. Representative blots are shown in the same order as on the graph. No significant affects were observed ($p > 0.05$) for diabetes or exercise. N = 8, 8, 7, 6 for C, CX, D and DX respectively.

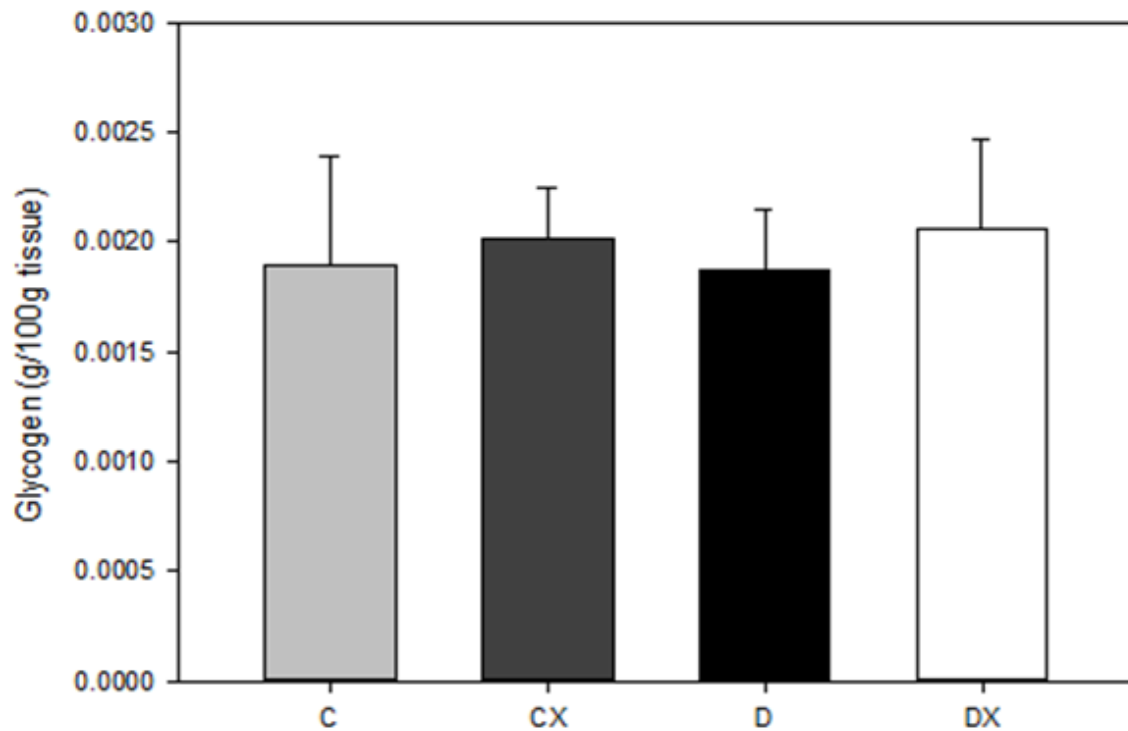


Figure 2.10 Post Insulin Clamp Hepatic Glycogen Content. Glycogen contents are presented as mean \pm SE. No significant effects for diabetes or exercise were detected ($p > 0.05$). $n = 8, 8, 8, 6$ for C, CX, D and DX respectively.

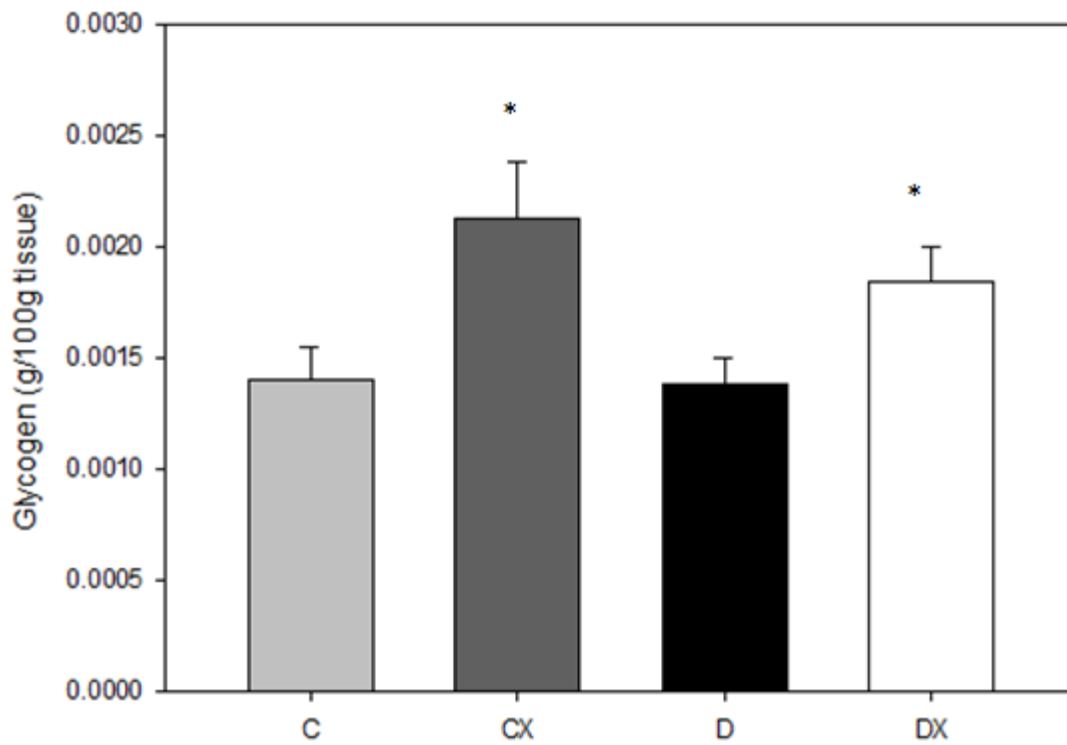


Figure 2.11 Post Insulin Clamp Soleus Glycogen Content. Glycogen contents are presented as mean \pm SE. An asterisk (*) indicates a significant main effect of exercise ($p < 0.05$). No significant effects of diabetes ($p > 0.05$) were observed. $n = 8, 8, 7, 6$ for C, CX, D and DX respectively.

2.4 Discussion

Results of the current study indicate that 10 weeks of aerobic exercise training is not able to reduce the hepatic glycogen deficit seen in rats with T1DM. Furthermore, proteins comprising the direct and indirect pathway for glycogen synthesis, GLUT2, GK, GS and PEPCK, are all present at levels equal to or in excess of those seen in healthy control animals. Finally, hepatic insulin signaling appears to be intact in these animals as indicated by increases in IR content and equivalent GSK-3 β phosphorylation following insulin stimulation.

Early work has demonstrated that aerobic exercise training is able to increase hepatic glycogen stores in healthy female rats by approximately 75%²⁷. Although mechanistic details were not examined, it was suggested that increases in food intake by the exercised animals could be driving the increase in glycogen storage²⁷. Additional work from Galbo et al. (1979) demonstrated increased hepatic glycogen following aerobic training and noted higher basal GS and lower glycogen phosphorylase (GP) activities in trained relative to untrained animals^{26,28}. It is unclear why the control-exercised animals in the current study did not increase their hepatic glycogen stores. Despite sex differences between studies, male and female rats have been shown not to differ on both basal and post exercise liver glycogen levels³⁹. Differences in animal sacrifice time post final exercise bout as well as exercise modality/duration of training may also account for discrepancies. Both of the abovementioned studies demonstrating an exercise-mediated increase in hepatic glycogen utilized swimming for up to 6 hours per day in contrast to the 1 hour of running used in the present study; total training volume over the duration of the study was also much greater and could account for the discrepancy⁴⁰.

In agreement with this argument, rats with T1DM in the current study had significantly less liver glycogen than their healthy controls and this was not improved with exercise training. Leme et al (2009) also demonstrated no increase in liver glycogen in animals with T1DM or in healthy animals submitted to swim training³⁰. Animals in that study however, swam for 1 hour per day and were sacrificed 2 days post exercise, as opposed to three days as done by Galbo^{26,30}.

While it is understood that insulin promotes glycogen storage through the activation of GS, insulin treatment in the current study was unable to restore/improve glycogen storage in rats with T1DM. It is possible that, because normoglycemia was never achieved, treatment was insufficient to restore glycogen levels. Furthermore, previous work from our laboratory demonstrated that T1DM-exercised animals require less insulin than unexercised-T1DM, which may account for the lack of glycogen storage⁴¹. Additionally, subcutaneous insulin pellets utilized in this study release insulin at a constant rate which has been shown to produce different effects upon insulin signaling and subsequently, GS activation, than the pulsatile release seen from a healthy pancreas which may account for the lack of treatment effect⁴².

Work from Gannon and others has suggested that short term T1DM (lasting 8 days) results in an increase in GS activity^{19,43-45}. In contrast, Rao and coworkers has demonstrated that STZ-induced, insulin treated T1DM animals have equivalent GS activity and protein content to healthy animals after three weeks of diabetes²⁰. To date no studies have examined the effects of long term T1DM on GS activity. However, these aforementioned works do suggest GS activity may actually decrease overtime, which, if continued over the long term may produce lowered activity. Temporary increases in activity may also be indicative of a latency period in which the disease has not fully developed and is consistent with studies demonstrating improvements and even some reversals when interventions (insulin, exercise) are initiated very early in the progression of the disease^{22,46}. To date no studies have been done examining if exercise training can increase GS and/or decrease GP activities in patients with T1DM; however, a lack of hepatic glycogen in diabetic-exercised animals suggests that GS activity might be lowered.

