The Cardiovascular Benefits of Regular Exercise in Type 1 Diabetes Mellitus and the Risk of Exercise-induced Hypoglycemia

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Graduate Program in Kinesiology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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Abstract

Type 1 diabetes mellitus (T1DM) is associated with compromised glycemic control and a heightened risk for cardiovascular disease. The common treatment of T1DM with strict glycemic control through intensive insulin therapy can be problematic (weight gain, insulin resistance, hypoglycemia). Regular exercise is known to improve cardiovascular health, yet most individuals with T1DM remain sedentary, and identify the risk of exercise-induced hypoglycemia as a significant barrier. The investigation into the use of different forms of exercise (higher intensity, resistance) for preventing exercise-induced hypoglycemia in populations with T1DM has been promising, however, little work has investigated their cardiovascular benefit or whether the risk of exercise-induced hypoglycemia changes over the course of exercise training. As such, using a novel insulin-treated rat model of T1DM the objectives of this dissertation were: (1) to determine whether the risk of hypoglycemia in response to different exercise modalities changes over the course of training in T1DM, (2) to characterize which exercise modality provides the largest amount of cardiovascular protection (as determined by recovery from an ischemia-reperfusion injury and fine-wire vascular myography), while assessing risk for exercise-induced hypoglycemia, and (3) to explore whether exercise training, when paired with modest glycemic control, results in larger cardiovascular protection than stringent glycemic control alone. The main findings of these collective studies were as follows; (1) the magnitude of the abrupt decline in blood glucose in response to different exercise modalities remains consistent after exercise training and infrequently reaches hypoglycemic concentrations if blood glucose concentrations are elevated prior to exercise in T1DM rats, (2) both exercise-induced fluctuations in blood glucose and the amount of cardiovascular protection obtained from regular exercise training appears to be modality-specific; however, results suggest that high intensity aerobic exercise provides the largest amount of cardiovascular protection (increased recovery from ischemia-reperfusion injury, vascular insulin sensitivity, and glycemic control), and (3) maintaining more modest glycemic control may provide similar cardiovascular benefits as stricter glycemic control when combined with regular exercise. Overall, less of a reliance on strict glycemic could allow for exercise to be performed safely (and providing cardiovascular benefits), while preventing complications associated with intensive insulin therapy.

Keywords: diabetes, exercise training, hypoglycemia, insulin sensitivity, cardiovascular disease
Co-Authorship Statement
The following is a list of co-authors that contributed to this dissertation (see the appendix for the details pertaining to published material):

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Earl G. Noble (study design, revisions of manuscript)
C.W. James Melling (study design, writing and revisions of manuscript)

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Jaume Padilla (data collection and analysis)
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Dedication

To my family: without your love and encouragement I would never have reached this point of my education.
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This dissertation would not have been completed without the collaborative effort of a number of my colleagues. First, I would like to thank my supervisor Dr. Jamie Melling. I would never have imagined nine years ago, sitting in your undergraduate anatomy lecture, that I would ever reach this point in my education. Your knowledge and support (both in the lab and on the golf course) allowed me not only grow as a scientist but as a person. I would also like to acknowledge Dr. Earl Noble, who has been there every step of the way. Your passion and excitement for research are qualities that every scientist should possess.

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end products</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AT&lt;sub&gt;high&lt;/sub&gt;</td>
<td>High intensity aerobic exercise training</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary units</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BB</td>
<td>Biobreeder</td>
</tr>
<tr>
<td>C</td>
<td>Control non-T1DM rat</td>
</tr>
<tr>
<td>C-AT&lt;sub&gt;mod&lt;/sub&gt;</td>
<td>Control non-T1DM rat and moderate-intensity aerobic exercise training</td>
</tr>
<tr>
<td>CIT</td>
<td>Conventional insulin treatment</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>Cu/Zn SOD</td>
<td>Copper-zinc superoxide dismutase</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>D-ART</td>
<td>Diabetic resistance and high-intensity aerobic exercise training</td>
</tr>
<tr>
<td>D-AT&lt;sub&gt;high&lt;/sub&gt;</td>
<td>Diabetic high-intensity aerobic exercise training</td>
</tr>
<tr>
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<td>Diabetic low-intensity aerobic exercise training</td>
</tr>
<tr>
<td>D-AT&lt;sub&gt;mod&lt;/sub&gt;</td>
<td>Diabetic moderate-intensity aerobic exercise training</td>
</tr>
<tr>
<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
</tr>
<tr>
<td>D-CIT</td>
<td>Sedentary diabetic conventional insulin therapy</td>
</tr>
<tr>
<td>D-IIT</td>
<td>Sedentary diabetic intensive insulin therapy</td>
</tr>
<tr>
<td>D-RT</td>
<td>Diabetic resistance exercise training</td>
</tr>
<tr>
<td>EDHF</td>
<td>Endothelium-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>EDIC</td>
<td>Epidemiology of Diabetes Interventions and Complications</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>eu</td>
<td>Energy Units</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter 4</td>
</tr>
<tr>
<td>G6P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Term</td>
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<tr>
<td>--------------</td>
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<tr>
<td>G6Pase</td>
<td>Glucose-6-phosphotase</td>
</tr>
<tr>
<td>HBA\textsubscript{lc}</td>
<td>Glycosylated hemoglobin</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>Hsp70</td>
<td>Heat shock protein 70</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>IIT</td>
<td>Intensive insulin treatment</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>I-R</td>
<td>Ischemia-reperfusion</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LVEDP</td>
<td>Left ventricular end-diastolic pressure</td>
</tr>
<tr>
<td>LVDP</td>
<td>Left ventricular developed pressure</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MHR</td>
<td>Maximal heart rate</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium-calcium exchanger</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>P\textsubscript{i}</td>
<td>Phosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarcoendoplasmic reticulum calcium transport ATPase</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Resistance exercise training</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>+dp/dt</td>
<td>Maximal rate of contraction</td>
</tr>
<tr>
<td>-dp/dt</td>
<td>Maximal rate of relaxation</td>
</tr>
</tbody>
</table>
CHAPTER 1

1 « Introduction »

1.1 Type 1 Diabetes Mellitus

Diabetes is identified by the inability of the body to produce insulin, or the failure of the body to utilize insulin properly. The disease is categorized into two types; Type 1 diabetes (previously insulin-dependent or juvenile diabetes) and Type 2 diabetes mellitus (previously insulin-independent diabetes). Type 2 diabetes mellitus (T2DM) is the most common form of diabetes and is characterized by the development of insulin insensitivity (insulin resistance), a condition in which the body does not respond efficiently to normal levels of insulin and occurs as a result of a number of factors including aging, obesity or physical inactivity (115). As such, the body is unable to effectively utilize glucose in the blood, resulting in chronically elevated levels of blood glucose (hyperglycemia) (115). Type 1 diabetes mellitus (T1DM) is characterized by the autoimmune destruction of the insulin producing β-cells of the pancreas, which develops into an overall insulin deficiency. The onset of T1DM typically occurs during childhood or adolescence, although it is now recognized that the disease can develop during adulthood (4). Genetic factors are related to the development of T1DM and a number of genes have been identified (HLA region on chromosome 6P21, PTPN22; which encodes the lymphoid protein tyrosine phosphatase, IL2RA; Allelic variation in the interleukin (IL)-2 receptor-α gene (IL2RA), CTLA-4; cytotoxic T lymphocyte-associated protein 4, and the insulin gene) that make individuals more susceptible to the disease (8). Investigation into the triggers of T1DM has primarily implicated viral infections, although evidence exists for environmental (cow’s milk, wheat proteins, vitamin D) and bacterial factors (8).
Nonetheless, the development of T1DM appears to be a complex interplay between a number of factors (8). The overall incidence of individuals with T1DM are increasing, with areas of Canada representing the highest reported worldwide (92).

1.2 Treatment of T1DM

In the late 1800’s and early 1900’s the primary cause of mortality for patients with diabetes was ketoacidosis (diabetic coma)(59). By the 1920’s cardiovascular-renal disease had become the primary cause of death for patients with diabetes (59), which currently remains the leading cause of diabetes-related death to date (67, 85). Identified as a disease of high blood glucose concentrations (hyperglycemia), the original treatment option for diabetes was intermittent fasting (58). The largest breakthrough in the treatment of diabetes came in 1921 when Fredrick Banting and Charles Best discovered and successfully isolated insulin from the pancreata of dogs (5). Subsequently, insulin was genetically engineered from E. coli bacteria and the first biosynthetic insulin became commercially available in 1982 (46).

Although hyperglycemia had been implicated in the pathogenesis of diabetic complications it was not until the Diabetes Control and Complications Trial (DCCT) that this view became widely accepted. The DCCT investigated the importance of strict glycemic control through intensive insulin therapy (IIT) on the prevention of diabetes-related complications, while comparing this strategy to the traditional and more moderate means of glycemic control known as conventional insulin therapy (CIT)(121). The two cohorts of patients in the DCCT utilized either one or two insulin injections per day (CIT), or three or more insulin injections daily designed to achieve normoglycemia (IIT). As a result, blood glucose concentrations ranged from 9 to 15mmol/L in patients utilizing CIT, and significantly lower (<9mmol/L) in patients utilizing IIT. Results of the DCCT
demonstrated that significant reductions in retinopathy, nephropathy, and neuropathy were evident in patients following IIT in comparison to those utilizing CIT (121). These results were so convincing that due to ethical considerations the study was terminated early and all patients were switched to IIT. An observational study was completed following the DCCT termed the Epidemiology of Diabetes Interventions and Complications (EDIC), whereby patients originally using CIT in the DCCT (now reassigned to IIT) were continually monitored for overall cardiovascular health. Results from the EDIC provided further evidence that prescribing to IIT significantly reduces the risk of cardiovascular disease onset with diabetes (91). Further, the EDIC also revealed a phenomenon termed “metabolic memory”, in that those patients who were initially prescribed IIT during the DCCT had a reduced incidence of cardiovascular disease that persisted for up to 30 years. This level of protection was not evident in patients originally placed in the CIT cohort, suggesting that IIT should be initiated as early as possible into the progression of T1DM in order to obtain maximal cardiovascular benefits (34). Both the DCCT and EDIC have demonstrated a causal relationship between hyperglycemia and the progression of microvascular complications (retinopathy, neuropathy, etc.) associated with T1DM (34, 91, 123). However, the utilization of IIT requires constant attention to blood glucose concentrations and insulin dosing, as well as the close monitoring of daily eating habits and activity levels. Patients in the DCCT and EDIC undergoing IIT experienced a threefold increase in severe hypoglycemic episodes, highlighting the difficulty in intensively managing glycemia (121, 122). Despite these difficulties, the encouraging results of the DCCT and EDIC have formed the basis for the treatment of T1DM and its complications for the past 30 years.
1.3 Cardiovascular Disease in T1DM

In populations with T1DM, there remains a tenfold increase in cardiovascular disease-related mortality that is not entirely accounted for by traditional risk factors such as hypertension, dyslipidemia, obesity, or smoking (67, 85, 113). The EDIC found a 42 percent reduction in the risk of experiencing cardiovascular disease and a 57 percent reduced risk of nonfatal myocardial infarction, stroke, or death from cardiovascular disease when patients were prescribed IIT (123). However, the suitability of IIT for all patients with T1DM has recently been challenged (22). Many patients prescribing to IIT often experience difficulty maintaining strict glycemic control whereby insulin overcorrection and hypoglycemia is common (28, 122). In addition to the increased risk of hypoglycemia, the amount of weight gain that can be associated with IIT has raised significant concerns (106), since it is often accompanied by insulin resistance, higher waist:hip ratios, and a more atherogenic lipid profile (22). Some researchers and clinicians have challenged the findings of the DCCT and EDIC, suggesting that the subjects in these trials were not representative of the patient population with T1DM. Indeed, the risk of cardiovascular disease for patients included in the DCCT was minimal, as individuals with established vascular disease, or exhibiting obesity, hypertension, or hypercholesterolemia were excluded from participation in the study (121). Two more recently completed clinical trials, the Action to Control Cardiovascular Risk in Diabetes (ACCORD) and the Action diabetes and Vascular disease: Preterax and Diamicron MR controlled evaluation (ADVANCE), found no benefit of strict glycemic control for preventing cardiovascular events in patients already at a heightened risk of cardiovascular disease (119, 120). In fact, the intensive glycemic control group in the ACCORD trial experienced an increase in cardiovascular disease associated death, which was attributed
to weight gain and the increased frequency of hypoglycemia (119). The equivocal findings of the DCCT, EDIC, ADVANCE and ACCORD studies has led many practitioners, researchers, and patients to question which insulin treatment strategy is ideal in order to limit the susceptibility of patients with T1DM to cardiovascular disease.

1.3.1 Ischemic Heart Disease

Ischemic heart disease (coronary artery disease) is one of the more widely cited complications of cardiovascular disease in patients with T1DM (85, 96, 113). The diabetic heart is more susceptible to ischemic heart disease due to the development of micro- and macrovasculature dysfunction, as well as diabetic cardiomyopathy (130). The heart relies on sufficient blood flow from the coronary arteries to provide the necessary oxygen for oxidative phosphorylation, since glycolysis alone is unable to produce enough ATP for its continuous contraction and relaxation. A disruption in blood flow during myocardial ischemia is debilitating to the heart tissue, as a lack of oxygen leads to the accumulation of ADP, AMP and Pi, and a decrease in pH (due to the accumulation of lactic acid)(69). The degree of damage to the myocardium is related to the total length of ischemia, where prolonged ischemia can cause irreversible damage and cell death. Cardiac reperfusion following ischemia can further exacerbate damage to the myocardium, largely due to increases in intracellular calcium, reactive oxygen species (ROS), and inflammatory processes (69). Ischemic reperfusion also causes an attenuation of endothelium-dependent vasodilation, resulting from the inactivation of nitric oxide (NO) by superoxide and reduced NO bioavailability (100). In addition, production of endothelin by the endothelium is increased during reperfusion leading to greater vasoconstriction (100).
The term “diabetic cardiomyopathy” characterizes diabetic-related changes in the structure and function of the myocardium that occur in the absence of coronary artery disease or hypertension. The prominent feature of diabetic cardiomyopathy is cardiac hypertrophy; specifically, increased left ventricular mass and wall thickness, which occurs concomitant with impaired systolic and diastolic function (33). Diastolic dysfunction usually occurs earlier in the course of diabetes, while systolic dysfunction generally develops as the disease progresses (14). Dysfunction in Ca\textsuperscript{2+} handling and homeostasis in cardiomyocytes is recognized to be a contributing factor for diastolic dysfunction in diabetic cardiomyopathy (108, 141); although numerous other mechanisms have been proposed (for review (14)). Hyperglycemia has been shown to directly contribute to many of the pathologies associated with diabetic cardiomyopathy, such as cardiac hypertrophy, fibrosis, cardiac autonomic neuropathy, and changes in structure and function of the myocardium (141).

Evidence examining the causal factors of diabetic cardiomyopathy in T1DM has been generated primarily from experimental studies using streptozotocin (STZ)-induced T1DM rats. The hearts of these insulin-deficient STZ rats typically experience diminished ventricular compliance, cardiomyocyte contractility, and strength of contraction (108, 128). Interestingly, in untreated STZ-induced diabetic rats the myocardium can experience atrophy, as opposed to the hypertrophy typically observed in clinical diabetes (118). It appears that the lack of insulin in the STZ-treated animals is responsible for these differential changes to the heart, as the supplementation of insulin to STZ diabetic rats results in pathological hypertrophy (79). This suggests that while untreated STZ-diabetic rats provide insight into how hyperglycemia influences the function of the
myocardium these studies may not be translational to clinical populations with T1DM, as patients with T1DM are seldom absent of some form of insulin treatment.

1.3.2 The Vasculature

The prevalence of cardiovascular disease in populations with T1DM can largely be attributed to dysfunction of the micro- and macrovasculature (24, 70). Microvasculature (small resistance arteries, arterioles and capillaries) disease generally results in retinopathy, nephropathy and neuropathy, while macrovasculature disease affects the peripheral arteries, coronary arteries, and cerebrovasculature. Historically, vascular dysfunction in T1DM has been primarily attributed to chronic hyperglycemia (70); however, there is emerging evidence that the development of insulin resistance is also associated with vascular dysfunction in T1DM (22, 35, 49, 98, 106). Several studies have demonstrated that T1DM-related alterations in insulin signalling can contribute to vascular dysfunction (6, 19, 95, 132).

1.3.3 Endothelial Dysfunction

The unicellular inner layer of blood vessels, the endothelium, is actively involved in the regulation of vascular permeability, inflammation, coagulation, and vascular tone (75). In order to regulate vascular tone, and subsequently blood flow, the endothelium can release vasodilators such as NO, prostacyclin and endothelium-derived hyperpolarizing factors (EDHF), as well as vasoconstrictors like endothelin-1 (ET-1) and angiotensin II (116). One of the most important molecules produced by the endothelium is NO, which is constitutively synthesized by endothelial nitric oxide synthase (eNOS) from L-arginine (24). Nitric oxide exerts its vasodilatory effects on vascular smooth muscle cells by reducing intracellular calcium, as a result of the activation of guanylate cyclase and the increased production of cyclic guanosine monophosphate (cGMP)(7). Aside from its
vasodilatory effects, NO also has numerous protective benefits such as preventing platelet and leukocyte interaction on the vascular wall, inhibiting smooth muscle cell proliferation and migration, and regulating inflammatory processes (7, 24).

Endothelial dysfunction occurs when there is an imbalance in endothelium-derived relaxing and contracting factors, which results from a reduction in the bioavailability of the vasodilator NO or the favouring of molecular pathways involved in vasoconstriction, inflammation, or thrombosis (24, 116). T1DM is typically associated with some level of endothelial dysfunction, as demonstrated by the reduced production and bioavailability of NO (32, 57, 64, 65, 102). Endothelial dysfunction often precedes other evidence of vascular disease and is a primary factor contributing to cardiovascular disease and its co-morbidities, such as atherosclerosis, arteriosclerosis, hypertension, peripheral neuropathy, and metabolic dysfunction (65, 88).

Chronic hyperglycemia can have detrimental effects on most cells in the body, especially vascular endothelial cells, a type of cell that is not entirely reliant on insulin for glucose uptake (3, 60). It has been shown that the intracellular glucose concentrations of endothelial cells mirror that of their extracellular environment (60). When exposed to high blood glucose concentrations, comparable to levels experienced by patients with T1DM, endothelial cells experience a large influx of glucose, which ultimately results in an increase in ROS. Increased ROS, such as superoxide anion, can inactivate NO by forming peroxynitrite (93), which in turn, can cause the uncoupling of eNOS and decreased production of NO (84). The overproduction of ROS by the mitochondria in response to hyperglycemia potentiates endothelial dysfunction by increasing polyol pathway flux, intracellular advanced glycation end products (AGE) formation, protein kinase C (PKC) activation, and hexosamine pathway flux (see review (45)). Thus, a
significant driving force in the pathogenesis of vascular complications in T1DM is believed to be hyperglycemia-related endothelial dysfunction and oxidative stress (24, 112, 116). Indeed, the degree of endothelial dysfunction in T1DM has been demonstrated to be closely related to HbA₁c, a marker of long-term glycemic control (111).

1.3.4 Vascular Insulin Resistance

Secondary to its metabolic effects, insulin can act as an important vasodilator (9, 19, 88). Endothelium-dependent vasodilation occurs following the binding of the insulin molecule to its receptor and the activation of the phosphatidylinositol 3-kinase (PI3K)-Akt signalling pathway. Subsequently, NO is produced by the phosphorylated form of eNOS. Insulin can also result in vasodilation by acting directly on vascular smooth muscle cells by modulating Ca²⁺ handling and sensitivity (9). It is believed that insulin-induced vasodilation occurs in order to assist in the delivery of insulin and glucose to skeletal muscle (75, 88). Conversely, insulin can also result in endothelium-dependent vasoconstriction by activating the Ras/mitogen-activated protein kinase (MAPK) pathway, which subsequently releases ET-1.

The vascular actions of insulin can be diminished in populations with T1DM (6, 19, 104, 132). Insulin resistance in the vasculature is characterized by diminished PI3K-Akt signalling (vasodilation) and increased MAPK signalling (vasoconstriction)(97). Deficient actions of insulin on the vasculature often occurs early in the course of endothelial dysfunction, making it both an early biomarker, as well as a promising avenue for the early treatment of vascular disease development (39, 65, 88). The implications of vascular insulin resistance are abundant. A larger reliance on the MAPK insulin signalling pathway is detrimental for vascular health due to an increase in the binding of adhesion molecules, inflammation, and net vasoconstriction (56, 87, 97). Further,
impaired insulin-induced dilation in resistance arteries results in the reduced delivery of glucose and insulin to skeletal muscle, which in turn can compromise glycemic control (19, 66, 87). In the macrovasculature, vascular insulin resistance can cause a predisposition to atherosclerosis (107). Aortic cells lacking the insulin receptor gene experience accelerated progression of atherosclerosis in the absence of elevated plasma lipids or hypertension (107). Vascular insulin resistance is specific to the vessel, in that impaired insulin-induced vasodilation in T1DM can occur even without the presence of whole-body insulin resistance (132).

1.4 Exercise and T1DM

A sedentary lifestyle has been shown to contribute to a number of diseases that plague our society and physical activity has been demonstrated to prevent a number of chronic diseases, including many diabetic-related pathologies (12). Prior to the discovery of insulin in 1921, in the late 1800’s regular exercise was suggested as a treatment option by one of the first practitioners treating patients with diabetes, Apollinaire Bouchardat (58, 59). In a recent systematic review of the literature it was demonstrated that sufficient evidence exists for clinicians to advocate regular physical activity for the management of T1DM (20). The Canadian Diabetes Association recommends that individuals with T1DM participate in 150 minutes of moderate intensity aerobic exercise (50-70% of maximal heart rate) weekly (over at least 3 days)(2). It is also suggested that resistance exercise be incorporated at least twice per week (2). The benefits of regular exercise for populations with T1DM are widespread and include: improved physical fitness, reduced insulin requirement, improved lipid levels and endothelial function, increased insulin sensitivity, reduced cardiovascular disease, and decreased mortality (20, 137).
1.5 Cardiovascular Benefits of Exercise for T1DM

Difficulties have arisen when investigating the development of diabetic cardiomyopathy in exercising populations, since dynamic exercise stimulates cardiac hypertrophy via many of the same pathways involved in pathological remodelling of the heart (82). Typically, increased left ventricular diameter and wall thickness occurs following aerobic exercise training due to volume overload and increases in blood pressure, a process known as eccentric hypertrophy (82). These processes occur to increase maximal cardiac output and reduce maximal heart rate during exercise. In contrast, wall thickness is increased following resistance exercise due to increases in systolic and diastolic blood pressure, a process known as concentric hypertrophy, which results in a small increase in maximal cardiac output (82). In insulin treated STZ-diabetic rats our laboratory has shown that aerobic exercise training results in beneficial growth of the heart, while sedentary T1DM rats develop pathological hypertrophy (79). Further, insulin-treated T1DM rats demonstrated increased constitutive levels of an important cardioprotective factor, Hsp70, which has been shown to protect the myocardium from an ischemic-reperfusion injury (79, 80, 99). In fact, myocardial Hsp70 has been shown to positively correlate ($r=0.97$) with a reduced infarct size following ischemia (53). Data showing the cardioprotective benefit of other modes of exercise in T1DM is limited. Although resistance exercise has been shown to provide protection from ischemic-reperfusion injuries in non-T1DM rats (114), evidence is still needed to determine the cardioprotective role of this form of exercise in populations with T1DM.

Slowed calcium clearance and abnormal cardiomyocyte excitation-contraction coupling is evident in T1DM (103, 108). Exercise training has been shown to increase the contractile force of the heart, largely through increased cardiac hypertrophy and
improvements in contractility of the cardiomyocyte. Further, exercise-induced enhancements in the contractility of the cardiomyocyte can occur due to the increased ability to transport calcium, as well as the improved response of myofilaments to calcium (61). Both aerobic or resistance exercise have been shown to increase sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) expression, an essential Ca\(^{2+}\) transporter (81, 125). Due to the substantial role insulin resistance can have on cardiomyocyte dysfunction, it is believed that the insulin sensitizing effect of regular exercise can have a beneficial impact on Ca\(^{2+}\) handling in T1DM (133).

The vascular benefits of aerobic exercise are believed to occur as a result of an increase in shear stress (or stretch) on the vessel, resulting in changes in endothelial cell gene expression and phenotype. However, questions still remain regarding the mechanosensory mechanisms governing these benefits (131). Instead of improving endothelial cell gene expression and phenotype, some argue that the changes to the endothelium that occurs with long-term exercise training are necessary to maintain a ‘normal’ endothelial cell phenotype (12). Nonetheless, regular exercise training results in positive arterial remodeling and increased endothelium-dependent vasodilation in the vascular beds of active skeletal muscles, as well as larger conduit arteries (131, 134).

Regular exercise has been shown to improve vascular and endothelial function in different vascular beds of human populations with T1DM (41, 74). These findings have been supported in animals models of T1DM where increased endothelium-dependent vasodilation was evident following both aerobic and resistance exercise training (18, 32, 86, 90, 95). Increased endothelium-dependent vasodilation in response to exercise training occurs largely as a result of the increased bioavailability of nitric oxide, through the upregulation of eNOS (32, 90, 95, 134). Vascular function is also increased indirectly
by improving lipid levels (increased HDL, decreasing LDL and triglycerides) (20). Although oxidative stress (e.g. ROS accumulation) due to chronic hyperglycemia is a significant factor contributing to endothelial dysfunction (84, 93, 111), it is not entirely clear whether improved glycemic control following exercise training is driving enhanced vascular function. There are a lack of studies providing evidence for improved HbA1c following regular exercise in patients with T1DM (20, 137).

Clinically, HbA1c, a marker of long-term glycemic control, is one of the more widely used predictors for the development of cardiovascular disease. In populations with T2DM, exercise is well established to reduce HbA1c levels (126); however, similar results in subjects with T1DM have been conflicting (20, 137). For example, Yardley et al. (137) conducted a systematic review and meta-analysis of the literature and determined that although results are promising there is currently a scarcity of well-designed studies to ascertain whether regular exercise training can reduce HbA1c. Reported improvements in glycemia following regular exercise have generally occurred following higher intensities of aerobic and resistance exercise (17, 38). However, the influence of exercise on HbA1c may be difficult to distinguish in clinical studies since physically active individuals with T1DM typically display higher HbA1c. In comparison to less active patients with T1DM, exercising patients with T1DM often intentionally elevate blood glucose levels prior to, and following exercise, in order to mitigate the risk of hypoglycemia (31).

Individuals with T1DM have also demonstrated impairments in the vascular actions of insulin (6, 19, 104, 132). While exercise training has been shown to have a positive role in enhancing vascular insulin sensitivity in a variety of populations (50, 83, 94, 97), few have studied how exercise can modulate the vascular actions of insulin in
T1DM. Work from our laboratory has shown an improvement in insulin-stimulated nerve arterial vasodilation in insulin treated STZ-diabetic rats following long-term aerobic exercise training (95). Similar results were demonstrated in untreated STZ-diabetic rats, where aerobic exercise increased insulin signalling in the cutaneous microvasculature (50). However, research in the area of T1DM-related vascular insulin resistance has focused on the microvasculature and aerobic treadmill training only, ignoring other exercise modalities (i.e. resistance exercise). Limited research conducted in humans has suggested that exercise training appears to only improve insulin sensitivity in the vasculature supplying active skeletal muscles. For example, following one-legged aerobic or resistance training in humans with T2DM the increase in leg blood flow during an euglycemic-hyperinsulinemic clamp is larger in the trained leg (30, 51). It is believed that shear stress may be modulating the insulin sensitizing effects of exercise on the vasculature (97). Further, the insulin sensitizing effects of aerobic exercise results in an increase in NO-induced vasodilation, as well as a reduction in insulin-stimulated ET-1 constriction (25, 97). Concurrent with increased insulin-stimulated nerve arterial vasodilation our laboratory has reported elevations in eNOS protein content following exercise training in T1DM rats (95). In models of T2DM, others have also shown a downregulation of molecular pathways involved in ET-1 production following exercise training (25, 83). Consequently, improvements in the vascular actions of insulin with regular exercise training could have positive implications on glycemic control by increasing insulin and glucose delivery to skeletal muscle and reducing the risk of atherosclerosis (19, 66, 87, 107). However, improvements in the vascular actions of insulin following exercise training are likely not a result of improved glycemia itself, since no improvements in insulin-stimulated vasodilation are evident following
metformin treatment in T2DM rats that exhibit lowered glycemia and body mass (26). This would suggest exercise training is essential to increase vascular insulin sensitivity in populations with diabetes and improving glycemic control alone is insufficient.

