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Insulin Resistance and T1D: The Effect of Exercise on Skeletal Muscle Lipid Metabolism

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

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Abstract

Insulin resistance is a characteristic of Type 2 Diabetes (T2D) and metabolic syndrome, and until recently has not been considered in the context of Type 1 Diabetes (T1D). A new classification, Double Diabetes, encompasses the presence of insulin resistance in T1D. Unfortunately, these individuals are at significantly higher risk of cardiovascular complications than those with T1D alone. In T2D, abnormal skeletal muscle lipid metabolism and the accumulation of insulin desensitizing lipid metabolites contribute the development of insulin resistance. There is some evidence to suggest this occurs in the context of T1D as well. Exercise training is an effective means to enhance insulin sensitivity, however, the fear of hypoglycemia remains a significant barrier in T1D. Using our rodent model of T1D, the objectives of this dissertation were as follows: 1) To determine whether insulin resistance is associated with increased skeletal muscle lipid content, and whether this is ameliorated with aerobic exercise 2) to compare the efficacy of combined aerobic and resistance exercise compared to aerobic exercise itself on skeletal muscle oxidative capacity and lipid metabolism, and 3) to compare the effects of the optimal exercise modality (combined exercise training) and the current recommendation of intensive insulin therapy on insulin resistance and the composition of skeletal muscle lipid. The findings of this dissertation were as follows: 1) There is an association between insulin resistance and the accumulation of intramyocellular lipid metabolites, which is ameliorated with aerobic exercise training. 2) Muscle lipid accumulation in sedentary T1D animals may result from a reduced oxidative capacity, coupled to greater lipid uptake. Aerobic exercise is sufficient to restore oxidative capacity, however combined exercise induces more robust improvements to oxidative capacity. 3) Combined exercise significantly enhances insulin sensitivity, while intensive insulin therapy is sufficient to prevent the drastic decline in insulin sensitivity observed with conventional insulin treatment. Further, muscle lipid composition differs between sedentary and exercised diabetic animals. These findings indicate a reduction in oxidative capacity, coupled to increased lipid accumulation and insulin resistance in sedentary T1D animals. Combined exercise is an effective modality to enhance insulin sensitivity and modify muscle lipid metabolism.

Keywords: diabetes, exercise, insulin resistance, lipid, insulin therapy

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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Dedication

To Mom, Dad, and James. So much love and so many thanks.

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List of Abbreviations

(H+L)-HRP	Heavy chain + light chain horseradish-peroxidase conjugated
4E-BP1	Eukaryotic translation factor 4E-binding protein
AA	Arachidonic acid
AC8	Adenylyl cyclase 8
ACC	Acetyl CoA carboxylase
ACC β	Acetyl CoA carboxylase beta
ACS	Acyl Coa Synthase
ADP	Adenosine diphosphate
AGE	Advanced glycation end product
AGPAT	Acylglycerolphosphate acyltransferase
AIRE	Autoimmune regulatory gene (human)
AMPK	5' adenosine monophosphate-activated protein kinase
APC	Antigen presenting cell
APS-1	Autoimmune polyendocrinopathy-candidiasis-ectodermal
AS160	Akt substrate of 160 kDa
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BMI	Body mass index
C	Control sedentary
CaMKK	Calcium/calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
cAMP	Cyclic adenosine monophosphate
CAN	Cardiac autonomic neuropathy
CD	Diabetic + conventional insulin treatment + sedentary
CD36	Fatty acid transporter CD36
CD8	Cytotoxic T-cell
CPT-1	Carnitine palmitoyltransferase-1
CPT-2	Carnitine palmitoyl transferase 2

CREB	cAMP-response element-binding protein
CS	Citrate synthase
CTLA4	Cytotoxic T lymphocyte associated protein
CVB	Coxsackievirus B
CVD	Cardiovascular disease
DAE	Diabetic + insulin+ aerobic exercise
DARE	Diabetes + insulin + aerobic + resistance exercise
DCCT	Diabetes Control and Complications Trial
DCM	Diabetic cardiomyopathy
DGAT	Diacylglycerol acyltransferase
DHA	Docosahexaenoic acid
DIT	Diabetes + intensive insulin treatment + sedentary
DNA	Deoxyribonucleic acid
DTNB	5,5'-Dithiobis (2-nitrobenzoic acid)
EDIC	Epidemiology of Diabetes Interventions and Complications
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
eIF2B	Eukaryotic translation initiation factor 2B
EPAC ₂	Exchange protein activated by cAMP
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
FABP	Fatty acid binding protein
FACoA	Fatty acyl-CoA
FAD	Flavin adenine dinucleotide
FADH ₂	Flavin adenine dinucleotide
FAS	Fatty acid synthase
FATP1	Fatty acid transporter 1
FATP4	Fatty acid transporter 4
G1P	Glucose-1-phosphate
G6P	Glucose-6-phosphate
GAP	Rab-GTPase-activating protein

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEF	Glucose transporter 4 enhancer factor
GIR	Glucose infusion rate
GLUT4	Glucose transporter 4
GPAT	Glycerol-3-phosphate acyltransferase
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HbA1c	Glycated hemoglobin
HDAC4/5-MEF2	Histone deacetylase 4/5-myocyte enhancer factor 2
HDL	High density lipoprotein
HLA	Human leukocyte antigen
HLA	Human Lymphocyte Antigen
HNF1 α	Homeobox A
HOMA-IR	Homeostatic model of insulin resistance
HPLC	High performance liquid chromatography
HSL	Hormone sensitivity lipase
IFIH1	Interferon-induced helicase C domain-containing protein 1
IGF-1	Insulin-like growth factor 1
IKK β	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL2RA	Interleukin-2 receptor- α
IMCL	Intramyocellular lipid
IMP	Inosine monophosphate
IMTG	Intramyocellular triacylglycerol
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
IR	Insulin receptor
IRE1	Inositol-requiring endoplasmic reticulum-to-nucleus signalling protein 1
IRS-1	Insulin receptor substrate 1
JNK	c-Jun N-terminal kinase
LC-MS	Liquid chromatography coupled to mass spectroscopy
LD	Lipid droplet
LDL	Low density lipoprotein

LYP	Lymphoid protein tyrosine phosphatase
MAPK	Mitogen-activated protein kinase
MGL	Monoacylglycerol lipase
MHC	Major histocompatibility complex
mRNA	messenger RNA
mtDNA	Mitochondrial DNA
mTOR	Mechanistic target of rapamycin
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide
NF κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NRF1	Nuclear respiratory factor 1
NRF2	Nuclear respiratory factor 2
OPLS-DA	Orthogonal partial least squares discriminant analysis
ORO	Oil red O
p38 MAPK	p38 mitogen-activated protein kinase
p70S6k	Ribosomal protein s6 kinase beta-1
PARP	Poly ADP ribose polymerase
PCA	Principle component analysis
PDE3B	Phosphodiesterase 3B
PDK1	Phosphoinositide-dependent kinase 1
PDK1	Protein-dependent kinase-1
PGC-1 α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	Phosphatidylinositol 3-kinase pathway
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP ₃	Phosphatidylinositol 3,4,5-triphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKA	Protein kinase A
PKA	Protein kinase A
PKB	Protein kinase B (also Akt)
PKC	Protein kinase C

PP1	Protein phosphatase 1
PPAR α	Peroxisome proliferator-activated receptor alpha
PTPN22	Lymphoid protein tyrosine phosphatase
PUFA	Polyunsaturated fatty acid
R-ORB	Redox optimized reactive oxygen species balance
ROS	Reactive oxygen species
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SERCA	Sarcoplasmic reticulum calcium ATPase
SFA	Saturated fatty acid
SIRT1	Sirtuin 1
SOD	Superoxide dismutase
SPT-1	Serine palmitoyl transferase 1
SRCa ²⁺	Sarcoplasmic reticulum calcium
SREBP-1C	Sterol regulatory element binding transcription factor 1 C
STZ	Streptozocin
T1D	Type 1 Diabetes Mellitus
T2D	Type 2 Diabetes Mellitus
T3D	Type 3 diabetes
TAG	Triacylglycerol
TBS	Tris buffered saline
TCF7L2	Transcription factor 7 like 2
TCR	T Cell receptor
Tfam	Transcription factor A, mitochondrial
TNF- α	Tissue necrosis factor alpha
TORC1	Target of rapamycin complex 1
TORC2	Target of rapamycin complex 2
TORC3	Target of rapamycin complex 3
TRAF2	TNF receptor-associated factor 2
TTBS	Tris buffered saline with Tween20
UCP-2	Uncoupling protein 2

UDP-glucose	Uridine diphosphate-glucose
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoproteins
VNTR	Variable number tandem repeats
VO _{2max}	Maximal oxygen consumption

CHAPTER 1

1 « Introduction »

1.1 Type 1 Diabetes Mellitus

One of the earliest observations of Diabetes is recorded on the Ebers Papyrus dated back to 1552 BCE. While the disease had yet to be named, an Egyptian physician had recorded treatments for “the passing of too much urine”¹. By 1922, following the conception of insulin by Frederick Banting, pharmaceutical company Eli Lilly & Co. struck a deal for the mass production of insulin, allowing patients to manage the disease and serving as the primary treatment strategy for Type 1 Diabetes to this day. In 1993, the ten-year Diabetes Control and its Complications Trial was published, which confirmed the effectiveness of insulin for managing blood glucose and reducing the complications associated with Type 1 Diabetes¹. This year, a study reported successful interspecies transplantation of a pancreas, suggesting transplantable human organs grown in animals may one day become a reality for the treatment of Diabetes². Such novel findings continue to advance our understanding of the disease and drive the scientific community to search for a Diabetes cure. Despite over a century of these advancements, approximately 3.4 million Canadians with Diabetes require lifelong medication for other diabetes-related illnesses, consistent insulin treatment and glucose monitoring, and despite this, still experience an increased risk of cardiovascular disease^{3,4}.

There are two types of Diabetes, Type 1 Diabetes Mellitus (T1D) and Type 2 Diabetes Mellitus (T2D). Normally, circulating blood glucose is maintained at approximately 3-5 mM by pancreatic insulin secretion, which promotes glucose uptake into tissues for storage or metabolism⁵. T2D occurs when the pancreas is unable to produce sufficient insulin, or when

tissues become resistant to the insulin molecule, resulting in chronically elevated blood glucose termed hyperglycemia. T2D often results from lifestyle and genetic factors, and for many individuals is manageable through lifestyle modifications such as diet and physical activity as well as medication ⁶. T1D, however, results from autoimmune mediated pancreatic beta cell (β -cell) destruction and the subsequent inability to produce insulin ⁷. T1D patients often present with weight loss, increased thirst and urination, fatigue, recurring infections, blurred vision, and slow healing wounds ⁸. Following diagnosis, patients require exogenous insulin to promote glucose uptake into tissues and prevent chronic hyperglycemia.

Multiple genes that predispose individuals to T1D have been identified. Several of these genes are involved in immune function, such as Class II Human Lymphocyte Antigen (HLA) genes ⁷. These genes code for proteins present on immune cells critical to the immune response, and are the greatest T1D risk predictors. Similarly, lymphoid protein tyrosine phosphatase (PTPN22), cytotoxic T lymphocyte associated protein (CTLA4) and interferon-induced helicase C domain-containing protein 1 (IFIH1) are genes coding for immune factors implicated in the onset of T1D ⁷. Short variable number tandem (VNTR) repeats within the insulin gene itself are associated with a higher risk of T1D onset ⁷.

There are two predominant theories to explain the pathogenesis of T1D. In the “fertile field hypothesis”, it is suggested that an initial infection may stimulate the development of autoreactive CD8 immune cells, which destroy infected cells ⁹. Upon a subsequent infection, or other environmental trigger, the autoimmune response is robust and may initiate β -cell destruction, due to potential structural similarities between viral and pancreatic antigens ⁷.

The “accelerator hypothesis” suggests that T1D and T2D are the same disease set against different genetic backgrounds ¹⁰. It has been generally accepted that T1D results from an

autoimmune attack on pancreatic β -cells, and that T2D is a metabolic disorder. According to the accelerator hypothesis, the only difference between T1D and T2D is the age of onset, determined by the tempo of β -cell loss, not the cause. According to this hypothesis, the onset of diabetes is determined by insulin resistance (the “driver”) and genotype (the “modulator”). For individuals with T1D, a high genetic susceptibility requires little environmental pressure, such as insulin resistance, to result in β -cell failure ¹¹.

It likely that the onset of T1D results from a combination of genetic and environmental factors, resulting in the autoimmune-mediated destruction of insulin-producing pancreatic β -cells. In the absence of endogenous insulin, hyperglycemia results, necessitating insulin therapy.