In the current study, proteins involved in the direct pathway of glycogen synthesis were measured to assess if the liver had the capacity to synthesize glycogen. No change in hepatic GLUT2 protein was detected which is consistent with work in other diabetic models. Furthermore, this passive transporter is not thought to be the rate limiting step governing hepatic glucose uptake or glycogen synthesis in healthy subjects^{10,47,48}. However, work in

pancreatectomized diabetic animals has demonstrated increases in GLUT2 protein in the liver with exercise training, a result not supported in the current study⁴⁹. It was suggested that because these animals had lower circulating glucose levels (one factor known to modulate GLUT2 production) than their untrained controls, which may explain why it was not observed in the current study⁴⁹. GLUT2 mRNA however, has been shown to decrease with training and exhibit a negative correlation with hepatic glycogen, suggesting that increases in GLUT2 may not be required for glycogen storage⁵⁰.

GK was found to be increased in both diabetic groups, with no modulation with exercise. STZ induced T1DM has been shown to dramatically reduce GK activity which is subsequently normalized with insulin treatment^{51,52}. Furthermore, adenoviral overexpression of GK in rats with T1DM resulted in a decreased fasting blood glucose, improved glucose clearance during a tolerance test and increased hepatic glycogen content²¹. GK overexpression has also been shown to increase liver glycogen stores in healthy animals, demonstrative of its control over glycogen synthesis¹⁷. One potential reason why the increase in GK content was unable to stimulate glycogen storage is that despite increased protein, activity may still be decreased in T1DM. This may occur via glucokinase regulating protein (GKRP), which is increased under instances of high glucose but is unaffected by insulin⁵³. Under current conditions in which insulin was present but insufficient to produce normoglycemia, it is conceivable the GKRP might be produced in excess and subsequently suppress GK activity as a result of hyperglycemia^{13,54-56}. Park and colleagues have demonstrated increases in GK content with training in diabetic animals; however, these animals were not receiving exogenous insulin as were the animals in the current study⁴⁹. The lack of exercise-induced increase could be a result of exercise and insulin working through a common mechanism with regards to GK expression.

Although direct comparison between GS protein content as measured in the current experiment and its activity are unfounded, changes in protein content have been linked to changes in glycogen storage capacity^{17,57,58}. The current study has reported an increase in GS protein

content in the livers of DX animals, which, in combination with increased GK content suggest that the capacity to store glycogen is present in these animals.

The indirect pathway of glycogen synthesis is also known to be perturbed in T1DM, such that it accounts for a larger proportion of glycogen synthetic precursors than it does in a healthy state^{6-8,22}. As flux through the gluconeogenic pathway is largely governed by PEPCK protein content, data in the current study agrees with this dogma as it is elevated in T1DM, indicative of increased gluconeogenesis^{22,59,60}. Insulin is the primary factor controlling PEPCK production, however, previous work using insulin-treated T1DM has also concluded that insulin treatment is unable to correct the increase in protein^{22,60,61}. Inability of the liver to properly decrease expression of gluconeogenic proteins may be indicative of a resistant state which is unable to be altered with aerobic exercise.

In addition to dysregulation of glycogenic proteins, it has been shown that patients with T1DM suffer from an overdriven sympathetic nervous system^{62,63}. These findings are supported by previous data from our laboratory using this model of T1DM demonstrating that basal neuropeptide-Y concentrations are elevated in both exercise and sedentary T1DM rats (unpublished data). Furthermore, higher levels of glucagon are present within pancreatic islets⁵ and, following aerobic exercise training, the glucagon response to an acute bout of exercise-induced reductions in glycemia is increased relative to pretraining (unpublished data). This is consistent with the hyperglucagonemia known to contribute to hyperglycemia in T1DM patients⁶⁴. Both sympathetic agents (neuropeptide Y (NPY), epinephrine etc.) and glucagon activate hepatic glycogen degradation and prevent its synthesis, which may contribute to an overall hepatic glycogen deficit¹⁰.

Another well-known characteristic of T1DM is hepatic insulin resistance^{24,65}. If an insulin signal is not propagating downstream (IRS-AKT-GSK pathway), phosphorylase is likely to remain active, and consequently, GS will not be effectively activated which will further hinder glycogen synthesis^{25,49,66}. To address this, an insulin clamp was done and markers of hepatic

insulin signaling were measured. Glucose infusion rates required to maintain basal blood glucose concentrations were higher in both exercised groups compared to sedentary levels; and the control-exercised animals required the highest rates of glucose infusion, consistent with a) insulin resistance in T1DM and b) improvements in whole-body insulin sensitivity with exercise training in both healthy and T1DM subjects^{24,67,68}.

Interestingly, basal hepatic insulin receptor content was found to be increased in the T1DM rats of the current study. Insulin receptor content is known to fluctuate based upon circulating insulin levels, with hyperinsulinemia and hypoinsulinemia decreasing and increasing receptors, respectively^{69,70}. T1DM rats in the current study were treated with insulin and previous work using this model of T1DM found that circulating levels were equivalent to that of control animals, so it is unclear why IR content is elevated⁴¹. It is possible that differences exist between endogenous rat insulin and the human insulin that the T1DM animals are treated with in their ability to bind and/or activate the IR. The importance of the insulin receptor in hepatic insulin resistance has been demonstrated by liver insulin receptor knockout (LIRKO) mice which demonstrate impairments in glucose clearance and suppression of hepatic glucose production, as well as insulin signaling defects with regards to activation of its substrates IRS 1 and 2⁷¹. Interestingly, upregulation of insulin receptors has also been shown to improve insulin sensitivity and glucose clearance in cultured human hepatocytes⁷². This appears to contradict the widely accepted notion that T1DM suffer from hepatic insulin resistance²⁴. Reasons for this discrepancy are unclear, however, the etiology of hepatic insulin resistance is also illusive in T1DM so it is possible that defects more distal in the signaling pathway are the root cause and receptors are being upregulated in an attempt to compensate.

To assess insulin signal transduction, insulin induced inhibition of GSK-3 β , a key effector upon GS, was measured following the hyperinsulinemic clamp. No change in GSK-3 β phosphorylation was detected in T1DM rats in comparison to non-T1DM, suggesting that the insulin signaling pathway is not perturbed in T1DM. Although mechanistic studies in T1DM are

lacking, recent work using another model of hepatic insulin resistance, in which hepatic NF κ B is hyperactive, showed decreases in GSK-3 β phosphorylation following insulin stimulation²⁵. That same model found decreased hepatic glycogen storage as well as impaired suppression of hepatic glucose production, two known features of T1DM^{24,25}. While this appears to suggest normal hepatic sensitivity in the current study, it is important to note that modulation of GSK is not the only way insulin modulates glycogen synthesis. Many other protein kinases including adenosine monophosphate-dependent protein kinase (AMPK), Protein Kinase A, AKT (also known as protein kinase B), and calmodulin induced protein kinase (CamK) among others are able to phosphorylate GS and are also modulated by insulin^{10,15,73,74}. GSK-3 β phosphorylates residues known to be important to GS activity yet can only do so when other residues have already be phosphorylated (referred to as hierarchal phosphorylation) by other proteins. Alterations in any of the above mentioned factors could, therefore, also affect GSK-3 β 's ability to modulate GS activity^{10,73}.

As GS is most active in its unphosphorylated state, defects in its dephosphorylation will also strongly modulate its ability to synthesize glycogen. Protein phosphatase 1 (PP1), the primary phosphatase of hepatic GS, is also altered in T1DM⁷⁵. PP1 activity towards GS was also shown to be dependent on the degree of insulin deficiency⁷⁵. More recent works have demonstrated that a subunit of PP1, G_L, that targets PP1 to a glycogen granule, was absent in T1DM⁷⁶. This, however, was also corrected with insulin therapy⁷⁶. As previously mentioned, animals in the current study were not given sufficient insulin to achieve normoglycemia so it is unclear whether or not PP1 and/or G_L deficiencies play a role in the glycogen deficit.

Insulin stimulated glycogen synthesis has also been shown to stem from AKT-mediated inactivation of GP. GSK-3 β is a downstream target of AKT; however, constitutively active AKT mutants stimulate glycogen synthesis in cultured hepatocytes; even in the presence of the GSK-3 β inhibitor SB-216763^{66,77}. Furthermore, cells with overactive AKT had decreased GP activity and greater levels of glycogen synthesis than in GSK-3 β inhibited cells alone^{66,77}. It should be noted

that GSK-3 β is still an important regulator of GS activity, but active GS alone does not appear to be sufficient to drive glycogen synthesis ⁶⁶. Although the mechanism by which this occurs is not understood, it suggests that glycogen accumulation can be hindered despite normal GSK-3 β phosphorylation in response to insulin.