Despite all the aforementioned evidence supporting exercise prescription for patients with T1DM, some research suggests that exercise does not promote the same cardiovascular benefit as seen in non-diabetic populations. For example, a cross-sectional study conducted by Mason et al. (74) found that while regular exercise can protect against cardiovascular disease progression, the improvement in vascular function in T1DM may not occur to the same extent as individuals without diabetes. Indeed, a retrospective analysis of the DCCT found no evidence that physical activity improves microvascular outcomes in patients with T1DM (71).

1.6 Risks Associated with Exercise

One of the important revelations from the DCCT is that accompanying IIT is an increase in the risk of hypoglycemia (121, 122). While individuals with T1DM should partake in regular exercise in order to prevent the progression of cardiovascular complications, one of the most frequently identified causes of severe hypoglycemia is exercise (10). In fact, fear of hypoglycemia is reported as the largest barrier to regular physical activity for individuals with T1DM (13).

Maintaining blood glucose homeostasis is critical in order for the body to sustain metabolism. The brain, arguably the body’s most important organ relies almost entirely on circulating blood glucose. A person weighing 70kg relies on the continuous supply of approximately 4 grams of glucose in the blood for the optimal function of all cells in the body (129). Maintaining this concentration of blood glucose is accomplished at the expense of hepatic and muscle glycogen stores. Hepatic glycogen, through the process of
glycogenolysis, is responsible for increasing blood glucose concentrations by releasing glucose from glucose-6-phosphate (G6P) via the enzyme glucose-6-phosphotase (G6Pase). Since skeletal muscle lacks the enzyme G6Pase, muscle glycogen contributes to blood glucose homeostasis by sparing blood glucose and supplying working skeletal muscle with enough G6P to maintain glycolysis. Once glycogen stores are depleted, blood glucose concentrations are maintained by gluconeogenesis, the process of producing glucose from other sources besides glycogen, like the by-products lactate, glycerol or amino acids. The release of pancreatic hormones, insulin and glucagon, play an important role in the regulation of these processes, whereby insulin stimulates glycogen synthesis and glucagon initiates glycogen breakdown and the production of blood glucose.

1.6.1 Exercise-induced Hypoglycemia

The increased occurrence of hypoglycemia in patients with T1DM can be attributed to many factors. Intensive exogenous insulin regimes frequently cause hypoglycemia due to insulin overcorrection (insulin-induced hypoglycemia) (27, 76). Exercise can further increase the risk of hypoglycemia due to its insulin sensitizing effects (49) or through the increased mobilization of insulin from subcutaneous stores (40). The glucoregulatory defenses against hypoglycemia are also disturbed in T1DM. Shortly after the diagnosis of diabetes, the inability of the pancreas to secrete glucagon in response to hypoglycemia is evident (44). Although glucagon levels are still evident in the blood during exercise, the release of the hormone can be impaired during exercise and into recovery from exercise (76). Further, glucoregulatory defenses are altered following multiple bouts of exercise or prior hypoglycemia, as it has been reported that antecedent exercise or hypoglycemia reduces the release of glucagon during a subsequent exercise
session (29, 42, 43). Epinephrine, secreted from the adrenal gland, can also result in blood glucose production and is considered the last defense against hypoglycemia (27). Unfortunately, the epinephrine response to hypoglycemia is also diminished following a prior bout of hypoglycemia or exercise in populations with T1DM (29, 42, 43). Potentiating the risk of hypoglycemia, and secondary to impaired hormonal glucoregulation, is that patients with T1DM exhibit a deficiency in hepatic glycogen stores (11, 54, 76). Our laboratory has shown that in insulin-treated T1DM rats, several key enzymes involved in hepatic glycogen synthesis (glycogen synthase, glucokinase, phosphoenolpyruvate carboxykinase, etc.) are still present, and in some cases are even elevated compared to non-T1DM (76). These data suggest that other factors, such as the mechanisms governing hepatic glycogen storage and breakdown, may be altered in T1DM (76). Glycogen deficiencies can be detrimental when attempting to prevent hypoglycemia during exercise, since blood glucose production in response to both hypoglycemia and exercise originates predominantly from hepatic glycogen stores (62, 101, 127). The risk of hypoglycemia with regular exercise is not limited to during or immediately after the exercise; individuals with T1DM experience a heightened risk of hypoglycemia eight to ten hours after an exercise bout (78, 124). Indeed, the risk of nocturnal hypoglycemia is increased in those with T1DM and is amplified following exercise (55, 72, 117).

1.6.2 Exercise Prescription for T1DM

Due to the increased frequency of exercise-induced hypoglycemia, many research groups have attempted to determine the ideal exercise regime that could mitigate hypoglycemic risk. In populations with T1DM, moderate aerobic exercise leads to significant acute reductions in blood glucose, especially if the exercise is prolonged (16,
55, 135, 138). Due to its unique effects on glucose regulation, increasing the intensity at which exercise is performed has garnered the most attention in the literature (73). High-intensity exercise in patients with T1DM results in elevations in blood glucose concentrations due to a significant rise in catecholamines, which could be advantageous to exercising patients with T1DM to mitigate the development of hypoglycemia (73). Indeed, performing as little as a ten second sprint prior to moderate intensity exercise can diminish the magnitude of the immediate drop in blood glucose evident with moderate intensity aerobic exercise alone (15, 16). Brief bouts of high-intensity exercise over short durations has also provided benefits for attenuating the drop in blood glucose concentrations (1, 47, 48, 55). While these types of high intensity exercises are beneficial for preventing hypoglycemia acutely, there is evidence that they may increase the risk of nocturnal hyperglycemia (72). Further, when partaking in higher intensities of exercise individuals are at a heightened risk for the development of ketoacidosis (109). More recently, the benefits of resistance exercise for attenuating hypoglycemia have been highlighted (139). Resistance exercise results in similar acute glycemic benefits as high-intensity exercise and relies on similar anaerobic fuel sources. In contrast to higher intensities of aerobic exercise, resistance exercise does result in acute reductions in blood glucose, however, the drop in blood glucose is more gradual and does not appear to reach hypoglycemic blood glucose concentrations (135). Lastly, the integration of resistance and aerobic exercise has also showed promise. Indeed, prior resistance exercise attenuates hypoglycemia risk during subsequent aerobic exercise, believed to be due to the increased secretion of glucoregulatory hormones during resistance exercise (138, 140).
1.6.3 Exercise Training and Glucoregulation

While the use of different forms of exercise (higher intensity, resistance) for preventing exercise-induced hypoglycemia in populations with T1DM has been promising (15, 16, 135, 140), little work has investigated their cardiovascular benefit. In this regard, studies have generally focused on the blood glucose response to a single bout of exercise, whereas the manifestation of hypoglycemia following exercise training, the form of exercise that would be effective for cardioprotection, is unknown. Many factors responsible for governing blood glucose control change over the course of exercise training. In T1DM, exercise training is known to result in alterations to insulin sensitivity and insulin dosing (35, 49), increased glucagon sensitivity and secretion during recovery from exercise (37, 68, 76), increased epinephrine secretion during exercise (76), and positive alterations to insulin-producing pancreatic β-cells (21, 23, 52). Further, the response of most glucoregulatory hormones have been shown to be blunted following a previous bout of exercise or hypoglycemia (29, 42, 43). Specifically, high intensity aerobic exercise has been shown to impact the glucose counterregulatory responses to subsequent hypoglycemia (77). While glycogen metabolism is also known to change over the course of exercise training, it appears that in T1DM aerobic exercise training is unable to increase hepatic glycogen stores, despite increases in hepatic glycogen synthase (76). On the other hand, resistance exercise has been shown to increase hepatic glycogen content in non-T1DM animals (105). While it is not clear whether resistance exercise can improve hepatic glycogen stores in patients with T1DM, resistance exercise has been shown to alleviate exercise-induced hypoglycemia in population with T1DM (136).
1.7 Rationale

While strict glycemic control has been shown to provide cardiovascular benefits, mounting evidence has identified complications associated with this treatment strategy (risk of hypoglycemia, insulin resistance, weight gain). Regular physical activity is well-established to have numerous health benefits, particularly relating to cardiovascular health, yet hypoglycemia remains a significant barrier to its prescription (12). Utilizing different modalities of exercise (higher intensity exercise or resistance exercise) has provided optimism for limiting the risk of exercise-induced hypoglycemia in patients with T1DM; however, the optimal intensity, duration or modality of exercise which provides the largest degree of cardiovascular protection has yet to be determined (20). In order for physical activity to become a common practice for individuals with T1DM, the overall cardiovascular benefit and risk of hypoglycemia development within each modality of exercise must be established.

1.8 Thesis Objectives

1) To assess the risk of hypoglycemia development following different modes of acute exercise in T1DM and to establish whether this risk changes over the course of training.

2) To determine which modality of exercise training provides the largest degree of cardiovascular protection in T1DM.

3) To explore whether certain modalities of exercise training, when paired with modest glycemic control, results in larger cardiovascular protection than intensive insulin therapy alone.
1.9 Overview and Thesis Development

It is clear from the current literature that chronic hyperglycemia often experienced by individuals with T1DM contributes to a heightened risk of cardiovascular disease (123). While this is supported in studies using the T1DM model induced by STZ, it is not entirely clear if the pathology of the disease evident with this experimental model is the same as that occurring in patients with T1DM. Further, STZ-induced T1DM animals exhibit blood glucose concentrations that are at least two-fold higher than patients with T1DM (110, 121), challenging our understanding of how glycemia may impact the development of cardiovascular disease. Indeed, due to the countless actions of insulin in the human body, it is important that insulin treatment be accounted for in experimental models of T1DM (63, 87). Individuals with T1DM that have the greatest risk for developing cardiovascular disease have difficulty achieving rigorous glycemic control through intensive insulin therapy (IIT), and thus, are often referred to as poorly controlled patients with T1DM (123). As such, the focus of this dissertation was to examine the impact of regular exercise on this particular population of T1DM, using a novel insulin-treated, yet poorly controlled rat model of T1DM, believed to be more representative of human populations with T1DM (79, 110, 121). This model has been shown to display moderate hyperglycemia, comparable blood glucose fluctuations during exercise (76), and similar cardiovascular pathologies to human populations with T1DM (79, 89).

The objectives of this thesis were to determine the ideal exercise regime which produces the greatest cardiovascular protection for populations with T1DM, while assessing the risk of exercise-induced hypoglycemia following exercise training. Initially, due to the paucity of data investigating whether the risk of exercise-induced hypoglycemia changes over the course of exercise training, it was determined whether the
magnitude of the acute reduction in blood glucose in response to exercise was changed over the course of exercise training in T1DM rats (*Chapter 2*). A moderate aerobic exercise was chosen in the first study since our laboratory had shown that this exercise regime provided a cardiovascular benefit to T1DM rats (79). In this first study, the results demonstrated that the abrupt reduction in blood glucose concentrations in response to moderate aerobic exercise remained similar throughout the duration of training. In a follow up study (*Chapter 3*), it was determined whether fluctuations in blood glucose in response to different exercise modalities (high intensity aerobic, low intensity aerobic, and resistance) were variable and whether a particular form of exercise provided a larger amount of cardioprotection. In this study, exercise training lasted for six weeks, a time period that was chosen because we had previously established that this duration of aerobic and resistance exercise training was sufficient to improve insulin sensitivity (49). It was concluded that high intensity aerobic exercise provided the largest amount of cardioprotection and that this exercise regime appears to be manageable provided that it is accompanied by less stringent glycemic control (CIT). While short-term resistance exercise did not result in improved cardioprotection, it was determined that a prolonged training period may be needed for this type of training modality to improve cardioprotection (36, 114). Accordingly, in the third study of this dissertation (*Chapter 4*) it was investigated whether prolonging resistance exercise training, or the integration of aerobic exercise with resistance exercise, would lead to similar cardioprotection as high intensity aerobic exercise alone. Secondly, and perhaps more importantly, it was also determined whether the degree of cardioprotection provided by these different exercise regimes (when paired with less stringent glycemic control) was larger than simply improving glycemia through intensive insulin therapy. Lastly, concurrent with the third
study (*Chapter 4*), vasomotor function was also assessed in the femoral artery following both aerobic and resistance exercise training, which in turn, was compared exclusively to intensive insulin therapy alone (*Chapter 5*). This final chapter was completed in order to determine if the magnitude of cardioprotection following different modalities of exercise training was similar in the peripheral vasculature and not specific to the heart itself.
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CHAPTER 2

2 «Morphological assessment of pancreatic islet hormone content following aerobic exercise training in rats with poorly controlled Type 1 diabetes mellitus»

2.1 Introduction

Type 1 diabetes mellitus (T1DM) is characterized by the autoimmune destruction of insulin producing β-cells within pancreatic islets, resulting in the requirement of exogenous insulin in order to limit chronic hyperglycemia and prevent long-term complications (43). Accompanying insulin supplementation is the risk of overcorrection, leading to an increased incidence of severe hypoglycemia (43, 44). Further, the lack of insulin management in T1DM often leads to the development of insulin insensitivity and increased cardiovascular risk (18, 24). Regular exercise has been shown to improve many diabetes-related complications including enhanced glucose tolerance and improved cardiac function in T1DM (18, 33). However, due to a lack of insulin control and heightened incidence of hypoglycemia during and following exercise, many patients with T1DM refrain from exercise (3, 32).

Insulin and glucagon are the two most important hormonal regulators of blood glucose concentrations in response to hypoglycemia, as well as glucose production during moderate intensity aerobic exercise (8, 11, 29, 47). While a reduction in islet insulin content and its release from pancreatic islets is well characterized in T1DM, impaired glucagon release from islet α-cells is poorly understood. Shortly after the onset of the disease, secretion of glucagon is absent upon exposure to hypoglycemia, concomitant with an overall decrease in the hepatic response to glucagon (17, 36). Interestingly,
impaired glucagon function occurs despite postprandial hyperglucagonemia becoming more apparent as the disease progresses (6). Experimentally, α-cell expansion is evident in streptozotocin (STZ)-induced rats with T1DM in comparison to non-diabetic rats (27).

It is well recognized that following aerobic training both insulin and glucagon sensitivity are improved (13, 18, 25). It is unknown if exercise training can improve pancreatic islet hormone content in populations with T1DM. Findings have supported a role of aerobic training in improving insulin content within pancreatic islets of STZ-induced T1DM animals; however, research to date has been completed in severely hyperglycemic animals not treated with exogenous insulin (10, 20). This model is not reflective of the exercising population with T1DM, as patients are not likely to partake in exercise without accompanying exogenous insulin treatment. Further, most patients with T1DM display moderate levels of blood glucose even in the presence of insulin supplementation, due to the difficulty of maintaining tightly regulated blood glucose concentrations (42). Exercise training has been shown to limit the progression of type 2 diabetes onset by inducing β-cell hyperplasia, by stimulating proliferation and suppressing apoptosis through the activation of the insulin/IGF-1 signaling cascade (9). Further, exercise training can stimulate islet growth and survival pathways in non-diabetic rats (7). It has yet to be established if glucagon content in T1DM pancreatic islets is normalized following aerobic training; although differences in glucagon sensitivity have been reported following training. Drouin et al.(13) reported that exercise trained subjects demonstrate an increased hepatic sensitivity to glucagon, while Légaré et al.(25) reported an increased density of glucagon receptors in the liver of swim trained rats. Further work is needed to better understand the impact of aerobic training in the restoration of pancreatic islet morphology, particularly in insulin-treated rats with T1DM.
Given the risks associated with exercise-induced hypoglycemia, studies examining blood glucose concentrations in response to exercise are acute in duration (a single bout of daily exercise)(1, 22, 49). Our group recently demonstrated that exercise training of various modalities is beneficial for improving insulin sensitivity measures and insulin dosage requirements in rats with T1DM (18). It is unknown if the increase in insulin sensitivity as a result of exercise may result in an increased risk of exercise-induced hypoglycemia, as exogenous insulin is a contributing factor in hypoglycemia development in response to exercise (5, 12). It is unknown if acute reductions in blood glucose as a result of exercise may change following exercise training in populations with T1DM.

Using a model in which rats are moderately hyperglycemic and insulin-treated, the purpose of the present investigation was to: 1) determine the impact of a 10-week aerobic training program on its ability to normalize pancreatic islet composition; 2) examine if the acute exercise-mediated reduction in blood glucose is altered over the course of exercise training. It was hypothesized that 10 weeks of exercise training would lead to restoration of pancreatic insulin and glucagon content; however, exercise training will cause exercise-induced reductions in blood glucose to increase in magnitude.

2.2 Methods

*Ethics Approval and Animals.* This study was approved by the Research Ethics Board of The University of Western Ontario in accordance with the guidelines of the Canadian Council on Animal Care. Eight-week old, male Sprague-Dawley rats were obtained from Charles River Laboratories (St. Constant, QC, Canada), housed in standard rat cages, and maintained on a 12-hour light/dark cycle at a constant temperature and
humidity. Rats were allowed access to standard rat chow and water *ad libitum*. Twenty-two rats were randomly assigned to either non-T1DM sedentary control (*C*; *n*=5), non-T1DM moderate aerobic exercise (*C-AT* <sub>mod</sub>; *n*=5), T1DM sedentary (*D-CIT*; *n*=6), T1DM moderate aerobic exercise (*D-AT* <sub>mod</sub>; *n*=6).

*Diabetes Induction.* A multiple low-dose STZ-induced T1DM model was employed. T1DM was induced by intraperitoneal injection of STZ (20mg/kg; Sigma-Aldrich, Oakville, ON, Canada) dissolved in citrate buffer (0.1 M, pH 4.5) on five consecutive days. This multiple low-dose model of T1DM has been established to elicit immune-mediated destruction of pancreatic β-cells similar to the normal pathogenesis of a patient with T1DM (33, 35). All injections were sterile, filtered (0.2µm), and occurred within 5 minutes of STZ being dissolved in the citrate buffer. Diabetes was confirmed following two consecutive blood glucose concentrations of >18mM. Following T1DM confirmation, an insulin pellet (1 pellet; 2U insulin/day; Linplant, Linshin Canada Canada, Inc., Toronto, ON, Canada) was implanted subcutaneously in the abdomen. Blood glucose concentrations were closely monitored throughout the study (see below) and insulin pellets were adjusted to maintain blood glucose within a moderate glycemic range (9-15mM). This blood glucose range is typically observed in patients with T1DM prior to the initiation of exercise and undergoing conventional insulin therapy (CIT) (39, 43).

*Exercise Protocol.* Animals were gradually introduced to the exercise training program with familiarization to the treadmill on two separate occasions (15m/min, 2% grade for 15 minutes). Subsequently, training consisted of treadmill running for one hour each day at a 2% grade, 5 days a week for a total of 10 weeks. The 10 weeks of treadmill training consisted of 17m/min (week 1), 24m/min (week 2), and subsequently 27m/min
for the completion of the study (week 3-10). Treadmill running at 27m/min represents approximately 70-80% of a STZ-induced diabetic rat’s VO$_2$ max (40). Continuous running was encouraged by compressed air or tactile stimulus when rats reached the back of the treadmill and broke a photoelectric beam.

_Blood Glucose Concentrations._ Non-fasted blood glucose concentrations were obtained from the saphenous vein at the beginning of and following exercise training using a Freestyle Lite Blood Glucose Monitoring System (Abbott Diabetes Inc., Mississauga, ON, Canada). Blood glucose concentrations were also recorded prior to, and immediately following an acute bout of treadmill training, during weeks 1, 4, 7 and 10 of the aerobic training period.

_Tissue collection and immunohistochemistry._ Pancreatic tissue was extracted from animals four days following the last exercise bout. Pancreata were fixed in 10% normal buffered formalin for 72 hours at 4°C and placed in ethanol prior to tissue processing and paraffinization. Paraffin embedded tissue was sectioned at 5µm and mounted on positively charged slides (Fischer Scientific Superfrost Plus #12-550-15). Immunohistochemical staining was conducted using the avidin-biotin complex technique. Paraffin embedded pancreata were deparaffinized and hydrated in xylene and graded ethanol solutions. Following deparaffinization, pancreata were subjected to heat-induced epitope retrieval (antigen retrieval buffer: 10mM sodium citrate buffer, pH 6, 0.05% Tween-20). Slides were then quenched in 3% hydrogen peroxide solution for 20 minutes, washed in phosphate buffered saline (PBS) for 5 minutes and blocked in 10% goat serum overnight at 4°C. All antibodies were diluted with 1% goat serum and incubated for one hour at room temperature: insulin (1:100; Dako: A0564), glucagon (1:4000; abcam: k79bB10) and Ki67 (1:100; abcam: ab16667). Corresponding biotinylated secondary
antibodies were diluted in 1% goat serum (1:200, Vector Laboratories Inc.) and incubated for 1 hour at room temperature. Sections were then treated with Vectastain solution (Vectastain ABC Elite Kit, PK-6100 Vector Laboratories Inc.) for 1 hour at room temperate prior to being exposed to diaminobenzidine (DAB; Vector Laboratories, SK-4100) for 7 minutes. Tissues were then counterstained with hematoxylin (Sigma-Alderich hematoxylin solution: GHS-3).

**Immunohistochemistry Quantification.** Insulin and glucagon within pancreatic islets were examined by measuring the cumulative signal strength of images, a quantitative immunochemistry technique established by Matkowskyj and colleagues (30, 31). Approximately 30 islets from each animal were visualized using a Zeiss Axiovert S100 inverted microscope in combination with a Sony PowerHAD (DXC950-3CCD color) camera. For each islet analyzed, two serial secretions of pancreata within 5µm were used, with only one section being exposed to primary antibody. In order to account for possible visual differences in exocrine tissue between stained and unstained images, and to make backgrounds more homogenous, each islet was placed on a black background. A black pixel is represented by a zero in each of the three (red, blue, green) colour channels. All islets were cropped using Photoshop (Adobe Photoshop CS6) and placed on a black background. Consistent with Matkowskyj and colleagues (30, 31) the amount of ‘energy units (eu)’ from the image of a non-stained islet was subtracted from the eu of the same stained islet using Matlab (Mathworks, Version R2009a). Differences in islet size were accounted for by dividing eu by the number of pixels making up each individual islet. Ki67 positive cells within pancreatic islets were counted by two observers blind to experimental conditions. Pancreatic islet area and diameter were determined for each individual cropped islet with the mean islet diameter in the current
study being ~50µm. Small islets were classified as having a diameter of 50µm or less while large islets had a diameter of greater than 50µm.

**Western Blotting.** Red vastus muscle (100 mg) was homogenized in a 1:10 ratio of homogenizing buffer (100 mmol/L NaCl, 50 mmol/L Tris base, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA and 1% Triton × 100, adjusted to pH ~7.5%, 1% phosphatase inhibitor and 1% protease inhibitor) and protein concentrations were determined using the Bradford method (4). Homogenates were mixed with equal volumes of sample buffer (0.5 M Tris base, 13% glycerol, 0.5% SDS, 13% β-mercaptoethanol and bromophenol blue) and separated according to their molecular weight on 10% acrylamide separating gel and 4% acrylamide stacking gel. Each gel contained a molecular weight marker (Bio-Rad: catalog no. 161-0373) to determine the molecular weight of the proteins as well as a soleus muscle, which served as a positive control to permit the standardization of each band across the various gels. After electrophoresis, proteins were transferred to nitrocellulose membranes and blocked in 5% milk blotto (BioRad 170-6404) in Tris-buffered saline (TBS; 10 mmol/L Tris, 100 mmol/L NaCl, 0.1% Tween-20, pH 7.5) for 2 hours. Membranes were incubated with primary antibodies specific to GLUT4 (rabbit mAb anti-GLUT4, Cell Signaling 2213, 1:1000) and insulin receptor (rabbit mAb anti-insulin receptor beta, Cell Signaling 3025, 1:1000) in TTBS (0.01% Tween 20 and TBS) with 2% milk blotto. Membranes were then washed and incubated for 1 hour with horseradish peroxidase-conjugated secondary antibody specific for rabbit IgG (goat anti-rabbit IgG-HRP conjugate, BioRad, 1:5000) diluted in TTBS with 2% non-fat dry milk. Following 3 washes in TTBS for 10 minutes membranes were developed using chemiluminescent substrate (BioRad Western C Enhanced Chemiluminescent Kit, 170-5070). Protein bands were captured using a BioRad Chemidoc XRS imager and optical
densities were quantified using BioRad Quantity One software (Bio-Rad, Hercules, CA, USA). Optical densities were normalized to the soleus muscle positive control on each blot.

**Statistical Analysis.** Body weight, blood glucose concentrations, GLUT4 and insulin receptor protein content, islet hormone content (insulin and glucagon) and islet area were compared using a two-way ANOVA. Blood glucose concentrations in response to aerobic exercise were analyzed using a two-way repeated measure ANOVA. Insulin and glucagon content, as well as Ki67 positive cells based on islet size, were compared using a three-way ANOVA. A Tukey’s post hoc test was performed with a significance level set at P<0.05. All data are expressed as mean ± SE and statistical analysis was performed using SigmaPlot 11 statistical software.

2.3 Results

*Animal weights and blood glucose.* All rats gained weight following the completion of the 10-week aerobic exercise training program (p<0.05). C weighed significantly more than both D-CIT and D-AT\textsubscript{mod} (p<0.05), while D-AT\textsubscript{mod} weighed significantly less than C-AT\textsubscript{mod} (p<0.05; Table 2.1). All rats began the experiment at similar blood glucose concentration, while at the completion of the aerobic training program blood glucose concentrations of D-CIT and D-AT\textsubscript{mod} were significantly higher than both C and C-AT\textsubscript{mod} (p<0.05). This was not influenced by aerobic training (p>0.05). The acute reduction in blood glucose concentrations in response to a single bout of aerobic exercise were significantly different between C-AT\textsubscript{mod} and D-AT\textsubscript{mod} (Figure 2.1; p<0.05). D-AT\textsubscript{mod} demonstrated a significant reduction in blood glucose concentrations after each exercise bout, while C-AT\textsubscript{mod} demonstrated significant increases in blood
glucose concentrations compared to pre-exercise (p<0.05). These acute changes in post-exercise blood glucose concentrations were not altered over the course of 10 weeks of exercise training in C-AT\textsubscript{mod} or D-AT\textsubscript{mod} rats (P>0.05).

*Pancreatic islet insulin and glucagon content.* Pancreatic islet insulin content was significantly reduced in rats with T1DM compared to rats without (p<0.05; Figure 2.2), while islet glucagon content was significantly increased in rats with T1DM compared to rats without (p<0.05; Figure 2.3). The aerobic exercise program did not significantly alter insulin or glucagon content in rats with or without T1DM (p>0.05).