1.1.2 Insulin for the Management of Type 1 Diabetes Mellitus

The discovery of insulin by Banting and Best was critical to the management of T1D ¹². For T1D patients, insulin is now self-administered via subcutaneous injection or via surgically implanted insulin pump devices. Without sufficient insulin (termed hypoinsulinemia), hyperglycemia results, and is an underlying factor in the development of several complications including retinopathy, nephropathy, neuropathy and increased risk of cardiovascular disease ¹³. The Diabetes Control and Complications Trial tested the “glucose hypothesis”, which suggests hyperglycemia is the primary cause of complications in T1D. This trial compared the effects of conventional and intensive insulin treatment on cardiovascular complications. Briefly, patients with T1D were randomized into two different insulin treatment groups. The conventional insulin therapy group was instructed to maintain HbA1c (glycated hemoglobin, a measure of average blood glucose for approximately three months) at or below 13%, with the use of insulin injections. The intensive insulin therapy group maintained significantly more

rigid glycemic control, striving to maintain HbA1c at approximately 6.5% with the use of multiple daily insulin injections ¹⁴. Findings from the trial were the basis for recommending an intensive insulin regime to patients with T1D, as intensive insulin treatment resulted in significant improvements in retinopathy, microalbuminuria, neuropathy and microvascular events ¹⁴. The findings were so convincing that the trial was stopped, and all conventional insulin treatment patients were switched to an intensive treatment. Following the re-organization of subjects, patients were followed from 1994 to 2017 (anticipated completion date for primary outcome measure) as part of the Epidemiology of Diabetes Interventions and Complications (EDIC) trial. This trial examined measures of atherosclerosis, including carotid intima media thickness and coronary artery calcification. Despite converting all DCCT patients to intensive insulin therapy, differences in these outcomes between the intensive only patients and the newly converted patients widened over the first four years of the EDIC trail, suggesting an effect of prior metabolic control on complications, termed metabolic memory ¹⁴.

More recent work has closely examined the DCCT and EDIC trials, and has identified several limitations. Most notably, the exclusion criteria for the DCCT, which prevented individuals with cardiovascular disease, hypertension, dyslipidemia, neuropathy and those taking lipid-lowering drugs from participating in the trial ¹⁵. It has been suggested that the patients in the DCCT were otherwise healthy, and not representative of a T1D population ¹⁵. Still, within this group of patients, evidence suggests factors other than glycemic control are associated with adverse cardiovascular outcomes. For example, metabolic syndrome and insulin resistance have been identified as better predictors of cardiovascular risk compared to HbA1c ¹⁵. Analysis of a subgroup of DCCT patients revealed a synergistic role of hyperglycemia and abnormal lipid partitioning, whereby individuals with increased low density lipoproteins (LDLs), which are modified by advance glycation end products (AGEs)

are at a significantly increased risk of high carotid intima-media thickness ¹⁵. Further, DCCT patients who were prescribed intensive treatment presented with an increased occurrence of metabolic syndrome, and greater insulin resistance at baseline was associated with increased risk of micro- and macrovascular complications ¹⁶.

While evidence highlighted in the DCCT and EDIC trials would appear to have confirmed the “glucose hypothesis”, emerging studies have begun to question these outcomes and identify limitations. It remains to be determined whether stringent glycemic control is the optimal approach to reduce cardiovascular disease risk in T1D, despite DCCT findings providing the rationale for the current prescription of intensive insulin therapy for T1D patients.

1.1.3 Type 1 Diabetes: Pathophysiology

T1D manifests when a genetic predisposition is triggered by a precipitating event, such as viral or bacterial infection, or dietary allergens. In rare circumstances, monogenic forms of T1D present, whereby mutation of a single gene when accompanied by other immune conditions, disrupts regulatory pathways. Examples include IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked), which causes dysfunction of regulatory T cells and approximately 80% of children with this mutation will develop T1D ⁷. Further, APS-1 (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) results in mutations in the transcription factor AIRE, an autoimmune regulator and 20% of these individuals go on to develop T1D ⁷.

Human Leukocyte Antigen (HLA) genes are a locus of genes that encode proteins on the surface of cells for immune regulation. This locus is present on the HLA region of the chromosome 6p21 (IDDM1), and is divided into class I and class II genes ⁷. Class II genes have a promoter and pre-initiation complex, and are the strongest genetic determinants of risk

for the development of T1D. Individuals with DRB1*1501-DQA1*0102-DQB1*0602 haplotype are protected against T1D, while those with the DR3/4-DQ8 heterozygous haplotype are susceptible to developing T1D¹⁷. Class I genes have a less prominent association with T1D, however the HLA-B*39, HLA-A*02, and HLA-A*0201 are associated with T1D¹⁸⁻²⁰.

The insulin gene, at the IDDM2 locus on chromosome 11 is considered a primary autoantigen in T1D²¹. The degree of susceptibility depends on the number of variable tandem repeat polymorphisms in the promoter region, with VNTR type I individuals having shorter repeats significantly increasing the risk of T1D²². VNTR type 1 reduces insulin transcription in the thymus, reducing tolerance and promoting the onset of T1D^{7,23}. PTPN22, encoding lymphoid protein tyrosine phosphatase (LYP), IL2RA (interleukin (IL)-2 receptor- α) and CTLA-4 (cytotoxic T lymphocyte-associated protein 4) are also genes implicated in the onset of T1D^{7,24}.

Lastly, a gene for a sensor of viral infection, IFIH1 (interferon-induced helicase 1) may contribute to the development of T1D²⁵. Increased function of IFIH1 may increase recognition of specific viruses, exacerbating antiviral immunity and the production of type 1 interferons⁷. Viruses are an important precipitating factor in the pathogenesis of T1D, and are a component of the “Fertile Field” hypothesis⁹. In individuals with this genetic phenotype, a precipitating event can trigger the onset of T1D. Viruses including coxsackie virus, enteroviruses, rotavirus, and congenital rubella are all associated with the development of T1D^{7,26-28}. The Fertile Field hypothesis suggests that a viral or bacterial infection causes pancreatic β -cells to upregulate interferon (IFN α) and MHC Class I genes, which expose cells to attack by autoreactive T cells with specificity for antigens in the pancreas. In this sense, an infection leads to a transient period, in which the pancreas is a “fertile field” for autoimmune cell development^{7,29}. A subsequent series of immune events leads to the generation of autoreactive immune cells,

resulting in pancreatic β -cell destruction and the cessation of insulin production. T1D results, and patients require exogenous insulin to maintain normoglycemia ⁷.

1.2 Skeletal Muscle Metabolism

1.2.1. Insulin Signalling and Glucose Metabolism

In healthy pancreatic β -cells, insulin is synthesized when mRNA is translated into preproinsulin. Preproinsulin is inserted into the endoplasmic reticulum, where its signal peptide is cleaved, generating proinsulin. The peptide is cleaved again via endopeptidases resulting in free C-peptide and insulin that accumulates in the cytoplasm of the β -cell, which are subsequently released into circulation. This molecular process occurs in response to increased blood glucose, which enters the beta cell via glucose transporters (GLUT). Glucose enters glycolysis followed by subsequent metabolism in the mitochondria, and an increased adenosine triphosphate: adenosine diphosphate ratio (ATP:ADP). The increased ratio triggers membrane depolarization, calcium enters the β -cell and interacts with GTP (guanosine triphosphate) and AC8 (adenylyl cyclase 8), activating cAMP (cyclic AMP) which activates PKA (protein kinase A) and EPAC₂ (a guanine nucleotide exchange factor) leading to insulin exocytosis ³⁰. Insulin binding to its receptor initiates a variety of signalling pathways in skeletal muscle, including GLUT4 translocation for glucose uptake. Skeletal muscle contraction also initiates signalling to enhance glucose uptake (see below).

Skeletal muscle metabolism is a complex and coordinated system, driven by demand for energy in the form of ATP. To resynthesize ATP, an intricate cycle of enzymes facilitates the breakdown of substrates including glucose and lipid. Regulation of substrate use depends on a variety of factors, including sarcoplasmic reticulum calcium (SRCa²⁺), ATP breakdown products, substrate availability and circulating hormones ³¹. When muscle energy

requirements are low, substrate entering the muscle cell is converted to glycogen (glucose storage), diacylglycerol (DAG) and triacylglycerol (TAG) lipids (fatty acid storage).

Glucose entry into skeletal muscle during contraction is regulated by delivery, transport and metabolism³². Delivery of glucose to the working muscle depends on blood flow and capillary recruitment within the muscle, while transport and metabolism depend on the quantity of transporter proteins and enzymes as well as their activity. Skeletal muscle glucose uptake relies primarily on the GLUT4 transporter, which translocates to the membrane in response to contraction signalling as well as insulin signalling.

During contraction, increased intracellular calcium (Ca^{2+}) and an increased AMP:ATP ratio activates AMPK via Ca^{2+} /calmodulin-dependent protein kinase (CaMKK), resulting in phosphorylation of Akt substrate of 160 kDa (AS160) and TBC1D1, increasing 14-3-3 binding to these proteins³³. This also inhibits Rab-GTPase-activating protein (GAP) activity toward Rab isoforms, promoting conversion to more active GTP-loaded Rab, stimulating GLUT4 translocation and fusion with the plasma membrane and subsequent glucose uptake³³.

Insulin signalling is similar to skeletal muscle contraction signalling, as insulin binding the insulin receptor (IR) promotes GLUT4 translocation and muscle glucose uptake. This occurs, however, through the phosphatidylinositol (PI) 3-kinase pathway. The binding of insulin to its receptor activates and phosphorylates it, resulting in the phosphorylation and recruitment of insulin receptor substrate 1 (IRS-1)³⁴. IRS proteins interact with the p85 regulatory subunit of PI3-kinase, activating and targeting the molecule to the plasma membrane, where it generates phosphatidylinositol 3,4,5-triphosphate (PIP_3)³⁴. PIP_3 activates the serine/threonine kinase PDK1, which phosphorylates AKT1 promoting GLUT4 translocation to the membrane³⁴. Other signals, such as nitric oxide (NO) and reactive oxygen

species (ROS) are increased with muscle contraction and promote GLUT4 translocation to the membrane and increased glucose uptake ³².

Within skeletal muscle, glucose may be oxidized to produce ATP for working muscle, or stored as glycogen. Upon entry into the muscle cell, glucose is phosphorylated and converted to glucose-6-phosphate (G6P) by hexokinase ³⁵. G6P may then proceed through the glycolytic and oxidative pathways to produce ATP. When energy demand is low, insulin activates protein phosphatase 1 (PP1), which dephosphorylates and activates glycogen synthase promoting storage of glucose as glycogen ³⁵. G6P is converted to glucose-1-phosphate (G1P), then uridine diphosphate-glucose (UDP-glucose) by UDP-glucose-phosphorylase, and assembled into glycogen by glycogen synthase ³⁵.

1.2.2 Lipid Metabolism

In skeletal muscle, lipid metabolism consists of the oxidation of free fatty acids from circulation, esterification and storage of free fatty acids, and breakdown of intramyocellular lipid stores.

Acyl-CoA is formed by the addition of CoA to a free fatty acid, which enters the cell by a variety of mechanisms. Circulating chylomicrons and very low density lipoproteins (VLDLs) are too large to cross the skeletal muscle membrane, and require enzymatic processing. Lipoprotein lipase hydrolyzes the TAG component in circulating chylomicrons and very low density lipoproteins (VLDLs), releasing free fatty acids into the cytosol for metabolism ³⁶. Circulating free fatty acids are smaller than chylomicrons and VLDLs, and cross the muscle membrane via transporters, or through a “flip-flop” mechanism of diffusion ³⁷. CD36 (also called FAT, fatty acid translocase) is a fatty acid transport protein expressed in skeletal muscle. CD36 translocates from an endosomal compartment to the muscle membrane

via AMPK signalling during muscle contraction as well as via PI3K mediated signalling following insulin binding ³⁸. Therefore, CD36 translocation and free fatty acid uptake mirrors the mechanisms of GLUT4 translocation and glucose uptake. Fatty acid binding protein (FABP), and fatty acid transporters (FATP1 and FATP4) are co-expressed with CD36 and translocate to the skeletal muscle membrane in response to muscle contraction ³⁹. These transport proteins facilitate fatty acid uptake under conditions of lower or normal circulating lipid concentrations. However, during high circulating concentrations fatty acids at the outer leaflet of the plasma membrane could enhance diffusion fluxes as these molecules “flip-flop” from the outer to inner leaflet of the bilayer ³⁷.

In the cytosol, free fatty acids form Acyl-CoA via Acyl CoA Synthase (ACS). This Acyl-CoA may now undergo β -oxidation, or may be stored as intramyocellular lipid, specifically, TAG. β -oxidation is the hydrolysis of fatty Acyl CoA to produce Acetyl CoA (which enters the Krebs cycle) and the coenzymes NADH and FADH₂, leading to ATP generation. When free fatty acids are present in excess, or cellular energy demand is low, they may be esterified for storage within skeletal muscle. Two key enzymes for intramyocellular lipid esterification are glycerol-3-phosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT), which regulate the first committed step and final step, respectively ⁴⁰. GPAT catalyzes the acylation of sn-glycerol-3-phosphate (from glycolysis) at the *sn*-1 position, resulting in lysophosphatidic acid, while DGAT covalently links diacylglycerol and a fatty acyl-CoA ⁴¹.