Differences between the current study and other published works may also stem from measurement technique. Assessment of insulin-induced suppression of hepatic glucose production gives a measure of hepatic response to insulin, but does not decipher the mechanistic details with respect to the known direct and indirect effects of insulin on the liver ⁷⁸. The direct effects of insulin are the alteration of hepatic glycogenolysis (discussed above) and gluconeogenesis, which is largely mediated through an AKT-mediated inhibition of PEPCK transcription ⁷⁸. The indirect effects of insulin include its manipulation of circulating levels of glucagon, non-esterified fatty acids (due to decreased adipose and skeletal muscle lipolysis), gluconeogenic precursor glycerol, as well as neural signals stemming from the hypothalamus ⁷⁸. It has also been suggested that the indirect effects of insulin are greater effectors on hepatic glucose production in rodents than its direct effects, making comparisons between the current study and other existing works quite confounded ⁷⁹.

In addition to insulin signaling, post-clamp glycogen was measured in both the liver and soleus muscle to assess where glucose is being stored during an insulin stimulus. Soleus glycogen was significantly increased in exercise-trained groups, consistent with improvements in peripheral insulin sensitivity following training. All groups had equivalent liver glycogen levels following the clamp, which is consistent with what is known to occur following an overnight fast ⁹. Furthermore, euglycemic clamps do not stimulate glucose uptake via the GLUT2 transporter and, subsequently, do not stimulate significant hepatic glycogen storage ^{9,80,81}. This is an interesting finding as the control and diabetic groups were clamped at different blood glucose concentrations. Control and diabetic animals were clamped at 5mM and 15mM blood glucoses respectively, in an attempt to assess insulin sensitivity at their resting blood glucose

concentration. However, the T1DM animals were clamped in what is typically considered a hyperglycemic, not a euglycemic, range. Contradictory to the current study, previous works using clamps in this range have shown increases in liver glycogen following a hyperglycemic clamp in animals with T1DM⁸². Differences in the model of T1DM (STZ vs pancreatectomy), as well as disease duration, may account for this discrepancy; however, another possibility does exist. As has been posited for other conditions such as baroreflex sensitivity in hypertension, it is possible that, following sufficient duration of T1DM, a “resetting” of the homeostatic resting blood glucose may occur⁸³. If, for example, the liver in these diabetic animals perceives 15mM as a new normoglycemia, it is unlikely that glucose would be taken up during the insulin clamp and glycogen would be stored. This would account for lack of storage in the present study and also lend support to tighter regulation of resting blood glucose being beneficial in T1DM¹. Future works examining intracellular glucose and glucose-metabolite levels in these animals would help elucidate why hyperglycemia is unable to stimulate hepatic glycogen synthesis. Furthermore, use of this model in conjunction with more intensive insulin treatment and normoglycemia will help to determine if this is the case.

2.5 Conclusion

In conclusion, the current study demonstrates that 10 weeks of aerobic exercise training is not able to improve the deficit in hepatic glycogen content in rats with T1DM. This was also associated with a lack of training-derived improvements in GLUT2, GK and PEPCK protein contents, and increases in GS protein in exercised T1DM animals. These data indicates that the capacity to synthesize glycogen is not hindered in these animals and may suggest that metabolic signaling deficits are somehow hindering synthesis. However, GSK-3 β inactivation following an insulin clamp was unchanged with either exercise training or T1DM, suggesting that an insulin signal is capable of reaching the key effectors on GS activation. Of course the possibility remains that other protein kinases capable of phosphorylating GS may be deregulated in T1DM, and

provides avenues for future investigation. Additionally, other insulin functions, such as modulation of GS phosphatases as well as GP activity and gluconeogenic flux may all play a role in hindering glycogen synthesis. Finally, it is possible that a “resetting” of blood glucose homeostasis is hindering glucose uptake such that glycogenic substrates are not present to drive glucose metabolism towards storage. All of these results indicate the complexity of glycogen storage and, despite the known benefits of exercise upon T1DM-related complications, suggests that exercise training is not an effective way to improve hepatic glycogen storage in T1DM rats.

2.6 Reference List

1. Group, T. D. C. and C. T. R. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* **329**, 977–986 (1993).
2. Chiang, J. L., Kirkman, M. S., Laffel, L. M. B. & Peters, A. L. Type 1 diabetes through the life span: A position statement of the american diabetes association. *Diabetes Care* **37**, 2034–2054 (2014).
3. Brazeau, A. S., Rabasa-Lhoret, R., Strychar, I. & Mircescu, H. Barriers to physical activity among patients with type 1 diabetes. *Diabetes Care* **31**, 2108–2109 (2008).
4. Riddell, M. C. & Perkins, B. A. Type 1 diabetes and vigorous exercise: Applications of exercise physiology to patient management. *J Diabetes* **30**, 63–71 (2006).
5. McDonald, M. W., Murray, M. R., Hall, K. E., Noble, E. G. & Melling, C. J. Morphological assessment of pancreatic islet hormone content following aerobic exercise training in rats with poorly controlled Type 1 diabetes mellitus. *Islets* **6**, 1–9 (2014).
6. Hwang, J. H. *et al.* Impaired net hepatic glycogen synthesis in insulin-dependent diabetic subjects during mixed meal ingestion. A ¹³C nuclear magnetic resonance spectroscopy study. *J Clin Invest* **95**, 783–787 (1995).
7. Bischof, M. G. *et al.* Effects of short-term improvement of insulin treatment and glycemia on hepatic glycogen metabolism in type 1 diabetes. *Diabetes* **50**, 392–8 (2001).
8. Bischof, M. G. *et al.* Hepatic glycogen metabolism in type 1 diabetes after long-term near normoglycemia. *Diabetes* **51**, 49–54 (2002).
9. Kishore, P. *et al.* Role of hepatic glycogen breakdown in defective counterregulation of hypoglycemia in intensively treated type 1 diabetes. *Diabetes* **55**, 659–666 (2006).
10. Bollen, M., Keppens, S. & Stalmans, W. Specific features of glycogen metabolism in the liver. *Biochem J* **336**, 19–31 (1998).
11. Kahn, B. B. Facilitative glucose transporters: regulatory mechanisms and dysregulation in diabetes. *J Clin Invest* **89**, 1367–1374 (1992).
12. Shepherd, P. R. & Kahn, B. B. Glucose transporters and insulin action. *N Engl J Med* **341**, 248–257 (1999).
13. Agius, L. Glucokinase and the molecular aspects of liver glycogen metabolism. *Biochem J* **414**, 1–18 (2008).
14. Von Wilamowitz-Moellendorff, A. *et al.* Glucose-6-phosphate-mediated activation of liver glycogen synthase plays a key role in hepatic glycogen synthesis. *Diabetes* **62**, 4070–4082 (2013).

15. Villar-Palasi, C. & Guinovart, J. J. The role of glucose 6-phosphate in the control of glycogen synthase. *FASEB J* **11**, 544–558 (1997).
16. Gomis, R. R. *et al.* Glucose 6-phosphate produced by gluconeogenesis and by glucokinase is equally effective in activating hepatic glycogen synthase. *J Biol Chem* **278**, 9740–9746 (2003).
17. Gomis, R. R., Ferrer, J. C. & Guinovart, J. J. Shared control of hepatic glycogen synthesis by glycogen synthase and glucokinase. *Biochem J* **351**, 811–816 (2000).
18. Libal-Weksler, Y., Gotlibovitz, O., Stark, a H. & Madar, Z. Diet and diabetic state modify glycogen synthase activity and expression in rat hepatocytes. *J Nutr Biochem* **12**, 458–464 (2001).
19. Gannon, M. C. & Nuttall, F. Q. Effect of feeding, fasting, and diabetes on liver glycogen synthase activity, protein, and mRNA in rats. *Diabetologia* **40**, 758–63 (1997).
20. Rao, P. V., Pugazhenti, S. & Khandelwal, R. L. The effects of streptozotocin-induced diabetes and insulin supplementation on expression of the glycogen phosphorylase gene in rat liver. *J Biol Chem* **270**, 24955–24960 (1995).
21. Morral, N. *et al.* Adenovirus-mediated expression of glucokinase in the liver as an adjuvant treatment for type 1 diabetes. *Hum Gene Ther* **13**, 1561–1570 (2002).
22. Soares, A. F. *et al.* Restoration of direct pathway glycogen synthesis flux in the STZ-diabetes rat model by insulin administration. *Am J Physiol Endocrinol Metab* **303**, E875–885 (2012).
23. DeFronzo, R. A., Hendler, R. & Simonson, D. Insulin resistance is a prominent feature of insulin-dependent diabetes. *Diabetes* **31**, 795–801 (1982).
24. Bergman, B. C. *et al.* Features of hepatic and skeletal muscle insulin resistance unique to type 1 diabetes. *J Clin Endocrinol Metab* **97**, 1663–1672 (2012).
25. Cai, D. *et al.* Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med* **11**, 183–190 (2005).
26. Galbo, H., Saugmann, P. & Richter, E. a. Increased hepatic glycogen synthetase and decreased phosphorylase in trained rats. *Acta Physiol Scand* **107**, 269–272 (1979).
27. Baldwin, K. M., Fitts, R. H., Booth, F. W., Winder, W. W. & Holloszy, J. O. Depletion of muscle and liver glycogen during exercise Protective effect of training. *Pflugers Arch* **354**, 203–212 (1975).
28. Cunha, T. S., Tanno, A. P., Costa Sampaio Moura, M. J. & Marcondes, F. K. Influence of high-intensity exercise training and anabolic androgenic steroid treatment on rat tissue glycogen content. *Life Sci*. **77**, 1030–1043 (2005).