*Comparisons of pancreatic islets by size.* T1DM led to a significant reduction in pancreatic islet size (p<0.05), independent of aerobic training (p>0.05; Figure 2.4). There was no significant three-way interaction for islet insulin or glucagon content between T1DM, aerobic training and islet size (p>0.05). There was a main effect of islet size on hormone content, as smaller islets had significantly more insulin and glucagon than large islets (p<0.05; Figure 2.5). Large islets had significantly more Ki67 positive cells than small islets based on the three-way ANOVA (p<0.05; Figure 2.6). Further, there were also main effects of diabetes and exercise (p<0.05). However, there was no significant interactions for Ki67 positive cells and islet size, diabetes or exercise (p>0.05).

*GLUT4 and insulin receptor protein content.* Exercise training led to a significant increase in GLUT4 protein content in rats with T1DM (p<0.05; Figure 2.7). There were no significant differences in insulin receptor protein content across experimental groups (p>0.05).
Table 2.1: Animal characteristics. (*) indicates a significant difference from pre-training (p<0.05). (†) indicates a significant difference from non-T1DM sedentary rats (C) following training (p<0.05). (#) indicates a significant difference from non-T1DM exercised rats (C-AT\textsubscript{mod}) following training period (p<0.05). Data are expressed as a mean ± SE for each group.

<table>
<thead>
<tr>
<th></th>
<th>Weight (g)</th>
<th>Blood Glucose Conc. (mM)</th>
<th>Pre Training</th>
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<tr>
<td>C (n=5)</td>
<td>338.9 ± 5.3</td>
<td>631.2 ± 26*</td>
<td>5 ± 0.1</td>
<td>4.6 ± 0.2</td>
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<tr>
<td>C-AT\textsubscript{mod} (n=5)</td>
<td>350.6 ± 6.1</td>
<td>596.5 ± 16.8*</td>
<td>5.6 ± 0.3</td>
<td>4.1 ± 0.6</td>
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<tr>
<td>D-CIT (n=6)</td>
<td>345.4 ± 3.1</td>
<td>561.8 ± 11.5*†</td>
<td>6.4 ± 0.5</td>
<td>12.2 ± 1*†#</td>
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<tr>
<td>D-AT\textsubscript{mod} (n=6)</td>
<td>341.7 ± 3.2</td>
<td>526.6 ± 14.7*†#</td>
<td>6 ± 0.3</td>
<td>11.6 ± 1.6*†#</td>
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Figure 2.1. The change in blood glucose concentrations from pre to post exercise. The change in blood glucose concentrations in response to exercise was significantly different between non-T1DM exercised rats (C-AT\textsubscript{mod}) and T1DM exercised rats (D-AT\textsubscript{mod}; P<0.05). A significant increase in blood glucose was evident in response to exercise in C-AT\textsubscript{mod} rats while a significant decrease was evident in D-AT\textsubscript{mod} rats (P<0.05). The change in blood glucose concentration following exercise was not significantly changed at any point during training in C-AT\textsubscript{mod} or D-AT\textsubscript{mod} rats (P>0.05). Data are expressed as a mean ± SE for each group.
**Figure 2.2.** Representative images of pancreatic islet insulin (A) and insulin quantification (B). Pancreatic islets from both sedentary and exercised rats with T1DM had significantly less insulin staining than sedentary and exercised non-T1DM rats (P<0.05). (*) indicates a significant difference from C. (#) indicates a significant difference from C-AT\textsubscript{mod} (P<0.05). Data are expressed as a mean ± SE for each group (40x magnification; Bar=50 µm).

**Figure 2.3.** Representative images of pancreatic islet glucagon (A) and glucagon quantification (B). Pancreatic islets from both sedentary and exercised rats with T1DM had significantly more glucagon staining than sedentary and exercised non-T1DM rats (P<0.05). (*) indicates a significant difference from C. (#) indicates a significant difference from C-AT\textsubscript{mod} (P<0.05). Data are expressed as a mean ± SE for each group (40x magnification; Bar=50 µm).
Figure 2.4. Pancreatic islet area. Pancreatic islets from both sedentary and exercised rats with T1DM were significantly smaller than sedentary and exercised non-T1DM rats (P<0.05). (*) indicates a significant difference from C. (#) indicates a significant difference from C-AT\textsubscript{mod} (P<0.05). Data are expressed as a mean ± SE for each group.

Figure 2.5. Pancreatic islet insulin (A) and glucagon (B) staining by diameter (small islets < 50 μm). Small islets had significantly more insulin and glucagon staining than larger islets. (*) indicates a significant difference (P<0.05). Data are expressed as a mean ± SE for each group.
Figure 2.6. Representative Ki67 stain (A) and number of Ki67 positive cells per islet (B) based on islet diameter (small islets < 50 µm). Large islets had significantly more Ki67 positive cells. (*) indicates a significant main effect of islet size (P<0.05). Both diabetes and exercise training had a significant main effect on Ki67 positive cells (P<0.05). Data are expressed as a mean ± SE for each group (40x magnification; Bar=50µm).

Figure 2.7. GLUT4 (A) and insulin receptor (B) protein content in the red vastus muscle. Exercise training resulted in a significant increase in GLUT4 protein content in rats with T1DM (P<0.05). No change in insulin receptor protein content was evident across experimental groups (P>0.05). (*) indicates a significant difference compared to D-CIT (P<0.05). Data are expressed as a mean ± SE for each group.
2.4 Discussion

While exercise training has been shown to increase insulin sensitivity in patients with T1DM, it is unknown if this is accompanied by increased islet insulin content. Coskun et al. (10) determined via immunohistochemistry that aerobic training has positive effects on pancreatic β-cells during the induction phase of T1DM through STZ injections. Further, it has been reported that following 6 weeks of free-wheel training, STZ-induced T1DM mice demonstrated elevated levels of islet insulin staining, as well as basal insulin release from isolated islets (20). Contrary to these findings, the present investigation found that 10 weeks of aerobic training failed to significantly alter insulin content in pancreatic islets. A possible explanation for these discrepancies might be the time at which the exercise regime was initiated. In the study by Coskun et al. (10), treadmill exercise was initiated prior to, and during STZ administration, while free-wheel exercise in the Huang et al. (20) study took place just three days following STZ administration. Indeed, similar to the current study, Howarth et al. (19) found that treadmill exercise initiated one week after the last STZ injection had no effect on the distribution of insulin within islets. These findings may suggest that aerobic exercise can have a positive influence on islet insulin content if exercise is initiated early in the course of T1DM development. Further, the role of exercise may be better suited to alleviate β-cell degradation, rather than reversal of insulin loss after T1DM onset (10).

The current study also significantly differs from others with regard to the blood glucose level at which T1DM rats were maintained. In the present study both exercise trained and sedentary rats with T1DM were treated with subcutaneous insulin pellets in order to maintain blood glucose in a moderately hyperglycemic range (9-15-mM). This
range is within that typically observed for patients with T1DM treated with conventional insulin therapy (43). Further, individuals with T1DM are typically educated to refrain from exercise when blood glucose concentrations are greater than 14mM, as elevated blood glucose likely implies elevated ketones which can become detrimental and exacerbated by exercise (39). It is suggested that T1DM populations begin an exercise bout when blood glucose concentrations are between 5-13.9mM (39), however, most studies utilize animal models of T1DM in the absence of insulin supplementation and are severely hyperglycemic. In contrary to Coskun et al. (10), in the current investigation rats with T1DM were insulin treated and had similar blood glucose levels in both sedentary and trained groups. In this respect, exercise training may not have increased islet insulin content in the Coskun et al. (10) study but rather sedentary diabetic rats experienced larger insulin degradation as a result of their higher glycemic level (10). Indeed, prolonged exposure of isolated islets to high glucose concentrations greatly reduces islet insulin content (15).

It is well-recognized that T1DM is associated with an insulin deficiency within pancreatic islets; however, there is still uncertainty whether islet glucagon content may change over the course of diabetes. Some evidence has suggested there is little change in islet α-cells and glucagon content in both genetically-induced Biobreeder (BB) rats and chemically-induced STZ rats with T1DM (37), while others have shown significant increases in islet glucagon (19, 20, 27, 38). Consistent with the latter studies, we demonstrate that rats with T1DM had significantly more glucagon content per islet than rats without T1DM. The potential role that increased glucagon content in the pancreatic islet may have in exacerbating hyperglycemia has historical context. In 1975, Unger and Orci (46) proposed the bihormonal-hypothesis which suggests that chronic hyperglycemia
in T1DM is due to an insulin deficiency combined with an excess in circulating glucagon. T1DM is typically associated with postprandial hyperglucagonemia as the disease progresses, which may be reflected by the increased pancreatic glucagon content demonstrated in the present study (6). Elevated levels of glucagon as T1DM progresses has been suggested as a potential therapeutic target in the early course of T1DM (6). As such, the potential impact that aerobic training may have in reducing pancreatic islet glucagon content and subsequently hyperglucagonemia may prove useful as a therapeutic strategy.

Exercise training has been shown to increase glucagon sensitivity through changes in glucagon receptor density and increased hepatic glucose production in response to a glucagon bolus (13, 25). Despite these changes it is unknown how glucagon content within pancreatic islets may change following exercise training. In the current study, the aerobic training program did not alter glucagon content in pancreatic islets of rats with T1DM. While these results were surprising, they may suggest that islet hormone content may not be the primary determinant governing its release and sensitivity; rather, neural or hormonal factors regulating islet hormone release may be of greater importance. This is supported by the fact that extremely low amounts of glucagon producing α-cells are still capable of maintaining glycemic control (45). Further, in comparison to rats without diabetes, STZ-induced T1DM animals exhibit a two-fold greater glucagon response to epinephrine, which is returned to normal levels following 10 weeks of aerobic training (34). Interestingly, this training-mediated alteration in glucagon release was also not explained by islet glucagon or insulin content, and similarly to the current findings, islet hormone content remained unchanged as a result of the training program (34).
Exercise-induced reductions in blood glucose occurs frequently in individuals with T1DM (3). Similarly, in the present study rats with T1DM frequently experienced significant exercise-induced reductions in blood glucose, while rats without T1DM experienced a rise in blood glucose concentrations. The different blood glucose responses to exercise between rats with and without T1DM likely occurred due to an inability to inhibit blood insulin concentrations in rats with T1DM, concomitant with increased insulin sensitivity as a result of the exercise (41, 50). The normal physiological response to exercise is to decrease the insulin to glucagon ratio, therefore promoting glucose production (47). However, similarly to the exercising T1DM population, rats with T1DM are unable to reduce exogenous insulin in circulation and thus blood glucose levels fall.

A significant portion of individuals with T1DM have difficulty regulating insulin dosing and suffer from insulin insensitivity and insulin resistance. While it is unclear as to the mechanisms by which T1DM individuals develop insulin resistance, they are disassociated from mechanisms-related to T2DM (2). As a consequence, insulin overcorrection is an unavoidable contributing factor to most hypoglycemic episodes (44). Further, insulin therapy is also problematic during glucose demanding states whereby circulating insulin levels must be intensely regulated (i.e. exercise)(5, 12). Exogenous insulin can potentiate the glucose demand of exercise and increase blood glucose clearance. Our laboratory has shown that exercise, regardless of modality, results in an increase in insulin sensitivity, shown by reduced exogenous insulin requirements, increased glucose clearance to a glucose tolerance test, as well as alterations in GLUT4 in skeletal muscle (18, 33). Exercise has also been shown to increase subcutaneous insulin absorption, which can ultimately lead to more significant insulin-induced reductions in blood glucose (12). As such, it was hypothesized that concomitant with the expected
increase in insulin sensitivity from exercise training, the reduction in blood glucose in response to an exercise bout would become larger in depth, ultimately increasing the frequency of exercise-induced hypoglycemia. Contrary to our hypothesis, the reduction in blood glucose which occurred following an exercise bout remained the same throughout 10 weeks of training. This exercise-induced reduction in blood glucose occurred despite elevations in GLUT4 being evident following exercise training in rats with T1DM. The increase in GLUT4 protein content is likely even larger than shown in the current study, as translocation of GLUT4 to the cell membrane is transient and is typically greatly reduced by 48 hours post-exercise (16). A possible explanation for this finding is that increases in glucagon sensitivity likely occurred concurrently with insulin sensitivity. Indeed, it has been shown that aerobic training can increase the response of gluconeogenesis and glycogenolysis to glucagon (13, 14). This expected increase likely attenuated significant reductions in blood glucose, as glucagon itself accounts for more than 60% of glucose production during exercise (47, 48). In summary, these results and the findings of others suggest that regulation of blood glucose post-exercise is multifaceted and that several factors must be considered when attempting to improve the metabolic response to exercise in patients with T1DM.

While aerobic training was unable to alter pancreatic islet hormone content in rats with T1DM, evidence exists which suggests that pancreatic islets respond differently based on their size (21, 26, 28). Early reports suggested that the bulk of islet endocrine function comes from larger islets and small islets had little functional importance (23). However, to date this view has been challenged by evidence suggesting that small islets contain more insulin, are superior in insulin secretion, and have better transplantation outcomes compared to large islets (21, 26, 28). The majority of this work cites larger
increases in C-peptide and insulin secretion from small islets in isolated islet preparations; therefore, it was important to explore different islet subpopulations in an *in vivo* setting (21, 26, 28). Results from the present study support the view that small islets (<50µm in diameter) in rats with and without T1DM have significantly more insulin content per islet than larger islets. Huang et al. (21) also observed *in vivo* through immunochemistry that small islets had more insulin content than larger islets in rats without T1DM; however, until now this observation had not been demonstrated in T1DM pancreatic islets. Less information is available as to the distribution of glucagon with respect to islet size. MacGregor et al. (28) observed that glucagon content within small and large islets was distributed similarly between both subpopulations. However, to our knowledge, this is the first experiment to demonstrate that glucagon content was higher in smaller islets in rats with and without T1DM. Ultimately, the differences in the amount of insulin and glucagon content in different sizes of pancreatic islets supports the view that different subpopulations of pancreatic islets exist. Further, this suggests that the significant increase in glucagon content within the pancreatic islets of rats with T1DM may exist due the presence of more small islets than large. In order to determine if the increase in islet hormone content in small islets occurred as a result of increased cell proliferation the number of Ki67 positive cells were examined. While Ki67 positive cells were significantly higher in large islets, cell proliferation was likely occurring at the same rate between small and large islets, as proliferating cells within islets were minimal. Despite cell proliferation not occurring at a high rate, islets may be undergoing dedifferentiation or hypertrophy. Indeed, others have shown that small islets consistently had a higher cell density when compared to larger islets (21). Further work is required to determine the
implications of islet size on both insulin and glucagon release, although it does not appear that aerobic training is able to alter changes in islet size in rats with T1DM.

To date, studies examining alterations in islet hormone content following aerobic training were conducted in T1DM animals without insulin supplementation, an environment not representative of human population with T1DM. Using a conventional insulin therapy model, the present investigation determined whether 10 weeks of aerobic training could restore T1DM pancreatic islet hormone composition to similar levels as age matched rats without T1DM. It was concluded that 10 weeks of exercise training was unable to improve insulin and glucagon content in pancreatic islets. Further, the current study supports the view that different islet subpopulations, based on size, may exist. Although no islet composition changes were evident following exercise training, the magnitude of exercise-induced blood glucose reductions remained constant across the 10 weeks of training.
2.5 References


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42. The Diabetes Control and Complications Trial Research Group. Epidemiology


CHAPTER 3

3 «Ischemia-reperfusion injury and hypoglycemia risk in insulin-treated T1DM rats following different modalities of regular exercise»

3.1 Introduction

Ischemic heart disease is a major complication that is associated with significant morbidity and mortality, particularly for patients with Type 1 Diabetes Mellitus (T1DM) (36). Experimental evidence supports the view that populations with T1DM are more susceptible to ischemia, as hearts from streptozotocin (STZ)-induced diabetic models are more sensitive to damage following ischemic-reperfusion injury (I/R-injury), as demonstrated by increased infarct size (15). Regular exercise can improve cardiovascular function, as well as other diabetes-related complications including bone health, body composition, kidney function, insulin sensitivity, and overall quality of life (8, 10, 21, 25, 31). Despite these benefits, many patients with T1DM refrain from participating in regular exercise, largely due to the increased risk of experiencing hypoglycemia. Exercise is the most frequently identified cause of known hypoglycemia, while fear of experiencing a hypoglycemic event is the number one barrier for exercise participation for patients with diabetes (5, 6).

The Canadian Diabetes Association guidelines advise patients with diabetes to participate in regular exercise of moderate intensity (50-70% maximal heart rate (MHR)) to vigorous intensity (>70% MHR), at least 3 times a week for a total of 150 minutes (1, 51). However, there is a lack of evidence that regular exercise is capable of improving the primary clinical treatment outcome measure, HbA1c, which has led many to question
whether exercise is as advantageous to patients with T1DM as it is for those with Type 2 diabetes mellitus (8, 28). In fact, patients with T1DM regularly compensate their glucose management by elevating pre-exercise blood glucose concentrations through a reduction in insulin dosage to offset the risk of hypoglycemia (47). In a recent study, we examined the role of a ten-week moderate intensity aerobic exercise training program on cardiac function in poorly controlled STZ-induced T1DM rats. (31). Maintaining T1DM rats in a moderately hyperglycemic range through insulin supplementation was conducted to measure the cardiovascular benefit of regular aerobic exercise in a T1DM model that more accurately reflects those individuals with T1DM who exercise (31, 47, 50). Regular moderate intensity aerobic exercise led to significant improvements in cardiovascular function, as evidenced by an improved ratio of the early (E) and late (A) ventricular filling velocities (E/A ratio: a marker of left ventricle diastolic function), as well as improvements in other diabetic complications including bone density, insulin sensitivity measures and body composition (31). In rats with T1DM, regular aerobic exercise also led to a significant increase in Hsp70 protein content in the heart to the same degree as non-diabetic exercised rats (31). Elevated Hsp70 is believed to combat diabetes-related damage in many organ systems through enhanced antioxidant defenses, which provides a potential mechanism by which exercise may offset hyperglycemic-related oxidative stress (37, 38).

Promising clinical data have shown that higher levels of exercise intensity, either aerobic or resistance exercise, may alleviate the onset of hypoglycemia in T1DM subjects through increased secretion of glucose counter-regulatory defenses (1, 48, 49). However, few studies have examined the cardiovascular benefits of these forms of exercise. In fact, many practitioners encourage patients with diabetes to avoid intense exercise, as vigorous physical activity can acutely and transiently increase the risk of a cardiovascular event (46).
However, a recent study in patients with coronary heart disease reported that although a low risk of experiencing a cardiovascular event is present, given the tremendous advantages over lower intensity exercise, high intensity exercise should be considered in a coronary heart disease rehabilitation setting (41). Indeed, our laboratory has shown that higher intensities of regular aerobic exercise in rats with T1DM leads to improvements in vasorelaxation responsiveness, insulin dose requirements and insulin sensitivity measures in comparison to lower intensities of exercise (18, 34). Experimental work is needed to further understand the cardiovascular benefits associated with different modalities of exercise, while taking into consideration which of these exercise modalities provide the lowest risk of exercise-mediated hypoglycemia.

The present study employed an insulin-treated STZ model of T1DM in which rats were maintained at moderately elevated blood glucose concentrations, which more accurately reflects the exercising patient population of T1DM. Using this model, we examined which mode of exercise training elicited the greatest cardiovascular benefit, as determined by the recovery from ischemia-reperfusion (I/R) injury. Secondly, we measured the blood glucose concentration of rats with T1DM prior to, and following exercise, in order to examine which mode of regular exercise delivers the lowest risk of hypoglycemia development. It was hypothesized that higher intensities of regular exercise such as resistance and high intensity aerobic exercise would lead to the greatest cardiovascular benefit while limiting the onset of exercise-mediated hypoglycemia during the course of the exercise program.

3.2 Methods
Eight-week old male Sprague-Dawley rats were obtained from Charles River Laboratories, housed two per cage and maintained on a 12-hour dark/light cycle at a constant temperature (20 ±1°C) and relative humidity (50%). Rats were allowed access to standard rat chow (Prolab-RMH-3000; PMI Nutrition International: 22% crude protein, 5% crude fat, 5% fiber, 6% ash) and water ad libitum. Ethics approval for the participation of rats in this study was acquired through the Research Ethics Board of the University of Western Ontario, which is in accordance with the guidelines of the Canadian Council on Animal Care.

**Experimental groups.** Fifty rats were randomly assigned to one of five groups: 1) Non-diabetic sedentary control (C; n=10), 2) diabetic sedentary control (D-CIT; n=10), 3) diabetic resistance exercise (D-RT; n=10), 4) diabetic low intensity aerobic exercise (D-AT\text{low}; n=10) and 5) diabetic high intensity aerobic exercise (D-AT\text{high}; n=10).

**Diabetes Induction.** Upon arrival rats were housed for a minimum of five days to allow them to become familiar with their new surroundings. T1DM was induced by administering 20 mg/kg of filtered (0.2µm) streptozotocin (STZ; Sigma Alderich, Oakville, Ontario) dissolved in a citrate buffer (0.1M, pH 4.5) via intraperitoneal (IP) injection on five consecutive days. Diabetes was confirmed by measuring two consecutive blood glucose concentrations of greater than 18mM. If diabetes confirmation was not obtained following five injections, the animals were given subsequent 20mg/kg STZ-IP injections until two readings of 18mM were obtained. Following the confirmation of diabetes, insulin pellets (LinShin, Toronto, Ontario) were implanted subcutaneously above the abdomen. According to manufactures instruction, each insulin pellet slowly releases insulin at an approximate rate of two International Units (IU) per day. Insulin pellet dosages were then monitored for 1 week and adjusted in order to obtain daily non-fasting blood glucose
concentrations in the range of 9-15mM, representative of conventional insulin therapy (CIT).

**Exercise Protocols.** The exercise protocols consisted of six weeks of regular exercise (5 days per week, starting at 9am). D-RT rats were required to climb a ladder with a weighted bag secured to the proximal portion of their tail. The ladder was 1.1 m tall on an 80-degree incline with 2 cm spacing between rungs. This protocol describes an animal model of resistance exercise that closely resembles the exercise parameters and physiological adaptations observed in humans who participate in resistance training (19). During the first week of the exercise intervention, D-RT rats were familiarized to the weighted ladder climb. To do so, rats performed 10 climbs per day with varying weights attached to their tails (5%, 15%, 20%, and 35% of each rat’s body mass). Between each climb, rats were allowed to rest for 120 seconds in a 20 cm$^3$ darkened box placed at the top of the ladder. The rest of the resistance training intervention (week two to six) consisted of rats carrying 50%, 75%, 90% and 100% of their maximal lifting capacity for the first four climbs. Subsequent climbs were performed at 100% of their maximal lifting capacity until rats reached exhaustion (unable to finish a climb despite tactile stimulation to haunches). To determine maximal lifting capacity, rats were initially required to climb carrying 75% of their body weight. Thirty grams of weight was then added to each subsequent climb until rats reached exhaustion. The weight carried prior to exhaustion was marked as the new maximal lifting capacity. Maximal lifting capacity was determined and checked every four exercise sessions. During the first week of exercise, D-AT$_{low}$ were familiarized on the motor-driven treadmill (6 percent grade) at progressive running speeds of 7 m/min for 10 minutes, 11 m/min for 10 minutes, 13 m/min for 30 minutes and 15 m/min for 10 minutes. Following familiarization (Weeks 2-6), D-AT$_{low}$ rats exercised for 1 hour per day at a speed
of 15 m/min (6 percent grade). Week one of D-AT_{high} familiarization consisted of progressive running on the motor-driven treadmill (6 percent grade) at 7 m/min for 10 minutes, 15 m/min for 10 minutes, 21 m/min for 30 minutes and 24 m/min for 10 minutes.

Following familiarization (Weeks 2–6), D-AT_{high} rats ran for 1 hour per day at a speed of 27 m/min (6 percent grade). The exercise intensity chosen for D-AT_{low} and D-AT_{high} was approximately 50-60% and 70-80% of VO_{2max}, respectively (3). Continuous running during the aerobic exercise sessions was encouraged by small blasts of compressed air when rats broke a photoelectric beam close to the rear of the treadmill belt.

**Body Weights and Blood Glucose Concentrations.** Body weights and blood glucose concentrations for C, D-CIT, D-RT, D-AT_{low} and D-AT_{high} were measured prior to the STZ administration and exercise intervention, as well as prior to the last exercise session. Post-exercise blood glucose measures were collected every 15 minutes for 2 hours following an exercise bout and occurred during week three and week six of the exercise intervention. Weeks three and week six of the exercise interventions were selected as the early and late phases, respectively. To remove any stress-related changes in blood glucose responses to exercise, week three was selected as the early time phase, as animals have become familiar with the mechanics of the exercise by this stage and require little encouragement to complete the one hour exercise session. Blood was obtained from the saphenous vein (50 ul drop) and blood glucose concentrations were detected using a One Touch Ultra 2 Blood Glucose Monitoring System (Lifescan Canada Ltd, Burnaby, BC) and One Touch test strips (Lifescan Canada Ltd, Burnaby, BC). Following blood collection at sacrifice, samples were clotted at room temperature for at least 30 minutes and then centrifuged at 10,000 rpm for 15 minutes at 4°C. Aliquots of serum were collected and analyzed for HDL cholesterol, total cholesterol, and triglycerides using a Hitachi 911 analyzer. Exogenous insulin
concentrations were measured by ELISA (Alpco, Salem, NH: Catalog #80-INSRT-E01) following the exercise intervention.

Heart collection and Langendorff preparation. Eighteen hours after their last exercise bout animals were anaesthetized via intraperitoneal injection of sodium pentobarbital (65mg/kg) and sacrificed. Hearts were extracted and immediately arrested by placing them in ice cold Krebs-Henseleit buffer (KHB). Hearts were cannulated for unpaced retrograde aortic constant flow perfusion (15 ml/minute) of coronary arteries with KHB (120mM NaCl, 4.63mM KCl, 1.17mM KH₂PO₄, 1.25mM CaCl₂, 1.2mM MgCl₂, 20mM NaHCO₃, and 8mM glucose gassed with 95% O₂, and 5% CO₂) maintained at 37°C (37). Hearts were equilibrated for 30 minutes to determine baseline function (pre-ischemic value) and then flow was terminated for 50 minutes to induce ischemia. Following ischemia, hearts were subsequently reperfused for 30 minutes at 15 mL/minute. Measures of left ventricular mechanical function were measured throughout the 30 minute reperfusion period. These measures of left mechanical function include left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), maximal rate of contraction (+dP/dt) and maximal rate of relaxation (-dP/dt). Each cardiac functional measure obtained during the reperfusion period was then converted to a percentage of pre-ischemic values. Immediately following the ischemia-reperfusion protocol, hearts were removed from the cannula and the left ventricle was dissected, frozen in liquid nitrogen, and stored at -70°C until analyzed.