Finally, in addition to free fatty acid oxidation and esterification, skeletal muscle may metabolize lipid stores for ATP generation. Intramyocellular triacylglycerol (IMTG) is a large source of substrate during exercise ⁴². Hormone sensitive lipase (HSL) is an enzyme

regulator of lipolysis, and is activated during exercise in response to calcium activation of protein kinase C (PKC), which stimulates extracellular regulated kinase (ERK)⁴³. HSL is also activated by epinephrine via protein kinase A (PKA)-mediated mechanisms⁴³. During lipolysis, adipose triglyceride lipase (ATGL) cleaves the first fatty acid off a triglyceride molecule, producing diacylglycerol (DAG) and a free fatty acid⁴⁴. DAG is subsequently cleaved by HSL producing a free fatty acid and a monoacylglycerol, which is finally cleaved by monoacylglycerol lipase (MGL) releasing glycerol and a fatty acid⁴⁴. The cleaved fatty acids may now enter the mitochondria for oxidation and generation of ATP for contraction.

1.2.3 Glucose and Lipid Overload

More recently, an interplay between glucose, lipids and thermogenesis within skeletal muscle has been suggested⁴⁵. Excess glucose and fatty acid oxidation results in significant production of acetyl-CoA, which may overload the Krebs cycle, producing excess citrate. Citrate is an allosteric activator of acetyl-CoA carboxylase (ACC), which catalyzes the production of malonyl-CoA, a precursor in fatty acid synthesis. Further, via citrate lyase, citrate will provide acetyl-CoA for ACC. This results in *de novo* lipogenesis within the muscle. A futile cycle has been proposed, whereby PI3K activity increases glucose entry into the cell, and, as described, drives *de novo* lipogenesis. AMP-activated protein kinase (AMPK) however, phosphorylates ACC resulting in approximately 50-60% inhibition of activity and a subsequent reduction in malonyl-CoA. AMPK also disinhibits carnitine palmitoyltransferase-1 (CPT-1) an enzyme responsible for the transport of fatty acids into the mitochondria for oxidation. Increased lipid oxidation produces acetyl-CoA, which overloads Krebs and drives *de novo* lipogenesis, continuing the futile cycle.

1.3 Insulin Resistance

As early as 1936, the concept of insulin resistance began to develop, as it was observed that some patients required larger insulin doses than others to achieve similar effects on blood glucose⁴⁶. Insulin resistance occurs when a “normal” or physiological concentration of insulin produces a less than normal response, and may stem from alterations prior to insulin-insulin receptor interaction, alterations in intracellular signalling, or alterations to the insulin receptor itself⁴⁷.

The development of insulin resistance is determined by a variety of measures, including measures of fasting glucose and insulin, insulin tolerance tests and oral glucose tolerance tests. The hyperinsulinemic-euglycemic clamp, developed by DeFronzo et al. is considered the “gold standard” for assessing insulin resistance⁴⁸. This test involves fasting patients overnight, followed by an intravenous infusion of insulin at a constant rate to achieve hyperinsulinemia. Insulin infusion will stimulate tissue uptake of glucose, and a subsequent reduction in blood glucose. Blood glucose is measured at set intervals (usually every five to ten minutes) and dextrose is infused simultaneously at a variable rate to reach normoglycemia. A greater glucose infusion rate (GIR) suggests greater insulin action and sensitivity, while a lower GIR is indicative of reduced insulin action.

Insulin resistance is a physiological state that contributes to the pathophysiology of several cardiovascular and metabolic diseases⁴⁹. It is a hallmark of T2D, and has been observed among individuals with T1D, resulting in a state of Double Diabetes^{15,16,50}. Double Diabetes occurs when patients with T2D also present with autoimmune markers against pancreatic β -cells, or when T1D patients develop characteristics such as insulin resistance and central adiposity^{15,50}. Further, T1D individuals with a family history of T2D are at a

significantly higher risk of developing Double Diabetes ¹⁵. Among many T1D patients, hyperglycemia as a result of T1D, and dyslipidemia, the result of central adiposity, may act synergistically to substantially increase the risk of coronary heart disease (CHD) ¹⁵. Cleland (2012) employs findings from the FinnDiane (Finnish Diabetic Nephropathy) study to illustrate the emergence of different phenotypes associated with significantly increased mortality ^{15,51}. Those with poor glycemic control, coupled to insulin resistance and an atherogenic lipid profile were at significantly greater risk of CHD ^{15,51}.

1.3.1 Insulin Resistance: Pathophysiology

The pathophysiology of insulin resistance is complex, as there are a variety of mechanisms that impair insulin signalling, with the possibility of multiple factors acting simultaneously. Excessive substrate oxidation, lipid intermediates, an altered cellular redox environment, endoplasmic reticulum stress, and cytokines and macrophages have all been implicated in the development of insulin resistance ⁵²⁻⁵⁴.

Chronically high circulating glucose contributes to skeletal muscle insulin resistance via reactive oxygen species (ROS) formation. Excess oxidation produces electron leak from the mitochondria and subsequent ROS formation ⁵⁵. ROS are neutralized by cellular antioxidant systems, such as glutathione coupled to NADH, which neutralizes H₂O₂ to 2H₂O ⁵⁴. However, accumulation of ROS may overwhelm the antioxidant system and ROS that are not neutralized negatively influence the insulin signal, particularly via JNK (c-Jun N-terminal kinase) phosphorylation of IRS-1 ⁵⁵.

Ceramides are formed from the interaction between a saturated fatty acid (ie. palmitoyl CoA) and L-serine to form dihydrosphingosine via serine palmitoyl transferase 1 (SPT-1) ⁵⁶. Dihydrosphingosine is then converted to dihydroceramide and finally, ceramide ⁵⁶. Ceramides

interfere with insulin signalling at the level of PKB/Akt, inhibiting GLUT4 translocation, as PKB/Akt stimulates AS160⁵⁷. In addition to interfering with the insulin signal, ceramide activation of PKB/Akt reduces glycogen synthesis via inhibition of glycogen synthase kinase 3 (GSK3), and enhances lipogenesis and reduces lipolysis via stimulation of phosphodiesterase 3B (PDE3B)⁵⁶. This initiates cAMP and PKA signalling, and subsequent activation of fatty acid synthase and inactivation of acetyl CoA carboxylase and acetyl CoA lyase.

DAGs are generated from free fatty acids that enter the muscle cell through transporters such as CD36, FATPs, FABP, etc., through hydrolysis of lipoproteins via lipoprotein lipase, or by passive diffusion across the membrane. Upon entry into the cell, fatty acids are converted to fatty acyl-CoAs (FACoA), via acetyl CoA synthase⁵⁸. FACoAs may then enter β -oxidation, then the mitochondria via carnitine shuttles for oxidation. However, when lipolysis is inhibited, or energy demands are low, excess FACoAs combine with glyceraldehyde-3-phosphate (G3P), and through a series of enzymatic reactions involving glycerol-palmitate acyltransferase (GPAT) and acylglycerolphosphate acyltransferase (AGPAT) to produce DAG⁵⁸. DAGs contribute to insulin resistance by impairing insulin signalling via interaction with PKC θ ⁵⁹. PKC θ phosphorylates the insulin receptor at Serine 307, which impairs insulin binding to initiate autophosphorylation of the receptor and activation of IRS-1⁴⁹. This subsequently fails to activate PI3K, and the signalling cascade that follows to mediate GLUT4 translocation. Interestingly, despite its negative effect on the insulin signal, increased skeletal muscle DAG content is observed in individuals with normal or enhanced insulin sensitivity, such as endurance athletes⁵³. It is important to note, however, that endurance training upregulates PGC-1 α and mitochondrial content within the muscle⁶⁰. Further, exercise training may enhance “metabolic channeling” of lipids, which refers to the direct delivery of fatty acids (following TAG and DAG hydrolysis) to the mitochondria from the lipid droplet (LD)⁵⁴.

Metabolic channeling also includes the enzymes of β -oxidation, as they are organized into multienzyme complexes; a series of catalytic reactions and the transfer of intermediates between enzymes which prevents the diffusion into the aqueous environment ⁵⁴. Lastly, the composition of DAG may determine its effect on insulin signalling, as DAGs composed of saturated fatty acids (ie.di-C18:0) are associated with insulin resistance ⁶¹. Therefore, endurance trained muscle is better equipped to oxidize intramuscular DAG, and the saturation of DAG may differ in this population, perhaps preserving insulin signalling in these individuals.

Excess circulating free fatty acids may also contribute to the onset of insulin resistance through the formation of acylcarnitines. Increased free fatty acids in skeletal muscle are converted to acyl-CoA via ACS. Acyl-CoAs are converted to acylcarnitines via CPT-1, and enter the inner mitochondrial membrane. Here, via CPT-2, the addition of a CoA and removal of carnitine results in acylcarnitine conversion to acyl-CoA, which may then undergo oxidation. Under conditions of excessive free fatty acid accumulation in skeletal muscle, the Krebs cycle is outpaced by fatty acid oxidation and acyl carnitines accumulate and are transported out of the mitochondria ⁵⁴. Acylcarnitines interact with nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B), an inflammatory factor that exerts negative effects on insulin signalling via JNK ⁶².

When circulating free fatty acids enter skeletal muscle and are not diverted toward storage, they are oxidized. This is an essential function of muscle metabolism and an enormous source of ATP for working muscle. However, in excess, free fatty acid oxidation stresses the cellular redox environment, which disrupts the redox optimized ROS balance (R-ORB), and subsequently impairs skeletal muscle insulin signalling ⁵⁴. The nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂) reducing equivalents resulting

from oxidation of fatty acids reach the mitochondrial electron transport chain where they donate electrons, which stimulates the transport of hydrogen ions across the membrane. This is essential for ATP production, as it generates a proton pump. As hydrogen passes via ATP synthase, the energy converts ADP to ATP. Excess flux of substrate through oxidative pathways leads to an accumulation of reducing equivalents at the entry point of the electron transport chain. Increased electron leak and formation of reactive ROS (H_2O_2) results. It is also important to note that excess skeletal muscle fatty acids may bind peroxisome proliferator-activated receptor alpha ($PPAR\alpha$) and upregulate acetyl CoA carboxylase and fatty acid synthase⁶³.

In addition to altering the redox environment, excess fatty acid flux into the skeletal muscle cell places an increased demand on cellular machinery, leading to the accumulation of misfolded proteins from the endoplasmic reticulum (ER). Unfolded protein accumulation stimulates phosphorylation of the unfolded protein response sensor inositol-requiring ER-to-nucleus signalling protein 1 (IRE1). IRE1 forms a complex with adaptor protein TNF receptor-associated factor 2 (TRAF2) and activates JNK, which inhibits IRS-1 and impairs insulin signalling⁶⁴. Further, saturated fatty acids are not as freely converted to triglycerides as unsaturated fatty acids and may travel to the ER resulting in alterations to its morphology and function (ER stress)⁶⁵.

In addition to abnormal substrate metabolism, inflammatory cytokines and macrophages have been shown to drive the development of skeletal muscle insulin resistance. Tissue necrosis factor alpha ($TNF-\alpha$), a proinflammatory cytokine, exerts negative effects on insulin sensitivity⁵². The saturated fatty acid palmitate stimulates PKC θ and activates NF- κ B, which increases $TNF-\alpha$ gene expression⁶⁶. $TNF-\alpha$ alters AKT, AS160 and glucose transport in skeletal muscle, and may also interfere with insulin signalling via activation of

sphingomyelinase and subsequent production of ceramides⁶⁶⁻⁶⁸. Inflammatory cytokines are also released by macrophages. In response to circulating fatty acids, a macrophage may become M1 polarized, its inflammatory phenotype^{52,66}. When these macrophages are M1 polarized within the skeletal muscle fibres, they secrete inflammatory cytokines and exert a paracrine effect on muscle cells. Interestingly, these macrophages may be located near the skeletal muscle fibre, or be present in adipose tissue infiltrating muscle fibres⁶⁶.

1.3.2 Type 2 Diabetes: Pathophysiology

In T2D, insulin resistance may be coupled to insufficient insulin production. T2D is the product of a gene-environment interaction, with a variety of genes predisposing an individual to obesity and insulin resistance, and eventually T2D⁶⁹.

Leptin is a hormone that is particularly important in the development of obesity. Leptin functions as an antilipogenic hormone in target tissues, and is also able to cross the blood-brain-barrier, exerting effects in the hypothalamus⁶³. Leptin binding to its receptors in the hypothalamus results in reduced food intake, which would slow or prevent the onset of obesity. However, saturation of transport across the blood-brain-barrier occurs when circulating leptin concentration is lower than what is observed in obesity, suggesting this system may be exhausted and the protective effects may be lost⁶³. In other target tissues, leptin binds its receptor and stimulates PPAR α , CPT-1, UCP-2, and genes of fatty acid oxidation, increasing oxidation and uncoupling to compensate for increased caloric intake^{70,71}.

In circumstances of uncompensated caloric excess contributing to obesity, circulating leptin is insufficient to increase oxidation and uncoupling, and excess fatty acids activate lipogenic genes including SREPB-1C, ACC, and FAS, increasing TAG, DAG, and ceramide production⁷¹. These lipid intermediates may exert negative effects on the insulin signal

(particularly at IRS-1). This reduces insulin-stimulated glucose uptake and glycogen synthesis, as is observed in T2D⁷². Further, chronically elevated circulating glucose depresses GLUT4 transporter content in skeletal muscle, contributing to reduced muscle glucose uptake⁷³. Historically, these lipogenic genes and handling of excess fatty acid and glucose was advantageous, however with our increasingly sedentary modern lifestyle, they now predispose individuals to insulin resistance and T2D⁷⁴.