29. Prestes, J. *et al.* Resistance training and glycogen content in ovariectomized rats. *Int J Sport. Med* **33**, 550–554 (2012).
30. Leme, J. A. C. A. *et al.* Long-term physical training increases liver IGF-I in diabetic rats. *Growth Horm IGF Res* **19**, 262–266 (2009).
31. Petersen, K. F., Price, T. B. & Bergeron, R. Regulation of net hepatic glycogenolysis and gluconeogenesis during exercise: impact of type 1 diabetes. *J Clin Endocrinol Metab* **89**, 4656–4664 (2004).
32. O'Brien, B. A., Harmon, B. V, Cameron, D. P. & Allan, D. J. Beta-cell apoptosis is responsible for the development of IDDM in the multiple low-dose streptozotocin model. *J Pathol* **178**, 176–181 (1996).
33. Bolli, G. B. How to ameliorate the problem of hypoglycemia in intensive as well as nonintensive treatment of type 1 diabetes. *Diabetes Care* **22**, B43–52 (1999).
34. Bedford, T. & Tipton, C. Maximum oxygen consumption of rats and its changes with various experimental procedures. *J Appl Physiol* **47**, 1278–1283 (1979).
35. Deforonzo, R. A., Tobin, J. D. & Andres, R. Glucose clamp technique : a method for quantifying insulin secretion and resistance. *Am J Physiol Endocrinol Metab* **273**, 214–223 (1979).
36. Lo, S., Russel, J. C. & Taylor, A. W. Determination of glycogen in small tissue samples. *J Appl Physiol* **28**, 234–236 (1970).
37. Olver, T. D. *et al.* Exercise training enhances insulin-stimulated nerve arterial vasodilation in rats with insulin-treated experimental diabetes. *Am J Physiol Regul Integr Comp Physiol* **306**, 941–950 (2014).
38. Mcdonald, M. The effect of consecutive bouts of aerobic exercise on exercise-induced hypoglycemia in type 1 diabetic rats. (2012).
39. Ivey, P. A. & Gaesser, G. A. Postexercise muscle and liver glycogen metabolism in male and female rats. *J Appl Physiol* **62**, 1250–1254 (1987).
40. James, D. E., Kraegen, E. W. & Chisholm, D. J. Effects of exercise training on in vivo insulin action in individual tissues of the rat. *J Clin Invest* **75**, 657–666 (1985).
41. Melling, C. W. J. *et al.* A model of poorly controlled type 1 diabetes mellitus and its treatment with aerobic exercise training. *Diabetes Metab.* **39**, 226–235 (2013).
42. Matveyenko, A. V *et al.* Pulsatile portal vein insulin delivery enhances hepatic insulin action and signaling. *Diabetes* **61**, 2269–2279 (2012).
43. Bahnaks, B. R. & Gold, A. H. Effects of alloxan diabetes on the turnover of rat liver glycogen synthase. *J Biol Chem* **257**, 8775–8780 (1982).

44. Hornbrook, K. R. Synthesis of liver glycogen in starved alloxan diabetic rats. *Diabetes* **19**, 916–923 (1970).
45. Akatsuka, A., Singh, T. J. & Huang, K.-P. Comparison of the Liver glycogen synthase from normal and streptozotocin-induced diabetic rats. *Arch Biochem Biophys* **220**, 426–434 (1983).
46. Huang, H.-H. *et al.* Exercise increases insulin content and basal secretion in pancreatic islets in type 1 diabetic mice. *Ex Diabetes Res* **2011**, 1–10 (2011).
47. Thorens, B. & Mueckler, M. Glucose transporters in the 21st century. *Am J Physiol Endocrinol Metab* **298**, E141–145 (2010).
48. Thorens, B., Gerard, N. & Deriaz, N. GLUT2 surface expression and intracellular transport via the constitutive pathway in pancreatic beta cells and insulinoma: evidence for a block in trans-Golgi network exit by brefeldin A. *J Cell Biol* **123**, 1687–1694 (1993).
49. Park, S., Hong, S. M. & Ahn, I. S. Exendin-4 and exercise improve hepatic glucose homeostasis by promoting insulin signaling in diabetic rats. *Metabolism* **59**, 123–133 (2010).
50. Morifuji, M., Sakai, K., Sanbongi, C. & Sugiura, K. Dietary whey protein increases liver and skeletal muscle glycogen levels in exercise-trained rats. *Br J Nutr* **93**, 439–445 (2007).
51. Weinhouse, S. Regulation of glucokinase in liver. *Cur Top Cell Regul* **11**, 1–50 (1976).
52. Iynedjianl, P. B., Gjinovci, A. & Renold, A. E. Stimulation by insulin of glucokinase gene transcription in liver of diabetic rats. *J Biol Chem* **263**, 740–744 (1988).
53. Van Schaftingen, E., Detheux, M. & Da Cunha, M. V. Short-term control of glucokinase activity: role of a regulatory protein. *FASEB J* **8**, 414–419 (1994).
54. Farrelly, D. *et al.* Mice mutant for glucokinase regulatory protein exhibit decreased liver glucokinase: A sequestration mechanism in metabolic regulation. *Proc Nat Acad Sci USA* **96**, 14511–14516 (1999).
55. Iynedjiang, P. B. & Jotterand, D. Transcriptional induction of glucokinase gene by insulin in cultured liver cells and its repression by the glucagon-CAMP System. *J Biol Chem* **264**, 21824–21829 (1989).
56. Ma, L., Robinson, L. N. & Towle, H. C. ChREBP**Mlx* is the principal mediator of glucose-induced gene expression in the liver. *J Biol Chem* **281**, 28721–30 (2006).
57. Roesler, W. J. & Khandelwal, R. L. Quantitation of glycogen synthase and phosphorylase protein in mouse liver: correlation between enzymatic protein and enzyme activity. *Arch Biochem Biophys* **244**, 397–407 (1986).

58. Weinstein, D. a, Correia, C. E., Saunders, A. C. & Wolfsdorf, J. I. Hepatic glycogen synthase deficiency: an infrequently recognized cause of ketotic hypoglycemia. *Mol. Genet. Metab.* **87**, 284–288 (2006).
59. Lemaigre, F. P. & Rousseau, G. G. Transcriptional control of genes that regulate glycolysis and gluconeogenesis in adult liver. *Biochem J* **303**, 1–14 (1994).
60. Pilkis, S. J. & Granner, D. K. Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. *Annu Rev Physiol* **54**, 885–909 (1992).
61. Pilkis, S. J. & El-Maghrabi, M. R. Hormonal regulation of hepatic gluconeogenesis and glycolysis. *Ann Rev Biochem* **57**, 755–783 (1988).
62. Perin, P. C., Maule, S. & Quadri, R. Sympathetic nervous system, diabetes, and hypertension. *Clin Exp Hypertens.* **23**, 45–55 (2001).
63. Iyngkaran, P., Anavekar, N., Majoni, W. & Thomas, M. C. The role and management of sympathetic overactivity in cardiovascular and renal complications of diabetes. *Diabetes Metab.* **39**, 290–8 (2013).
64. Farhy, L. S. *et al.* Association of basal hyperglucagonemia with impaired glucagon counterregulation in type 1 diabetes. *Front. Physiol.* **3**, 40 (2012).
65. DeFronzo, R. A., Simonson, D. & Ferrannini, E. Hepatic and peripheral insulin resistance: a common feature of type 2 (non-insulin-dependent) and type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* **23**, 313–319 (1982).
66. Aiston, S., Coghlan, M. P. & Agius, L. Inactivation of phosphorylase is a major component of the mechanism by which insulin stimulates hepatic glycogen synthesis. *Eur J Biochem* **270**, 2773–2781 (2003).
67. Hall, K. E. *et al.* The role of resistance and aerobic exercise training on insulin sensitivity measures in STZ-induced Type 1 diabetic rodents. *Metabolism* 1–11 (2013). doi:10.1016/j.metabol.2013.05.012
68. Goodyear, L. & Kahn, B. Exercise, glucose transport, and insulin sensitivity. *Annu Rev Med* **49**, 235–261 (1998).
69. Soll, A. H., Kahn, C. R. & Neville, D. M. Insulin receptor deficiency in genetic and acquired obesity. *J Clin Invest* **56**, 769–780 (1975).
70. Gavin, J. R., Roth, J., Neville, D. M., Meyts, P. D. E. & Buellt, D. N. Insulin-dependent regulation of insulin receptor concentrations : A direct demonstration in cell culture. *Proc Nat Acad Sci USA* **6**, 84–88 (1974).
71. Michael, M. D. *et al.* Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Mol Cell* **6**, 87–97 (2000).