Western blotting. Left ventricles were homogenized in a 1:10 (weight:volume) ratio of buffer (100mM NaCl, 50mM Tris base, 0.1mM EDTA, 0.1mM EGTA and 1% Triton-X 100, adjusted to pH ~7.5, 1% phosphatase inhibitor and 1% protease inhibitor) using a polytron, and centrifuged at 14000 rpm for 20 minutes. The Bradford protein assay was
used to calculate total protein concentration in each homogenate sample. Polyacrylamide gels were composed of a 10% acrylamide separating gel and 4% acrylamide stacking gel. Homogenates were placed in a 1:1 ratio of sample buffer (0.5M Tris base, 13% glycerol, 0.05% SDS, 13% 2-beta-mercaptoethanol, and bromophenol blue) to homogenates. Each gel contained a molecular weight marker to determine the molecular weight of the proteins. Electrophoresis was then performed and proteins were run at a constant voltage of 150V for 1.5 hours in running buffer (25mM Tris base, 200mM glycine, and 0.1% SDS, pH ~8.3). Gels were placed in transfer buffer (10% running buffer, 20% methanol, 70% ddH2O) and proteins were electrophoretically transferred to nitrocellulose membranes at 70V for 1.5 hours. The membranes were blocked in 5% non-fat dry milk (BioRad, Mississauga, ON 170-6404) in Tris buffered saline (TTBS; 10mM Tris, 100mM NaCl, 0.1 % Tween-20, pH 7.5) for 1 hour. Membranes were then incubated overnight at 4°C with primary antibodies specific to Hsp70 (Enzo Life Sciences Inc., Farmingdale, NY; 1:4000), MnSOD (Enzo Life Sciences Inc., Farmingdale, NY; 1:2000), and Cu/Zn SOD (Enzo Life Sciences Inc., Farmingdale, NY; 1:2000) in TTBS with 2% non-fat dry milk. After primary antibody incubation, nitrocellulose membranes were washed in TTBS 3 times for 10 minutes. Membranes were then incubated for 1 hour with horseradish peroxidase-conjugated secondary antibody specific for rabbit IgG (goat anti-rabbit IgG-HRP conjugate, BioRad, Mississauga, ON; 1:5000) diluted in TTBS with 2% non-fat dry milk. Membranes were again washed in TTBS 3 times for 10 minutes and subsequently imaged using chemiluminescent detection. A luminol-based chemiluminescent substrate (BioRad, Mississauga, ON; Western C Enhanced Chemiluminescent Kit, 170-5070) was placed on the membranes, allowing protein bands to become visible. Protein bands were captured using a BioRad Chemidoc XRS imager and optical densities were quantified using BioRad
Quantity One software. Optical densities were normalized to a soleus positive control as well as β-actin.

Data analysis. Body weight, blood lipids, exogenous insulin, glucose concentrations, Langendorff measures (AUC), Hsp70, MnSOD and Cu/Zn SOD protein content were compared via one-way ANOVA. Post-exercise blood glucose concentrations were compared using a two-way repeated measures analysis of variance (ANOVA). When a significant effect was found, a least squares difference post hoc test was performed. A significance level was set at p<0.05. All data are expressed as mean ± SE. All statistical analysis was performed using SigmaPlot and SigmaStat computer software.

3.3 Results

Animal Characteristics. In comparison to C, all diabetic animals (D-CIT, D-RT, D-AT\textsubscript{low} and D-AT\textsubscript{high}) demonstrated significantly lower mean body weight, while demonstrating significantly higher blood glucose concentrations prior to both the onset of the exercise intervention and the last bout of exercise (p<0.05; Table 3.1). These measures did not differ across the diabetic groups (p>0.05). Regular exercise reduced exogenous insulin requirements independent of the exercise modality (p<0.05). Several components of the blood lipid panel were significantly altered as a result of diabetes in comparison to non-diabetic animals (p<0.05; Table 3.1). Triglyceride and LDL cholesterol were significantly lower than C animals (p<0.05), and were not altered as a result of exercise training (p>0.05). Total cholesterol was reduced in D-RT and D-AT\textsubscript{low}, and the lowest in D-AT\textsubscript{low} (p<0.05). Total cholesterol:HDL was significantly lower in the exercise trained (D-RT, D-AT\textsubscript{low}, and D-AT\textsubscript{high}) animals and lowest in the D-AT\textsubscript{low} animals (p<0.05).
Left ventricle mechanical performance. Compared to C, diabetic groups (D-CIT, D-AT\textsubscript{low}, D-RT and D-AT\textsubscript{high}) demonstrated greater cardiac functional performance in post-ischemic left ventricular developed pressure (LVDP), maximal rate of contraction (+dP/dt) and maximal rate of relaxation (-dP/dt) (Figure 3.1; \(p<0.05\)). The demonstrated protective effects of exercise, as assessed by left ventricular performance, were not consistent across each of the modalities of exercise. D-AT\textsubscript{high} animals showed the greatest positive benefit in cardiovascular recovery in comparison to D-CIT and D-RT in all four variables examined (LVDP, LVEDP, +dP/dt, -dP/dt) (\(p<0.05\)). Both the D-AT\textsubscript{high} and D-CIT animals had significantly lower left ventricular end-diastolic pressure (LVEDP), which was not significantly different amongst the rest of the groups (\(p<0.05\)).

SDS-PAGE and Western blot analysis. Compared to both C and D-CIT, D-AT\textsubscript{high} rats exhibited higher cardiac Hsp70 expression (Figure 3.2; \(p<0.05\)). Hsp70 content of both D-RT and D-AT\textsubscript{low} trained rats were not significantly different from either C or D-CIT (\(p>0.05\)). There were no differences in MnSOD across experimental groups (Figure 3.3A; \(p>0.05\)). Compared to C, both D-AT\textsubscript{low} and D-AT\textsubscript{high} had significantly higher expression of Cu/Zn SOD, while D-AT\textsubscript{low} had significantly elevated levels of Cu/Zn SOD in comparison to D-CIT (Figure 3.3B; \(p<0.05\)).

Exercise-mediated blood glucose reductions. At week three, blood glucose concentrations in D-RT animals declined slowly following the exercise, reaching significantly lower concentrations (vs. pre-exercise) at 45 minutes post-exercise, and remained significantly lower until 110 minutes post-exercise (Figure 3.4A; \(p<0.05\)). In D-AT\textsubscript{low} and D-AT\textsubscript{high} animals, the decline in blood glucose concentrations in response to exercise during week three was evident immediately following the exercise session (Figure 3.4B and 3.4C; \(p<0.05\)). The reduction in blood glucose concentrations of D-AT\textsubscript{low} rats
remained significantly lower (vs. pre-exercise) at 120 minutes post-exercise, while D-AT\textsubscript{high} rats returned to pre-exercise blood glucose concentrations by 90 minutes post-exercise (p<0.05). At week six of training, D-RT rats demonstrated a delayed blood glucose response to exercise in comparison to the week three exercise session, whereby a significant drop in blood glucose was not evident until 60 minutes post-exercise (p<0.05). Similarly, D-AT\textsubscript{high} rats demonstrated an altered blood glucose response to exercise at week six, exhibiting a quicker return to pre-exercise blood glucose concentrations by 60 minutes (p<0.05). At week six, a drop in blood glucose was absent in D-AT\textsubscript{low} rats, but rather an increase in blood glucose was evident at 30, 105 and 120 minutes post-exercise (p<0.05).

Despite a reduction in blood glucose concentration being apparent across the different modalities of exercise, the extent of the drop in blood glucose did not approach hypoglycemic concentrations (<3.0mM) in either week three or six of exercise training.
<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>D-CIT</th>
<th>D-RT</th>
<th>D-AT&lt;sub&gt;low&lt;/sub&gt;</th>
<th>D-AT&lt;sub&gt;high&lt;/sub&gt;</th>
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</thead>
<tbody>
<tr>
<td><strong>Blood glucose (mM)</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Pre-STZ administration</td>
<td>5.6 ± 0.09</td>
<td>5.8 ± 0.2</td>
<td>5.8 ± 0.2</td>
<td>5.3 ± 0.2</td>
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<td>Pre-exercise intervention</td>
<td>5.6 ± 0.1</td>
<td>11.8 ± 1.2*</td>
<td>14.5 ± 1.5*</td>
<td>12.0 ± 1.7*</td>
<td>15.2 ± 0.5*</td>
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<tr>
<td>Prior to the last bout of exercise</td>
<td>5.13 ± 0.15</td>
<td>15.45 ± 0.84*</td>
<td>15.27 ± 0.75*</td>
<td>13.2 ± 1.24*</td>
<td>14.81 ± 0.76*</td>
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<td><strong>Body weight (g)</strong></td>
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<tr>
<td>Pre-STZ administration</td>
<td>366 ± 7.88</td>
<td>371 ± 9.09</td>
<td>365 ± 6.94</td>
<td>345 ± 7.10</td>
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<tr>
<td>Pre-exercise intervention</td>
<td>408 ± 7.90</td>
<td>376 ± 5.81*</td>
<td>372 ± 5.34*</td>
<td>369 ± 7.33*</td>
<td>365 ± 8.48*</td>
</tr>
<tr>
<td>Prior to the last bout of exercise</td>
<td>574 ± 9.05</td>
<td>471 ± 9.05*</td>
<td>452 ± 11.16*</td>
<td>448 ± 21.72*</td>
<td>438 ± 8.43*</td>
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<tr>
<td><strong>Exogenous insulin (IU)</strong></td>
<td></td>
<td>25.41 ± 10.58</td>
<td>11.90 ± 3.62*</td>
<td>14.53 ± 7.46*</td>
<td>11.00 ± 3.77*</td>
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<tr>
<td>Total cholesterol (mM)</td>
<td>1.81 ± 0.13</td>
<td>1.63 ± 0.11</td>
<td>1.42 ± 0.07 *</td>
<td>1.33 ± 0.07 *#</td>
<td>1.59 ± 0.03</td>
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<tr>
<td>Triglycerides (mM)</td>
<td>1.72 ± 0.13</td>
<td>1.09 ± 0.14*</td>
<td>1.16 ± 0.20 *</td>
<td>0.857 ± 0.18*</td>
<td>1.09 ± 0.13*</td>
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<td>HDL cholesterol (mM)</td>
<td>0.78 ± 0.16</td>
<td>1.11 ± 0.15</td>
<td>1.10 ± 0.08</td>
<td>0.98 ± 0.06</td>
<td>1.03 ± 0.03</td>
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<tr>
<td>Total cholesterol:HDL</td>
<td>1.90 ± 0.3</td>
<td>1.67 ± 0.09</td>
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<td>1.38 ± 0.05*#</td>
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<td>LDL cholesterol (mM)</td>
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<td>0.84 ± 0.21*</td>
<td>0.84 ± 0.32*</td>
<td>0.45 ± 0.15*</td>
<td>0.77 ± 0.18*</td>
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</table>

Data are means ± SE; n=10. (*) significantly different from C (p<0.05). (#) significantly different from D-CIT (p<0.05).

**Table 3.1.** General animal characteristics at the completion of the study.
Figure 3.1. Effect of T1DM and exercise training modality on left ventricle mechanical function. The data are presented in time course format (A) and area under the curve measurement (B). Compared with control, diabetic stress and exercise training lead to enhancement in post-ischemic LVDP, +dP/dt, –dP/dt but not LVEDP in rat hearts. (*) different than C (P<0.05), (†) different than D-CIT (P<0.05), (‡) different than D-RT (P<0.05), (ψ) different than D-AT_high (P<0.05). Data presented as a mean ± SE.
Figure 3.2. Effect of T1DM and exercise training modality on left ventricle Hsp70 protein content. High intensity aerobic exercise training led to an enhancement in Hsp70 expression in rat hearts compared to sedentary rats. (*) different than C (P<0.05), (†) different than D-CIT (P<0.05). Data presented as a mean ± SE.

Figure 3.3. Effect of diabetic stress and training on left ventricle MnSOD (A) and Cu/Zn SOD (B) protein content. No change in MnSOD was evident across experimental groups while both low and high intensity exercise aerobic training led to elevations in Cu/Zn SOD when compared to sedentary control rats. Low intensity aerobic exercise also demonstrated significantly more Cu/Zn SOD compared to sedentary diabetic rats. (*) different than C (P<0.05), (†) different than D-CIT (P<0.05). Data presented as a mean ± SE.
Figure 3.4. Two-hour post-exercise blood glucose measures. Blood glucose was measured in (A) D-RT (B) D-AT\text{low} and (C) D-AT\text{high} every 15 minutes for 2 hours post-exercise. D-RT and D-AT\text{high} demonstrated no significant difference in post-exercise blood glucose response between week 3 and week 6; D-AT\text{low} demonstrated an attenuated blood glucose response at week 6 compared to week 3 (p<0.05). (*) indicates a significant difference in blood glucose from pre-exercise (-15 minutes) at week 3 (P<0.05), (†) indicates a significant difference in blood glucose from pre-exercise (-15 minutes) at week 6 (P<0.05). Data presented as a mean ± SE.
3.4 Discussion

Using an insulin-treated model of T1DM, the purpose of the present study was to investigate the cardiovascular benefit of several different exercise regimes which have been previously shown clinically to minimize the onset of hypoglycemia by exercise (29, 48). Here, we demonstrate that the extent of exercise-related protection from ischemia-reperfusion (I/R) damage appears to be dependent on the exercise modality, whereby high intensity aerobic exercise demonstrates the greatest recovery following an ischemic insult. Secondly, although insulin-treated rats with T1DM demonstrated a significant drop in blood glucose concentration regardless of exercise modality, blood glucose concentrations failed to reach hypoglycemic concentrations (<3.0mM) and aerobic exercise was associated with the fastest return to pre-exercise glucose concentrations, especially with longer periods of regular exercise. These findings would suggest that each exercise modality can be performed safely, at least under conditions in which blood glucose concentrations are elevated before exercise is initiated; however, the greatest cardiovascular benefit is evident following high intensity aerobic exercise.

In partial agreement with our hypothesis, these results demonstrate an intensity-related effect on the reduction of I/R-injury in aerobically trained rats. These findings would support previous literature in otherwise healthy individuals in which exercise performed at higher relative intensities elicited greater cardiovascular benefit than moderate intensity levels of exercise, independent of the volume of activity (26). Less information is available with regards to the impact of higher levels of exercise intensity in patients with T1DM, a population well characterized to be at greater risk for cardiovascular disease (36). In line with these findings, our laboratory has previously reported that high
Intensity aerobic exercise training is able to restore the diabetes-related loss of nerve arterial vasodilation, decreased vascular responsiveness, and insulin sensitivity (18, 34, 35). In contrast to high intensity aerobic training, we found little evidence of cardiovascular protection from I/R-injury following six weeks of resistance exercise (D-RT) and low-intensity aerobic exercise (D-ATlow) in comparison to sedentary diabetic (D-CIT) rats. To our knowledge, the present investigation is one of the first studies to have measured I/R-injury in resistance exercised animals with T1DM. Doustar et al. (12) examined the impact of a 4-week resistance training program on I/R-injury in healthy rats and reported no exercise-related benefits. However, in a subsequent study it was demonstrated that 12 weeks of resistance training led to significant improvements in coronary flow, developed pressure, diastolic pressure and infarct size, suggesting that this form of exercise can be effective for cardiac protection (42). Thus, the current findings may be reflective of the slower beneficial adaptations this form of exercise training may have in comparison to aerobic exercise. Indeed, endurance exercise has been shown to elicit rapid cardiovascular adaptations following as little as 6 days of training (16, 17). While it is difficult to extrapolate these reports to our model of T1DM, it is plausible that lengthening the resistance training program may lead to enhanced cardiac protection to a similar degree as high intensity exercise.

Our laboratory, as well as others, have demonstrated that exercise-related increases in Hsp70 can protect the myocardium against I/R injury (4, 20, 32, 37). It has been established that Hsp70 protein is reduced in several tissues including the heart in severely hyperglycemic rats with STZ-induced T1DM (2). Further, the elevated expression of Hsp70 in the heart, which normally occurs following moderate intensity aerobic exercise, is suppressed in untreated STZ-induced T1DM animals (2). Using the same insulin-treated
diabetic model used in the current study we recently reported that rats with T1DM are able to elicit elevations in constitutive Hsp70 content in the heart to similar levels as non-T1DM controls following moderate intensity exercise training (31). These discrepancies are likely due to the use of insulin supplementation in our rat model of T1DM. It has been established that insulin treatment alone can increase Hsp70 expression, as well as enhance myocardial recovery of contractile function post-ischemic injury (27). Here, we demonstrate that the elevations in heart Hsp70 expression were modality specific and positively associated with the level of cardiovascular protection. High intensity aerobic exercise (D-AT\textsubscript{high}) demonstrated a significant elevation in Hsp70 and exhibited the largest recovery from I/R injury. In contrast, low intensity regular aerobic exercise (D-AT\textsubscript{low}) did not exhibit evidence of improved cardioprotection, nor did this exercise regime display significant increases in heart Hsp70. Indeed, it has been reported that an intensity dependent threshold exists in the expression of Hsp70 following aerobic exercise in otherwise healthy animals (33).

A notable finding of the current study is that in comparison to C animals, D-CIT animals demonstrated some level of protection from I/R injury. This ischemia resistance has been demonstrated in STZ models of T1DM elsewhere in the literature (24, 44) and may be due to the duration and severity of the diabetic state (for detailed review, see (39)). Enhanced antioxidant defenses may contribute to this heightened cardioprotection, as it has been reported that animals with STZ-induced T1DM demonstrate elevations in antioxidant defenses, such as catalase, Mn-SOD, and Glutathione S Transferase (GST), which are evident as early as two weeks following STZ-administration (23, 45). Although rats with T1DM in the present study did not exhibit changes in cardiac MnSOD or Cu/Zn SOD protein, it is possible that their activity or other antioxidant defenses may be elevated and contribute to increased cardioprotection (40). It has been shown that hearts of rats with
T1DM demonstrate a decrease in the accumulation of glycolytic products during ischemia (lactate and protons), which has also been proposed to be beneficial during an ischemic event (14). Further, T1DM hearts have been shown to have alterations in intracellular calcium signaling, which has been shown to induce protection in the normal heart (40). It is important to note that enhanced protection from I/R injury has also been reported in patients with T1DM undergoing forearm ischemia (13). Increased nitric oxide production coupled with changes in vascular diameter may also contribute to improved cardiovascular function in the early stages of streptozotocin induced diabetes (9). Lastly, rats with T1DM in the current study were supplemented with insulin, which may have put them in a heightened protective state in comparison to control sedentary animals (27). Nonetheless, the current data provide evidence that high intensity aerobic exercise can greatly improve cardiovascular function in insulin-treated rats with T1DM. Further work is still required to fully understand the susceptibility of patients with T1DM to damage during ischemic stress and the impact of insulin treatment on the severity of the stress.

While benefits in cardiovascular function and protection from I/R damage are evident following high intensity aerobic exercise, a primary consideration when prescribing exercise to populations with T1DM is the elevated risk of experiencing hypoglycemia. Exercise-mediated reductions in blood glucose differed based on the exercise modality, an observation well documented in patient populations with T1DM (11, 22, 48). In the present investigation we observed that at week three of the exercise intervention both aerobic exercise modalities (low and high intensity) led to a significant drop in blood glucose immediately post-exercise. In contrast, resistance exercise resulted in a more gradual decline in blood glucose, which did not return to pre-exercise values until approximately four hours post-exercise (data from 2-4 hours not shown). This gradual decline in blood
glucose in response to resistance exercise may be more beneficial for patients with T1DM, as it provides ample time to combat the drop in blood glucose following exercise. Precautionary measures such as carbohydrate ingestion or a decrease in insulin dosage could mitigate the reduction in blood glucose altogether. Managing the immediate drop in blood glucose which occurs in response to aerobic exercise is more challenging, and as such, patients often compensate by elevating blood glucose concentrations prior to exercise through carbohydrate ingestion or insulin dosage adjustments (47, 50). However, it is important to note that despite the rapid drop in blood glucose, exercising rats with T1DM failed to reach hypoglycemic blood glucose concentrations (<3.0mM) when they started with moderately elevated blood glucose concentrations. As such, maintaining blood glucose concentrations at a higher target range prior to exercise, significantly reduces the risk of hypoglycemia associated with aerobic exercise.

In a recent publication, our group demonstrated that the immediate exercise-induced reduction of blood glucose in response to moderate intensity aerobic exercise is unaltered after ten weeks of training (30). Here, it appears that the magnitude of blood glucose reduction in response to different exercise modalities, and the return to pre-exercise blood glucose concentrations, may adapt during the course of the six-week exercise intervention. For instance, an elevation in blood glucose was evident post-exercise following six weeks of low intensity aerobic exercise rather than a significant decline in blood glucose, which was apparent at week three. This is of particular interest given that insulin sensitivity is heightened following exercise training and it is unlikely that a robust sympathetic response would be present at this level of exercise intensity (18, 43). However, it is likely that a reduction in the reliance on blood glucose as an energy source occurred, as running speed was consistent across the treatment period. Thus, the relative exercise intensity likely
declined towards the later stages of the experiment, which in turn may have led to a shift in fuel source from primarily carbohydrate to fat (7). On the other hand, high intensity aerobic and resistance exercised rats displayed a smaller blood glucose adaption post-exercise following the exercise intervention. It is plausible that the higher level of intensity in these exercise modalities led to a similar, or even increased (resistance exercise), glucose demand during the course of the exercise intervention period.

In summary, we demonstrate that the extent of exercise-related protection from I/R damage appears to be dependent on exercise modality, whereby higher intensities of aerobic exercise demonstrate the greatest recovery following an ischemic insult. Secondly, although the magnitude and time course is different, a significant decrease in blood glucose is apparent following each modality of exercise. Maintaining rats at a higher target blood glucose concentration prior to exercise may reduce the risk of post-exercise hypoglycemia. These findings would suggest that each exercise modality can be performed safely when combined with moderately elevated blood glucose; however, the greatest benefits are evident following high intensity aerobic exercise.
3.5 References


14. Feuvray D, Lopaschuk GD. Controversies on the sensitivity of the diabetic heart


4.1 Introduction

Individuals with Type 1 diabetes mellitus (T1DM) exhibit a heightened risk for cardiovascular disease (CVD) not entirely accounted for by traditional risk factors (hyperglycemia, obesity, hypertension, dyslipidemia, and smoking)(39). Since the advent of the Diabetes Control and Complications Trial (DCCT) the predominant treatment to limit diabetic complications such as CVD, has been intensive insulin therapy (IIT)(26). Regular exercise is also well-recognized for its cardiovascular benefits for populations with T1DM (5, 20, 22). However, both IIT and exercise potentiate the risk of hypoglycemia, especially when attempted collectively (4, 26). Over 60% of individuals with T1DM are inactive (30), with fear of hypoglycemia identified as their largest barrier to exercise participation (4). Further, accumulating evidence suggests that while IIT decreases HbA$_1c$ it can also lead to weight gain and insulin resistance development, leading many to question HbA$_1c$ as a marker for cardiovascular risk (7).

Individuals with T1DM often intentionally elevate their blood glucose concentrations prior to exercise through changes in insulin dosing and/or carbohydrate ingestion in order to counteract the risk of hypoglycemia (48). Indeed, it has been shown that physically active individuals with T1DM exhibit higher HbA$_1c$ values compared to sedentary patients with T1DM (8). The reduced focus on glycemic control, characteristic of a more conventional style of insulin therapy (CIT)(41), may be counterproductive
since elevations in glycemia (HbA1c) are known to increase the risk of cardiovascular complications (26). Moreover, a retrospective analysis of the DCCT found no evidence that physical activity improves microvascular outcomes (19). Contrary to this work, our group has demonstrated that the combination of CIT and high-intensity aerobic exercise training (AT\textsubscript{high}) in experimental T1DM rats, not only decreases the risk of exercise-induced hypoglycemia (20), but numerous cardiovascular benefits are apparent such as increased recovery from an ischemic insult (20), reduction in cardiovascular autonomic dysfunction (15), improvement in systolic and diastolic heart function (22), and improved vascular reactivity (25, 27). Additional work is needed to better evaluate the cardiovascular benefits and risks associated with regular exercise in physically active individuals with T1DM that often prescribe to less stringent glycemic control (i.e. CIT). Specifically, these studies need to compare the importance of regular physical activity versus IIT alone, the predominant treatment option for individuals with T1DM (26, 41) which is typically accompanied by a more sedentary lifestyle (8).

At both the experimental (21) and clinic level (3), it has been shown that poor glycemic control in patients with T1DM leads to hepatic glycogen deficiencies. Thus, the restoration of hepatic glycogen content could represent a mechanism for combatting hypoglycemia, as hepatic glycogen is the predominant source of blood glucose during exercise (44) and insulin overcorrection (18). Our laboratory has recently shown that ten weeks of AT\textsubscript{high} fails to normalize hepatic glycogen in T1DM rats despite significantly elevated levels of hepatic glycogenic storage enzymes (21). In contrast, resistance training (RT) has been shown to increase hepatic glycogen content in rats (32), while also alleviating the risk of exercise-induced hypoglycemia in T1DM (20, 46). Recent work has also shown that compared to abrupt reductions in blood glucose with aerobic
exercise, RT results in less of an initial decline in blood glucose (46, 47). While still allowing for the cardiovascular benefits associated with regular aerobic exercise, the integration of RT with aerobic exercise may allow individuals with T1DM to exercise safely by reducing the risk of hypoglycemia development. In fact, the Canadian Diabetes Association recommends that RT be incorporated into aerobic exercise regimes at least twice a week (1).

The objective of the present study was two-fold: 1) to determine if 12 weeks of CIT paired with AT_{high} results in larger cardioprotection from an ischemia-reperfusion (I-R) injury than IIT alone; 2) to establish if the combination of RT with AT_{high} results in similar cardioprotection as AT_{high} alone, while also limiting abrupt reductions in blood glucose in response to exercise. It was hypothesized that CIT paired with AT_{high} results in similar levels of cardioprotection as IIT alone and that comparable levels of cardioprotection would be evident between both combined exercise training (AT_{high} and RT) and AT_{high} alone. Further, the integration of RT with AT_{high} would augment hepatic glycogen storage and prevent abrupt reductions in blood glucose in response to aerobic exercise.

4.2 Methods

This study was approved by the Research Ethics Board of the University of Western Ontario which is in compliance with the guidelines of the Canadian Council on Animal Care. Eight-week old male Sprague-Dawley rats were obtained from Charles River Laboratories, provided standard rat chow ad libitum, and housed in pairs at a standard temperature and humidity (21.5°C and 50% humidity).
**Experimental Protocol.** Sprague-Dawley rats were randomly divided into one of five diabetic groups: conventional insulin therapy (D-CIT; n=12), intensive insulin therapy (D-IIT; n=12), high-intensity aerobic exercise training (D-AT_{high}; n=8), resistance exercise training (D-RT; n=8) and combination aerobic/resistance exercise training (D-ART; n=8). During week one, T1DM was induced after five consecutive daily injections of streptozotocin (Sigma-Aldrich; 20mg/kg; dissolved in 0.1M citrate buffer, pH 4.5) and T1DM was confirmed after two non-fasting blood glucose concentrations greater than 18mM. After diabetes confirmation, subcutaneous insulin pellets (Linshin, Toronto, Canada) were implanted in the abdomen (week two of study). Through insulin pellet adjustments it was intended to maintain blood glucose concentrations in D-CIT, D-AT_{high}, D-RT and D-ART between 9-15mM and D-IIT between 4-9mM. Exercise training occurred five times a week over a twelve-week period. D-AT_{high} rats exercised on a motorized treadmill at 27m/min (six percent grade) for one hour. Continuous running was encouraged by small blasts of compressed air at the rear of the treadmill. In D-RT rats, resistance training consisted of climbing a vertical ladder with weights secured to the proximal portion of the tail, as previously described (20). Briefly, familiarization occurred the week prior to training and consisted of 10 climbs a day with varying weights attached (5%, 15%, 20% and 35% of each rat’s body mass). Regular resistance training sessions consisted of incremental increases in weight (50%, 75%, and 90% of maximal lifting capacity) followed by 100% of their maximal lifting capacity until exhaustion (unable to finish climb despite tactile stimulation to haunches). Maximum lifting capacity was calculated every fourth exercise session and was determined by sequentially adding 30 grams of weight to the rat’s tail until exhaustion (starting at 75% of their body mass). In
D-ART rats, exercise training consisted of alternating daily between the aerobic and resistance exercises.