Genetic susceptibility to T2D also occurs at the level of the pancreatic β -cells. In a normal β -cell, obesity and insulin resistance contribute to increased cell function and growth⁷¹. As a result, hyperinsulinemia maintains normal glucose tolerance. However, a variety of genes render β -cells susceptible to dysfunction and apoptosis (HNF1 α , TCF7L2, mitochondrial genes, etc.)⁶⁹. Often, by the time of T2D diagnosis, there is a 50% decrease in the number pancreatic β -cells, and the remaining cells function at or below 25% of functional capacity⁶⁹. The progressive nature of T2D can be described as the following cycle: a continuous decline in β -cell function results in decreased insulin secretion and a subsequent increase in blood glucose, which contributes to β -cell toxicity⁶⁹. Impaired insulin release also contributes to the onset of insulin resistance at the level of the hypothalamus, stimulating increased food intake (as discussed above). Further, hypoinsulinemia removes inhibition of adipose tissue lipolysis, increasing the release of fatty acids into circulation which impair insulin signalling in β -cells and skeletal muscle, resulting in insulin resistance and T2D⁶⁹.

1.3.3 “Double Diabetes”: Type 1 Diabetes and Insulin Resistance

Type 1 and Type 2 Diabetes have long been considered two distinct metabolic diseases, with T1D being a genetic, autoimmune disease and T2D being an interaction between predisposing genetic factors and the environment. In 2001, the concept of “Double Diabetes”

was formulated ⁷⁵. Double Diabetes refers to T1D autoantibody positive individuals who are also insulin resistant ⁷⁵. These individuals differ from T1D patients as they have a lower frequency of MHC genes, and an increase in genes associated with the risk of T2D ⁷⁶. Unfortunately, Double Diabetes is associated with an increased risk of cardiovascular complications even compared to non-insulin resistant T1D patients ¹⁵.

In 2009, Wilkin proposed a hypothesis for the development of Double Diabetes termed “The Accelerator Hypothesis”. This proposed that T1D and T2D are the same disorder of insulin resistance, set against different genetic backgrounds with a different rate of progression ^{10,11}. According to this hypothesis, T1D is thought to develop in individuals who are extremely genetically susceptible, who are subject to our obesity-promoting environment. This manifests as a high degree of insulin resistance, coupled to a strong immune response in pancreatic islets, leading to the onset of T1D. The rate of β -cell loss determines when the disease presents, with a moderate loss manifesting as adult onset diabetes, whereas a rapid loss results in childhood onset diabetes ¹¹. Regardless of the time of onset, insulin resistance (the “driver”) increases β -cell stress via glucotoxicity and lipotoxicity, which increases the immune response in genetically predisposed (the “modulator”) individual ¹¹. Wilkin provides support for this theory from a variety of epidemiological studies. For instance, the increasing rate of T1D diagnoses parallels an increasing rate of childhood obesity ^{77,78}. Further, children who are insulin resistant prior to developing T1D and those with the highest insulin resistance at baseline are more likely to develop T1D than those who are less insulin resistant ^{79–81}.

Perhaps the most interesting tenet of this hypothesis is the interaction between predisposing genes and the environment. This suggests that while our susceptibility to T1D may be fixed in terms of genetics, we have the capacity to alter our environment. Increasing

physical activity, for instance, is a powerful method to reduce insulin resistance, and therefore may protect against Double Diabetes. The role of exercise training in T1D will be discussed in the subsequent pages.

1.4 Exercise and T1D

It is well-established that regular physical activity reduces the risk of chronic disease and improves lifespan and quality of life across a variety of populations, including T1D ⁸². Many of these outcomes are the result of improved metabolic and cardiovascular health, such as improved insulin sensitivity and cardiac function, as our lab has previously reported ^{83,84}. Others have observed improvements to dyslipidemia, BMI, waist circumference, and aerobic fitness following exercise training in T1D patients ⁸⁵⁻⁸⁹. Further, studies examining the effects of different exercise modalities in T1D suggest that resistance training alone or in combination with aerobic exercise may improve HbA1c and cholesterol and is associated with a reduced risk of hypoglycemia ⁹⁰⁻⁹².

1.4.1 Exercise Recommendations for T1D patients

The Canadian Diabetes Association currently recommends that individuals with T1D engage in at least 150 minutes of aerobic exercise, in addition to a minimum of two bouts of resistance training each week ⁹³. However, a major limitation to individuals with T1D participating in exercise is a fear of hypoglycemia ⁹⁴. Post-exercise hypoglycemia occurs as the result of a reliance on exogenous insulin coupled to tissue (particularly skeletal muscle) glucose uptake. In a nondiabetic individual, endogenous insulin production is suppressed, while in those with T1D, the reliance on exogenous insulin may result in higher circulating insulin during and following exercise. Therefore, this may exacerbate tissue glucose uptake in the post-exercise recovery period resulting in hypoglycemia.

There are, however, a variety of recommendations for preventing post-exercise hypoglycemia, including ingestion of glucose to enhance blood glucose, in addition to reducing the insulin dose ⁹⁵. While this strategy reduces the risk of post-exercise hypoglycemia, there is the risk of overcompensating and compromising long-term blood glucose control (HbA1c) and weight gain ⁹⁶. More recent studies are examining various exercise modalities and the incorporation of brief high intensity exercise and resistance training to reduce the risk of hypoglycemia without ingesting additional glucose.

Brief and intermittent high intensity exercise (such as sprinting for a few seconds) has been shown to increase circulating epinephrine, norepinephrine, growth hormone and lactate ⁹⁶⁻¹⁰⁰. The catecholamines epinephrine and norepinephrine stimulate hepatic glucose production, and may contribute to a slower decline in blood glucose during exercise via increased rate of glucose appearance ^{96,100,101}. This concept was observed in a study of individuals with T1D that compared the effects of a 30 minute bout of cycling either at 40% VO_{2max} continuously or cycling at 40% VO_{2max} with a 4 second sprint every 2 minutes ¹⁰¹. Results showed a reduced blood glucose decline following exercise in the group incorporating the short sprints, and is likely the result of catecholamine-induced increases in hepatic glucose production ^{100,101}. These findings suggest the inclusion of brief high intensity bouts of exercise may be advantageous for T1D patients engaging in exercise.

Resistance training is also gaining recognition as an exercise modality that may be safer than traditional continuous aerobic exercise for individuals with T1D. As has been observed with intermittent high intensity exercise, resistance exercise stimulates increased circulating epinephrine, norepinephrine, growth hormone and lactate ^{96,102-104}. Yardley et al. have conducted several studies examining the effects of aerobic and resistance exercise in T1D patients and has observed that resistance exercise is associated with a smaller initial decline in

blood glucose than aerobic exercise ¹⁰⁵. In an earlier study, T1D patients engaged in 45 minutes of running at approximately 60% $\text{VO}_{2\text{max}}$ followed by 45 minutes of resistance training or the resistance training preceding the running ¹⁰⁵. Performing the resistance exercise first produced an attenuated decline in blood glucose during exercise, and reduced the frequency of hypoglycemic events ⁹². It is hypothesized that the increase in circulating epinephrine occurring during resistance exercise stimulates increased glycogen use during exercise, preserving blood glucose ^{92,106}.

1.4.2 Skeletal Muscle: Adaptations to Aerobic Exercise

Increased mitochondrial biogenesis is a hallmark adaptation to aerobic endurance exercise training. The Krebs cycle and β -oxidation occur exclusively within the mitochondria, making this organelle essential to the cell's energy supply. In 1967, Holloszy conducted one of the earliest studies to demonstrate increased skeletal muscle mitochondrial protein content following training in rats. Today, the effects of endurance training on mitochondrial biogenesis are well documented ^{31,107-109}.

A variety of stimuli occurring during endurance exercise initiate the transcription of mitochondrial genes. Many of these stimuli activate peroxisome proliferator-activated receptor-gamma coactivator (PGC-1 α), a co-transcriptional regulator. PGC-1 α activates several transcription factors, including NRF-1 and NRF-2 which interact with Tfam to drive transcription and replication of mtDNA ^{110,111}. Factors activating PGC-1 α include AMPK, nitric oxide (NO), sirtuin 1 (SIRT1), target of rapamycin complex 1/2/3 (TORC1/TORC2/TORC3), and p38 mitogen-activated protein kinases (p38 MAPK) among others ¹¹⁰.

Aerobic exercise results in greater energy turnover within the cell, and greater conversion of ATP to ADP and AMP. These factors activate AMPK following binding to the γ subunit¹¹². It has also been shown that AMPK has a glycogen binding domain, and is more active when glycogen is low, especially during exercise¹¹². AMPK is a regulator of cellular anabolism and catabolism, and, through activation of PGC-1 α and SIRT1, induces mitochondrial biogenesis and promotes an oxidative fiber type¹¹². AMPK directly phosphorylates PGC-1 α , while SIRT1 deacetylates PGC-1 α ¹¹². Similar to AMPK, SIRT1 may function as a sensor, becoming active during changes in the redox balance between NAD⁺ and NADH¹¹³. Greater mitochondrial content bestows a greater ability to oxidize substrate, and provide energy for contracting muscle. PGC-1 α also moderates substrate flux through its interaction with peroxisome proliferator activated-receptors (PPARs). PPARs bind with retinoid X receptors (RXRs) to DNA sequences and upregulate gene expression of a variety of genes associated with skeletal muscle lipid oxidation and fatty acid uptake^{31,111}. Therefore, exercise training promotes a greater capacity to oxidize metabolic substrates.

In addition to developing a greater capacity to oxidize substrate, exercise promotes increased skeletal muscle glucose uptake from the circulation. AS160 is an AMPK target, and when phosphorylated, it aids in the translocation of GLUT4 to the membrane³². Skeletal muscle contraction has been shown to increase GLUT4 translocation and subsequent glucose uptake. Our lab and others have observed increases in skeletal muscle GLUT4 protein content following aerobic exercise training^{83,114,115}. AMPK in addition to the CaMKII kinase, both activated during muscle contraction, regulate GLUT4 transcription via HDAC4/5-MEF2 (histone deacetylase 4/5-myocyte enhancer factor 2) and MEF2-GEF (GLUT4 enhancer factor) interactions³².

GLUT4 is also essential to glucose storage (as glycogen) in skeletal muscle. Following uptake into the cell, hexokinase phosphorylates glucose to produce glucose-6-phosphate (G6P). G6P is then converted to UDP-glucose, which, via glycogen synthase, is converted to glycogen for storage in skeletal muscle. Increased GLUT4 protein content has been correlated to greater glycogen storage following exercise^{32,116,117}.

Metabolic changes in skeletal muscle following exercise training are not limited to glucose metabolism, as substantial improvements to lipid metabolism are observed. During exercise, activation of AMPK inhibits ACC β (acetyl CoA carboxylase beta), which catalyzes the synthesis of Malonyl-CoA. Malonyl-CoA inhibits CPT-1 and fatty acid transport into the mitochondria for oxidation. Therefore, inhibition of Malonyl-CoA synthesis leads to increased fatty acid transport and subsequent oxidation¹¹⁸. This enhanced lipid oxidation is coupled to greater lipid storage and synthesis of IMCL¹¹⁹. While excess skeletal muscle lipid content may be detrimental, sequestering fatty acids in neutral TAG molecules and lipid droplets is protective to the cell. Synthesis of these neutral lipid pools sequesters fatty acids and prevents them from becoming harmful intermediates, and serves as an energy reserve for oxidation during exercise.

Perhaps one of the most clinically relevant physiological adaptations to exercise is enhanced insulin sensitivity. Greater insulin sensitivity results in improved glucose uptake and regulation of blood glucose, preventing the negative effects of hyperglycemia in a variety of tissues. There are several potential mechanisms underlying this effect, including greater GLUT4 protein content and/or translocation, or reduced interference with the insulin signal via reduced ROS production, DAG and ceramide content¹²⁰⁻¹²². Enhanced insulin sensitivity has been observed immediately post-exercise, and may be the result of decreased skeletal muscle glycogen content, AMPK activation, AS160 phosphorylation and enhanced lipid oxidation¹²³⁻

¹²⁸. In addition to acute improvements to insulin sensitivity, aerobic exercise training elicits more long lasting effects¹²⁹.

1.4.3 Skeletal Muscle: Adaptations to Resistance Exercise

The American College of Sports Medicine defines resistance training as exercising a muscle or muscle group against external resistance. Some of the well-understood adaptations to resistance training include skeletal muscle hypertrophy, fibre type conversion, increased capillary to fibre ratio and increased fat free mass¹³⁰.