72. Kong, W.-J. *et al.* Berberine reduces insulin resistance through protein kinase C-dependent up-regulation of insulin receptor expression. *Metab* **58**, 109–119 (2009).
73. Imazu, M., Strickland, W. G., Chrisman, T. D. & Exton, J. H. Phosphorylation and inactivation of liver glycogen synthase by liver protein kinases. *J Biol Chem* **259**, 1813–1821 (1984).
74. Taniguchi, C. M., Emanuelli, B. & Kahn, C. R. Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* **7**, 85–96 (2006).
75. Miller, T. B., Vicalvi, J. J. & Garnache, A. K. Alteration of hepatic glycogen synthase phosphatase activity by insulin deficiency. *Am J Physiol* **240**, 539–543 (1981).
76. Doherty, M. J., Cadefau, J., Stalmans, W., Bollen, M. & Cohen, P. T. W. Loss of hepatic glycogen-binding subunit (GL) of protein phosphatase 1 underlies deficient glycogen synthesis in insulin-dependent diabetic rats and in adrenalectomized starved rats. *Biochem J* **333**, 253–257 (1998).
77. Aiston, S., Hampson, L. J., Arden, C., Iynedjian, P. B. & Agius, L. The role of protein kinase B/Akt in insulin-induced inactivation of phosphorylase in rat hepatocytes. *Diabetologia* **49**, 174–82 (2006).
78. Girard, J. The Inhibitory effects of insulin on hepatic glucose production are both direct and indirect. *Diabetes* **55**, 65–69 (2006).
79. Cherrington, A. D. The role of hepatic insulin receptors in the regulation of glucose production. *J Clin Invest* **115**, 1136–1139 (2005).
80. Shulman, G. I., DeFronzo, R. A. & Rossetti, L. Differential effect of hyperglycemia and hyperinsulinemia on pathways of hepatic glycogen repletion. *Am J Physiol Endocrinol Metab* **260**, 731–735 (1991).
81. Lisato, G., Cusin, I., Tiengo, A., Del Prato, S. & Jeanrenaud, B. The contribution of hyperglycemia and hypoinsulinemia to insulin resistance of streptozotocin-diabetic rats. *Diabetologia* **35**, 310–315 (1992).
82. Giaccari, A. & Rossetti, L. Predominant role of gluconeogenesis in the hepatic glycogen repletion of diabetic rats. *J Clin Invest* **89**, 36–45 (1992).
83. Xie, P., McDowell, T. S., Chapleau, M. W., Hajduczuk, G. & Abboud, F. M. Rapid Baroreceptor resetting in chronic hypertension implications for normalization of arterial pressure. *Hypertension* **17**, 72–79 (1991).

Appendix A

A1, Streptozotocin Induction

REVISION DATE: 13/06/2012

PURPOSE:

To induce Type I diabetes in rats

MATERIALS:

Gloves
 Lab Coat
 Streptozotocin (STZ)
 5X Stock Citric Acid/Citrate Buffer
 - Anhydrous Citric Acid
 - Sodium Citrate Dihydrate
 - MilliQ Deionized Water
 13M HCl
 3 Falcon Tube
 Sterile Filter

EQUIPMENT:

Biological Safety Cabinet
 Weigh Scale
 pH Meter

PROCEDURE:

Preparing 5X Citric Acid/Citrate Buffer

1. For a pH 4.6 buffer at 765 mM (5X stock solution), in a beaker, Add
 - i. 13.8g Anhydrous Citric Acid (Sigma) or 15.1g Citric Acid Monohydrate
 - ii. 23.8g Sodium Citrate Dihydrate (Sigma)
 - Mix into iii. 175mL of MilliQ water
 The pH should be at 4.6, Add HCl or NaOH to adjust (do not over-shoot pH)
2. Once the proper pH is obtained, add MilliQ water until you are close to the 200 ml mark (pH will move slightly). If satisfied with the pH, adjust volume in a 250 ml graduated cylinder and filter in a 0.2µm filter.
3. Store at room temperature. This is your 5X stock solution.

Making up Streptozotocin (STZ) for Injection

****NOTE** Animals should be pre-weighed prior to making up STZ to ensure accurate amounts of STZ to be prepared.

1. Using pre-made buffer, put 1 mL of buffer in a 50 mL Falcon Tube and add 4 mL of distilled water filtered through a 0.2µm syringe filter. Check the pH. This gives you a working concentration of 153 mM
2. The desired pH is between 4.5-4.7. Under the fume hood, add 1 drop at a time of concentrated HCl to the buffer, checking pH in between until desired pH is reached.
3. Once pH is reached, add 1 mL distilled water (sterile filtered through a 0.2µm syringe filter as before). If pH is below 4.5, restart.

4. Weigh out an appropriate amount of STZ for the number of animals (see calculations below) that will be injected in a 15 minute time frame.

Ex. Rats will be injected at 20mg/kg, so for 10 animals at an ideal weight of 200g (avg. weight of rats to be injected), you will require a minimum of 40mg.

$$20\text{mg/kg} \times 0.2\text{kg} = 4\text{mg per animal}$$

The amount of STZ weighed out should be more than the minimum as some solution will be lost in filtering. (4mg (per animal) X 12 rats = 48mg total (0.048g)

5. Dissolve the STZ into buffer (keeping in mind a comfortable injection volume). Shake to dissolve powder (approx. 1min). Sterile filter using a 0.2 μ m syringe filter.

$$\text{Ex. } 48\text{mg STZ} \div 3 \text{ mL buffer} = 16\text{mg/mL solution}$$

$$4\text{mg} \div 16\text{mg/mL solution} = 0.25\text{mL}$$

6. STZ is time dependent and must be used within 15 minutes

Injecting and Follow-Up of the Animals

1. Promptly inject each rat with the solution (intraperitoneal) at a dosage rate of 20mg/mL (in this example, 0.25mL). Do not use anymore STZ solution more than 15 minutes after it has been dissolved in the sodium citrate buffer.
2. Dispose of any container having come into contact with the STZ (in either powder or dissolved form) into a biohazardous waste receptacle. Dispose of needles into a sharps container.
3. Return injected rats to their cage. Record the date of STZ injection and add a biohazard label to the cage (leave biohazard label on cage for at least 3 days following the last injection).
4. Repeat this procedure the following day.
5. Check blood glucose daily. Diabetes is achieved with two non-fasting blood glucose readings of >18 mmol Diabetes should be achieved after 5-8 injections (i.p. 20mg/kg).

Reference:

Low dose STZ induction protocol. Animal Models of Diabetic Complications Consortium AMDCC Protocols.2003

A2, Insulin Pellet Implant

REVISION DATE: 13/06/2012

MATERIALS:

LinShin LinPlant Insulin Pellet
Rat anesthetic - Isoflurane
Ampicillin
Sterile water
1ml syringe with 25 g needle
10% providone-iodine solution
gauze (or swab)
Tissue forceps
Scalpel handle and blades (or scissors)
Silk suture
Needle drivers

EQUIPMENT:

Isofluorane Anaesthetic Machine
Hair clippers
Heat lamp

Special Safety:

Must don lab coat and gloves before handling rodents. Any bite or scratch that breaks the skin must be thoroughly scrubbed with soap and water (report to Occupational Health and Safety).

PROCEDURE:

Pellet implantation (for a rat):

1. Anesthetize the animal using the isofluorane machine by placing it in the induction chamber. Set isofluorane to 4-5% with an O₂ flow rate of 1L/min. Open the stopcock valve so gas reaches the chamber. Keep in chamber until the animal is unconscious.
2. Remove the animal and place its nose in the nose cone, reduce the isofluorane to 3% to maintain the plane of anesthesia.
3. Shave the area where the pellet is to be implanted.
4. Using gauze (or a swab), apply 10% providone-iodine solution to the skin, followed by 70% ethanol, to disinfect the site of insertion.
5. Hold the skin with forceps and make a subcutaneous incision.
6. Cleanse a 12g trocar with 10% providone-iodine solution and insert it through the puncture site to a depth of at least 2 cm.
7. Using forceps, briefly immerse the pellet in 10% providone-iodine solution, rinse with saline and insert into the subcutaneous region.
8. Use 1 pellet for the first 350g of body weight.

9. Pinch the skin closed after the last pellet is inserted. Place a drop of 10 % providone-iodine solution over the opening.
10. Close the incision by suturing.
11. Place the animal under a heat lamp and monitor until it recovers from anesthesia.
12. Record on the cage card that insulin pellets have been implanted.

Pellet removal:

1. Anesthetize the animal as described above for implantation.
2. Shave and palpate the area of implantation to locate pellets. Sterilize this area by applying 10% providone-iodine solution followed by 70% ethanol.
3. Using a scalpel (or scissors), make an incision through the skin superficial to the location of the pellets.
4. Using forceps, remove the pellet. Some connective tissue may need to be cut away using scissors. Discard the pellet.
5. Close the incision by suturing.
6. Place the animal under a heat lamp and monitor until it recovers from anesthesia.
7. Record on the cage card that the pellets have been removed.