**Blood Analysis.** Blood samples were taken over two consecutive days from the saphenous vein during the last week of exercise training (pre/post exercise) to determine if antecedent AT<sub>high</sub> or RT altered the blood glucose response to a subsequent exercise bout (37). In D-ART, this measure was conducted at week 11 and week 12 of training to determine if performing AT<sub>high</sub> (or RT) first had an effect on glucoregulation following a subsequent bout of RT (or AT<sub>high</sub>). Blood glucose concentrations were detected using a One Touch Ultra 2 Blood Glucose Monitoring System (Lifescan Canada Ltd, Burnaby, BC, Canada) and One Touch test strips (Lifescan Canada Ltd). Epinephrine concentrations prior to, and after exercise were determined via ELISA (Cusabio, Catalog #CSB-E08678r). Fructosamine concentrations were determined using the procedure outlined by Oppel et al. (28). Briefly, serum samples taken at the completion of the study were added to a carbonate buffer (pH 10.8) containing 0.25mM nitroblue tetrazolium (NBT) at 37°C. Following a 20 minute incubation at 37°C the reaction was read at 530nm and compared to standards of 1-deoxy,1-morpholinofructose (DMF; Sigma-Aldrich) and albumin (40g/l).

**Langendorf Heart Preparation.** Three days following the last exercise bout all rats were anaesthetized with isoflurane and hearts were extracted and placed in cold Krebs-Henseleit buffer (KHB; 120mM NaCl, 4.63mM KCl, 1.17mM KH<sub>2</sub>PO<sub>4</sub>, 1.25mM CaCl<sub>2</sub>, 1.2mM MgCl<sub>2</sub>, 20mM NaHCO<sub>3</sub>, and 8mM glucose). Hearts were rapidly cannulated for unpaced retrograde perfusion of KHB (37°C; gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>) at 15ml/min. A small water-filled latex balloon was inserted through the mitral valve and
into the left ventricle. Hearts were equilibrated to the preparation for 30 minutes (pre-ischemia) followed by the termination of flow for 50 minutes. Subsequently, reperfusion occurred for a total of 30 minutes at 15m/min. Left ventricle pressures (LVDP, left ventricle developed pressure; LVEDP, left ventricle end-diastolic pressure) were measured with a pressure transducer (Statham Gould P23ID) and the rate of pressure development (+dp/dt) and relaxation (-dp/dt) were obtained using a Powerlab 8/30 Data Acquisition System and analyzed by Labchart 7.0 Pro Software (ADInstruments, Colorado Springs, Colorado, USA). Area under the curve (AUC) was determined for the pressure curves of each rat in the study in order to correlate measures to glycemic control and insulin resistance.

**Glucose Tolerance Test.** Intravenous glucose tolerance tests (IVGTT) were conducted following training after an 8-12 hour fast and consisted of a sterile injection (1g/kg) of dextrose solution (50% dextrose, 50% ddH2O) into the lateral tail vein. Blood glucose concentrations were measured at 5, 10, 20, 30, and 40 minutes post-injection and area under the curves (AUC) were determined for each individual rat. Prior to the IVGTT, blood samples were taken from the saphenous vein and exogenous insulin concentrations were measured via ELISA (Alpco, Salem, NH: Catalog # 80-INSHU-E01.1). The measure of insulin resistance was considered the AUC of the IVGTT multiplied by exogenous insulin concentration. We have previously reported that when using this T1DM model, sedentary rats can become insulin resistant and require substantial more exogenous insulin in order to maintain the desired blood glucose concentrations (16, 21). Accordingly, when determining the insulin resistance measure the amount of circulating insulin present in the rat during the IVGTT was factored into the calculation.
**Western Blotting.** Liver and left ventricles were homogenized in buffer (100mM NaCl, 50mM, Tris base, 0.1mM EDTA, 0.1 EGTA, pH ~7.5) using a polytron and total protein concentrations were determined by the Bradford protein assay. Homogenates (40-80µg of protein) were mixed with equal volumes of sample buffer (0.125M Tris, 20% Glycerol, 4% SDS, 10% β-mercaptoethanol, 0.015% Bromophenol blue, pH ~6.8), separated by SDS-PAGE (4% stacking, 10% separating) and transferred to nitrocellulose membranes. Membranes were blocked in 5% non-fat dairy milk in TTBS (10mM Tris, 100mM NaCl, 0.1% Tween-20, pH 7.5) for 1 hour and incubated overnight at 4°C with primary antibodies (Cell Signaling: Hsp70 1:4000, glycogen synthase 1:1000; abcam: glycogen phosphorylase 1:2000, SERCA2 1:1000; Santa Cruz: glucose-6-phosphotase 1:200) diluted in TTBS with 2% non-fat dairy milk. Following washes in TTBS, membranes were exposed to corresponding secondary antibodies (IgG-HRP conjugated, Bio-rad) in TTBS with 2% non-fat dairy milk for 1 hour at room temperature. After successive washes in TTBS, protein bands were visualized with a luminol-based chemuminescent substrate (Western C Enhanced Chemiluminescent Kit; Bio-rad), imaged with the Chemidoc XRS System (Bio-rad), and analyzed with Quantity One Software (Bio-rad). Optical densities were normalized to a consistent non-T1DM control sample and subsequently β-actin.

**Statistical Analysis.** Body mass, blood glucose, fructosamine, exogenous insulin, insulin resistance and Western blot data were compared using a one-way analysis of variance (ANOVA). Langendorf measures were compared using a two-way repeated measures ANOVA. Blood glucose concentrations and epinephrine concentrations in response to exercise, and over consecutive days, were compared using a two-way
repeated measures ANOVA. Relationships between left ventricular mechanical performance and fructosamine or insulin resistance were determined via Pearson correlation. When a significant effect was found, a least squares difference post hoc test was performed and significance was set at p<0.05. All data are presented as a mean ± standard error. All statistical analyses were completed using GraphPad Prism 6.

4.3 Results

Animal Characteristics. Blood glucose concentrations were lower in D-IIT compared to D-CIT, D-AT\textsubscript{high} and D-ART, and lower in D-RT compared to D-ART (Table 4.1; p<0.05). Body mass was higher in D-IIT compared to D-AT\textsubscript{high}, D-RT, and D-ART (p<0.05). Fructosamine concentrations were lower in D-IIT and D-AT\textsubscript{high} compared to D-CIT and D-ART (p<0.05). Exogenous insulin concentrations were lower in D-RT compared to D-IIT, and lower in D-ART compared to D-CIT and D-IIT (p<0.05). The insulin resistance measure was higher in D-IIT compared to D-AT\textsubscript{high}, D-RT and D-ART (p<0.05), D-CIT compared to D-ART (p<0.05), and D-AT\textsubscript{high} compared to D-RT and D-ART (p<0.05).

Left ventricular mechanical performance. For the first objective of the study we compared left ventricular mechanical performance following ischemia in D-CIT, D-AT\textsubscript{high} and D-IIT. There was a significant increase in LVDP in D-AT\textsubscript{high} compared to D-CIT (Figure 4.1A; p<0.05). No difference in LVDP was observed between D-AT\textsubscript{high} and D-IIT (p>0.05), and LVDP between D-CIT and D-IIT did not reach significance (p=0.052). LVEDP was lower in D-IIT compared to D-CIT (Figure 4.1B; p<0.05), while D-AT\textsubscript{high} was lower than D-CIT at 5 and 10 minutes during reperfusion (p<0.05). There was no difference in +dp/dt or –dp/dt across any of the groups (Figure 4.1C/D; p>0.05).
For the second objective of the study we compared left ventricle mechanical performance following ischemia in D-AT$_{high}$, D-RT, and D-ART. There were no differences in LVDP or LVEDP between D-AT$_{high}$, D-RT, or D-ART (Figure 4.2A/B; p>0.05). D-ART had a higher +dp/dt at 25 and 30 minutes during reperfusion (Figure 4.2C; p<0.05), while D-AT$_{high}$ had a slower –dp/dt than both D-RT and D-ART (Figure 4.2D; p<0.05).

Molecular Analysis. In comparison to both D-CIT and D-IIT, an elevation in left ventricle Hsp70 content was evident in D-AT$_{high}$ (Figure 4.3A; p<0.05), while no differences were evident in SERCA2 between D-CIT, D-IIT or D-AT$_{high}$ (Figure 4.3B; p>0.0.05) Differences existed between exercise regimes in that D-AT$_{high}$ resulted in higher left ventricle Hsp70 compared to D-RT (p<0.05), but did not differ significantly from D-ART (Figure 4.3A; p>0.05). No differences in SERCA2 expression was evident among exercise regimes (Figure 4.3B; p>0.05).

Correlations of left ventricular mechanical performance. There was a significant correlation between the AUC of LVDP and fructosamine concentration (Table 4.2; p=0.01; r=-0.4), while no correlation was evident between the AUC of LVEDP, +dp/dt, -dp/dt and fructosamine concentration (p>0.05). There was a significant correlation between the AUC of +dp/dt and insulin resistance (p=0.03; r=-0.4), but no correlation between insulin resistance and the AUC of LVDP, LVEDP, or –dp/dt (p>0.05).

Hepatic glycogen content and regulatory enzymes. Hepatic glycogen content was lower in D-AT$_{high}$, D-RT and D-ART compared to both D-CIT and D-IIT (Figure 4.4A; p<0.05). No differences in glycogen synthase, glycogen phosphorylase, and glycogen-6-phosphotase were apparent between experimental groups (Figure 4B/C/D; p>0.05).
Exercise-mediated changes in blood glucose. Significant declines in blood glucose concentrations following exercise were apparent in D-AT\textsubscript{high} at day 1 and day 2 of exercise (week 12 of training; Table 4.3; p<0.05). No change in blood glucose concentrations were apparent following RT at day 1 or day 2 (week 12 of training; p>0.05). In D-ART, significant declines in blood glucose concentrations were apparent only on AT\textsubscript{high} days (both week 11 and 12 of training; p<0.05). In D-AT\textsubscript{high}, no change in epinephrine concentrations were evident from pre to post exercise (week 12 of training; p>0.05; Table 4.4), and epinephrine concentrations were similar between day 1 or day 2 (week 12 of training; p>0.05). In D-RT, no change in epinephrine concentrations were evident from pre to post exercise (week 12 of training; p>0.05); however, epinephrine concentrations were lower overall on day 2 compared to day 1 (week 12 of training; p<0.05). In D-ART, when RT occurred the day before AT\textsubscript{high} (week 11 of training), epinephrine concentrations were reduced overall during AT\textsubscript{high} (p<0.05). In D-ART, no change in epinephrine occurred from pre to post exercise (both week 11 and 12 of training; p>0.05).
**Table 4.1.** General animal characteristics at the completion of the study

<table>
<thead>
<tr>
<th></th>
<th>D-CIT (g)</th>
<th>D-IIT (g)</th>
<th>D-AT\text{high} (g)</th>
<th>D-RT (g)</th>
<th>D-ART (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Mass</td>
<td>567±20</td>
<td>598±21\textsuperscript{3,4,5}</td>
<td>510±15</td>
<td>520±21</td>
<td>534±19</td>
</tr>
<tr>
<td>Blood glucose conc. (mM)</td>
<td>15.0±1.2</td>
<td>10.9±1.2\textsuperscript{1,3,5}</td>
<td>15.6±0.5</td>
<td>12.4±1.9\textsuperscript{5}</td>
<td>16.7±1.4</td>
</tr>
<tr>
<td>Fructosamine conc. (mM)</td>
<td>3.0±0.5</td>
<td>1.0±0.2\textsuperscript{1,5}</td>
<td>1.3±0.3\textsuperscript{1,5}</td>
<td>2.0±0.1</td>
<td>2.6±0.7</td>
</tr>
<tr>
<td>Exogenous Insulin (IU)</td>
<td>27.3±5.4</td>
<td>35.8±7.8</td>
<td>19.0±7.7</td>
<td>11.1±7.1\textsuperscript{2}</td>
<td>4.1±2.0\textsuperscript{1,2}</td>
</tr>
<tr>
<td>Insulin Resistance (AU)</td>
<td>10665±2078\textsuperscript{5}</td>
<td>16055±4558\textsuperscript{3,4,5}</td>
<td>4722±1988</td>
<td>1438±62\textsuperscript{3}</td>
<td>1260±601\textsuperscript{3}</td>
</tr>
</tbody>
</table>

Data are means ± SE

\textsuperscript{1}different from D-CIT; \textsuperscript{2}different from D-IIT; \textsuperscript{3}different from D-AT\text{high}; \textsuperscript{4}different from D-RT; \textsuperscript{5}different from D-ART
**Figure 4.1.** Left ventricle mechanical performance during ischemia-reperfusion. The data are presented in time course format. LVDP (A), LVEDP (B), +dP/dt (C), -dP/dt (D). (*) significant main effect (P<0.05); (φ) different from D-CIT (P<0.05). Data are presented as a mean ± SE.
Figure 4.2. Left ventricle mechanical performance during ischemia-reperfusion and following different modalities of exercise training. The data are presented in time course format. LVDP (A), LVEDP (B), +dP/dt (C), -dP/dt (D). (ψ) different from D-AT_{high} (P<0.05); (α) different from D-RT (P<0.05); (β) different from D-ART (P<0.05). Data are presented as a mean ± SE.

<table>
<thead>
<tr>
<th></th>
<th>vs. Fructosamine (mM)</th>
<th>vs. Insulin resistance (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p value</td>
<td>r</td>
</tr>
<tr>
<td>LVDP (AUC)</td>
<td>0.01*</td>
<td>-0.4</td>
</tr>
<tr>
<td>LVEDP (AUC)</td>
<td>0.8</td>
<td>-</td>
</tr>
<tr>
<td>+dp/dt (AUC)</td>
<td>0.7</td>
<td>-</td>
</tr>
<tr>
<td>-dp/dt (AUC)</td>
<td>0.8</td>
<td>-</td>
</tr>
</tbody>
</table>

*significant (p<0.05)

Table 4.2. Correlation of left ventricle mechanical performance on glycemia and insulin resistance.
Figure 4.3. Left ventricle Hsp70 (A) and SERCA2 (B) protein content. (φ) different from D-CIT; (#) different from D-IIT; (α) different from D-RT. Significance p<0.05. Data are presented as a mean ± SE.
Figure 4.4. Hepatic glycogen content (A), glycogen-6-phosphotase (B), glycogen synthase (MGS, muscle glycogen synthase; LGS, liver glycogen synthase) (C), and glycogen phosphorylase (D). (φ) different from D-CIT (P<0.05); (#) different from D-IIT (P<0.05). Data are presented as a mean ± SE.
<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th></th>
<th>Day 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-exercise</td>
<td>Post-exercise</td>
<td>Pre-exercise</td>
<td>Post-exercise</td>
</tr>
<tr>
<td><strong>D-AT&lt;sub&gt;high&lt;/sub&gt;</strong></td>
<td>15.0±0.4</td>
<td>8.0±1.1*</td>
<td>14.6±0.5</td>
<td>6.9±1.0*</td>
</tr>
<tr>
<td><strong>D-RT</strong></td>
<td>12.2±1.6</td>
<td>12.0±0.9</td>
<td>13.6±2.2</td>
<td>15.1±1.6</td>
</tr>
<tr>
<td><strong>D-ART</strong></td>
<td>14.9±1.6</td>
<td>15.6±1.2</td>
<td>15.4±1.7</td>
<td>8.0±1.8*</td>
</tr>
<tr>
<td>(week 11; RT then AT&lt;sub&gt;high&lt;/sub&gt;)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D-ART</strong></td>
<td>16.7±1.4</td>
<td>8.8±1.4*</td>
<td>15.2±2.0</td>
<td>15.6±1.2</td>
</tr>
</tbody>
</table>

Data are means ± SE

*significantly lower than pre-exercise (p<0.05)

**Table 4.3.** Blood glucose concentrations in response to exercise at week 11 or 12 of training

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th></th>
<th>Day 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-exercise</td>
<td>Post-exercise</td>
<td>Pre-exercise</td>
<td>Post-exercise</td>
</tr>
<tr>
<td><strong>D-AT&lt;sub&gt;high&lt;/sub&gt;</strong></td>
<td>254.3±44.6</td>
<td>93.1±24.9</td>
<td>237.0±43.8</td>
<td>298.0±109.1</td>
</tr>
<tr>
<td><strong>D-RT</strong></td>
<td>320.8±72.2</td>
<td>238.3±58.2</td>
<td>110.0±46.1*</td>
<td>136.3±66.3*</td>
</tr>
<tr>
<td><strong>D-ART</strong></td>
<td>254.3±107.2</td>
<td>150.8±98.0</td>
<td>38.7±9.3*</td>
<td>57.8±15.1*</td>
</tr>
<tr>
<td>(week 11; RT then AT&lt;sub&gt;high&lt;/sub&gt;)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D-ART</strong></td>
<td>331.0±169.8</td>
<td>112.6±45.7</td>
<td>202.2±33.0</td>
<td>184.6±41.8</td>
</tr>
</tbody>
</table>

Data are means ± SE

*significantly lower than day 1 (p<0.05)

**Table 4.4.** Epinephrine concentrations in response to exercise at week 11 or 12 of training
4.4 Discussion

The present investigation established that the magnitude of cardioprotection following I-R injury was similar between D-AT$_{\text{high}}$ (undergoing CIT) and D-IIT. This exercise-related cardioprotection does not appear to be exercise-specific; although each exercise modality may provide unique advantages. For example, the exercise-related protection from I-R injury experienced by D-ART was comparable to D-AT$_{\text{high}}$ and D-RT in many of the measures during the I-R protocol, while D-ART provided the largest increase in the rate of developed pressure. Further, D-AT$_{\text{high}}$ had the largest increase in cardioprotective Hsp70 expression and perhaps most importantly, the largest improvement in glycemia (fructosamine concentrations). Regardless of the exercise training modality, hepatic glycogen deficiency is still apparent in T1DM rats, which likely reflects the inability of RT to improve the rapid drop in blood glucose evident during aerobic exercise. However, it is important to note that T1DM rats in each of the different training modalities failed to reach hypoglycemic blood glucose concentrations (less than 3mM).

Clinically, stringent management of blood glucose concentrations through intensive insulin therapy is the primary treatment strategy in order to limit the progression of CVD (26). Indeed, D-IIT resulted in greater recovery from an I-R injury than D-CIT, supporting the deleterious effects of chronic hyperglycemia on the micro- and macrovasculature in individuals with T1DM (10, 41). During recovery from I-R injury, D-AT$_{\text{high}}$ showed improvements in cardiac functional recovery compared to D-CIT, while D-AT$_{\text{high}}$ and D-IIT displayed similar recovery values. In a previous study, we reported that six weeks of high intensity aerobic exercise led to significant improvements in I-R
functional recovery (20). Here, we demonstrate that this modality of exercise when combined with CIT can lead to comparable recovery from I-R injury as IIT alone. It is likely that glycemic control played a significant role in contributing to the increased cardioprotection of IIT and AT\textsubscript{high}, since both exhibited similar serum fructosamine concentrations. Indeed, the negative correlation between serum fructosamine, indicative of glycemic control, and LVDP would support this finding.

While it is well-recognized that regular exercise can improve glycemic control (lowered HbA\textsubscript{1c}) in Type 2 diabetes, results in T1DM have generally failed to show this glycemic benefit (5). A number of factors may contribute to this lack of evidence in previous studies, including the predominant use of adolescent subjects, the use of questionnaires to estimate activity levels, or the increased food consumption that is typically associated with the initiation of an exercise program (5). The current study measured fructosamine concentrations and demonstrated that D-AT\textsubscript{high} had similar levels of glycosylated protein in the blood as D-IIT. Although comparable to HbA\textsubscript{1c}, the measure of glycosylated hemoglobin, fructosamine is a measure of the amount of serum proteins that have undergone glycation and is thus, a better marker for shorter-term glycemic control (approximately two weeks). While there is a shortage of evidence supporting increased glycemic control in T1DM following aerobic exercise (5), exercise intensity appears to play a significant role as to whether glycemic benefits are obtained (5, 35). In the present study the aerobic exercise training program was intensive, representing approximately 70-80% of the rats VO\textsubscript{2max} (2). The potential ability of RT to improve glycemic control (determined by HbA\textsubscript{1c}) in populations with T1DM is inconclusive (47) and the present results would support work citing that it has no benefit on long-term glycemia (33). No improvement was evident in D-ART, despite
supplementing RT with AT$_{\text{high}}$, suggesting that the frequency of AT$_{\text{high}}$ may be an important factor to experience glycemic benefits.

In a previous report we demonstrated that six weeks of RT provided little protection against an I-R injury in T1DM rats (20). The current study demonstrated that longer term RT, conducted alone or paired with AT$_{\text{high}}$ (D-ART), is necessary in order to provide similar levels of cardioprotection as performing strictly AT$_{\text{high}}$. Indeed, it has been demonstrated in non-T1DM rats that short-term RT provides little cardioprotection (11), however, if the RT is prolonged the cardioprotective effects of this form of exercise becomes evident, as demonstrated by reduced infarct size following I-R injury (40). In this regard, longer term RT did provide some exercise-specific improvements following I-R injury in D-RT and D-ART. For example, the maximal rate of pressure development (+dp/dt) and relaxation (-dp/dt) in T1DM rats were significantly improved in experimental groups utilizing RT, that were not evident in D-AT$_{\text{high}}$. It has been reported that just a single bout of RT can improve the rate of left ventricular systolic pressure in hypertensive rats undergoing Langendorf-perfusion (13). Further, Melo et al. (23) reported faster cardiomyocyte contraction and relaxation in rats following eight weeks of RT, believed to be due to increased sarcoplasmic reticulum Ca$^{2+}$-ATPase (SERCA2a) expression. While neither +dp/dt or -dp/dt were altered in D-AT$_{\text{high}}$, these results may not be surprising as little change in Ca$^{2+}$ regulatory mechanisms are reported elsewhere in rat hearts following 12 weeks of treadmill training (9). Thus, these findings may support the incorporation of RT into the treatment of T1DM, since slowed Ca$^{2+}$ clearing and abnormal cardiomyocyte excitation-contraction coupling is prominent in T1DM (34).

The finding that rates of pressure development and relaxation were increased in D-RT and D-ART despite no improvement in glycemia (fructosamine), indicates that
other factors may contribute to changes in rates of pressure development. For example, cardiomyocytes from insulin resistant rats have demonstrated mechanical defects and impaired Ca\(^{2+}\) handling (12, 38). In the present investigation we report a negative correlation between the degree of insulin resistance and the rate of developed pressure. Indeed, the experimental groups that demonstrated the greatest insulin sensitivity, D-RT and D-ART, also displayed the quickest rates of pressure development and relaxation. In the insulin resistant state, impaired SERCA activity is well documented to contribute to cardiomyocyte dysfunction (45), and RT itself has been shown to increase SERCA expression (23). In the present study, SERCA2 expression was not changed as a result of RT or ART. This lack of change may not reflect changes in the activity levels of this enzyme, as impaired SERCA activity has been reported in insulin resistant animals despite normal protein content (45). Nonetheless, the implications of insulin resistance in the recovery from an I-R injury are significant and require further investigation, given the emerging evidence of “double diabetes”, a separate classification of patients with T1DM that exhibit both insulin deficiency and resistance (6).

In seeking to explain the mechanistic means by which a specific exercise training regime may prove to be more beneficial for the functional recovery of the heart during I-R injury, we examined Hsp70 protein expression in each of the groups (17, 29). In the current study we observed an increase in left ventricular Hsp70 content in D-AT\(_{\text{high}}\) compared to both sedentary T1DM groups (D-CIT and D-IIT). Our laboratory, as well as others, have established the importance of exercise-induced Hsp70 expression in recovery from I-R injury (17, 29). This finding is in line with previous work from our laboratory that demonstrated both short and long-term aerobic exercise can result in increased Hsp70 in the hearts of insulin-treated T1DM rats (20, 22). Further, we showed that D-AT\(_{\text{high}}\) had
higher Hsp70 expression than D-RT which supports an earlier finding by our laboratory (20). While it is not clear why differences in the expression of Hsp70 exists between exercise modalities, it may be reflective of exercise-specific modulation to Ca²⁺ handling or oxidative stress. While improvements in muscle contractility can be associated with the interaction of Hsp70 and SERCA (43), evidence suggests that Hsp70 may be more involved in oxidative stress following exercise (42). Indeed, Hsp70 only modestly binds to SERCA following exercise, in comparison to the Hsp70-SERCA binding in response to heat stress (14). We have previously shown that antioxidant enzymes are elevated in the myocardium following AT_high but not following RT (20). Further, increases in myocardial antioxidant defenses are likely associated with the duration and frequency of training (31) and exercise-induced elevations in Hsp70 are known to be intensity-dependent (24). Since D-ART demonstrated similar Hsp70 expression as D-AT_high, it is plausible that neither the same quantity or intensity of exercise was achieved in D-RT.

The largest barrier to exercise prescription for individuals with T1DM is exercise-induced hypoglycemia (4). Thus, independent of which exercise provides the largest cardiovascular benefit, the risk of exercise-induced hypoglycemia must also be considered. Similar to past findings (20), D-AT_high resulted in a significant drop in blood glucose immediately following exercise, while D-RT did not. Contrary to our hypothesis, the integration of RT and AT_high (D-ART) did not alter the abrupt drop in blood glucose in response to AT_high. It is conceivable that the lack of improvement in the blood glucose response to exercise in D-ART was a result of the inability of this form of exercise to improve hepatic glycogen levels. Recently, our group has demonstrated that both sedentary and exercised trained T1DM rats using CIT demonstrate hepatic glycogen deficiencies (21), similar to what has been reported using clinical populations (3). Despite
increased glycemic control in IIT there was no difference in hepatic glycogen content between D-IIT and D-CIT. Further, exercise trained T1DM rats, regardless of training modality, demonstrated significantly lower liver glycogen content. While these findings are contrary to previous work (21), it is likely that the amount of insulin in circulation in the exercise trained rats contributes to deficient hepatic glycogen. Both D-CIT and D-IIT had similar exogenous insulin concentrations, which would in turn regulate glycogen storage by increasing the activity of glycogen synthase (36). Moreover, independent of exercise modality, trained T1DM rats displayed the smallest amounts of hepatic glycogen, concurrent with the lowest exogenous insulin requirements. Despite the apparent cardiovascular benefits associated with regular exercise, the decreased hepatic glycogen content in trained T1DM rats could have implications for combatting hypoglycemia, since hepatic glycogen is a prominent source of blood glucose during glucose demanding states (18, 44).

The first objective of the present investigation was to determine if AT\textsubscript{high} coupled with CIT resulted in larger cardioprotective benefits than IIT alone. Findings presented here demonstrate that when CIT was paired with AT\textsubscript{high} the increase in cardioprotection from I-R injury was similar to that of D-IIT. In fact, the current findings may suggest that CIT with AT\textsubscript{high} may lead to a larger cardiac improvement in T1DM rats than IIT alone, given the increased expression of left ventricular Hsp70 with this form of exercise. While addressing our second objective, we determined that following long-term exercise training both D-ART and D-RT resulted in similar levels of overall cardioprotection as D-AT\textsubscript{high}; although each exercise training modality did appear to provide unique benefits. For example, improved glycemic control was only evident in D-AT\textsubscript{high}, while the largest improvements in insulin sensitivity measures were evident in exercises that utilize
resistance exercise (D-ART, D-RT). To this point, this study underlines the need to consider other factors besides glycemic control (i.e. insulin resistance) when tailoring an exercise treatment program for the patient with T1DM to reduce the risk of developing CVD.
4.5 References


26. **Nathan DM.** The diabetes control and complications trial/epidemiology of


CHAPTER 5

5 «High intensity aerobic exercise training improves insulin-induced vasorelaxation in a rat model of Type 1 diabetes mellitus»

5.1 Introduction

Type 1 diabetes mellitus (T1DM) is associated with an increased susceptibility to cardiovascular disease (CVD)(38). Both clinical and experimental T1DM are associated with impaired endothelial function, due to reduced production and bioavailability of nitric oxide (NO)(9, 18, 20, 34). Evidence suggests that vascular insulin resistance plays a central role in endothelial dysfunction, which is increasingly viewed as the primary factor contributing to CVD and its co-morbidities such as atherosclerosis, hypertension, peripheral neuropathy, and metabolic dysfunction (22, 29). Diminished vascular actions of insulin are evident in patients with T1DM (1, 35, 40) and the development of treatment strategies to improve vascular insulin sensitivity may provide an important means to mitigate the increased risk of cardiovascular disease in populations with T1DM. Indeed, impaired insulin-induced vasodilation is believed to play a pivotal role in the predisposition to atherosclerosis (36) and endothelial dysfunction (22, 29).