Resistance training stimulates increased circulating insulin-like growth factor 1 (IGF-1)¹³¹. IGF-1 initiates a signalling cascade which promotes skeletal muscle hypertrophy. Briefly, IGF-1 binds its receptor and activates tyrosine kinase, stimulating autophosphorylation creating insulin receptor substrate (IRS) docking sites. Phosphorylated IRS recruits and activates PI3K, which generates PIP3 from PIP2 and acts as docking site for phosphoinositide-dependent kinase 1 (PDK1) and Akt. PDK1 phosphorylates Akt which stimulates protein synthesis via activation of mTOR and TORC1/TORC2 which stimulate ribosomal protein s6 kinase beta-1 (p70S6k) and eukaryotic translation factor 4E-binding protein (4E-BP1) respectively, initiating protein translation and hypertrophy^{31,132}. Akt also phosphorylates and inactivates GSK3 β , which releases its inhibition on eukaryotic translation initiation factor 2B (eIF2B)¹³². Resistance training has also been shown to phosphorylate IKK β , linking cellular stress to the activation of mTORC1¹³³.

In addition to skeletal muscle hypertrophy, resistance training is emerging as an exercise modality associated with improvements to age-related skeletal muscle function decline, oxidative capacity and mitochondrial function and insulin sensitivity^{83,134,135}. Resistance training acutely increases insulin sensitivity, improves insulin sensitivity in

overweight individuals, and increases insulin-mediated suppression of endogenous glucose production^{136–138}. The effects of resistance training on insulin sensitivity appear to occur preferentially in skeletal muscle over the liver, and may be mediated by increases in key enzymes and proteins in glucose metabolism including hexokinase, GLUT4, Akt, insulin receptor, PKB, and glycogen synthase^{139,140}.

1.4.4 Skeletal Muscle: Adaptations to Combined Exercise

There is little doubt that exercise training, whether aerobic or resistance confers positive metabolic adaptations in skeletal muscle. However, engaging in a combination of these modalities was initially thought to impair fitness progression and improvement in each specific activity. More recent work suggests that a combination of aerobic and resistance training may produce substantial improvements to skeletal muscle metabolic health than either activity in isolation.

A 2015 study examined the effects of alternating days of running and resistance training on running performance¹⁴¹. This combined approach to training resulted in increased rating of muscular fatigue, muscle soreness and reduced maximum voluntary contraction. Further, concurrent training has been reported to hinder the capacity to develop strength, but not limit the magnitude of $\dot{V}O_{2\max}$ improvements¹⁴². This interaction between modalities has been termed the “interference effect”. More recent evidence suggests there is no interference between modalities, and the opposite may, in fact, occur. Short term-cycling followed by maximal knee extensions, has been shown to activate vascular endothelial growth factor (VEGF), cAMP-response element-binding protein (CREB), mechanistic target of rapamycin (mTOR) and inhibit JNK, NF κ B, and MAPK¹⁴³. Interestingly, these genes were upregulated in the cycling and knee extension (resistance exercise) group compared to the resistance

exercise only group, suggesting that combining modalities increases expression of genes for myofibre growth and oxidative capacity¹⁴³.

The interference effect may be a significant consideration for athletes striving for optimal performance. However, for individuals engaging in less rigorous exercise participation, combining modalities may induce significant benefits. For example, citrate synthase (CS) activity is a marker for oxidative capacity and has shown robust improvements in activity following combined exercise training as opposed to either aerobic or resistance training in isolation^{143–145}. The benefits of combined training have also been observed in the study of individuals with T2D, as mitochondrial content and ability to metabolize substrate has also been shown to increase to a greater degree following combined exercise compared to aerobic exercise among T2D patients¹⁴⁶. Further, combined training is associated with reductions in HbA1c and cholesterol and increased high density lipoprotein (HDL) among T2D patients¹⁴⁷.

These studies of combined exercise training suggest that the combination of aerobic and resistance exercise may elicit significant improvements to skeletal muscle metabolic health. Resistance exercise may therefore be a powerful adjunct to aerobic exercise in the management of skeletal muscle metabolic health and disease.

1.4.5 Cardiovascular Benefits of Exercise

The myocardium undergoes hypertrophy in response to exercise or as a pathological adaptation to disease. Previous work from our lab has shown that sedentary diabetic animals develop pathological cardiac hypertrophy, while exercise-trained animals show functional cardiac hypertrophy in response to exercise¹⁴⁸. Hypertrophy occurring in the sedentary animals is a characteristic of T1D termed diabetic cardiomyopathy (DCM)^{149,150}. DCM

consists of ventricular dysfunction, abnormal intracellular calcium homeostasis and contributes to myogenic cardiac dysfunctions^{149,150}. The development of DCM occurs via decreased mitochondrial respiration, decreased pyruvate dehydrogenase activity, dysfunctional contractile and regulatory proteins, impaired calcium homeostasis^{149,151,152}. On a larger scale, this dysfunction results from oxidative stress, inflammation, and cardiac autonomic neuropathy (CAN)^{153–156}.

Exercise training has been shown to improve and even prevent the development of DCM¹⁵⁷. Exercise has been shown to reduce contractile dysfunction and cardiac mortality in T2D patients with insulin resistance^{149,158}. One of the most robust physiological adaptations following exercise training is increased cardiac output^{149,159,160}. Mechanisms underlying this adaptation include increased cardiac pumping function and increased autonomic response, as diabetes is associated with depressed catecholamine responsiveness^{149,161–163}.

Oxidative stress contributes to DCM via DNA damage. Hyperglycemia drives increased superoxide (O_2^-) formation via the electron transport chain^{153,164,165}. Superoxide dismutase (SOD) then increases nitric oxide (NO), leading to peroxynitrite ($ONOO^-$) formation. $ONOO^-$ induces DNA damage, slows glycolysis, the electron transport chain and ATP production^{153,165}. Exercise training alleviates oxidative stress through enhancement of myocardial antioxidant systems as well as reducing cytochrome c leakage¹⁶⁶. In streptozotocin (STZ)-induced DCM, exercise increases the expression of extracellular SOD and attenuates oxidative stress¹⁶⁷.

Hyperglycemia is also associated with an increase in inflammatory cytokines, which damage the vascular endothelium and increased the risk of cardiovascular events^{153,155}.

Exercise reduces circulating inflammatory cytokines, and in diabetic rodents, has been shown to reduce tissue necrosis factor alpha (TNF- α)¹⁶⁸.

Individuals with poorly managed T1D often develop cardiac autonomic neuropathy (CAN), consisting of cardiac sympathetic dysinnervation^{153,156}. CAN is associated with cardiovascular morbidity and mortality in T1D patients and is implicated in the pathogenesis of diabetic nephropathy^{169–171}. In rodent studies, low intensity resistance training attenuates CAN, and among individuals with T2D, six months of exercise training significantly improves CAN^{172,173}.

There is some disagreement as to whether patients with T1D achieve the same cardiovascular benefits from exercise as other populations. In a retrospective analysis of the DCCT, exercise was shown to have no effect on improvements in microvascular outcomes for T1D patients¹⁷⁴. However, a large study of T1D patients in Germany and Austria found an inverse association between self-reported levels of physical activity and HbA1c, diabetic ketoacidosis, dyslipidemia and hypertension¹⁷⁵. It is possible, however, that no effect was observed for patients in the DCCT based on the inclusion criteria of the trial, as these T1D patients had no other complications¹⁵. Given that this retrospective analysis reports no harm to patients with T1D engaging in exercise, it should remain a valuable tool for the management of T1D and for the maintenance of metabolic and cardiovascular health.

1.5 Rationale

Approximately 20% of T1D patients go on to develop insulin resistance, resulting in what has been termed “Double Diabetes”⁷. Cardiovascular disease is the leading cause of morbidity and mortality among individuals with T1D and individuals with Double Diabetes are at an even greater risk than those with T1D alone⁵⁰. In T2D and metabolic syndrome,

abnormal skeletal muscle lipid metabolism and accumulation of intramyocellular lipids and lipid metabolites have been shown to impair insulin signalling and contribute the development of insulin resistance, which contributes to the development of cardiovascular disease ^{3,176}. It is unclear to what extent skeletal muscle lipid metabolism is altered in the context of T1D. In T2D, exercise training has been shown to alleviate lipid accumulation and is associated with reduced insulin resistance ¹⁷⁶. Despite the numerous benefits of exercise, intensive insulin therapy is currently recommended for the management of T1D ¹⁷⁷. However, intensive insulin therapy is associated with an increased risk of hypoglycemic events, and may contribute to the development of insulin resistance ^{15,16}.

Given the role of exercise training in restoring skeletal muscle lipid metabolism and insulin sensitivity in T2D, it is possible these effects will be observed in T1D. However, aerobic exercise is associated with significant risk of hypoglycemia, and we have observed significant post-exercise declines in blood glucose in T1D animals that is not improved following ten weeks of aerobic exercise training ¹⁷⁸. Recent findings reveal the beneficial effects of combined (aerobic and resistance) exercise for the reduction of post-exercise hypoglycemia ^{91,92,96}. Resistance exercise performed before aerobic exercise maintains plasma glucose during the aerobic exercise bout, and reduces the risk of post-exercise hypoglycemia ^{91,92}. Further, it is possible that the incorporation of both modalities as combined training would confer the greatest improvements to insulin sensitivity and skeletal muscle lipid metabolism, while reducing the risk of post-exercise hypoglycemia. Emerging findings suggest resistance and aerobic exercise may act synergistically to enhance the adaptations to exercise, and that combined exercise training may be superior to aerobic exercise alone for the improvement of metabolic health ¹⁴³⁻¹⁴⁵. Therefore, to restore or enhance insulin sensitivity in the context of T1D, it is necessary to examine the underlying molecular mechanisms, and the

effect of insulin therapy and different exercise modalities on these processes. It is hypothesized that abnormal skeletal muscle lipid accumulation will contribute to the development of insulin resistance, and that exercise training will enhance insulin sensitivity through the amelioration of muscle lipid metabolism.

1.6 Thesis Objectives

1. To determine whether insulin resistance in T1D is associated with increased skeletal muscle lipid metabolites, and whether this is ameliorated with aerobic exercise training.
2. To examine whether combined (aerobic + resistance) exercise training improves skeletal muscle oxidative capacity and lipid metabolism to the same degree as aerobic exercise training alone in T1D.
3. To compare the effects of combined exercise and the current recommendation of intensive insulin therapy on insulin resistance and skeletal muscle lipid composition in T1D.

1.7 Overview and Thesis Development

The negative effects of intramyocellular lipids and lipid metabolites on the insulin signalling cascade are well-understood in the context obesity and T2D ^{176,179,180}. It is not as well understood in the context of T1D, although some have demonstrated an association between intramyocellular lipid content and insulin resistance among T1D patients ¹⁸¹. Given the rise of “Double Diabetes”, it is important to examine the mechanisms underlying the development of insulin resistance, and whether exercise is an effective means to enhance insulin sensitivity. The focus of this dissertation was to examine the effects of different exercise modalities and insulin therapy on skeletal muscle lipid accumulation and insulin

resistance. To do so, we used a rat model of STZ-induced, insulin treated T1D, which is considered representative of humans with T1D ¹⁴⁸.

The first objective of this thesis was to determine whether increased muscle lipid accumulation was associated with insulin resistance in our model of T1D and whether this was ameliorated with aerobic exercise training (*Chapter 2*). Using high performance liquid chromatography, coupled to time-of-flight mass spectrometry, we identified several lipid metabolites that differentiated skeletal muscle of sedentary, insulin resistant diabetic animals from healthy control animals and from aerobically trained diabetic animals. Exercise training also effectively restored insulin sensitivity in the sedentary diabetic animals to that of sedentary non-diabetic animals. These findings led us to examine whether combined exercise training induces comparable benefits compared to aerobic exercise, as it is associated with a reduced risk of hypoglycemia (*Chapter 3*) ^{92,105,143–145,147}. We observed impaired oxidative capacity, coupled to greater lipid accumulation in sedentary T1D animals. Aerobic exercise was sufficient to restore oxidative capacity in T1D animals to that of non-diabetic control animals, however, combined exercise training elicited significant improvements to oxidative capacity and lipid storage. We then considered the importance of insulin therapy for individuals with T1D and sought to compare the gold standard intensive management to combined exercise in a conventionally treated group (*Chapter 4*). The combined group is maintained in a higher glycemic range to mimic how patients with T1D manage their blood glucose in order to exercise safely ⁹⁶. The aim of this study was to examine the effects of the current recommended treatment (intensive insulin therapy) compared to combined exercise training on a direct assessment of insulin sensitivity via hyperinsulinemic-euglycemic clamp, and evaluate the composition of skeletal muscle lipid stores.

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CHAPTER 2

2 « Metabolomic Response of Skeletal Muscle to Aerobic Exercise Training in Insulin Resistant Type 1 Diabetic Rats »

2.1 Introduction

Type 1 Diabetes (T1D) is characterized by pancreatic β -cell destruction and the inability to produce insulin and maintain glycemic control. A relatively new manifestation of diabetes, termed “Double Diabetes” has been identified, whereby insulin deficiency is coupled with the development of insulin resistance. The combination of these risk factors is associated with a greater risk of cardiovascular disease than T1D or Type 2 Diabetes (T2D) alone ¹.