A3, Glycogen

REVISION DATE: 13/06/2012

MATERIALS:

Tissue samples
 Fume hood
 3 x 100mm glass test tubes
 Potassium hydroxide (KOH) pellets
 Sodium sulfate (Na_2SO_4)
 95% ethanol
 Glycogen powder
 Phenol crystals
 96-98 % Sulfuric Acid (H_2SO_4)
 490nm spectrophotometer

Standard Curve:

Prepare standard curve with stock glycogen solution (1mg/mL).

| Standard | [Glycogen] (mg/ml) | Volume (μl) | Water (μl) |
|----------|--------------------|--------------------------|-------------------------|
| 1 | 0.000 | 0 | 1000 |
| 2 | 0.005 | 5 | 995 |
| 3 | 0.010 | 10 | 990 |
| 4 | 0.050 | 50 | 950 |
| 5 | 0.100 | 100 | 900 |
| 6 | 0.200 | 200 | 800 |
| 7 | 0.300 | 300 | 700 |
| | 0.500 | 500 | 500 |
| | 1.000 | 1000 | 0 |

Colour Reaction:

Add 1 ml of 5 % phenol.

Rapidly add 5 ml of 96-98 % H_2SO_4 .

Let samples stand for 10 min.

Vortex and place in water bath (25 to 30°C) for *10 to 20 minutes*.

Read tubes at 490 nm.

Procedure:

1. Cut and weigh approximately 20mg of tissue samples and place in glass test tube.
2. Saturate 30% KOH stock solution with sodium sulfate (Na_2SO_4)

3. Add 0.5 ml of 30 % KOH saturated with Na₂SO₄. Make sure that tissue is completely submerged.
4. Put caps on tubes and immerse in boiling water bath until homogenous solution is obtained (*30 minutes*).
4. Place tubes on ice.
5. Precipitate glycogen with 1mL of 95 % ethanol for *30 minutes* (on ice).
6. Spin tubes at 840 x g (3 000 rpm on Sorval) for *20 to 30 minutes*.
7. Remove supernatants and immediately dissolve precipitates in 3mL ddH₂O. Do not allow precipitated to dry.
8. Pipette 1 ml glycogen solution into 3 separate glass test tubes (3 x 100mm).
- 3 separate tubes of glycogen solution are necessary for analysis in triplicates.
9. Add 1 ml of 5 % phenol.
10. Rapidly add 5 ml of 96-98 % H₂SO₄.
11. Let samples stand for 10 min.
12. Vortex and place in water bath (25 to 30°C) for *10 to 20 minutes*.
13. Read tubes at 490 nm.
14. Calculate glycogen content by:

$$\text{g of glycogen / 100g tissue} = \frac{A_{490}}{k} \times \frac{V}{v} \times \frac{10^{-4}}{W}$$

where, V = total volume of glycogen solution; v = volume of aliquot used in colour reaction; A₄₉₀ = absorbance at 490 nm; W = weight of tissue samples in grams; k = slope of standard curve; units = 1 per microgram glycogen.

SOLUTIONS:

| <i>Glycogen Stock Solution (1mg/mL)</i> | |
|---|--------------------|
| 100mL | ddH ₂ O |
| 100mg | Glycogen powder |

| <i>30% Potassium Hydroxide Stock Solution</i> | |
|---|--------------------------------------|
| 300g | Potassium Hydroxide Pellets (KOH) |
| 1L | ddH ₂ O |
| Reaction is exothermic; therefore, it might be necessary to put on ice. | |

SPEC. PROCEDURE:

1. Turn on ~20minutes before needed (TRANS)
2. Set desired λ & insert appropriate filter
3. Insert a blank tube (dH₂O)
4. With spec. on "TRANS", set to 0.000 using "zero" button & while pressing "zero set"
5. Release "zero set", but adjust value to 100.00 with spec set to "TRANS" using "100% T/OA" adjustments
6. Set spec from "TRANS" to "ABS" using LHS button
7. Check that "ABS" reading is 0.00; if not adjust with "100%T/OA"
8. Check that a true a zero reading has been reached by reading the abs values of 2 other blank tubes
9. Read 3 reagent tubes (blank) and record zero on the middle value using "100%T/OA"
10. Read all standard samples

Reference:

Lo, S.L., Russell, J.C., and Taylor, A.W. (1970) J Appl. Physiol, 28(2), 234-6.

A4, Western Blot

SEPARATING GEL (makes 4 10% gels)

- Remove samples and allow to thaw on ice.
- Clean mini-gel plates with 70% ethanol before use.
- Assemble gel cassettes and check for leaks.
- Prepare separating gel in a small beaker with continuous stirring.
 - Prepare a 10% ammonium persulfate (APS) solution; prepare fresh each time. Measure out 50mg of APS into a 1.5 mL Eppendorf tube and add 450 μ L of double distilled water (ddH₂O).
 - Add 15.992mL of ddH₂O
 - Add 13.333mL of Acrylamide solution¹; begin to stir
 - Add 10mL of Separating gel buffer²
 - Add 400 μ L sodium 10% SDS³
 - Let stir for a minimum of 10 min
 - Add 250 μ L of 10% APS solution and 25 μ L of tetramethylethylenediamine (TEMED) simultaneously and let stir for about 20 seconds.
- Quickly pour the gel into each cassette with a Pasteur pipet up to about 1.5cm from the top of the short plate.
- Try to remove most bubbles by tipping the apparatus and/or soaking them up with filter paper.
- Immediately overlay each gel with about 1cm of water-saturated butanol.
- Allow gel to polymerize 30-60 min
- Continue preparing samples:
 - Vortex samples and pipet appropriate volumes into a set of labeled tubes
 - Add an equal volume of 2X Lammeli SDS-PAGE sample buffer⁴ to each tube (each tube should now be a 1:1 mixture of homogenized sample to sample buffer)
 - Mix samples thoroughly, and centrifuge to pull all contents down if necessary.

STACKING GEL (makes 4 4% gels)

- Clean lane combs with 70% ethanol.
- Prepare the 4% stacking gel in small beaker with continuous stirring
 - Add 12.2 mL of ddH₂O
 - Add 2.6 mL of Acrylamide solution; begin to stir
 - Add 5 mL of Stacking gel buffer⁵
 - Add 200 μ L of 10% SDS
 - Let stir for a minimum of 10 min
- While stirring and once separating gel has polymerized, dump butanol overlay down the sink
- Dry exposed glass plates (above separating gel) with filter paper.
- After 10 min of stirring, add 125 μ L of 10% APS and 25 μ L of TEMED simultaneously and let stir for about 20 seconds.
- Quickly pour the gel into each cassette with a Pasteur pipet up to the top of the short plate.
- Carefully place lane combs in between the two plates, making sure no bubbles are trapped beneath it.
- Allow gel to polymerize for 30-60 min

- Continue preparing samples:
 - Bring water in a metal container to 75-80°C and place samples in water. Heating times vary (approximately 3-10min)
 - Remove from hot water and let cool at room temperature before loading onto gel.
 - Mix samples again by vortexing and centrifuge to pull down contents if necessary.
 - Prepare 1X running buffer⁶ (25mM Tris, 192 mM glycine, 0.1% SDS) from 10X running buffer and refrigerate. Approximately 1L is needed per electrophoresis unit.

STACKING GEL

- Once stacking gel has polymerized, carefully remove gel cassettes from apparatus and transfer to electrophoresis electrode modules.
 - Each BioRad Mini-Gel Protean Tetra Cell electrophoresis unit holds one “electrode” module and one “companion” module, and each module holds two gels. If running only two gels, you must use the “electrode” module.
 - Make sure each cassette is as far down as possible on the modules.
 - Make sure the short plate faces the inside of the module.
 - For electrophoresis to work, you need a closed-in compartment on the inside. If running an odd number of gels, you can place an unused cassette, but the “spacer” plate facing the inside, opposite to the lone gel in order to seal off the inside compartment.
 - **Make sure you can identify your gels
- Remove the lane combs and fill each well about halfway with 1X running buffer with a Pasteur pipet.
- Load the correct amount of each sample into each corresponding well using a micropipette
 - Based on 1:1 sample to sample buffer mixture, each well will contain a different volume of sample to equalize protein amounts.
 - Load 5 µL of chemiluminescent ladder (BioRad Precision Plus Protein Standards Kaleidoscope #161-0375 or #161-0324)
- Fill the inside compartment of the electrode modules with 1X running buffer to a level above the short plate to cover the wells.
- Fill the rest of the tank (outside the gel cassettes) with cold 1X running buffer to the same level.