Aside from its metabolic effects, insulin stimulates endothelium-dependent vasodilation, as well as vasoconstriction in order to regulate the delivery of insulin and glucose to skeletal muscle (2, 28). Briefly, endothelium-dependent insulin dilation occurs following the activation of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, which phosphorylates eNOS and increases the production of NO. Insulin can also lead to endothelium-dependent vasoconstriction by activating the Ras/mitogen-activated protein
kinase (MAPK) pathway, which subsequently releases endothelin-1 (ET-1). During states of vascular insulin resistance, there is a downregulation of the PI3K-Akt pathway and a favoring of the MAPK insulin signalling pathway, resulting in reduced NO availability, larger ET-1 production, and a net vasoconstriction (17, 28, 33).

It is well-established that poor glycemic control is a contributing factor to endothelial dysfunction in T1DM (20, 21, 37, 41). The findings of the Diabetes Control and Complications Trial (DCCT) clearly establishes that microvascular disease risk is significantly reduced in patients with T1DM following tight blood glucose management using an intensive insulin therapy (IIT) approach (39). Regular exercise has also been shown to improve the vascular actions of insulin (7, 25, 32, 33). However, patients with T1DM that adhere to a physically active lifestyle often maintain a higher level of glycemia, representative of a more conventional insulin therapy (CIT), in the attempt to mitigate the risk of hypoglycemia that can accompany exercise (8). Although a relationship exists between glycemic control (HbA1c) and the degree of endothelial dysfunction in T1DM (37), physical activity may be a more effective means to improve the vasoreactivity to insulin (6, 24, 25). Indeed, it has been reported in Type 2 diabetes mellitus (T2DM) that aerobic exercise training improves the vascular actions of insulin (24, 25), while metformin treatment alone does not enhance insulin-stimulated vasodilation, despite lowered HBA1c, body weight and body mass (6). These findings stress the importance of regular exercise in the improvement of vascular insulin sensitivity and suggest that lowering glycemia through intensive insulin therapy alone may not be sufficient to improve the vasoreactivity to insulin.

Due to the risk of hypoglycemia associated with aerobic exercise training in T1DM the use of other forms of exercise such as resistance exercise training (RT) has
gained attention \( (23, 42, 43) \). RT has been shown to have positive benefits on endothelial function \( (11, 14, 27) \) and vascular insulin sensitivity \( (16) \). For instance, it has been shown that insulin stimulated increases in leg blood flow are larger following both RT and aerobic exercise in subjects with T2DM \( (7, 16) \). It is not clear whether these same improvements are evident in patients with T1DM, as our group has reported that RT does not result in same level of improvements in vascular responsiveness as high intensity aerobic exercise \( \text{AT}_{\text{high}} \)(31). However, this may be reflective of the longer time it takes for beneficial vascular adaptations that RT may have in comparison to aerobic exercise. Nonetheless, the potential of RT to protect against exercise-induced hypoglycemia, while providing benefits to the vasculature in patients with T1DM is promising and should be further explored.

The purpose of the present study was to determine which modality of exercise \( \text{AT}_{\text{high}} \) or RT, when paired with CIT, results in superior femoral artery vasomotor function. Secondly, it was determined whether long-term \( \text{AT}_{\text{high}} \) or RT, when paired with CIT, results in superior vasomotor function compared to IIT, the standard treatment regime for T1DM. It was hypothesized that enhanced vasomotor function and insulin-induced vasorelaxation would be evident in both \( \text{AT}_{\text{high}} \) and RT. Further, \( \text{AT}_{\text{high}} \) and RT (paired with CIT) would result in superior vasomotor function compared to IIT alone.

5.2 Methods

All procedures were in compliance with the Canadian Council on Animal Care and were approved by the Research Ethics Board of the University of Western Ontario. Male Sprague-Dawley rats (8-weeks old) were obtained from Charles River Laboratories (Saint-Constant, Quebec, Canada). Rats were housed in pairs, at a constant humidity and
temperature (21.5°C and 50% humidity), and were provided water and standard rat chow *ad libitum*.

**Experimental Protocol.** Diabetic rats were randomly divided into intensive insulin treatment (D-IIT; n=10), conventional insulin treatment (D-CIT; n=10), high intensity aerobic exercise training (D-AT\textsubscript{high}; n=8), and resistance exercise training (D-RT; n=8). During week 1, T1DM was induced in all rats with multiple low doses of streptozotocin (Sigma-Aldrich; STZ; 20mg/kg; citrate buffer 0.1M, pH 4.5) over five consecutive days, and was confirmed after two consecutive non-fasting blood glucose concentrations greater than 18mM. A single rat in D-RT did not reach the desired blood glucose concentration and was excluded from the study. Starting during week 2, and for the duration of the study, exogenous insulin was provided to T1DM rats via subcutaneous insulin pellets (Linshin, Toronto, Canada). Through daily blood glucose monitoring and insulin pellet adjustment, blood glucose was maintained between 4-9mM in D-IIT and between 9-15mM in D-CIT, D-AT\textsubscript{high}, and D-RT. From week 3 to week 14 of the experimental protocol D-IIT and D-CIT remained sedentary and D-AT\textsubscript{high} and D-RT underwent their designated exercise training programs.

**Exercise Protocol.** In D-AT\textsubscript{high}, rats exercised on a motorized treadmill at 27m/min and 6% grade (1hr, 5 days/week). In D-RT, rats climbed a vertical ladder as previously described (13, 23). Briefly, rats were familiarized to the ladder during week 2 by climbing the ladder with increases in weight secured to the proximal portion of their tail (5%, 15%, 20%, and 35% of body mass). Regular RT sessions consisted of climbing the vertical ladder with incremental increases in weight attached to the tail (50%, 75%, 90% of maximal carrying capacity), followed by climbing with their maximal carrying
capacity until exhaustion. Every fourth day, maximal carrying capacity was determined by adding 30 grams of weight to their tail (beginning with 75% of their body mass) until rats reached exhaustion. Exhaustion was defined as failure to climb despite tactile stimulation to the haunches of the rat.

**Tissue Collection.** Three days after the last exercise bout rats were anaesthetized with isoflurane and euthanized via heart excision. Femoral arteries were carefully dissected and placed in cold Krebs-Henseleit buffer (118.1mM NaCl, 4.7mM KCl, 1.5mM CaCl₂, 1.2mM KH₂PO₄, 1.2mM MgSO₄, 11.1mM glucose, and 25mM NaHCO₃, pH 7.4). Vessels were cleaned of any connective or adipose tissue and were partitioned into ~2mm rings for *in vitro* isometric tension analysis. Remaining femoral arteries were flash frozen in liquid nitrogen for protein quantification.

**Blood Analysis.** Blood glucose concentrations were measured and recorded weekly using a One Touch Ultra 2 Blood Glucose Monitoring System (Lifescan Canada Ltd, Burnaby, BC, Canada) and One Touch test strips (Lifescan Canada Ltd). Serum endothelin-1 (Enzo Life Sciences, NY, USA; ADI-900-020A) and exogenous insulin concentrations (Alpco, Salem, NH: Catalog # 80-INSHU- E01.1) were determined via ELISA.

**Isometric Vascular Myography.** Vessel rings were carefully mounted on wire brackets connected to an isometric force transducer (GlobalTown, Microtech, Sarasota, FL) and were submerged in 5ml of Krebs-Henseleit buffer at 37°C. Vessel rings were washed every 15 minutes and gradually equilibrated over 1 hour to a baseline tension of ~1g, based on previous work (and pilot testing) from our laboratory (30, 31). Femoral arterial rings were pre-contracted with phenylephrine (PE; 3e⁻⁷M) and following a steady-
state level of contraction, dose-response curves for acetylcholine (ACh; half-log doses; \(1 \times 10^{-8.5} - 1 \times 10^{-5}\) M), sodium nitroprusside (SNP; whole-log doses; \(1 \times 10^{-11} - 1 \times 10^{-6}\) M), and insulin (whole-log doses; \(1 \times 10^{0} - 1 \times 10^{5}\) uIU/ml) were conducted. Thereafter, beginning with basal resting tension (~1 g), femoral rings underwent dose-response curves of PE (half-log doses; \(1 \times 10^{-8} - 1 \times 10^{-5}\) M). To evaluate the contribution of the endothelium, arterial rings were treated with nitric oxide inhibitor (NOS) NG-Monomethyl-L-arginine (L-NMMA; \(1 \times 10^{-5}\) M) for 10 minutes, pre-contracted with PE (\(3 \times 10^{-7}\) M), and following a basal steady-state level of contraction, were treated with either ACh (\(1 \times 10^{-6.5}\) M) or insulin (\(1 \times 10^{3}\) uIU/ml). Data were excluded if the response of 3 consecutive doses were ± 3 standard deviations from the mean.

*Western Blotting.* Triton X-100 tissue lysates were used to produce Western blot-ready Laemmli samples. Protein samples (10 \(\mu\)g/lane) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were probed with the following primary antibodies: eNOS (#610296; 1:500) and phospho-specific eNOS at Ser1177 (#61239; 1:250) from BD Biosciences, Akt (#4691; 1:500) phospho-specific Akt at Ser473 (#4061; 1:250), p44/42-MAPK (#4695; 1:500), phospho-specific p44/42-MAPK at Thr202/Tyr204 (#4370; 1:250) and GAPDH (#5174, 1:1000) from Cell Signaling, and endothelin-1 (#117757; 1:2000) from Abcam. Intensity of individual protein bands were quantified using FluoroChem HD2 (AlphaView, version 3.4.0.0), and expressed as ratio to control band of GAPDH.

*Statistical Analysis.* Weekly body mass and blood glucose concentrations were compared using a two-way repeated measures analysis of variance (ANOVA). Dose-responses were compared using a two-way repeated measures ANOVA. Differences in
vasorelaxation with L-NMMA, western blot data, systemic ET-1 concentrations, and insulin dose were determined using a one-way ANOVA. When a significance difference was evident, a least squares difference post-hoc test was performed with a significance level set at p<0.05. All data are presented as a mean ± standard error and all statistical analysis was completed using Graphpad Prism 6.

5.3 Results

*Animal Descriptives.* There was an interaction between experimental week and body mass in that D-IIT weighed more than D-AT\textsubscript{high} after week 7, and more than D-RT after week 8 (Figure 5.1A; p<0.05). Further, D-CIT weighed more than D-AT\textsubscript{high} at weeks 11 and 12, and more than D-RT at week 12 (p<0.05). There was an interaction between experimental week and blood glucose concentration in that D-IIT had lower blood glucose concentrations than D-CIT at every week but 1, 3, and 8, while D-IIT was lower than D-AT\textsubscript{high} at weeks 2 and 12 (Figure 5.1B; p<0.05). Further, D-CIT was higher than D-RT at weeks 2, 3, and 10, and higher than D-AT\textsubscript{high} at weeks 3, 4, and 7 (p<0.05). Exogenous insulin concentrations were lower in D-AT\textsubscript{high} and D-RT compared to D-IIT (Table 5.1; p<0.05) and no differences were evident in serum endothelin-1 (p>0.05).

*Isometric Vascular Myography.* No differences were observed across all doses of acetylcholine (Figure 5.2A; p>0.05). Insulin-induced vasorelaxation was greater in D-AT\textsubscript{high} at every dose of insulin compared to D-IIT, D-CIT, or D-RT (Figure 5.2B; p<0.05). In regards to endothelium-independent vasorelaxation, no differences were evident at any dose of sodium nitroprusside (Figure 5.2C; p>0.05). No difference in phenylephrine-induced contraction was evident across all groups (Figure 5.2D; p>0.05).
When vessels were treated with L-NMMA there was no difference in percent vasorelaxation following ACh or insulin dose (Table 5.1: p>0.05).

*Western Blotting.* In the femoral artery Phospho(Ser473) Akt/Total Akt was elevated in D-AT\textsubscript{high} compared to all other groups (Figure 5.3A; p<0.05). No differences were evident between Phospho(Ser1177) eNOS/Total eNOS (Figure 5.3B; p<0.05). Both D-IIT and D-AT\textsubscript{high} demonstrated elevated Phospho-p44/42 MAPK/Total p44/42 MAPK compared to D-CIT and D-RT (Figure 5.3C; p<0.05). No difference in ET-1 protein content was evident (Figure 5.3D; p>0.05).
Figure 5.1. Weekly body mass (A) and blood glucose concentrations (B) in T1DM rats. α; D-IIT different from D-AT\textsubscript{high}, β; D-IIT different from D-RT, Φ; D-AT\textsubscript{high} different from D-CIT, ψ; D-RT different from D-CIT; p<0.05. All data are means ± SE.

<table>
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<th>D-CIT</th>
<th>D-AT\textsubscript{high}</th>
<th>D-RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin dose (IU/kg)</td>
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<td>54.2±9.5</td>
<td>23.5±8\textsuperscript{1}</td>
<td>21.3±13.3\textsuperscript{1}</td>
</tr>
<tr>
<td>Serum ET-1 (pg/ml)</td>
<td>21.6 ± 4.4</td>
<td>22.2 ± 3.8</td>
<td>15.8 ± 2.9</td>
<td>19.8 ± 2.4</td>
</tr>
<tr>
<td>ACh + L-NMMA (%pre-contraction)</td>
<td>7.1 ± 4.0</td>
<td>4.9 ± 3.0</td>
<td>3.1 ± 1.7</td>
<td>4.2 ± 2.9</td>
</tr>
<tr>
<td>Insulin + L-NMMA (%pre-contraction)</td>
<td>4.8 ± 5.9</td>
<td>3.0 ± 3.8</td>
<td>4.6 ± 4.4</td>
<td>-4.6 ± 1.7</td>
</tr>
</tbody>
</table>

All data are means ± SE. \textsuperscript{1}different from D-IIT (p<0.05).

Table 5.1. Blood analysis and L-NMMA data
Figure 5.2. Dose response curves for acetylcholine (A), insulin (B), sodium nitroprusside (C), and phenylephrine (D) in the femoral artery of T1DM rats. *different from all groups; p<0.05; All data are means ± SE.
Figure 5.3. Phospho-specific Akt/Akt (A), phospho-specific eNOS/eNOS (B), phospho-specific MAPK/MAPK (C), and ET-1 (D) in the femoral artery of T1DM rats. *different from all groups, α; different from D-CIT, β; different from D-RT; p<0.05. All data are means ± SE.
5.4 Discussion

Findings demonstrate that vasomotor function in the femoral artery was similar in sedentary T1DM rats undergoing either CIT or IIT, despite different ranges of blood glucose maintained in these two groups. Acetylcholine-induced vasorelaxation was similar across experimental groups, while only long-term AT\textsubscript{high} resulted in increased insulin-induced vasorelaxation and was accompanied by an increase in the active phosphorylated form of Akt. This study emphasizes the importance of AT\textsubscript{high} for the improvement of vascular insulin sensitivity in T1DM, which may be an important factor in mitigating T1DM-related endothelial dysfunction and overall cardiovascular risk in this population.

The T1DM model used in the current investigation has demonstrated endothelial dysfunction at varying levels (30, 32). In the current study we report no difference in vascular responsiveness between sedentary T1DM rats (D-CIT and D-IIT) that were different only with regards to blood glucose concentrations and insulin treatment strategies. This finding would challenge the belief that glycemia, as measured by HbA\textsubscript{1c}, is closely related to the degree of endothelial dysfunction (37). Indeed, studies examining endothelial function in experimental models of T1DM have been contradictory, whereby some studies demonstrate an enhancement in endothelium-dependent vasodilation (34), while others have shown impairments (for review see (4)). Differences in disease duration, artery examined, or the use of insulin supplementation may explain many of these discrepancies. Despite different blood glucose levels in D-IIT and D-CIT, similar amounts of insulin administration were used between these two sedentary groups, which may account for the comparable levels of acetylcholine-stimulated vasorelaxation.
reported in the current study. Indeed, short-term insulin supplementation in T1DM rats has been shown to attenuate endothelial dysfunction by inducing the expression of eNOS (21), which may also explain why eNOS expression was similar between these two sedentary groups (D-IIT and D-CIT). Further, comparable levels of acetylcholine-induced vasorelaxation has been reported elsewhere in STZ-T1DM rats when animals are treated with either low or high doses of insulin, even when these same experimental groups differed dramatically in blood glucose concentrations (19).

No difference in insulin-stimulated vasorelaxation was evident between D-CIT and D-IIT in the current study. This finding is supported by evidence in T2DM rats, whereby metformin failed to enhance insulin-stimulated vasodilation despite lowered HBA$_{1c}$ (6). Increased expression of the active phosphorylated form of MAPK was only evident in D-IIT (compared to D-CIT), suggesting that the femoral artery of D-IIT rats may be in the beginning stages of vascular insulin resistance. It is well established that hyperinsulinemia leads to the selective inhibition of PI3K and the inability of insulin to stimulate eNOS, which in turn, may cause a functional shift of insulin to a mitogenic role in the vessel (26). Indeed, despite comparable expression levels of eNOS in all of the experimental groups, the phosphorylated active form of MAPK was higher in D-IIT. It is unclear whether this is an indication of pathology to the vessels, as ET-1, the vasoconstrictive factor activated by MAPK, was not elevated. Additional work is warranted to discern whether the increases in the phosphorylated form of MAPK in D-IIT is detrimental to the vasculature of these animals (5).

Given that the benefits of aerobic exercise and RT on endothelium-dependent vasodilation in T1DM has been established both clinically (12) and experimentally (3, 9, 12, 27), the inability of both exercise regimes to improve acetylcholine-induced
vasorelaxation in the current study is surprising. However, these discrepancies may be explained in part by the use of insulin supplementation in the current study. We have previously reported that both $AT_{\text{high}}$ and RT increased whole-body insulin sensitivity in T1DM rats (13), which results in a reduction in the required insulin dose that is necessary to maintain a desired blood glucose concentration. Further, both insulin treatment (21) and exercise training (9, 31, 32) are well established to increase eNOS expression in T1DM. Thus, it is conceivable that the benefits of exercise training on acetylcholine-induced vasorelaxation may have been masked by the increase in the insulin dosage necessary to maintain the desired blood glucose ranges in both sedentary T1DM groups (D-IIT and D-CIT). Insulin treatment alone can restore endothelial-dependent vasorelaxation in STZ rats (19–21). It is important to note that others have reported that $AT_{\text{high}}$ fails to improve NO-dependent relaxation in the aorta of STZ rats (44).

The main finding of the current study was that $AT_{\text{high}}$ resulted in significant increases in insulin-induced vasorelaxation that appears to be Akt-dependent. The ability of regular exercise to enhance the vascular actions of insulin within resistance and conduit arteries in different populations has recently been reviewed (33). However, few studies have determined the implications of exercise training on the vascular actions of insulin in T1DM (15, 32). Using this insulin-treated model of T1DM our group has shown that ten-weeks of $AT_{\text{high}}$ can alleviate diabetes-related impairments in insulin-stimulated vasa nervorum dilation, which also coincides with improved motor nerve conduction velocity (32). Further, others have shown an increase in cutaneous blood flow in response to local microinjections of insulin in exercise trained STZ-T1DM rats (15). To our knowledge, this is the first study to demonstrate that $AT_{\text{high}}$ can increase vascular insulin sensitivity in conduit arteries of T1DM rats, which could have large implications
for a patient population at a heightened risk for vascular disease (4, 18, 38). In contrast to AT\textsubscript{high}, RT failed to demonstrate an improvement in insulin-induced vasorelaxation. This work does not support previous evidence that used healthy rats to demonstrate an increase in insulin-induced vasodilation immediately following a single bout of RT (11). These discrepancies may be explained by the fact that arteries in the present investigation were not harvested until three days following the last exercise bout, which may have mitigated any acute benefits of RT. Nonetheless, it has been hypothesized that increases in vascular insulin sensitivity following exercise training occurs as a result of shear stress caused by exercise-induced hyperemia (33). It is plausible that an increase in the sensitivity to insulin following AT\textsubscript{high} only may be a result of a lack of exercise-induced hyperemia associated with this RT protocol.

Endothelial dysfunction often precedes vascular complications, while deficiencies in vascular insulin signalling often occurs early in the progression of endothelial dysfunction (10, 22, 29). Here, the results demonstrate that AT\textsubscript{high}, when paired with more modest glycemic control (CIT), can drastically increase insulin-induced vasorelaxation. Interestingly, more intensive glycemic control did not significantly improve vasomotor function. Increased vascular actions of insulin resulting from regular physical activity can have positive implications on both conduit and resistance arteries (33). It has been shown that insulin resistance in the vasculature is selective, in that only the vasodilatory PI3K/Akt pathway is disturbed, leaving the Ras/MAPK intact and possibly enhanced (26, 29). In the present study, the enhanced vasorelaxation response to insulin in the femoral artery was likely Akt-dependent, as the expression of the active phosphorylated form of Akt was only elevated in D-AT\textsubscript{high}. Further, D-AT\textsubscript{high} exhibited a higher expression of phosphorylated MAPK. Contrary to the elevation in phosphorylated MAPK evident in D-
IIT, the increased expression of phosphorylated MAPK in D-AT\textsubscript{high} is likely indicative of an overall increase in insulin signalling in the femoral artery. Indeed, despite D-AT\textsubscript{high} and D-RT having the same insulin dose, D-AT\textsubscript{high} demonstrated increased expression of both phosphorylated Akt and MAPK compared to D-RT.

In conclusion, long-term AT\textsubscript{high} results in enhanced insulin-induced vasorelaxation in the femoral artery, likely as a result of increased Akt expression. Findings presented here suggest that the inclusion of AT\textsubscript{high} into the treatment of T1DM, despite being accompanied by less reliance on glycemic control, can provide greater improvements in vascular insulin sensitivity than IIT alone. Long term clinical studies are warranted to investigate the importance of AT\textsubscript{high} in mitigating T1DM-related vascular insulin resistance and whether this benefit exceeds the well-established cardiovascular improvements reported with lifelong IIT.
5.5 Reference List


15. **Heidarianpour A, Hajizadeh S, Khoshbaten A, Niaki AG, Bigdili MR,**


CHAPTER 6

6  «General Discussion»

6.1 Summary

The principal objective of this dissertation was to determine which exercise regime provides the greatest cardiovascular protection for populations with Type 1 diabetes mellitus (T1DM), while assessing the risk of exercise-induced hypoglycemia. Sufficient evidence exists for health practitioners to encourage regular exercise for the prevention of chronic disease (4). A recent review of the literature has recognized numerous benefits of exercise for patients with T1DM, most namely reduced insulin requirement, improved lipid levels, increased insulin sensitivity, and reduced cardiovascular disease and mortality (7). However, for the patient with T1DM, the ideal intensity, duration, or modality (aerobic/resistance) has yet to be fully established (7). Further, the largest barrier that patients must overcome when initiating an exercise program is exercise-induced hypoglycemia and the manifestation of hypoglycemia following exercise training is largely unknown (2, 5).

The main findings of these collective studies were as follows; (1) the magnitude of the abrupt decline in blood glucose in response to a single bout of exercise in T1DM rats remains consistent after exercise training and infrequently reaches hypoglycemic concentrations if blood glucose concentrations are elevated prior to exercise (Chapters 2, 3), (2) both exercise-induced fluctuations in blood glucose and the amount of cardiovascular protection obtained from regular exercise training appears to be modality-specific (Chapter 3), and (3) maintaining a more modest glycemic control (conventional insulin therapy) that is combined with regular exercise may provide similar (Chapter 4),
or even greater (Chapter 5), cardiovascular benefits than tight glycemic control through intensive insulin therapy.

In order to fulfil the objectives of this dissertation it was important that the acute blood glucose response to each exercise protocol in T1DM rats were comparable to data reported in exercising patients with T1DM. In T1DM rats, D-AT_{mod} and D-AT_{high} caused a significant reduction in blood glucose during exercise, while the D-RT protocol resulted in a more gradual decline in blood glucose concentrations during the recovery phase of exercise. Indeed, these acute exercise-induced changes in blood glucose mirror those responses reported in human populations with T1DM (25). Despite training-related changes in several glucoregulatory hormones (insulin, glucagon, and epinephrine) in T1DM rats (10, 14), the acute exercise-induced reduction in blood glucose was consistent throughout the training period, provided the exercise was of at least moderate intensity (Chapters 2, 3). Following short-term D-AT_{low} training the initial reduction in blood glucose in response to exercise was abolished, although this form of exercise training provided little cardioprotection. When blood glucose concentrations were elevated prior to the initiation of exercise, the risk of experiencing hypoglycemia appeared to be reduced during D-AT_{high} and D-RT, as exercising T1DM rats did not reach hypoglycemic blood glucose concentrations (<3mmol/l). In the clinical setting, through increased carbohydrate ingestion and insulin adjustment, the intentional elevation of blood glucose concentrations prior to exercise is one of the most practiced approaches for preventing hypoglycemia during exercise in patients with T1DM (21, 28). It has been reported that exercise should be initiated when blood glucose concentrations are between approximately 8-14 mmol/l, provided significant amounts of ketones are not present (28). As a consequence of frequent exercise, this blood glucose range is experienced
chronically by exercising patients with T1DM, and in turn, these patients are often characterized as using a more conventional insulin therapy strategy. Thus, the moderately glycemic T1DM rodent model used in these studies is reflective of the exercising patient with T1DM who utilizes a conventional insulin therapy strategy to offset the risk of hypoglycemia (23).

A high relative risk of mortality from ischemic injury to the heart exists in populations with T1DM (12). In fact, cardiovascular disease is the leading cause of death for both T1DM and T2DM (15). The second objective of this dissertation set out to determine which exercise modality would provide the largest degree of cardiovascular protection. By elevating blood glucose concentrations prior to exercise the use of both D-AT\textsubscript{high} and D-RT appears to be manageable for the patient with T1DM; however, based on the current findings the degree of cardiovascular protection they provide is duration and exercise-specific. Results demonstrated that following different modalities of short-term exercise training (D-AT\textsubscript{low}, D-AT\textsubscript{high}, or D-RT) the greatest protection following an ischemia-reperfusion (I-R) injury occurred following D-AT\textsubscript{high} (Chapter 3). These findings are supported by data in patients with T2DM from the ADVANCE (Action in Diabetes and Vascular Disease: Preterax and Diamicron modified release Controlled Evaluation) trial which showed that moderate to vigorous intensities of exercise are associated with a larger reduction in the incidence of cardiovascular events, microvascular complications, and all-cause mortality compared to a milder intensity (3).