While the etiology of insulin resistance in “Double Diabetes” remains to be determined, it is understood that it does not parallel other insulin resistant states (i.e. T2D or obesity), as patients with T1D often do not display associated factors such as obesity². It has been postulated that insulin resistance development in T1D stems from autoimmune-related mechanisms, while other suggest that chronic hyperglycemia and the accumulation of advanced glycation end products (AGEs) are causative factors ³⁻⁵. In this respect, glucotoxicity has been shown to lead to the activation of c-Jun N-terminal kinases (JNK) and protein kinase C (PKC) leading to disturbances in the insulin signalling pathway ^{6,7}. This role for hyperglycemia and cellular glucotoxicity has gained further support, as patients with T1D with a history of poor glycemic control are more likely to develop insulin resistance ⁶.

Increased intramyocellular lipid (IMCL) has also been implicated in the development of insulin resistance in T1D patients. This may also be a result of poor glycemic management, as glycosylated haemoglobin (HbA_{1c}) is correlated to IMCL in T1D ⁸. Others have also shown a positive relationship between insulin resistance and IMCL accumulation ^{9,10}. Similar to T2D lipotoxicity may play a role in the development of insulin resistance in T1D. In Zucker diabetic fatty rats, insufficient insulin leads to “metabolic overload” where mitochondrial function does not meet increased lipid flux ¹¹. In this case, free fatty acids may be converted to diacylglycerol (DAG) or metabolized into ceramides; both of which have been shown to interfere with insulin signalling ^{12,13}. We have established a rodent model of T1D using multiple low-dose streptozotocin (STZ)- treatment and insulin therapy to replicate poorly managed glycemic control observed in clinical T1D ¹⁴. In previous work, we have observed impairments in GLUT4 protein expression and the development of insulin resistance which were improved with high intensity aerobic exercise training ¹⁴⁻¹⁶. Concomitant with increases in insulin sensitivity we have shown that exercise training leads to reductions in blood lipid levels, decreased body mass, and decreased adipose tissue mass in comparison to sedentary T1D animals ^{15,17}. The purpose of this study was to determine whether insulin resistance development in a poorly controlled rodent model of T1D is associated with disturbances in lipid metabolism; and whether aerobic exercise-mediated improvements in insulin sensitivity are accompanied by changes in lipid metabolism. We hypothesized that several insulin-desensitizing lipid metabolites would be elevated in the skeletal muscle of insulin resistant T1D animals, indicative of impaired lipid metabolism, and that exercise training would restore insulin sensitivity and skeletal muscle lipid metabolism.

2.2 Methods

Ethics Approval and Animals. Eight-week old male Sprague Dawley rats (N=48) were obtained from Charles River Laboratories, and housed two per cage at constant temperature and humidity on a 12-h dark/light cycle. Rats had access to water and standard chow ad libitum. The experimental protocol followed the Principles of Laboratory Animal Care (US NH publication No. 83-85, revised 1985). Ethics approval was obtained through the University of Western Ontario Research Ethics Board, in accordance with Canadian Council on Animal Care guidelines.

Experimental Groups. Animals were randomly divided into three experimental groups; non-diabetic sedentary control (C, n=16), diabetic sedentary control (CD, n=16) and diabetic aerobic exercise (DAE, n=16). Upon completion of the training study, each group was divided into two subgroups. The first group (C, CD, DAE; n=8) underwent a hyperinsulinemic-euglycemic clamp prior to sacrifice while the second group (C, CD, DAE; n=8) did not undergo the clamp procedure but were sacrificed for tissue removal. The second group of animals did not undergo the clamp procedure in order to preserve the resting metabolic status of the muscle tissue for biochemical analysis.

Diabetes Induction. Upon arrival rats were housed for one week in order to familiarize with their surroundings. Following this period, T1D was induced with multiple low-dose STZ injections. STZ (20 mg/kg; Sigma Aldrich, Oakville, ON, Canada) was injected into the intraperitoneal cavity for five consecutive days¹⁸. Diabetes was confirmed by two consecutive non-fasting blood glucose readings of ≥ 18.0 mmol/L. Subsequently, insulin pellets (LinShin, Toronto, ON, Canada) were implanted subcutaneously. Insulin

pellet/dosages were monitored and adjusted throughout the 10-week experimental study to ensure daily non-fasting blood glucose concentrations of 9-15 mmol/L.

Exercise Training. Following the confirmation of diabetes and implantation of the insulin pellets, rats were familiarized on a motor-driven treadmill for one week prior to the onset of the exercise training program. This consisted of 15 minutes of progressive running up to 30 m/min for five days. Once familiarized, the exercise training program consisted of one hour of treadmill running at 27 m/min on a 6% gradient, five days per week for 10 weeks. This intensity of exercise has been shown to elicit 70-80% of VO_{2max} ¹⁹. To maintain continuous running, rats received small blasts of compressed air on their haunches if they broke a photoelectric beam at the rear of the treadmill.

Hyperinsulinemic-Euglycemic Clamp. To assess insulin resistance, eight animals from each group (a subgroup of C, CD, and DAE) underwent a hyperinsulinemic-euglycemic clamp three days following the final bout of exercise training. Prior to the clamp procedure animals were fasted for twelve hours and anaesthetized using isoflurane and an intraperitoneal injection of urethane (25 mg/kg)/ α -chloralose (4 mg/kg)^{20,21}. Once an analgesic plane was confirmed, isoflurane was removed and the urethane/ α -chloralose mixture maintained the anaesthesia. A catheter was surgically inserted into the right jugular vein for insulin and glucose infusion, and a second catheter was inserted into the right carotid artery for blood sampling and blood glucose measurement. Insulin (Eli Lilly, Toronto, ON, CAN) was infused at a constant rate of 10 mU/kg/min; 0.4 μ IU/mL. Glucose (EMD Millipore, Darmstadt, HE, Germany) was infused at 20 mg/kg/min, 0.2 g/mL and adjusted accordingly every 5 minutes until minute 20 and every 10 minutes thereafter to maintain the blood glucose concentration.

Tissue Collection. As noted above, eight animals in each group were euthanized for tissue collection three days following the final bout of exercise by isoflurane anaesthesia followed by exsanguination and cardiac excision. Due to tissue constraints, red portions of the gastrocnemius muscle were flash frozen for oil red O staining, soleus (primarily red) muscles were flash frozen for western blotting and the red portion of the tibialis anterior was flash frozen for thin layer chromatography.

Skeletal Muscle Metabolomics. 100 ± 3 mg of the red portion of the vastus muscle tissue was homogenized in 250 μ L of ice cold HPLC grade acetonitrile containing isatin (5 μ g/mL) and flurazepam (25 ng/mL) as internal standards for 2 minutes in an ice bath. Samples were vortexed, and centrifuged at 14000 rpm at 4 °C for 5 minutes. 120 μ L of supernatant was removed and diluted with 30 μ L of ultrapure water, for a final sample containing 80% acetonitrile. A control injection was generated by creating a pooled sample consisting of an equal volume of all injected samples. Samples were transferred to vials and 1 μ L was injected in triplicate from each vial. Injections were randomized to reduce error and pooled control samples were run every six injections. Chromatographic separation was performed on a Waters Acquity Ultra Performance Liquid Chromatograph system with separation achieved using an Acquity UPLC HSS T3 column (1.8 μ m particle size, 100 mm \times 2.1 mm). Column temperature was maintained at 45 °C in a Waters ACQUITY UPLC I-Class system (Waters, Milford, MA). The mobile flow was set to 0.45 ml/min and consisted of water (A) and acetonitrile (B), both containing 0.1% formic acid. UPLC conditions were as follows: 0–2 mins, 1–60% B; 2–6 mins 60–85% B; 6–8 mins 85–99% B; 8–10 mins 99–1% B. A Waters XevoTM G2S-QToFMS was used for mass spectrometry, and metabolites were measured in positive and negative electrospray

ionization mode. Capillary voltage and cone voltage were set at 2 kV and 40 V respectively, and the source temperature was 150 °C. Desolvation gas flow was set to 1200 L/h at 600 °C and the cone gas flow was 50 L/h. The data were acquired in centroid mode using an MSE method with an m/z range of 50–1200. Leucine-enkephalin (500 ng/mL) was used as the lockmass set at a flow rate of 10 µ L/min, measured every 10 seconds and averaged over 3 scans.

Histochemistry. Oil Red O staining for neutral lipids was performed on frozen sections of the red portion of the vastus muscle. Stock solution was prepared by combining 2.5 g of Oil Red O (Sigma Aldrich, Canada) and 400 ml isopropyl alcohol (99%) and stirred for 2h. Working solution was prepared by mixing 1.5 parts stock with 1 part ddH₂O, which was cooled and filtered. Slides were mounted in aqueous mounting media (10% PBS and 90% glycerol) and photographed using a Zeiss Axioskop Optical Microscope and Northern Eclipse software.

Thin Layer Chromatography. Total lipid extraction was performed on the red portion of the tibialis anterior muscle. 200-300 mg of minced tissue was submerged in chloroform:methanol (2:1, v:v) and placed in the dark for 1 h. The extract was then poured over Whatman filter paper into a glass tube. The extraction vessels were then rinsed and vortexed with 1 mL of chloroform: methanol solution, filtered and added to the original extract. Samples were dried under a steady air stream in a 30-40 °C water bath for approximately 1 h. The extract was weighed and diluted in 50 µL chloroform:methanol (2:1).

Lipid extracts and diacylglycerol (DAG) (1-Palmitoyl-2-Oleoyl-sn-Glycerol) Avanti Polar Lipids Inc. Alabama, USA) standard were run on glass plates with a silica gel matrix

(Analtech TLC uniplates, Sigma Aldrich, Canada) with a mobile phase consisting of toluene:methanol (7:30, v:v). Plates were visualized using Iodine ACS Reagent (Sigma Aldrich, Canada) in a closed glass chamber. Images were obtained using a flatbed scanner and analyzed using ImageJ.

Western Blotting. Soleus muscle from each group was homogenized in a 1:10 (w:v) 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% Tritonx100, pH 7.5), and a Bradford assay was used to determine total protein content. Polyacrylamide gels were composed of 10% acrylamide separating gel and 4% acrylamide stacking gel. Membranes were blocked in 5% non-fat dry milk and Tris buffered saline (TBS), then incubated overnight at 4°C in anti-hormone sensitive lipase (HSL) antibody (1:1000) (ab45422, Abcam, Cambridge MA, USA). Following secondary antibody incubation (BioRad goat-anti-rabbit IgG (H+L)-HRP conjugated 1662408, and goat-anti-mouse IgG (H+L)-HRP conjugated 1721101 as per manufacturer instructions), membranes were washed and visualized using a luminol-based chemiluminescent substrate (BioRad Western C Enhanced Chemiluminescent Kit, 170-5070) on a BioRad Chemidoc XRS imager. Densities were determined using Quantity One software.

Data Analysis. Multivariate analysis of LC-MS data was achieved with Waters Markerlynx with EZinfo 2.0 (Umetrics, Umea, Sweden) software packages. Following normalization to total marker intensity in Markerlynx, peak intensities were transferred to EZinfo. Pareto scaling dampened the selection of features with the highest variance. Principle component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed between control and diabetic control groups, and

diabetic control and diabetic exercise groups using EZinfo. Metabolites were identified using METLIN (<http://metlin.scripps.edu>) and HMDB (<http://www.hmdb.ca>) databases. Fragmentation patterns for each metabolite were compared to putative database compound fragmentation using MassFragment[®]. For Western Blots, hyperinsulinemic-euglycemic clamp and thin layer chromatography, group differences were tested using a one-way ANOVA and Tukey's post hoc test with a p value at $p < 0.05$.

2.3 Results

Animal Characteristics. Animal weights and blood glucose concentrations are shown for pre- and post-exercise training (Table 2.1). These values were obtained prior to the onset of training and again upon the completion of 10 weeks of training. CD and DAE animals had significantly higher blood glucose pre- and post-training. Body mass was significantly lower in DAE compared to C and CD pre- and post-training.

Hyperinsulinemic-Euglycemic Clamp. Glucose infusion rates were significantly lower in CD compared to C ($p < 0.05$) and in CD compared to DAE ($p < 0.05$), indicative of insulin resistance in the diabetic control animals (Fig. 2.1). The glucose infusion rate was not different between C and DAE ($p > 0.05$), indicative of an exercise-mediated improvement in insulin sensitivity.

Metabolomics. Metabolites in C and CD groups were described by OPLS-DA ($R^2(Y)=0.90$) with high predictive ability ($Q^2(Y)=0.58$). Those in CD and DAE groups were also described by OPLS-DA ($R^2(Y)=0.92$) with high predictive ability ($Q^2(Y)=0.51$). Analysis of the red portion of the gastrocnemius muscle revealed a significant increase in octadecenoic acid, palmitic acid, linoleic acid, arachidonic acid and docosahexaenoic acid

in CD compared to C. C demonstrated greater levels of adenosine diphosphate ribose (ADP Ribose), adenylosuccinic acid and pantothenic acid compared to CD. Octadecenoic acid, linoleic acid, arachidonic acid, docosahexaenoic acid and palmitic acid also demonstrated the largest factor of change in the CD compared to DAE groups. Significant increases in flavine adenine dinucleotide (FAD), pantetheine 4'-phosphate, pantothenic acid and ADP ribose were observed for DAE compared to CD. Certain metabolites were not identifiable (designated "unknown" in figures) likely due to poor fragmentation. (Figs 2.2 and 2.3) (Table 2.2).