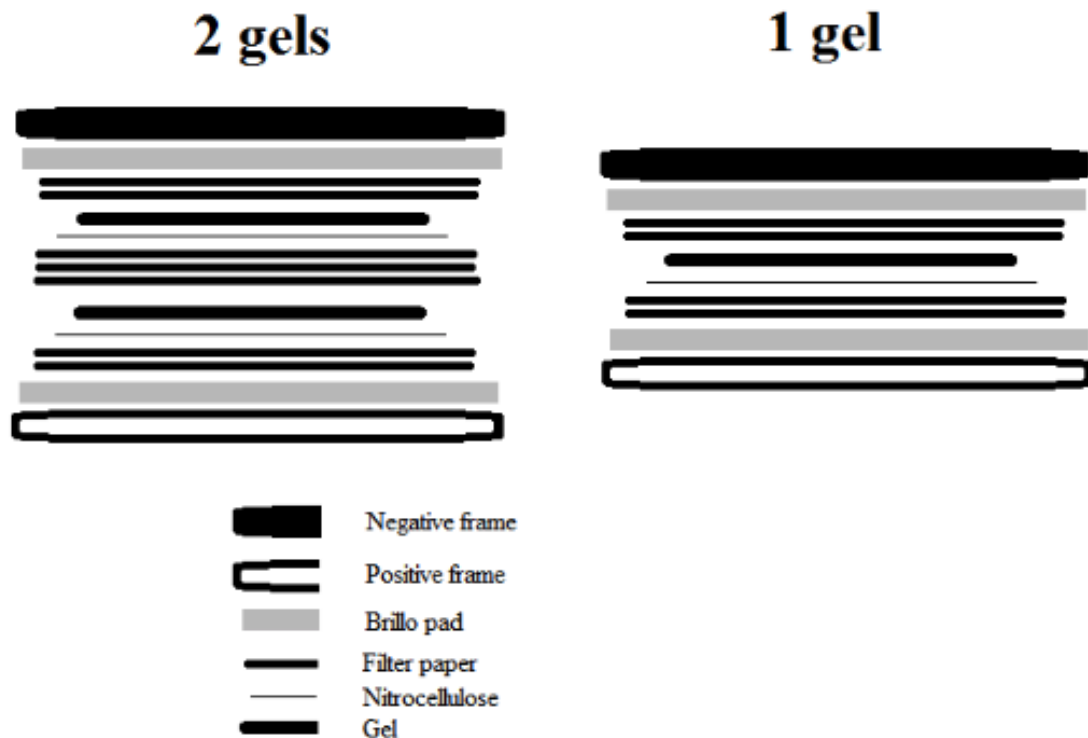
ELECTROPHORESIS

- Place electrophoresis unit into a second larger container and cover it. Ensure the electrodes match the lid: ie red to red and black to black.
- Fill container with ice
- Connect lid electrodes to the power unit.
- Generally, run at a low voltage (50-70) until the dye has passed through the stacking gel. Then turn it up to 110-125 for the remainder of the run.
- As this is taking place, prepare the transfer buffer⁷ (25 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol), and cut filter paper and nitrocellulose.
- Put a layer (arbitrary volume) of cold transfer buffer into a baking dish and soak the nitrocellulose, filter paper and brillo pads

TRANSFER

- Once electrophoresis is complete, unplug unit and remove lid.

- Using the “wedge” piece, separate the short and spacer plates to reveal the gel. Slice off the stacking gel.
- Dislodge the gel from the spacer plate and assemble the “sandwich” in the transfer apparatus:
 - Place the “positive” end (white) of the sandwich frame on the bottom.
 - Stack the individual components as follows: 1 brillo pad, 2 filter papers, 1 nitrocellulose piece, gel, 3 filter papers, 1 nitrocellulose piece, gel, 2 filter papers, 1 brillo. The “negative” end (black) of the sandwich frame is on top, and secures the sandwich.



- Apply pressure on the sandwich before closing to try to remove bubbles between the gel and nitrocellulose.
- Place the closed sandwich in the transfer electrode module and place this in the transfer tank. ****Remember to orient it correctly in the module.
- Place an ice pack in the tank and fill it with cold transfer buffer.
- Place the transfer unit into a second larger container and cover the unit with the lid.
 - Ensure electrodes match on the module and lid (ie. red to red and black to black)
- Fill container with ice.
- Connect the lid electrodes to the power unit.
- Generally, transfer is 70V for 90min
- While transfer is taking place, prepare 1X Tween TBS⁸ (TTBS; 10mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween-20) from 10X TBS, and blocking solution^{9a,b}.
 - Prepare 2L of TTBS for 4 blots
 - Select small, shallow containers to incubate blots
 - Prepare 25-30 mL of blocking solution per container.

BLOCKING

- Once transfer is complete, unplug from the power unit and remove the lid from the transfer tank.
- Remove each piece of nitrocellulose and place in the prepared blocking containers with blocking solution.
- Place only two pieces of nitrocellulose “blots” per container and ensure they are back-to-back.
- Allow to incubate for 1 hr at room temperature with gentle shaking (~40 rpm).
- Prepare the primary antibody solution^{10,11}
- When blocking is complete, wash blots once with TTBS
 - Dump blocking solution down the sink and replace with TTBS.
 - Allow to wash for 5 min at room temperature with vigorous shaking (~90 rpm).

PRIMARY ANTIBODY

- Dump TTBS down the sink and replace with prepared antibody solution.
- Allow blots to incubate overnight (12-16 hours) at 4°C with gentle shaking (~60 rpm).
- Before primary incubation is stopped, prepare the secondary antibody solution¹² in 50mL falcon tubes.
- Once primary antibody incubation is done, remove any unbound primary antibody with three successive washes and rinse in TTBS (10 min with vigorous shaking ~90rpm).

SECONDARY ANTIBODY

- Dump TTBS down the sink and replace with the previously prepared secondary antibody solution¹².
- Allow blots to incubate for 60 min at room temperature with gentle shaking (~40 rpm).
- Prepare 1X Tris buffered saline¹³ from 10X TBS
 - Prepare 100mL of TBS for 4 blots (2 containers).
- Once secondary antibody incubation is done, remove any unbound secondary with three successive washes and rinses in TTBS (10 min with vigorous shaking ~90 rpm).
- After last TTBS rinse, perform one final rinse with TBS¹³ and leave blots in an unspecified volume of TBS.

CHEMILUMINESCENT

- Turn on the BioRad Chemidoc XRS System, open the Quantity One 1-D analysis program
 - Select band analysis guide from the toolbar, and a pop-up with a list of items should appear on the top right of the screen
 - Click on “select scanner” and select “Chemidoc XRS”
 - In the control window, click “Live/Focus” and press “Epi White” on the chemidoc system.
- Clean the inside surface of the Chemidoc with 70% ethanol
- Prepare BioRad chemiluminescence substrate in a 1.5mL Eppendorf tube
 - Pipet 500 µL of Luminol solution and 500 µL of peroxide solution in the Eppendorf and mix.
- Place blots on transparency and pipet chemiluminescence solution over blot. Close transparency.
- Place blot in chemidoc drawer and align it to the center

- In the control window, check “highlight saturated pixels” box near the bottom, and click the “Open Iris” button repeatedly.
 - Saturated pixels will be displayed red, and there should be a “pulsating” ellipse of red pixels in the center of the display.
 - Continue pressing this button until the size of the ellipse is no longer noticeably increasing.
- Close the chemidoc door and press “Epi White” again to turn off the light.
- Click “freeze” in quantity one and click “Live Acquire”
- A pop-up appears and you can decide exposure parameters
- Allow the camera to detect chemiluminescence for the total exposure time.
 - Choose an image in which your bands of interest are just on the verge of saturation.

Solutions

| | Name | Components, concentrations, pH | Example amounts | Storage |
|----|-----------------------------------|---|--|----------------------------|
| 1. | Acrylamide solution | Acrylamide – 30% (w/v) Bis-acrylamide – 0.8% (w/v) | Acrylamide – 150 g Bis-acrylamide – 4 g Total V - 500 mL | 4°C |
| 2. | Separating gel buffer | Tris – 1.5 M SDS – 0.4 % (w/v) pH – 8.8 | Tris – 90.9 g SDS – 2 g Total V- 500 mL | 4°C |
| 3. | SDS solution | SDS – 10% (w/v) | SDS – 10 g Total V - 100 mL | Room temperature |
| 4. | 2X Laemmli SDS-PAGE sample buffer | Tris - 0.125 M Glycerol – 20% (v/v) SDS – 4% β –mercaptoethanol – 10% (v/v) Bromophenol blue – 0.015% (w/v) pH – 6.8 | Tris – 7.57 g Glycerol – 100 mL SDS – 20 g β –mercaptoethanol – 50 mL Bromophenol blue – 0.075 g Total V – 500 mL | Room temperature, fumehood |
| 5. | Stacking gel buffer | Tris – 0.5 M SDS – 0.4% (w/v) pH – 6.8 | Tris – 30.3 g SDS – 2 g Total V – 500 mL | 4°C |
| | 10X running buffer | Tris – 0.25 M Glycine – 1.92 M SDS – 1% (w/v) | Tris – 60.6 g Glycine – 288 g SDS – 20 g Total V – 2 L | 4°C |