While D-AT\textsubscript{high} may provide the greatest cardiovascular protection, the minimal decline in blood glucose with D-RT suggests that this form of exercise may be most manageable form of exercise in patients with T1DM. This finding is similar to clinical data, whereby the use of resistance exercise alone, or prior to a bout of aerobic exercise,
reduces the exercise-induced declines in blood glucose (25–27). Although the risk of exercise-induced hypoglycemia was minimal in D-RT, results presented here would suggest that in order for D-RT to provide cardioprotection the exercise must be continued long-term. Following just six weeks of resistance exercise it was found that the recovery from an I-R injury was similar to T1DM rats that remained sedentary (Chapter 3). However, after twelve weeks of training, the level of cardioprotection that resistance exercise provided during an I-R injury was comparable to D-AT\textsubscript{high} (Chapter 4). Further, the combination of aerobic and resistance exercises (D-ART) led to greater increases in the maximal rate of developed pressure and relaxation during the I-R injury compared to AT\textsubscript{high} alone (Chapter 4). While these results suggest that D-ART may be the superior exercise modality, it is of importance to note that D-AT\textsubscript{high} resulted in the largest increase in constitutive amounts of Hsp70 in the heart. Further, D-AT\textsubscript{high} was the only exercise modality to increase vascular insulin sensitivity (Chapter 5), which may indicate that this modality of exercise was the most advantageous in regards to the entire cardiovascular system. Although D-RT failed to increase vascular insulin sensitivity in the femoral artery, it is unknown if increases in insulin sensitivity were seen in other arteries. Increases in vascular insulin sensitivity following exercise training is recognized to occur specifically in the arteries supplying the active skeletal muscle (19). Secondly, it is not clear whether vascular insulin sensitivity improvements are evident following combined aerobic and resistance exercise training (D-ART). Studies examining vascular insulin responsiveness in multiple levels of the vasculature are warranted; particularly given that the greatest improvement in whole body insulin sensitivity was evident following this form of exercise training (Chapter 4). To this point, the results presented here would suggest each exercise training modality appears to provide unique benefits, underlining
the need to tailor an exercise treatment program to the individual patient with T1DM to best reduce their risk of cardiovascular disease development.

Many practitioners suggest that patients with diabetes should avoid intense forms of exercise due to the possible increased risk of a cardiovascular event (24). However, more recent evidence in a rehabilitation setting indicates that the risk of a cardiovascular event during exercise is relatively low, even in patients with coronary heart disease (22). Previously our laboratory has demonstrated numerous benefits of D-AT$_{\text{high}}$, such as reduction in cardiovascular autonomic neuropathy (9), improved micro- and macrovascular function (17, 18), and improved insulin sensitivity (8, 10). The current findings further add to this extensive list of potential benefits of this form of exercise training in patients with T1DM. Concurrent with increased recovery from I-R injury, D-AT$_{\text{high}}$ also led to increased left ventricular Hsp70 and SOD protein content, as well as improved glycemic control (lowered fructosamine levels) (Chapter 3, 4). Further, D-AT$_{\text{high}}$ resulted in the increased sensitivity to insulin in the femoral artery following twelve-weeks of exercise training (Chapter 5). This exercise-related increase in vascular insulin sensitivity may have several implications on the prevention of cardiovascular disease development in patients with T1DM. For example, increases in vascular insulin sensitivity can reduce the risk of atherosclerosis (20), improve insulin and glucose delivery to skeletal muscle (6), and improve glycemic control (1, 19). Due to the relationship between vascular insulin resistance and endothelial dysfunction, the initiation of D-AT$_{\text{high}}$ shortly after the diagnosis of T1DM is a promising avenue for preventing the development of vascular disease (11, 13, 16). Secondly, the finding that D-AT$_{\text{high}}$ paired with a more conventional insulin therapy (CIT), actually improved glycemia (glycated fructosamine) to a comparable level as intensive insulin therapy (IIT) is extremely
promising. Taken together, the present work would strongly support the use of high intensity aerobic exercise and conventional insulin therapy in the prevention of cardiovascular complications associated with T1DM.

Initially (Chapter 2, 3), studies were designed to determine the optimal form of exercise training for the patient with T1DM. As the experiments of this thesis progressed and the cardiovascular benefits associated with $D$-$AT_{\text{high}}$ became extensive, it was important to then determine how these benefits of CIT and $D$-$AT_{\text{high}}$ (or D-RT) compared to IIT alone, the current ‘gold standard’ treatment of T1DM (Chapters 4, 5). These studies demonstrated that when CIT was paired with $D$-$AT_{\text{high}}$ the amount of cardioprotection from I-R injury was similar to IIT (without exercise training). In fact, results presented here would suggest that the amount of cardiovascular protection this treatment regime (CIT with $D$-$AT_{\text{high}}$) provides for a population with T1DM may be superior to IIT, due to increases in left ventricular Hsp70 protein content and heightened vascular insulin sensitivity (Chapters 4, 5). The results herein demonstrate that while hyperglycemia is a contributing factor in the development of cardiovascular complications other factors (i.e. insulin resistance) must be considered. Nonetheless, reduced reliance on strict glycemic control may allow for exercise to be performed safely (and providing cardiovascular benefits), while preventing complications associated with IIT (weight gain, insulin resistance, hypoglycemia).

6.2 Conclusions

The findings of this dissertation would strongly suggest that high intensity aerobic exercise may provide the largest amount of cardiovascular protection for individuals with T1DM. While high intensity aerobic exercise when accompanied with conventional insulin therapy appears to mitigate hypoglycemia onset, it does not eliminate the risk, and
such a regime requires careful monitoring of blood glucose concentrations during and following each exercise bout. Nonetheless, the minimal risk of hypoglycemia associated with high intensity aerobic exercise might be outweighed by the cardiovascular benefits associated with this form of exercise. Results from this body of work suggests that the cardiovascular benefits of high intensity aerobic exercise appear to be equal to or better than intensive insulin therapy alone.
6.3 Reference List


Appendix A. Multiple Low-dose Streptozotocin Protocol (rat)

**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)
   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 0.2µm syringe filter.

Ex. 48mg STZ ÷ 3 mL buffer = 16mg/mL solution

\[
4\text{mg} ÷ 16\text{mg/mL solution} = 0.25\text{mL}
\]

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).

REFERENCES:


Appendix B. Insulin Pellet Implantation (rat)

**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
- Isofluorane Anaesthetic Machine
- Hair clippers
- Heat lamp

**PROCEDURE:**

**Pellet implantation:**

1. Anesthetize the animal using the isoflurane machine by placing it in the induction chamber. Set isoflurane 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 isoflurane 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.

REFERENCES:

http://www.linshincanada.com
Appendix C. Intravenous Glucose Tolerance Test (rat)

**MATERIALS:**
- 15 ml Falcon tube
- D-glucose
- Distilled water
- 0.2 um syringe filter
- 5 ml vacutainer
- Lidocaine Cream
- 3 ml syringe
- Restraining towel
- Microcentrifuge tubes
- Vaseline
- 27 G needles
- 30 G needles
- Gauze squares
- Mettler balance
- Biological safety cabinet
- Heat lamp with 175 watt bulb
- Glucometer (Freestyle mini)
- Timer

**PROCEDURE:**

**Fasting Rats:**

1. Fast animals for 8-12 hours prior to the tolerance test

**Glucose preparation:**

1. On the morning of the test, dispense 5ml distilled water into a clean 15 ml Falcon tube.

2. Weigh out 5 grams of D-glucose, and add it to the tube containing the sterile water. Dissolve by vortexing to result in a 50% glucose solution.

3. In a biological safety cabinet, sterilize the glucose solution using a 0.2 um syringe filter.

4. Transfer the sterile glucose solution to a new sterile 15mL Falcon tube.

**Intravenous Glucose Tolerance Test (IVGTT):**

1. Place the cage containing the rat under a heat lamp. Remove the tube from the cage, and provide water for the rat. Warm the rat in this manner for 10 minutes.

2. Remove the rat from the cage and scrub the tail using soap and water.
3. Measure and record the rat’s weight.

4. Obtain a baseline blood glucose reading from saphenous vein and collect a baseline serum sample (keep blood sample on ice).

5. Return the rat to the cage and warm for an additional 20 minutes.

6. Place the rat’s tail in a container of warm tap water for 30-60 seconds.

7. One person should dry the tail and restrain the rat by wrapping it in a green drape so the tail is exposed. Occlude the lateral tail vein by applying pressure.

8. Using a 3 ml syringe and a 27 G needle, a second person will draw up the 50% glucose solution at a dose of 1g/kg (2 ml/kg). Locate the lateral tail vein and draw back on the plunger to ensure that the needle is within the vein.

9. Release the occlusion while the glucose solution is injected. Draw back the plunger a couple of times during the injection to ensure that the needle remains located within the vein.

10. Start the timer and return the rat to its cage. Continue warming it for the duration of the IVGTT.

11. Check and record the blood glucose values for the rat at 5, 10, 20, 30, 40, 50 and 60 minutes post glucose challenge. If by 60 minutes, the blood glucose value hasn’t returned to baseline, continue to check every 30 minutes until it does so (up to a maximum of 180 minutes).

12. Obtain a blood serum sample 10 minutes following D-glucose injection (store at -70°C until insulin analyses).

13. Upon completion of the IVGTT, remove the rat from under the heat lamp and return the tube to the cage. Place food on the wire lid of the cage, and ensure that the rat has access to water.

14. Note on the cage card that an IVGTT has been performed.

REFERENCE:
Appendix D. Fine-wire Vascular Myography

Protocol has been adapted from: Campos Oscar. Insulin sensitivity and the insulin-mediated vasorelaxation response in physically active and sedentary rats. University of Western Ontario: 2011.

Pharmacological agents tested:
Phenylephrine (PE), Acetylcholine (ACh), Sodium Nitroprusside (SNP), Insulin

Tissues tested:
Aorta, Carotid, Iliac, Femoral

Before you begin
Make sure you have the following materials:
1.5L of ddH2O maintained at 37° C in one beaker
0.5L of ddH2O maintained at 37° C in one Erlenmeyer flask
Pre-weighed portions of:
1) calcium chloride dihydrate (CaCl2·2H2O) – 0.441 ± 0.001 g
2) sodium bicarbonate (NaHCO3) – 4.201 ± 0.001 g
Pre-made 0.1 M ACh solution in 1.5 mL Eppendorf tube (stored in -20°C freezer)
Small printed 2 mm x 2 mm grid (or graphing paper)

Buffer preparation
1. Turn on heated water pump at the myography system.
2. Prepare the Krebs-Henseleit buffer
   a. Sequentially add the different components:
      1. 2 portions of pre-made modified 1L Krebs-Henseleit buffer powder (Sigma K3753)
      2. 1 portion (0.441 ± 0.001 g) of CaCl2·2H2O
      3. 1 portion (4.201 ± 0.001 g) of NaHCO3
   b. Pour warm ddH2O from the Erlenmeyer flask into the 2L beaker until the total volume while stirring is just about 2L.
      Check pH with an electronic pH meter if desired. pH should automatically be at around 7.4 after dissolution of NaHCO3.
3. Place the completed Krebs-Henseleit buffer bottle back into the 37°C water bath.

Washing
4. Prepare approximately 4L of ddH2O.
5. Add water to myography system and check to make sure the temperature of the bath is within the appropriate range (36.5 – 37.5° C). If not, then make a slight adjustment to the heated water pump setting.
6. Make sure to turn on the gas so that you push out any residual solution from the lines of the myography system.
7. Flush each organ bath with multiple washes.
To remove the last bit of fluid from the reservoir, you have to tip it as much as possible in the direction of flow. 

8. Perform two 2L flushes of the reservoir, tubing, and organ baths using ddH$_2$O.
9. Add the 2L of Krebs-Henseleit buffer to the reservoir and fill each organ bath.
   - Make sure to turn on the gas again and set to the appropriate flow rates.
   - The working pressure should be approximately 15 PSI.
   - Adjusting the individual organ bath, and reservoir gas flow rates is rather subjective. The guideline is that you want a steady flow of bubbles, but not enough that it will eventually disturb the vessel.

10. Leave the gas on for about 20 minutes (aerating the buffer may ultimately change the pH. Thus, pH may need to be adjusted to 7.4)

**Calibration**

11. As this is going on, calibrate the transducers
   1. If not already done, turn on the computer and PowerLab acquisition hardware, and log in.
   2. Hang each “upper leg” of the tissue holders on each of the transducers.
   3. Open the LabChart 7 program.
   4. Adjust the settings if not already saved
   5. Click on the “channel settings” button and a window with settings opens
   6. Change the number of active channels to “4” in the box near the bottom of the window and close the window.

*Optional:* Change the “sampling frequency” in the top right area of the window to 100 Hz (default is 1 kHz). There is no noticeable difference in using frequencies above 100 Hz. Secondly, sampling at a lower rate reduces computer resource usage (RAM) and makes files smaller when saved.

*Optional:* Drag each channel “value display” from the right hand side onto the main chart area, and resize to make the values clearly visible from a distance.

7. Press the “Start” button in the lower right side of the window to start recording, and set the “display scale” (just below the chart area) to 5:1 or 10:1 (50:1 or 100:1 if using a 1 kHz sampling frequency).
   If voltages are not close to 0 V, adjust the “zeroing screw” on the BRAM-4 amplifier with a screwdriver. Turning the screw clockwise makes the voltage more negative, and counter clockwise makes it more positive.

8. If voltages are close to 0 V, then sequentially hang the 10 g weight on each of the tissue holder “upper leg” for a few seconds. Be very careful to ensure the weight is very steady and not swinging like a pendulum.

9. Once this is done, press the “Stop” button in the lower right side of the window to stop recording.

10. In each of the channel chart areas, highlight a portion of the recording in which the 10 g weight had been hung from the tissue holder upper leg. Make sure the highlight includes areas with and without the added 10 g weight.
In each of the channel “side bars” (on the right), the press on the lower drop-down menu and select “unit conversion”.

A window pops up with several settings and a representation of your highlighted area.

In the top right of this window select “g” as the unit you want to convert to from the drop-down box, and set the number of decimal places to 3.

Just to the left of “unit drop-down box”, set each of the points to 0 and 10 g.

In the recording shown at the bottom, select a region that corresponds to 0 g tension, and press on the “arrow” button to the left of the empty box corresponding to the 0 g row. Do the same for 10 g.

Do this for each of the channels.

Once the transducers are calibrated, wipe down all of the tissue holders (upper and lower legs) with 70% ethanol.

After about 20 minutes of aerating the buffer, collect about 200mL of Krebs-Henseleit buffer directly from the reservoir.

Turn off the gas flow, and quickly release the gas pressure from the oxy-manifold.

Place the collected amount of Krebs-Henseleit buffer on ice.

Dissection

Prepare the dissection area and tools needed. Both the previously collected Krebs-Henseleit buffer as well as some Petri dishes will be placed on ice.

- Find the dissection tools: “rat-tooth” tissue forceps, large sharp/blunt end scissors, medium blunt end scissors, small surgical scissors, 2 fine-tip curved dissection forceps, and a large haemostat.

- Set up about 6 – 8 small Petri dishes (without lids). On one Petri dish, tape a small pre-made 2 mm x 2 mm grid so that the grid can be seen through the bottom of the Petri dish.

- If collecting a piece of artery or other tissue for molecular analysis, find a Dewar vacuum flask and fill it with liquid nitrogen.

Anesthetize the rat with isoflurane gas. When the rat is fully unconscious and displays no reflexes the dissection can begin. Prior to heart exsanguination, remove at least 10mL of blood from the rat via the inferior vena cava.

Ring preparation

Prior to removing the vessel from the animal attempt to clear as much connective tissue as possible.

Once the artery is removed clean the vessel of remaining connective and adipose tissue.

When cleaning the vessel take great care not to directly handle the artery, or when pulling the connective tissue apart, to not stretch (in any direction).

During dissection, place the aorta in fresh Krebs-Henseleit buffer every 5 minutes.

When finished cleaning, place the artery into the dish with attached 2mm x 2mm grid.

Using a new razor blade, cut several (up to 4) 2 mm-long tubes/rings.
Mounting
25. Take the rings (for myography) to the myograph system along with a pair of fine-tip forceps.
26. Turn on the gas once again, and make sure any residual Krebs-Henseleit buffer is pushed out of the gas lines.
27. Empty each organ bath and refill with fresh Krebs-Henseleit buffer. Make sure the fresh buffer is warm (i.e. not at room temperature).
28. Start recording on LabChart (press “Start” in the lower right corner of the window). Very carefully, but quickly, mount each aortic ring onto the myograph system.

1) Place the upper leg of the myograph unit onto the force transducer.
2) Align the upper and lower legs (using the micrometers) so that the two tissue holder needles are touching. The upper leg needle may rest on the lower leg needle.
3) Once aligned, remove the upper leg, and manually push the lower leg back a few mm.
4) Pick up a ring with the upper leg. Place the upper leg, with ring, back onto the force transducer.
5) Align the upper and lower leg needles, and “slide” the lower leg needle into the lumen of the ring. An illustration of this process can be seen below.
6) Once mounted, quickly adjust the tension using the vertical micrometer to about 0.7 – 1 g.
7) Quickly and very carefully lower the myograph unit into the organ bath. Lower the unit until the ring is at about the center of the organ bath. Once in the buffer, readjust the tension to approximately 1 g.
8) Mount subsequent vessels.
Equilibration

29. Although the tension was set at initially at 1 g, this tension gradually decreases as the rings relax. Tensions may drop down as low as 0.4 g by 5 minutes.
30. Gradually increase tension over ~30 minutes (Aorta: 2 grams, Carotid: 1.5 grams, Iliac: 1.5 grams Femoral: 1 grams). During this process continually wash vessels every 5-10 minutes.
31. Allow the rings to remain stable at their desired tensions for ~30 minutes.
32. During this equilibration period, prepare PE, ACh, SNP and insulin dilution series in 1.5 mL Eppendorf tubes.

Phenylephrine (PE):
- PE needs to be made fresh. Measure out 0.041 ± 0.001g of PE and place into a 15 mL Falcon tube.
- Dissolve the PE in Krebs-Henseleit buffer (e.g. buffer left over from dissection) and top up to approximately 2mL. This creates a 0.1M (i.e. -1 log M) PE solution (0.1mol/l x 203.67g/mol = 20.367g/mol).

Acetylcholine (ACh):
- ACh stock (-1 log M) can be prepared ahead of time and frozen at -70°C. Measure out 0.018g of ACh and dissolve in 1mL buffer. This creates a 0.1M (i.e. -1 log M) ACh solution (0.1mol/l x 181.66g/mol = 18.166g/mol).

Sodium Nitroprusside (SNP):
- SNP needs to be made fresh. Measure out 0.0298g of SNP and dissolve in 1mL buffer. This creates a 0.1M (i.e. -1 log M) SNP solution (0.1mol/l x 298g/mol = 29.8g/mol).

L-NMMA:
- L-NMMA stock (-1 log M) can be prepared ahead of time and frozen at -70°C. Measure out 0.0248g of L-NMMA and dissolve in 1mL buffer. This creates a 0.1M (i.e. -1 log M) L-NMMA solution (0.1mol/l x 248.28g/mol = 24.829g/mol).

Insulin:
- Insulin used: Sigma 10516-5ml bovine insulin 10mg/ml

Create PE, ACh, SNP, Insulin series dilutions in Eppendorf tubes:
- For ten-fold dilutions (e.g. -1 to -2 log M), pipet 100 µL of solution (e.g. -1 log M) into 900 µL of Krebs-Henseleit buffer.
- For approximately three-fold dilutions (e.g. -3 to -3.5 log M), pipet 300 µL of solution (e.g. -3 log M) into 700 µL of Krebs-Henseleit buffer. (This dilution makes concentrations approximately -3.5 log M. The actual concentration is: -3.5229 log M).

Viability test
33. Once equilibration is complete, pipet 5 µL of -3.5 log M PE into each organ bath.
- After the last wash, make sure each organ bath has approximately 5 mL of buffer in it.
- The final concentration of PE in the organ bath is -6.5 log M.
- Make sure to add comments in the upper section of the chart view each time a new pharmacological agent is added or a wash is performed.

34. Wait for the tension to reach a new plateau.
- Tension should begin to increase within 10 seconds after the addition of PE.
- Tension increases in an exponential fashion and should plateau in approximately 10 – 20 minutes.

35. Once plateau is reached, pipet 5 µL of -3.5 log M ACh into each organ bath.
- The final concentration of ACh in the organ bath is -6.5 log M.

36. Again, wait for tension to reach a new plateau.
- Tension should begin to decrease within 10 seconds after the addition of ACh.
- Tension decreases exponentially and should plateau in approximately 5 – 10 minutes.

37. Once plateau is reached, begin a series of 4 washes/buffer changes at 5 minute intervals.
- By the end of these washes (i.e. 15 minutes), tension should return to a baseline level.
- If vessels failed to relax after ACh dose repeat steps 33-37. Occasionally, vessels will need this procedure to “awaken”.

**Concentration response curves**

38. At the end of this equilibration period, and after the wash, pipet 5µL of -3.5 M PE into each organ bath as before, and follow the same guidelines as before.
- Using a dose of -2M PE (-5M PE in bath) is best for pre-constricting the femoral artery.
- PE sometimes causes a greater contraction the second time.

39. Once plateau is reached, begin concentration-response experiments with ACh, SNP or insulin.

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<td>10</td>
</tr>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>10 000</td>
</tr>
<tr>
<td></td>
<td>100 000</td>
</tr>
</tbody>
</table>
40. For PE concentration curves after the vessel achieves a basal tone pipette the following doses of PE.

<table>
<thead>
<tr>
<th>PE Concentration(-logM)</th>
<th>-5</th>
<th>-4.5</th>
<th>-4</th>
<th>-3.5</th>
<th>-3</th>
<th>-2.5</th>
<th>-2</th>
</tr>
</thead>
</table>

41. To evaluate NO contribution concentration curves (or a single dose from the middle of the concentration curve) by adding L-NMMA (0.1 –logM; 0.4 –logM in bath) to bath prior at least 10 minutes prior to PE contraction.
Appendix E. Glycogen Quantification Protocol

MATERIALS:
Tissue samples
Fume hood
3 x 100mm glass test tubes
Potassium hydroxide (KOH) pellets
Sodium sulfate (Na₂SO₄)
95% ethanol
Glycogen powder
Phenol crystals
96-98 % Sulfuric Acid (H₂SO₄)
490nm spectrophotometer

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

<table>
<thead>
<tr>
<th>Standard</th>
<th>[Glycogen] (mg/ml)</th>
<th>Volume (µl)</th>
<th>Water (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.000</td>
<td>0</td>
<td>1000</td>
</tr>
<tr>
<td>2</td>
<td>0.005</td>
<td>5</td>
<td>995</td>
</tr>
<tr>
<td>3</td>
<td>0.010</td>
<td>10</td>
<td>990</td>
</tr>
<tr>
<td>4</td>
<td>0.050</td>
<td>50</td>
<td>950</td>
</tr>
<tr>
<td>5</td>
<td>0.100</td>
<td>100</td>
<td>900</td>
</tr>
<tr>
<td>6</td>
<td>0.200</td>
<td>200</td>
<td>800</td>
</tr>
<tr>
<td>7</td>
<td>0.300</td>
<td>300</td>
<td>700</td>
</tr>
<tr>
<td>8</td>
<td>0.500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>9</td>
<td>1.000</td>
<td>1000</td>
<td>0</td>
</tr>
</tbody>
</table>

Colour Reaction:
1. Add 1 ml of 5 % phenol.
2. Rapidly add 5 ml of 96-98 % H₂SO₄.
3. Let samples stand for 10 min.
4. Vortex and place in water bath (25 to 30°C) for 10 to 20 minutes.
5. 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$_2$SO$_4$)
3. Add 0.5 ml of 30 % KOH saturated with Na$_2$S0$_4$. 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).
5. Place tubes on ice.
6. Precipitate glycogen with 1ml of 95 % ethanol for 30 minutes (on ice).
7. Spin tubes at 840 x g (3 000 rpm on Sorval) for 20 to 30 minutes.
8. Remove supernatants and immediately dissolve precipitates in 3mL ddH$_2$O. Do not allow precipitated to dry.
9. 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.
10. Add 1 ml of 5 % phenol.
11. Rapidly add 5 ml of 96-98 % H$_2$SO$_4$.
12. Let samples stand for 10 min.
13. Vortex and place in water bath (25 to 30°C) for 10 to 20 minutes.
14. Read tubes at 490 nm.
15. Calculate glycogen content by:

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

where, V = total volume of glycogen solution; v = volume of aliquot used in colour reaction; $A_{490} = \text{absorbance at 490 mn}$; W = weight of tissue samples in grams; k = slope of standard curve; units = 1 per microgram glycogen.

SOLUTIONS:

<table>
<thead>
<tr>
<th>Glycogen Stock Solution (1mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100mL ddH$_2$O</td>
</tr>
<tr>
<td>100mg Glycogen powder</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>30% Potassium Hydroxide Stock Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>300g Potassium Hydroxide Pellets (KOH)</td>
</tr>
<tr>
<td>1L ddH$_2$O</td>
</tr>
</tbody>
</table>

Reaction is exothermic; therefore, it might be necessary to put on ice.
<table>
<thead>
<tr>
<th>5% Phenol Stock Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>50g Phenol crystals</td>
</tr>
<tr>
<td>1L ddH₂O</td>
</tr>
</tbody>
</table>

**SPEC PROCEDURE:**

1. Turn on ~20 minutes 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:**

APPENDIX F. Immunohistological Staining of Pancreatic Islets (glucagon or insulin)

**Tissue Preparation**
After tissue extraction pancreata were placed in formalin for 72hrs. Formalin was replaced every 24hrs with fresh formalin. After 72hrs, tissues were placed in 70% ethanol until the time of tissue processing. Tissue is processed and embedded in paraffin at Robarts Molecular Pathology Laboratory using the Leica ASP 300.

**Tissue Sectioning**
Initially blocks were trimmed at 10-15µm in order to expose tissue. Pancreata were sectioned at 5µm and placed on positively charged slides. Tissues on slides were incubated overnight at 37°C to reduce background staining.

**Protocol for Deparaffinization**
1. Deparaffinization is completed using the “auto-staining machine” at the Molecular Pathology Laboratory at Robarts (Program 11)
   a. The slide carriage was placed in 2 separate solutions of Xylene for 5 minutes each.
   b. The slide carriage was placed in 2 separate solutions of 100% ethanol for 2 minutes each.
   c. The slide carriage was placed in 1 solution of 95% ethanol for 1 minute.
   d. The slide carriage was placed in 1 solution of 70% ethanol for 1 minute.
   e. The slide carriage was placed in 1 ddH2O bath for 2 minutes.

2. Antigen retrieval is completed after deparaffinization (also at Molecular Pathology Laboratory at Robarts). **Duration 2 hours.**

<table>
<thead>
<tr>
<th>10mM Sodium Citrate Buffer Solution with 0.05% Tween-20</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000mL ddH2O</td>
</tr>
<tr>
<td>2.4g Trisodium Citrate (Labelled Sodium Citrate Dihydrate)</td>
</tr>
<tr>
<td>Adjust pH to 6.0</td>
</tr>
<tr>
<td>0.5mL Tween-20</td>
</tr>
<tr>
<td>Store at room temperature for 3 months or 4°C for long term storage</td>
</tr>
</tbody>
</table>

3. Slides were brought back to lab in PBS solution

**Immunostaining Protocol**
**Quenching:**
1. Place slides in a 3% **Hydrogen Peroxide** solution for 20 minutes (blocking of endogenous peroxidase) – must be prepared fresh
2. Wash slides in 1x in TPBS 2min.
3. Wash 2x PBS for 1min.
4. Add grease circles around tissue

**Blocking:**
1. Prepare enough blocking solution to cover all tissue (approx. 100μL per tissue)
2. Add 4 drops of avidin (approx. 180μL) to 1000μL 10% blocking solution²
3. Cover tissue with blocking solution and incubate for overnight in the fridge at 4°C.

**Primary:**
1. In 2 separate tubes, Add 4 drops of biotin (approx. 180μL) into tube containing 1000μL of 1% blocking solution
2. Add primary antibody (abcam Monoclonal Anti-glucagon antibody: 1:4000, Dako Polyclonal Guinea Pig Anti-Insulin: Dako: 1:100) to the first of the tubes (biotin/blocking solution)
3. Apply solution containing the primary antibody to frosted side of the microscope slide
4. Apply only the biotin/blocking solution to the tissue furthest from the frosted side of the microscope slide
5. Incubate slides for 1hr in the dark on the bench
6. Wash slides 1x in TPBS for 2min.
7. Wash slides 2x in PBS for 2min.