Neutral Lipid Staining. ORO staining for neutral lipids revealed darker stained fibres in skeletal muscle of the CD group. The C group showed minimal staining, only visible in a small number of fibres. The DAE group also showed minimal staining of fibres compared to the D group, but there appears to be more lipid accumulation compared to C (Fig. 2.4).

Diacylglycerol Content. Thin layer chromatography analysis of the red portion of the tibialis anterior muscle revealed significantly greater DAG content in CD compared to C ($p < 0.05$). DAE did not significantly differ from C ($p > 0.05$), suggesting exercise improved skeletal muscle DAG content (Fig. 2.5).

Hormone Sensitive Lipase Content. We quantified HSL protein to determine whether free fatty acid accumulation was a result of greater DAG and triacylglycerol (TAG) hydrolysis. No significant differences were observed for HSL protein content of the soleus muscle between groups ($p = 0.556$) (Fig. 2.6).

Group	Pre-Training		Post-Training	
	Blood glucose (mM)	Mass (g)	Blood glucose (mM)	Mass (g)
Control	4.6 ± 0.1	385 ± 6.4	4.2 ± 0.1	550 ± 11.7
Control Diabetic	10.2 ± 1.2*	304 ± 5.4*	16.2 ± 0.8*	460 ± 7.9*
Diabetic + Aerobic Exercise	9.4 ± 1.2*	292 ± 7.9*#	16.9 ± 0.5*	415 ± 12.1*#

Table 2.1. Animal characteristics at the onset and completion of the study. (*) indicates a main effect for diabetes ($p < 0.05$), (#) indicates a main effect of exercise ($p < 0.05$). Data are represented as mean \pm SE.

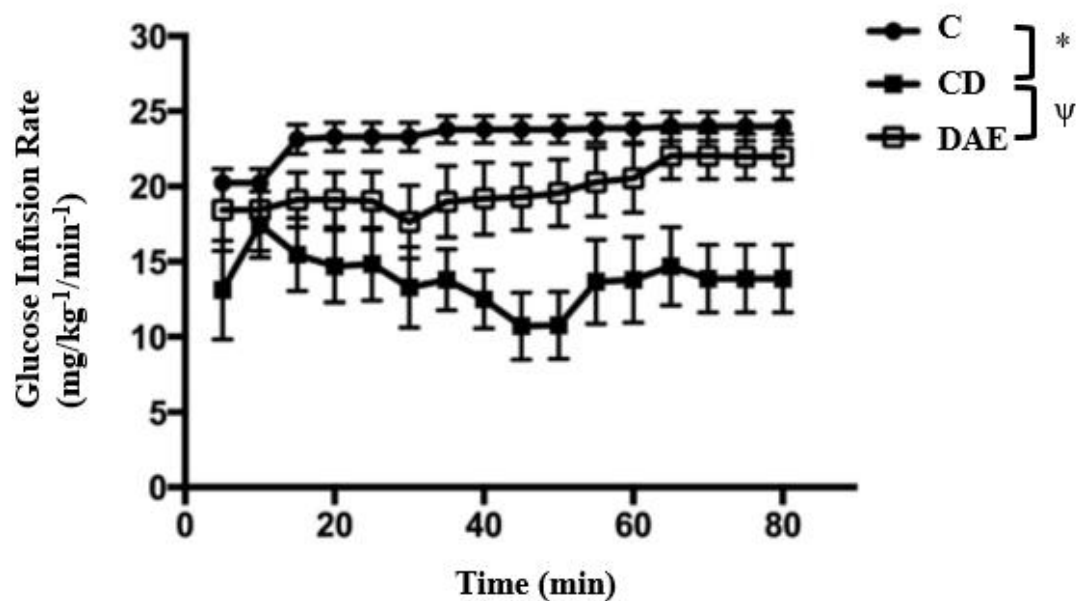


Figure 2.1. Hyperinsulinemic-euglycemic clamp assessment of insulin resistance. (*) Control diabetic (CD) significantly different from control. (ψ) Diabetes aerobic exercise (DAE) significantly different from control diabetic (CD). Data are expressed as mean \pm SE for each group.

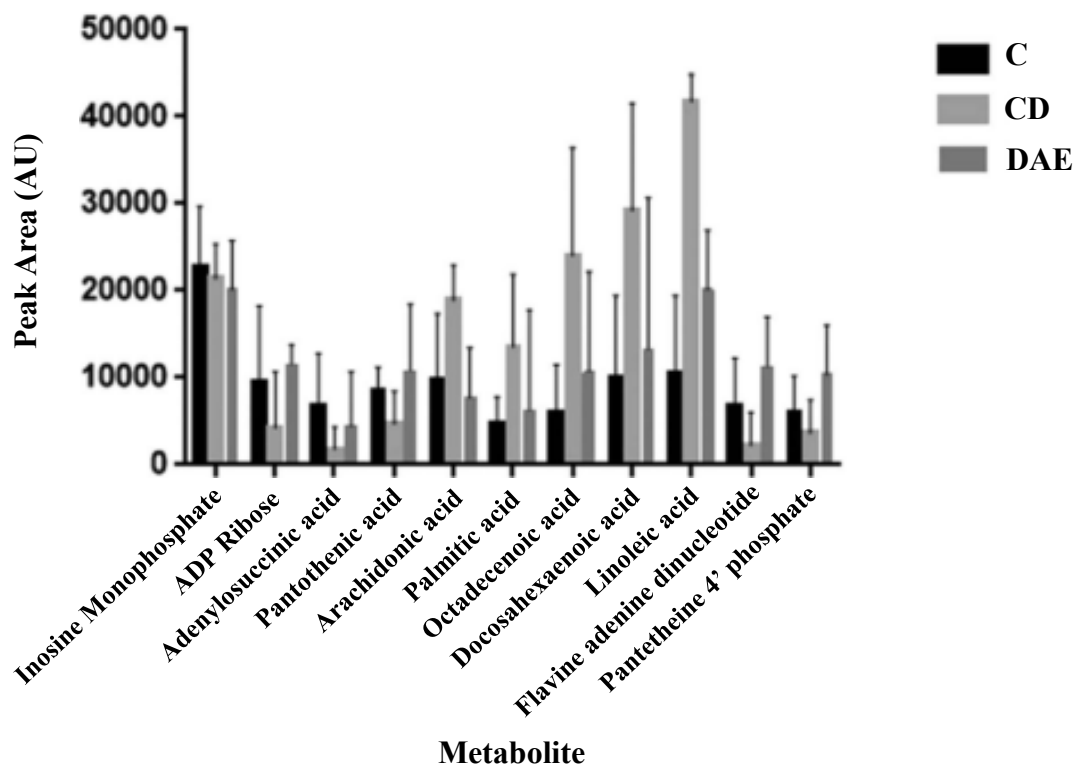


Figure 2.2. Metabolite peak area. Data represent average chromatogram peak area \pm SE for each identified metabolite.

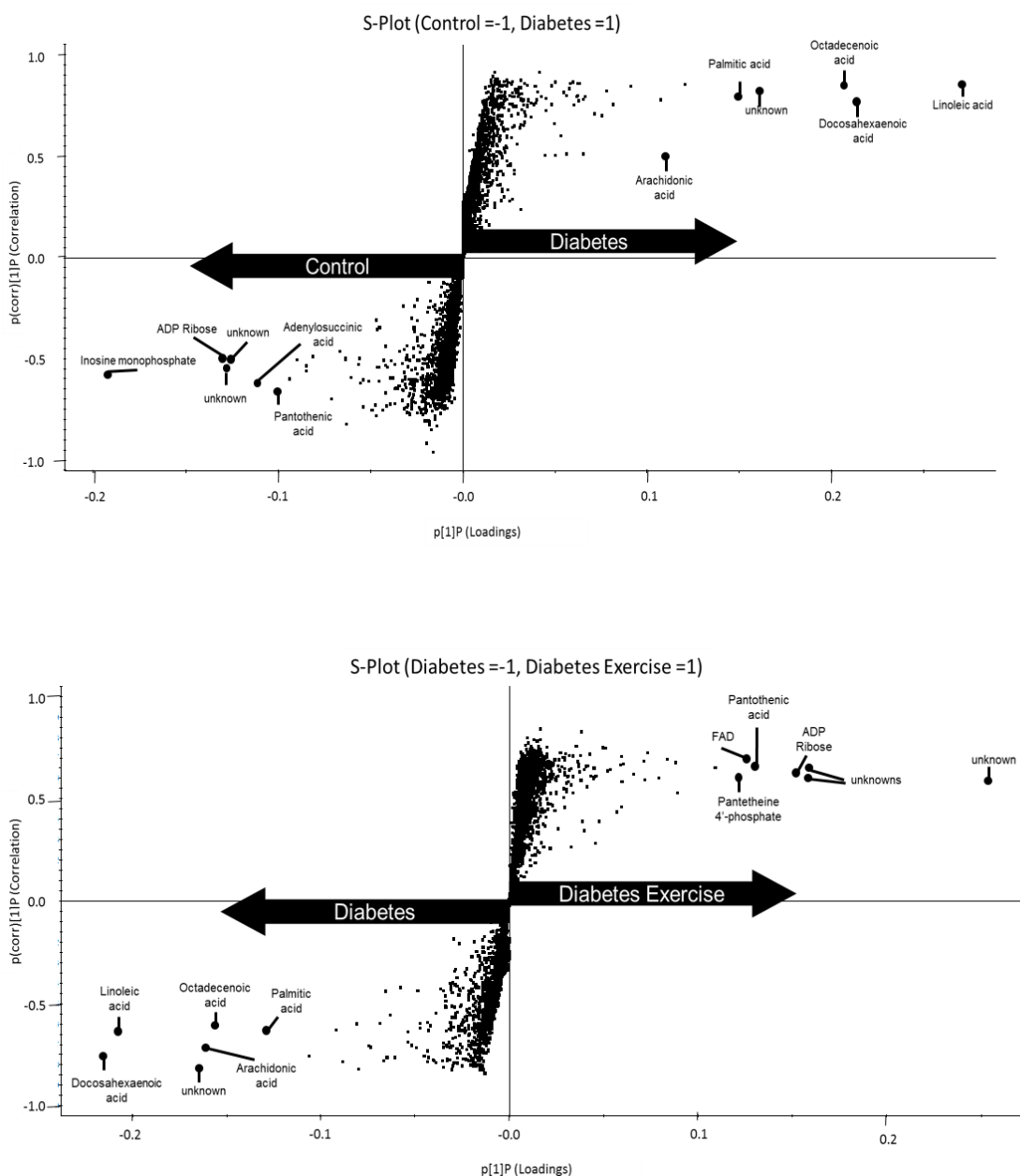


Figure 2.3. Untargeted metabolomics in the red portion of the gastrocnemius. S-plot comparison of control (C) and diabetic control (CD) (top). S-plot comparison of (CD) and diabetes exercise (DAE) (bottom). The S-plot is a visual method for identification of biomarkers. Variables farthest from the origin in the plot are deemed significant markers. Each biomarker is identified with the elemental composition from the accurate mass and comparison to fragmentation patterns from metabolite databases.

Identity	Empirical Formula	Mass (m/z)	t _R (min)	P[1]P	p(corr)[1]P	S-Plot VIP Score	Fold Change	Effect	ID level
Inosine monophosphate	C10H13N4O8P	347.0477	0.7	-0.190376	-0.593433	12.6789	5.1	↑ C vs D	2
ADP Ribose	C15H23N5O14P2	558.0647	0.65	-0.102561	-0.501781	7.15977	2.8	↑ C vs CD	2
Adenylosuccinic acid	C14H18N5O11P	462.0664	1.07	-0.114934	-0.612991	7.96882	7.8	↑ C vs CD	2
Pantothenic acid	C9H17NO5	218.1026	1.23	-0.103502	-0.65815	7.64157	20	↑ C vs CD	2
Arachidonic acid	C20H32O2	303.2326	6.68	0.111519	0.501134	7.64936	1.6	↑ CD vs C	2
				-0.165695	-0.716968		2.4	↑ CD vs DAE	
Palmitic acid	C16H32O2	255.2323	7.42	0.152801	0.800222	10.7176	2.6	↑ CD vs C	1
				-0.131925	-0.634719		2.2	↑ CD vs DAE	
Octadecenoic acid	C18H34O2	281.248	7.63	0.215684	0.844199	14.9743	3.6	↑ CD vs C	2
				-0.165018	-0.603556		2.2	↑ CD vs DAE	
Docosahexaenoic acid	C22H32O2	327.2325	6.5	0.221078	0.773611	16.1763	2.7	↑ CD vs C	2
Linoleic acid	C18H32O2	279.2322	6.82	0.279119	0.858839	19.7256	3.3	↑ CD vs C	2
				-0.216401	-0.648384		2.0	↑ CD vs DAE	
Flavine adenine dinucleotide (FAD)	C27H33P2N9O15	784.1504	1.33	0.133381	0.677607	6.84121	6.7	↑ DAE vs CD	2
Pantetheine 4'-phosphate	C11H23N2O7PS	357.0886	1.23	0.125091	0.597866	1.22198	3.0	↑ DAE vs CD	2
Unknown	-	347.0394	0.97	-0.140527	-0.529978	10.0537	3.2	↑ C vs CD	2
	-	-	-	0.175433	0.646263	-	3.7	↑ DAE vs CD	
Unknown	-	558.0647	0.65	-0.102561	-0.501781	7.15977	2.8	↑ C vs CD	2
	-	329.2481	6.88	-0.16972	-0.826997	11.8857	3.4	↑ CD vs C	
Unknown	-	-	-	-0.16972	-0.826997	-	3.3	↑ CD vs DAE	-
	-	347.0392	0.7	0.257514	0.590923	0.377651	1.9	↑ DAE vs CD	
Unknown	-	695.0872	1.23	0.154213	0.604018	1.221986	2.2	↑ DAE vs CD	2

Table 2.2. Metabolomic analysis. Metabolites that largely differentiate control (C), control diabetic (D) and exercise-trained diabetics (DAE) from the red portion of the gastrocnemius are presented.