| | | | | |
|-----|-----------------------------|--|--|-----------------------|
| 6. | 1X running buffer | 10X running buffer – 10% (v/v) | 10 X running buffer – 100 mL Total V – 1 L | 4°C |
| 7. | 1X transfer buffer | 10X running buffer – 10% (v/v) Methanol – 20% (v/v) | 10X running buffer – 100 mL Methanol – 200 mL (add last) Total V – 1 L | 4°C or colder |
| | 10X TBS | Tris – 0.1 M NaCl – 1 M pH – 7.5 | Tris – 24.2 g NaCl – 116.9 g Total V – 2 L | Room temperature |
| 8. | 1X TTBS | 10X TBS – 10% (v/v) Tween-20 – 0.1% (v/v) pH – 7.5 | 10X TBS – 200 mL Tween-20 – 2 mL Total V – 2 L | Room temperature |
| 9. | Blocking solution | a. Non-fat, dry milk protein – 5% (w/v) b. Bovine serum albumin – 5% (w/v) 1X TTBS – 95% | a. Non-fat, dry milk protein – 1.25 g b. Bovine serum albumin – 1.25 g 1X TTBS – top up to 25 mL | Room temperature |
| 10. | Primary antibody solution 1 | Non-fat, dry milk protein – 2% (w/v) 1X TTBS – 98% Antibody against protein of interest | Non-fat, dry milk protein – 0.4 g 1X TTBS – top up to 20 mL | 4°C (-20°C long term) |
| 11. | Primary antibody solution 2 | Bovine serum albumin – 5% (w/v) 1X TTBS – 95% Antibody against protein of interest | Bovine serum albumin – 1 g 1X TTBS – top up to 20 mL | 4°C (-20°C long term) |
| 12. | Secondary antibody solution | Non-fat, dry milk protein – 2% (w/v) 1X TTBS – 98% Antibody against primary antibody | Non-fat, dry milk protein – 0.4 g 1X TTBS – top up to 20 mL | Room temperature |
| 13. | 1X TBS | 10X TBS – 10% (v/v) pH – 7.5 | 10X TBS – 10 mL Total V – 100 mL | Room temperature |

SEPARATING GEL

| 10 % Gel | | | | |
|--|---------------|---------------|---------------|---------------|
| | 2 gels | 4 gels | 6 gels | 8 gels |
| ddH₂O | 7.996 mL | 15.992 mL | 23.988 mL | 31.984 mL |
| Acrylamide solution¹ | 6.667 mL | 13.333 mL | 20 mL | 26.667 mL |
| Separating gel buffer² | 5 mL | 10 mL | 15 mL | 20 mL |
| SDS solution³ | 200 µL | 400 µL | 600 µL | 800 µL |
| 10% APS solution | 125 µL | 250 µL | 375 µL | 500 µL |
| TEMED | 12.5 µL | 25 µL | 37.5 µL | 50 µL |
| Total Volume | 20 mL | 40 mL | 60 mL | 80 mL |

STACKING GEL

| 4 % Gel | | | | |
|--|---------------|---------------|---------------|---------------|
| | 2 gels | 4 gels | 6 gels | 8 gels |
| ddH₂O | 5.992 mL | 11.983 mL | 17.975 mL | 23.967 mL |
| Acrylamide solution¹ | 1.333 mL | 2.667 mL | 4 mL | 5.333 mL |
| Stacking gel buffer⁵ | 2.5 mL | 5 mL | 7.5 mL | 10 mL |
| SDS solution³ | 100 µL | 200 µL | 300 µL | 400 µL |
| 10% APS solution | 62.5 µL | 125 µL | 187.5 µL | 250 µL |
| TEMED | 12.5 µL | 25 µL | 37.5 µL | 50 µL |
| Total Volume | 10 mL | 20 mL | 30 mL | 40 mL |

Appendix B

B1, Ethics

| | |
|---|-------------------------------------|
| A. PROJECT/INVESTIGATOR INFORMATION | |
| Investigator Name: Earl Noble | |
| Project Title: <i>This is a NEW title</i> - Yes <input type="checkbox"/> | Current Protocol #: 2008-095 |
| Innovation to reduce cardiovascular complications of diabetes at the intersection of discovery, prevention and knowledge exchange | |

| | |
|---|------------|
| B. INVESTIGATOR DECLARATION | |
| <p>I. All animals used in this research project will be cared for in accordance with the recommendations of the Canadian Council on Animal Care and the requirements of the provincial legislation entitled, "The Animals for Research Act," of the Province of Ontario.</p> <p>II. I confirm that this <i>Animal Use Protocol</i> accurately represents the proposed animal use.</p> <p>III. I accept responsibility for procedures performed on animals in this project.</p> <p>IV. I will ensure that any individual who will perform any animal-related procedure(s) within this protocol will complete all related mandatory training AND will be made familiar with the contents of this document.</p> <p>1. I support the above declaration - YES <input checked="" type="checkbox"/> Today's Date: <i>mm/dd/yy</i>: October 30/09</p> <p>2. By checking 'YES' in this section, I authorize the submission of this form and its electronic delivery to auspc@uwo.ca YES <input checked="" type="checkbox"/> NO <input type="checkbox"/></p> | |
| AUS APPROVAL - AUS Office Use Only - Veterinary Authorization by Click Here Authorization Date (mm/dd/yy) / / | Signature: |

| | |
|--|--|
| C. CHANGES AT RENEWAL CONFIRMATION – Pick One Only | |
| <p><input checked="" type="checkbox"/> I confirm that NO CHANGES and/or NEW ELEMENTS to this AUP - other than staffing - have occurred since the last AUS Form submission.</p> <p><input type="checkbox"/> I confirm that all CHANGES and/or NEW ELEMENTS to this protocol not previously submitted WILL BE sent via a <i>Protocol Modification</i> form along with this <i>Protocol Renewal</i> Form.</p> | |

THE UNIVERSITY OF WESTERN ONTARIO – ANIMAL USE SUBCOMMITTEE
PROTOCOL RENEWAL FORM

Protocol # 2008-095 Noble

D. THREE R's PROGRESS REPORT

Describe any progress made with respect to the Three R's of replacement, reduction and refinement of animal use during the PAST PROTOCOL YEAR. See the CCAC 3 R's Microsite: <http://www.ccac.ca/en/alternatives/>

We have used less animals than anticipated in the past year because we have made more use of the issues generated by the animals that we employed.

- No additional animals were used during the past Protocol Year.
 No additional animals were requested during the past Protocol Year.

E. ANIMAL USE COMPLICATIONS PROGRESS REPORT

Describe any complications beyond the expected experimental outcomes and end points encountered relative to animal use – e.g. unpredicted outcomes and any unexpected animal pain, distress, or mortality - AND indicate measures being undertaken to resolve these complications for the PAST PROTOCOL YEAR.

- No complications were encountered relative to animal use during the past Protocol Year.

F. ANALGESIA ADMINISTRATION UPDATE

Choose One of the Following Statements:

1. Analgesics were administered as outlined within the *Animal Use Protocol* *If checked, please list all analgesics currently used below:

2. Analgesics are not indicated in this *Animal Use Protocol*

G. PROTOCOL PERSONNEL UPDATE

Complete for ALL Staff Working Under This Protocol for This Renewal Year

TRAINING INFORMATION

CCAC Mandated Training Requirements – All personnel working with live animals require CCAC mandated training including the 'Basic Animal Care & Use Web-CT Course' and related hands-on 'Workshops'. Completion of the 'Basic Animal Care & Use Web-CT Course' once every 5 years is mandatory for ALL personnel, including the Principal Investigator. The Animal Use Subcommittee will be informed of all personnel with incomplete training beyond 1 month of notice.

Workshop Enrolment Detail – The 'Workshop' requirements are determined by the species and procedures associated with each individual

Curriculum Vitae

Michael Murray

Education:

University of Western Ontario M.Sc. Kinesiology (2012-2015)

University of Western Ontario B.Sc. (Honours) Kinesiology (2008-2012)

Awards

Western Graduate Research Scholarship (WGRS; 2012-2014)

Edward Flinn Memorial Award, 2011 (\$1500)

Research Related Experience

Research Assistanceships (Jan 2015-Feb 2015)

Volunteer Position

Aortic Ring Myography in Rats with Type 2 Diabetes

Principle Investigator: Dr. Harold Laughlin

School of Veterinary Medicine, Department of Physiology, University of Missouri

Graduate Teaching Assistantship (Sept 2012-Dec 2012; Jan 2013-Apr 2013; Sept 2013-Dec 2013; Jan 2014-Apr 2014)

Exercise Physiology

Exercise Nutrition

Laboratory in Exercise Physiology

Proctor

Publications:

McDonald, M. W., **Murray, M. R.**, Hall, K. E., Noble, E. G., & Melling, C. J. (2014).

Morphological assessment of pancreatic islet hormone content following aerobic exercise training in rats with poorly controlled Type 1 diabetes mellitus. *Islets*, 6(1), 1-9.

doi:10.4161/isl.29221

Presentations:

Title: Alterations in hepatic glycogen metabolism following aerobic exercise training in rats with type 1 diabetes mellitus

Invited Co-presenter, Seminar, University of Missouri, Jan 21, 2015

Abstracts:

Metabolomic Response of Skeletal Muscle to Aerobic Exercise in a Model of Type 1 Diabetes, Dotzert, M.S.; **Murray, M.R.**; McDonald, M.; Noble, E.; Urquhart, B.L.; Melling, C.W.

[Abstract] American Diabetes Association Conference, 2015.

Resistance Exercise Induces Changes in Vasorelaxation Responses in Insulin-treated T1D Rats,

McDonald, M.; **Murray, M.R.**, Dotzert, M.S.; Noble, E.G.; Melling, C.W.J. [Abstract]

American Diabetes Association Conference, 2015.