**Secondary:**
1. Add secondary antibody (biotinylated anti-Mouse Goat) in a ratio of 1:400 (2.5μL) for glucagon or (biotinylated anti-Rabbit Goat) 1:200 (5 μL) for insulin to 1000μL 1% blocking solution
2. Apply solution containing the secondary antibody to all tissues
3. Incubate tissues for 30min. at room temperature in the dark

<table>
<thead>
<tr>
<th>Vectastain</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5mL</td>
<td>PBS</td>
</tr>
<tr>
<td>1 drop</td>
<td>Bottle A (Avidin)</td>
</tr>
<tr>
<td>1 drop</td>
<td>Bottle B (Biotin)</td>
</tr>
<tr>
<td></td>
<td>Must be prepared at least 30min. prior to use</td>
</tr>
</tbody>
</table>

4. Wash slides 1x in PBS for 5min.
5. Wash slides 2x in PBS for 2min.

---
² 3% Hydrogen Peroxide (per slide carriage)

<table>
<thead>
<tr>
<th>7.5mL</th>
<th>15mL</th>
<th>H₂O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>67.4mL</td>
<td>134.8mL</td>
<td>ddH₂O</td>
</tr>
</tbody>
</table>

ie. 1 part 30% H₂O₂ and 9 parts ddH₂O
Detection:
1. Apply 80µL of Vectastain to all tissues and incubate in the dark at room temperature for 30hr.
2. Wash slides 1x in PBS for 5min.
3. Wash slides 2x in PBS for 2min.
4. Prepare DAB solution:

<table>
<thead>
<tr>
<th>Diaminobenzine (DAB) Solution</th>
<th>Add in sequential order</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5mL ddH₂O</td>
<td></td>
</tr>
<tr>
<td>1 drop Buffer Solution</td>
<td></td>
</tr>
<tr>
<td>2 drop Diaminobenzidine (DAB)</td>
<td></td>
</tr>
<tr>
<td>1 drop Hydrogen Peroxide (H₂O₂)</td>
<td></td>
</tr>
<tr>
<td>1 drop Nickel (Ni)</td>
<td></td>
</tr>
</tbody>
</table>

5. Apply the DAB solution to all tissues for 6min.
6. Wash DAB off tissues by lightly squirting ddH₂O onto the slide and into a waste beaker
7. Place slides in ddH₂O and wash for at least 5min.
8. Dispose of the DAB contents into waste bottle in fume hood

Dehydration and Haematoxylin Counterstain:
1. Dehydration and counterstaining is best completed using the “auto-staining machine” at the Molecular Pathology Laboratory at Robarts (Program 4)

OR

1. Submerge slides in a Haematoxylin solution for 1min (nondiluted)
2. Wash slides with ddH₂O for 5min (2min in bath 1, 3min in bath 2 (fresh water)).
3. Place slides in 70% ethanol for 1min
4. Place slides in 95% ethanol for 1min
5. Place slides in 100% ethanol 2x for 3min
6. Place slides in Xylene 2x for 5min
**Solutions:**

### 10mM Sodium Citrate Buffer Solution with 0.05% Tween-20

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>1000mL</td>
<td>ddH₂O</td>
</tr>
<tr>
<td>Trisodium Citrate (Labelled Sodium Citrate Dihydrate)</td>
<td>2.4g</td>
<td></td>
</tr>
<tr>
<td>Adjust pH to 6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tween</td>
<td>0.5mL</td>
<td>Tween</td>
</tr>
</tbody>
</table>

Store at 4°C

### Phosphate Buffer Saline (PBS): 10x Stock Solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>1300mL</td>
<td>ddH₂O</td>
</tr>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>112g</td>
<td></td>
</tr>
<tr>
<td>Potassium Chloride (KCl)</td>
<td>2.8g</td>
<td></td>
</tr>
<tr>
<td>Disodium Monophosphate (Na₂HPO₄) – labeled as: Sodium Phosphate Dibasic Anhydrous</td>
<td>20.18g</td>
<td></td>
</tr>
<tr>
<td>Potassium Diphosphate (KH₂PO₄) – labeled as: Di-Potassium Hydrogen Orthophosphate</td>
<td>2.8g</td>
<td></td>
</tr>
<tr>
<td>Adjust pH to 7.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bring volume to 1400mL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Store at room temperature

### Tween-20 PBS (TPBS)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1L PBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100µL Tween-20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 10% Goat Serum Blocking Solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1mL Goat Serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9mL TPBS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Vectastain

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5mL PBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 drop Bottle A (Avidin)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 drop Bottle B (Biotin)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Prepare at least 30min. prior

### Diaminobenzine (DAB) Solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5mL ddH₂O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 drop Buffer Solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 drops Diaminobenzidine (DAB)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 drop Hydrogen Peroxide (H₂O₂)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 drop Nickel (Ni)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Add in sequential order

Working solution lasts up to 6hrs in the dark at 4°C
Appendix G. Homogenizing Protocol

**MATERIALS:**
- Glass plate to cut tissue  
- Razor blade  
- 1000 µl pipette  
- Homogenizing tubes  
- Bucket of ice to keep samples cold  
- Homogenizing buffer – make sure it’s fresh  
- Eppendorf tubes  
- Beaker ddH₂O to clean homogenizer between samples  
- Make sure to label tubes: both homogenizing and eppendorf tubes

1. Measure 0.1g sample (cut in freezer by surgery room).
2. Put in eppendorf tubes.
3. Add *homogenizing buffer*: 0.1g equals 0.1 ml @ 1x  
   Therefore 1ml @ 10x  
   1ml = 1000µl

   **Example:**

<table>
<thead>
<tr>
<th>sample</th>
<th>g of tissue</th>
<th>µl homogenizing buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>D - 1</td>
<td>0.0957</td>
<td>957</td>
</tr>
</tbody>
</table>

4. Complete steps 1-3 for each sample.
5. Homogenize each sample (never run homogenizer dry and always clean between samples).
6. Centrifuge homogenates
7. Pipette homogenized sample to eppendorf tubes and freeze at -70°C.
Appendix H. Bradford Protein Assay

1. Dilute 1 part of dye reagent with 4 parts water (Bio-Rad 500-0006, found in fridge).
   - 1 microplate requires 25ml dye reagent.

2. Filter the diluted solution through Whatman 1 filter paper.

3. Get a microplate and label.

4. Get a second set (different colour) of eppendorf tubes and label.

5. Add 5μl of sample with 95μL ddH₂O in separate eppendorf tubes.
   - An alternate to pipetting 0.5μl of sample and 9.5μl ddH₂O

<table>
<thead>
<tr>
<th>BSA : Water + 200μl reagent</th>
<th>Add 10μl of mixed sample + 200μl reagent (everything done in 3’s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>0</td>
<td>0 + 10</td>
</tr>
<tr>
<td>1</td>
<td>1 + 9</td>
</tr>
<tr>
<td>2</td>
<td>2 + 8</td>
</tr>
<tr>
<td>3</td>
<td>3 + 7</td>
</tr>
<tr>
<td>4</td>
<td>4 + 6</td>
</tr>
<tr>
<td>5</td>
<td>5 + 5</td>
</tr>
</tbody>
</table>

6. Shake microplate and Incubate at room temperature 5 to 60 minutes.
Appendix I. Western Blotting Protocol

**Tissue:** skeletal muscle, heart, liver

**Preparation of Samples**
1. Place the homogenized samples on ice.
2. Determine the amount of protein necessary to load in each well (initially a protein loading curve may be necessary).
3. The volume of homogenate needed is determined from the Bradford protein quantification assay (see Bradford protocol).
4. Label a new set of eppendorf tubes with the appropriate sample names.
5. Dilute the smallest possible volume of sample (determined from Bradford Assay) in *sample buffer.* (1:1 ratio, make sure to Vortex)
6. Prior to loading the sample into gel make sure to place the diluted sample in boiling water for up to 5 minutes (it may be necessary to poke holes in the tops of tubes).
7. Bring samples back to room temperature before loading in gel.

**Preparation of Gels**
1. Clean mini glass plates with 70% ethanol before use and then prepare gel cassette.
2. Prepare *separating gel* according to chart in relation to the number of gels and percent acrylamide to be used (*10% Separating Gel* works best for most proteins of interest; may be a different concentration when using very small or large proteins).

<table>
<thead>
<tr>
<th>ddH₂O</th>
<th>1 Gel</th>
<th>2 Gel</th>
<th>4 Gel</th>
<th>6 Gel</th>
<th>8 Gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 ml</td>
<td>8 ml</td>
<td>16 ml</td>
<td>24 ml</td>
<td>32 ml</td>
<td></td>
</tr>
<tr>
<td>30% Acrylamide (in fridge)</td>
<td>3.33 ml</td>
<td>6.67 ml</td>
<td>13.3 ml</td>
<td>20 ml</td>
<td>26.7 ml</td>
</tr>
<tr>
<td>Separating Buffer</td>
<td>2.5 ml</td>
<td>5 ml</td>
<td>10 ml</td>
<td>15 ml</td>
<td>20 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 µl</td>
<td>200 µl</td>
<td>400 µl</td>
<td>600 µl</td>
<td>800 µl</td>
</tr>
<tr>
<td>10% APS (in fridge: 0.1g in 1ml DDH₂O)</td>
<td>62.5 µl</td>
<td>125 µl</td>
<td>250 µl</td>
<td>375 µl</td>
<td>500 µl</td>
</tr>
<tr>
<td>TEMED (in fumehood)</td>
<td>6.25 µl</td>
<td>12.5 µl</td>
<td>25 µl</td>
<td>37.5 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

3. Pour *separating gel* using Pasteur pipette while trying to eliminate any bubbles (keep left over *separating gel* in beaker to make sure it polymerizes).
4. Immediately overlay the gel with water saturated isobutanol. This is done to remove bubbles and ensure a continuous charge from *separating* to *stacking gel.*
5. Wait for approximately 30-60 minutes until gel polymerizes and then rinse off overlay solution with ddH₂O and dry clean with filter paper.
6. Prepare and pour *stacking gel (4%)* according to the chart below:

<table>
<thead>
<tr>
<th>ddH₂O</th>
<th>1 Gel</th>
<th>2 Gel</th>
<th>4 Gel</th>
<th>6 Gel</th>
<th>8 Gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.05 ml</td>
<td>6.1 ml</td>
<td>12.2 ml</td>
<td>18.3 ml</td>
<td>24.4 ml</td>
<td></td>
</tr>
<tr>
<td>30% Acrylamide (in fridge)</td>
<td>0.65 ml</td>
<td>1.3 ml</td>
<td>2.6 ml</td>
<td>3.9 ml</td>
<td>5.2 ml</td>
</tr>
<tr>
<td>Stacking Buffer</td>
<td>1.25 ml</td>
<td>2.5 ml</td>
<td>5 ml</td>
<td>7.5 ml</td>
<td>10 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>50 µl</td>
<td>100 µl</td>
<td>200 µl</td>
<td>300 µl</td>
<td>400 µl</td>
</tr>
<tr>
<td>10% APS (in fridge)</td>
<td>31.3 µl</td>
<td>62.6 µl</td>
<td>125 µl</td>
<td>188 µl</td>
<td>250 µl</td>
</tr>
<tr>
<td>TEMED (in fume hood)</td>
<td>6.25 µl</td>
<td>12.5 µl</td>
<td>25 µl</td>
<td>37.5 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

7. Place the correct sized comb between the glass plates, making sure no air bubbles are trapped in the wells.
8. Prepare 1L of 1x *running buffer* per every 2 gels and store in refrigerator.
9. Once the *stacking gel* has polymerized (30-60 minutes), gently remove comb.
10. Fill wells with 1x *running buffer*. To allow for easier identification of wells when loading use a permanent marker to identify the bottom of wells.
11. Load correct amount of sample (determined from Bradford Assay) using micropipette with loading tip.
12. Once loading is complete place gels in running unit.
13. Fill running unit with cold 1x *running buffer* (make sure to fill the inside of the unit as well).
14. Run gels at 70V until through stacking portion of gel (~30 minutes) and then 120V until samples are fully through the gel.
15. Stop once dye (ie. Blue line) has moved completely through the gel.
16. During the running period prepare *transfer buffer* and keep in refrigerator.

<table>
<thead>
<tr>
<th>Transfer Buffer (4 BLOTS)</th>
<th>10x Running Buffer</th>
<th>100ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bring to 800ml with ddH₂O</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>200ml</td>
</tr>
</tbody>
</table>

**Transfer of Gels to Nitrocellulose**
1. Cut filter paper and nitrocellulose to appropriate size.
2. Soak filter paper, nitrocellulose and Brillo pads in cold *transfer buffer*.
3. Carefully separate gel from gel electrophoresis cassette and place in *transfer buffer*.
4. Assemble the transfer apparatus (“sandwich”) as shown below, making sure to remove all air bubbles between gel and nitrocellulose paper (bubbles will cause protein not to transfer).
5. Place “sandwich” in transfer holding tank. Fill tank with cold *transfer buffer*.
6. Connect to power supply and run at 70V for 1 ½ hours on ice.

**Blocking**
1. Prepare 1L 1x *TBS* per every 2 blots.
2. After transfer, remove gel and place in small container with 5% *blocking solution*. Incubate for 1 hour on shaker at room temperature.
3. After blocking prepare primary antibody (at least 15mL of solution).
4. Incubate blots (back-to-back) in primary antibody solution *overnight* at 4°C.
5. Once finished the 1º antibody solution can be stored at 4°C to be used within a week.
6. Wash blots 3x in TTBS for 5 minutes each.
7. Prepare secondary antibody (HRP – don’t use AP) solution (1:5000 a good starting point).
8. Incubate blots for *1 hour* on shaker at room temperature.
9. Wash blots 2x in TTBS and *1x* in TBS for *5 minutes* each wash.
WESTERN BLOTTING SOLUTIONS:

<table>
<thead>
<tr>
<th>Name</th>
<th>Components, concentrations, pH</th>
<th>Example amounts</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Acrylamide solution</td>
<td>Acrylamide – 30% (w/v)</td>
<td>Acrylamide – 150 g</td>
<td>4°C</td>
</tr>
<tr>
<td></td>
<td>Bis-acrylamide – 0.8% (w/v)</td>
<td>Bis-acrylamide – 4 g</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total V - 500 mL</td>
<td></td>
</tr>
<tr>
<td>2. Separating gel buffer</td>
<td>Tris – 1.5 M</td>
<td>Tris – 90.9 g</td>
<td>4°C</td>
</tr>
<tr>
<td></td>
<td>SDS – 0.4 % (w/v)</td>
<td>SDS – 2 g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH – 8.8</td>
<td>Total V - 500 mL</td>
<td></td>
</tr>
<tr>
<td>3. SDS solution</td>
<td>SDS – 10% (w/v)</td>
<td>SDS – 10 g</td>
<td>Room temperature</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total V - 100 mL</td>
<td></td>
</tr>
<tr>
<td>4. 2X Laemmli SDS-PAGE</td>
<td>Tris – 0.125 M</td>
<td>Tris – 7.57 g</td>
<td>Room temperature,</td>
</tr>
<tr>
<td>sample buffer</td>
<td>Glycerol – 20% (v/v)</td>
<td>Glycerol – 100 mL</td>
<td>fumehood</td>
</tr>
<tr>
<td></td>
<td>SDS – 4%</td>
<td>SDS – 20 g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>β-mercaptoethanol – 10% (v/v)</td>
<td>β-mercaptoethanol – 50 mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bromophenol blue – 0.015% (w/v)</td>
<td>Bromophenol blue – 0.075 g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH – 6.8</td>
<td>Total V – 500 mL</td>
<td></td>
</tr>
<tr>
<td>5. Stacking gel buffer</td>
<td>Tris – 0.5 M</td>
<td>Tris – 30.3 g</td>
<td>4°C</td>
</tr>
<tr>
<td></td>
<td>SDS – 0.4% (w/v)</td>
<td>SDS – 2 g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH – 6.8</td>
<td>Total V – 500 mL</td>
<td></td>
</tr>
<tr>
<td>10X running buffer</td>
<td>Tris – 0.25 M</td>
<td>Tris – 60.6 g</td>
<td>4°C</td>
</tr>
<tr>
<td></td>
<td>Glycine – 1.92 M</td>
<td>Glycine – 288 g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SDS – 1% (w/v)</td>
<td>SDS – 20 g</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total V – 2 L</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1X running buffer</td>
<td>10X running buffer – 10% (v/v)</td>
<td>10 X running buffer – 100 mL Total V – 1 L</td>
</tr>
<tr>
<td>---</td>
<td>------------------</td>
<td>--------------------------------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>6.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>1X transfer buffer</td>
<td>10X running buffer – 10% (v/v) Methanol – 20% (v/v)</td>
<td>10X running buffer – 100 mL Methanol – 200 mL (add last) Total V – 1 L</td>
</tr>
<tr>
<td>8.</td>
<td>10X TBS</td>
<td>Tris – 0.1 M NaCl – 1 M pH – 7.5</td>
<td>Tris – 24.2 g NaCl – 116.9 g Total V – 2 L</td>
</tr>
<tr>
<td>9.</td>
<td>Blocking solution</td>
<td>a. Non-fat, dry milk protein – 5% (w/v)</td>
<td>a. Non-fat, dry milk protein – 1.25 g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b. Bovine serum albumin – 5% (w/v)</td>
<td>b. Bovine serum albumin – 1.25 g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1X TTBS – 95%</td>
<td>1X TTBS – top up to 25 mL</td>
</tr>
<tr>
<td>10.</td>
<td>Primary antibody solution 1</td>
<td>Non-fat, dry milk protein – 2% (w/v)</td>
<td>Non-fat, dry milk protein – 0.4 g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1X TTBS – 98% Antibody against protein of interest</td>
<td>1X TTBS – top up to 20 mL</td>
</tr>
<tr>
<td>11.</td>
<td>Primary antibody solution 2</td>
<td>Bovine serum albumin – 5% (w/v)</td>
<td>Bovine serum albumin – 1 g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1X TTBS – 95% Antibody against protein of interest</td>
<td>1X TTBS – top up to 20 mL</td>
</tr>
<tr>
<td>12.</td>
<td>Secondary antibody solution</td>
<td>Non-fat, dry milk protein – 2% (w/v)</td>
<td>Non-fat, dry milk protein – 0.4 g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1X TTBS – 98% Antibody against primary antibody</td>
<td>1X TTBS – top up to 20 mL</td>
</tr>
<tr>
<td>13.</td>
<td>1X TBS</td>
<td>10X TBS – 10% (v/v) pH – 7.5</td>
<td>10X TBS – 10 mL Total V – 100 mL</td>
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</tbody>
</table>
SEPARATING GEL:

### 15 % Gel

<table>
<thead>
<tr>
<th></th>
<th>2 gels</th>
<th>4 gels</th>
<th>6 gels</th>
<th>8 gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>4.663 mL</td>
<td>9.325 mL</td>
<td>13.988 mL</td>
<td>18.65 mL</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>10 mL</td>
<td>20 mL</td>
<td>30 mL</td>
<td>40 mL</td>
</tr>
<tr>
<td>solution¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Separating gel</td>
<td>5 mL</td>
<td>10 mL</td>
<td>15 mL</td>
<td>20 mL</td>
</tr>
<tr>
<td>buffer²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDS solution³</td>
<td>200 µL</td>
<td>400 µL</td>
<td>600 µL</td>
<td>800 µL</td>
</tr>
<tr>
<td>10% APS solution</td>
<td>125 µL</td>
<td>250 µL</td>
<td>375 µL</td>
<td>500 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>12.5 µL</td>
<td>25 µL</td>
<td>37.5 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>20 mL</td>
<td>40 mL</td>
<td>60 mL</td>
<td>80 mL</td>
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### 12 % Gel

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<tbody>
<tr>
<td>ddH₂O</td>
<td>6.663 mL</td>
<td>13.325 mL</td>
<td>19.988 mL</td>
<td>26.65 mL</td>
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<tr>
<td>Acrylamide</td>
<td>8 mL</td>
<td>16 mL</td>
<td>24 mL</td>
<td>32 mL</td>
</tr>
<tr>
<td>solution¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Separating gel</td>
<td>5 mL</td>
<td>10 mL</td>
<td>15 mL</td>
<td>20 mL</td>
</tr>
<tr>
<td>buffer²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDS solution³</td>
<td>200 µL</td>
<td>400 µL</td>
<td>600 µL</td>
<td>800 µL</td>
</tr>
<tr>
<td>10% APS solution</td>
<td>125 µL</td>
<td>250 µL</td>
<td>375 µL</td>
<td>500 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>12.5 µL</td>
<td>25 µL</td>
<td>37.5 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>20 mL</td>
<td>40 mL</td>
<td>60 mL</td>
<td>80 mL</td>
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</table>
### 10% Gel

<table>
<thead>
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<th>6 gels</th>
<th>8 gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>7.996 mL</td>
<td>15.992 mL</td>
<td>23.988 mL</td>
<td>31.984 mL</td>
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<tr>
<td>Acrylamide solution¹</td>
<td>6.667 mL</td>
<td>13.333 mL</td>
<td>20 mL</td>
<td>26.667 mL</td>
</tr>
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<td>Separating gel buffer²</td>
<td>5 mL</td>
<td>10 mL</td>
<td>15 mL</td>
<td>20 mL</td>
</tr>
<tr>
<td>SDS solution³</td>
<td>200 µL</td>
<td>400 µL</td>
<td>600 µL</td>
<td>800 µL</td>
</tr>
<tr>
<td>10% APS solution</td>
<td>125 µL</td>
<td>250 µL</td>
<td>375 µL</td>
<td>500 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>12.5 µL</td>
<td>25 µL</td>
<td>37.5 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>20 mL</td>
<td>40 mL</td>
<td>60 mL</td>
<td>80 mL</td>
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### 7.5% Gel

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<th>8 gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>9.663 mL</td>
<td>19.325 mL</td>
<td>28.988 mL</td>
<td>38.65 mL</td>
</tr>
<tr>
<td>Acrylamide solution¹</td>
<td>5 mL</td>
<td>10 mL</td>
<td>15 mL</td>
<td>20 mL</td>
</tr>
<tr>
<td>Separating gel buffer²</td>
<td>5 mL</td>
<td>10 mL</td>
<td>15 mL</td>
<td>20 mL</td>
</tr>
<tr>
<td>SDS solution³</td>
<td>200 µL</td>
<td>400 µL</td>
<td>600 µL</td>
<td>800 µL</td>
</tr>
<tr>
<td>10% APS solution</td>
<td>125 µL</td>
<td>250 µL</td>
<td>375 µL</td>
<td>500 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>12.5 µL</td>
<td>25 µL</td>
<td>37.5 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>Total Volume</td>
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<td>40 mL</td>
<td>60 mL</td>
<td>80 mL</td>
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</table>

### 6% Gel

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</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>10.663 mL</td>
<td>21.325 mL</td>
<td>31.988 mL</td>
<td>42.65 mL</td>
</tr>
<tr>
<td>Acrylamide solution¹</td>
<td>4 mL</td>
<td>8 mL</td>
<td>12 mL</td>
<td>16 mL</td>
</tr>
<tr>
<td>Separating gel buffer²</td>
<td>5 mL</td>
<td>10 mL</td>
<td>15 mL</td>
<td>20 mL</td>
</tr>
<tr>
<td>SDS solution³</td>
<td>200 µL</td>
<td>400 µL</td>
<td>600 µL</td>
<td>800 µL</td>
</tr>
<tr>
<td>10% APS solution</td>
<td>125 µL</td>
<td>250 µL</td>
<td>375 µL</td>
<td>500 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>12.5 µL</td>
<td>25 µL</td>
<td>37.5 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>20 mL</td>
<td>40 mL</td>
<td>60 mL</td>
<td>80 mL</td>
</tr>
</tbody>
</table>
STACKING GEL:

<table>
<thead>
<tr>
<th></th>
<th>2 gels</th>
<th>4 gels</th>
<th>6 gels</th>
<th>8 gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>5.992 mL</td>
<td>11.983 mL</td>
<td>17.975 mL</td>
<td>23.967 mL</td>
</tr>
<tr>
<td>Acrylamide solution¹</td>
<td>1.333 mL</td>
<td>2.667 mL</td>
<td>4 mL</td>
<td>5.333 mL</td>
</tr>
<tr>
<td>Stacking gel buffer⁵</td>
<td>2.5 mL</td>
<td>5 mL</td>
<td>7.5 mL</td>
<td>10 mL</td>
</tr>
<tr>
<td>SDS solution³</td>
<td>100 µL</td>
<td>200 µL</td>
<td>300 µL</td>
<td>400 µL</td>
</tr>
<tr>
<td>10% APS solution</td>
<td>62.5 µL</td>
<td>125 µL</td>
<td>187.5 µL</td>
<td>250 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>12.5 µL</td>
<td>25 µL</td>
<td>37.5 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>10 mL</td>
<td>20 mL</td>
<td>30 mL</td>
<td>40 mL</td>
</tr>
</tbody>
</table>
Appendix J. Ethics Approval

Western

AUP Number: 2014-009
PI Name: Noble, Earl
AUP Title: Innovation To Reduce Muscular And Cardiovascular Complications Of Diabetes
Approval Date: 06/26/2014

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Innovation To Reduce Muscular And Cardiovascular Complications Of Diabetes" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2014-009::1

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care
Appendix K. Permission to Reproduce Published Materials

Published Dissertation Chapters:

Chapter 2: Morphological assessment of pancreatic islet hormone content following aerobic exercise training in rats with poorly controlled Type 1 diabetes mellitus

McDonald MW, Murray MR, Hall KE, Noble EG, Melling CJ. Morphological assessment of pancreatic islet hormone content following aerobic exercise training in rats with poorly controlled Type 1 diabetes mellitus. Islets 6: e27685, 2014.

“This is an Accepted Manuscript of an article published by Taylor & Francis in Islets on 2014-05-15, available online: http://dx.doi.org/10.4161/isl.29221.”

If you would like to “use my article internally within my institution or company” or “post my accepted manuscript on my departmental or personal website after publication” you must “include a link to the version of record”.


Chapter 3: Ischemia-reperfusion injury and hypoglycemia risk in insulin-treated T1DM rats following different modalities of regular exercise


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Excerpt from: http://physreports.physiology.org/reprints-permissions
Curriculum Vitae

Name: Matthew W. McDonald

Post-secondary Education and Degrees:
University of Western Ontario
London, Ontario, Canada
BA Kinesiology (Honours Specialization) 2006-2010

University of Western Ontario
London, Ontario, Canada
MSc Kinesiology 2010-2012

University of Western Ontario
London, Ontario, Canada
PhD Integrative Physiology of Exercise 2012-2016

Honours and Awards:
Martin Rothstein Postdoctoral Trainee Award 2016-2018
Canadian Partnership for Stroke Recovery
Ontario Graduate Scholarship 2011 2013-2016

Nominated for Graduate Student Teaching Award 2012

Related Work Experience:
Graduate Teaching Assistantship Indigenous Services 2013-2015

Graduate Teaching Assistantship Systemic Approach to Functional Anatomy 2010-2012

Research Assistantship, Get Fit for Active Living-Older Adults Community Exercise Program 2010

Publications:
Peer-reviewed (10):


Stathokostas L, **McDonald MW**, Little RM, Paterson DH. Flexibility of older adults aged 55-86 years and the influence of physical activity. *J Aging Res.* 2013;2013:743843.


*Abstracts (6 first author, 19 total)*:


McDonald MW, Grise KN, Noble EG, Melling CWJ. “Exercise training does not attenuate the exercise mediated reduction in blood glucose levels in streptozotocin-induced rats”, Applied Physiology, Nutrition and Metabolism (APNM) September, issue No. APNM 36(S2)): Presented in Quebec City, QC, September 2011.