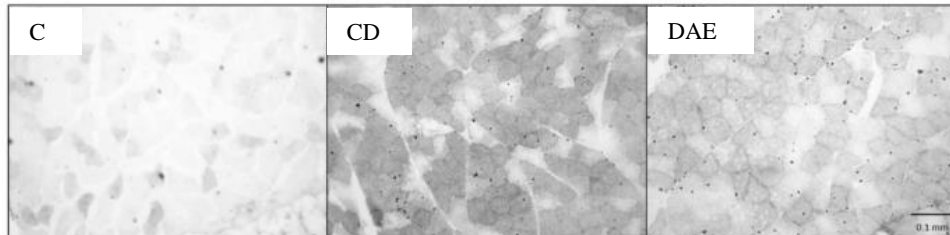


Figure 2.4. Oil Red O. Representative images of ORO staining in red vastus of sedentary control (C), control diabetic (CD) and diabetes aerobic exercise (DAE) groups.

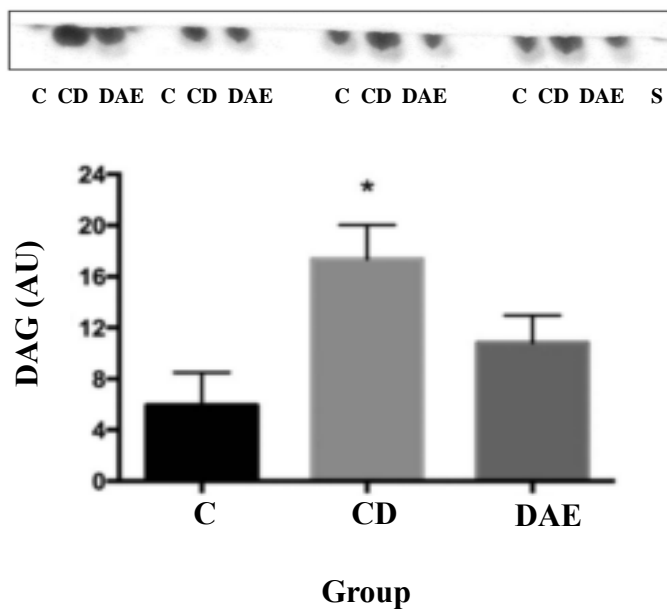


Figure 2.5. Thin layer chromatography quantification of DAG content in the red tibialis anterior muscle. (*) The CD group demonstrated significantly greater DAG content compared to C ($p < 0.05$). A representative chromatogram is shown. The representative chromatogram has been cropped to show molecule of interest. Data are expressed as mean \pm SE for each group.

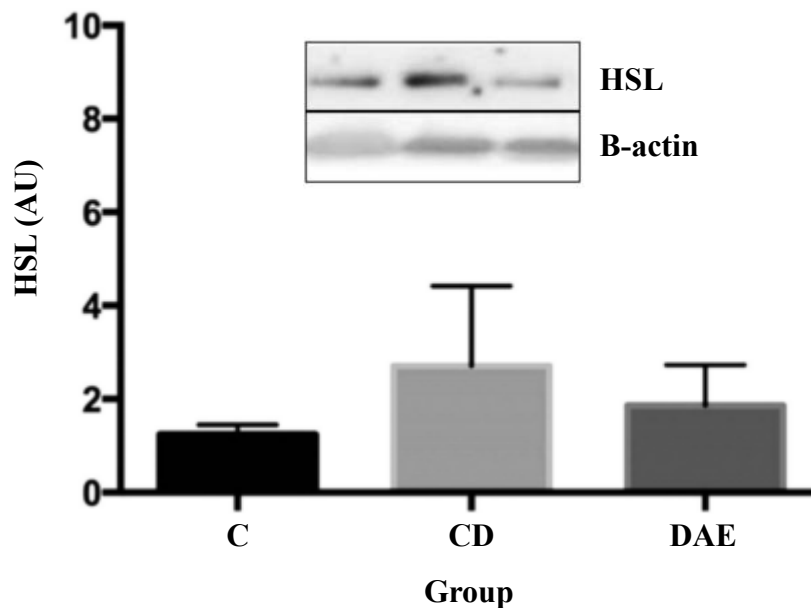


Figure 2.6. Hormone Sensitive Lipase protein content. Total soleus HSL protein content in sedentary control (C), control diabetic (CD), and diabetes aerobic exercise (DAE). There were no significant differences between groups ($p = 0.556$). Data are expressed as mean \pm SE for each group.

2.4 Discussion

In the current study, we annotated and identified multiple metabolites that differentiate skeletal muscle from insulin resistant (CD) animals from that of control (C) animals. Secondly, following aerobic exercise training, DAE animals showed improvements in insulin sensitivity as well as changes in metabolite levels such that they closely approximated those of healthy controls. Metabolites differentiating the CD group from C and DAE include arachidonic acid and palmitic acid. Given the involvement of these intermediates in inflammatory cytokine production and decreased GLUT4 translocation, these data suggest that insulin resistance in T1D elicits a shift toward the intramyocellular accumulation of insulin desensitizing, pro-inflammatory metabolites.

Arachidonic acid (AA) is a precursor to multiple inflammatory cytokines, many of which have been directly linked to the development of insulin resistance²². It has been reported that reductions in phospholipid membrane AA following omega-3 feeding in rats reduces the systemic inflammatory response induced by TNF- α ²³. TNF- α is responsible for the transcriptional suppression of genes relating to skeletal muscle glucose uptake including GLUT4 and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 α) and can directly influence insulin signalling²⁴. Palmitic acid, a saturated fatty acid (SFA), also impairs insulin signalling and is correlated to the development of insulin resistance²⁵. Palmitic acid, as well as other saturated fatty acids (SFAs), impair the insulin signal at many points, including reducing IRS-1 (insulin receptor substrate 1) and protein kinase B (PKB/Akt) phosphorylation²⁶. Lastly, linoleic acid, a polyunsaturated fatty acid (PUFA), also differentiated our CD from C and DAE groups. Linoleic acid has been shown to contribute to insulin resistance by reducing GLUT4 protein expression in

L6 muscle cells²⁷. Previously, we demonstrated that reductions in insulin sensitivity in T1D rats were coupled to changes in GLUT4 expression, which were normalized along with insulin sensitivity following six weeks of aerobic exercise training¹⁵. In line with these results, the current study demonstrates that, concomitant with improvements in insulin sensitivity, excess accumulation of palmitic acid, arachidonic acid, and linoleic acid were not evident in exercise-trained diabetic animals. This suggests that aerobic exercise training can ameliorate the insulin desensitizing effects of SFAs and PUFAs, possibly through a reduction in total skeletal muscle free fatty acid content²⁸.

Skeletal muscle from diabetic control animals displayed greater levels of docosahexaenoic acid (DHA) accumulation. DHA has been shown to decrease the transport efficiency of the sarcoplasmic reticulum (SR) Ca^{2+} -ATPase (SERCA) pump, through increased calcium (Ca^{2+}) leak. It is believed that DHA causes futile SERCA pumping thereby increasing the energy requirements to transport Ca^{2+} into the SR, as SR Ca^{2+} pumps account for 40-50% of resting metabolic rate in mouse skeletal muscle^{29,30}. Indeed, Ca^{2+} -ATPase activity and Ca^{2+} uptake has been shown to be elevated in cardiac and skeletal muscle in experimental T1D animals. The resultant increased intracellular Ca^{2+} is believed to play a role in the paradoxical increase in cardiac resistance to ischemia-reperfusion injury in T1D animals, while elevated intracellular Ca^{2+} may increase fatigability of skeletal muscle^{16,31,32}. Here, we observe that the increase in skeletal muscle DHA levels in diabetic animals is normalized following exercise training. While it cannot be ascertained whether a reduction in DHA is indicative of improved Ca^{2+} handling efficiency in exercise diabetic animals, a reduction in Ca^{2+} -ATPase activity and preserved

Ca²⁺ uptake has been reported following high-intensity exercise training in skeletal muscle of T1D patients ³³.

In addition to lowered DHA levels, exercised diabetic animals exhibited elevated levels of ADP ribose in comparison to diabetic control animals. ADP ribose is a Ca²⁺ mobilizing metabolite that has been shown to modulate the release of Ca²⁺ from the SR in skeletal muscle ³⁴. It has been shown that ADP-ribose accelerates cytosolic Ca²⁺ clearance via SERCA activity ³⁵. Elevated ADP ribose accumulation in exercised diabetic animals may be indicative of other alterations in Ca²⁺ mediated processes involving glucose uptake and metabolism ³⁶. For instance, ADP ribose formation is the primary signalling factor responsible for exercise mediated GLUT4 glucose uptake in high fat fed insulin-resistant mice. The increase in ADP ribose observed in the present study is likely indicative of exercise-mediated enhancement of GLUT4 translocation and improved glucose uptake as opposed to insulin mediated GLUT4 translocation ³⁷.

Cyclic ADP ribose is generated by ROS-mediated activation of poly ADP ribose polymerase (PARP), which splits NAD⁺ into nicotinic acid and ADP ribose ^{38,39}. Mitochondrial ROS-mediated activation of PARP contributes to ADP ribose polymers accumulating on glyceraldehyde 3-phosphate dehydrogenase (GAPDH), impeding the flow of glycolysis and activation of pro-apoptotic factors ³⁹. While this would seem contrary to the known benefits associated with exercise, it is plausible that excess lipid accumulation in diabetic animals may increase the pressure head point of entry into the mitochondria, elevating ROS production, and impairment of glucose metabolism via ADP ribose accumulation on GAPDH. It is yet to be determined how modifications of GAPDH and other nuclear proteins may impact skeletal muscle; however, it may reflect an increase in

the normal inflammatory process used to remove partially damaged muscle cells resulting from higher levels of exercise ⁴⁰.

Adenylosuccinic acid and inosine monophosphate (IMP) differentiated the C and CD groups, suggesting altered purine metabolism in T1D. Purine nucleotides act as key regulators of cell metabolism, serving as essential carriers of chemical energy such as ATP. Purine metabolite state modulates the AMP/ATP ratio and can impact mitochondrial function as well as AMPK activity which affects key cellular functions such as skeletal muscle oxidative capacity and glucose oxidation, hepatic glucose output and glucose sensitivity ⁴¹. Similarly, the elevation in pantothenic acid, a precursor to coenzyme A, would suggest that T1D led to a decrease in this cofactor critical for fatty acid metabolism. Pantothenic acid did not differentiate DAE from C, suggesting a restoration of aerobic metabolism with aerobic exercise training. In this respect, Flavin adenine dinucleotide (FAD), pantetheine 4'-phosphate, ADP ribose and pantothenic acid differentiate DAE from CD; indicative of improvements in oxidative pathways in our model of T1D. Enhanced free fatty acid oxidation could reduce lipid esterification as well as ceramide and DAG formation, preventing the inhibitory effects of these lipid species on the insulin signalling cascade, restoring insulin sensitivity.

While these findings identify key markers that dissociate control from diabetic animals, the mechanism underlying these differences in lipid metabolites remains unclear. It is plausible that a greater uptake of free fatty acids with insufficient oxidation may account for the observed changes ⁴². Hyperglycemia may also drive IMCL accumulation as CPT-1 and β -oxidation are inhibited in this state ⁴³. Moreover, circulating insulin hinders TAG and DAG hydrolysis and stimulates fatty acid esterification, potentially contributing

to these changes^{44,45}. In fact, we have previously shown significant reductions in the insulin requirement of aerobically trained animals compared to diabetic control animals¹⁶. Studies have shown impaired insulin-stimulated glucose transport and increased ceramide, DAG and TAG in skeletal muscle incubated in palmitate⁴⁶. Interestingly, a single bout of prior exercise protected the insulin signal and some redistribution of free fatty acids toward TAGs was observed⁴⁶. Here, we demonstrate an increase in DAG content in the red oxidative skeletal muscle of the CD group, which was normalized with aerobic exercise training. It has been shown that DAGs interfere with the insulin signal via PKC θ , and accumulation is associated with insulin resistance^{13,47}. Similarly, exercise has been shown to preserve the insulin signal and redistribute free fatty acids toward TAG storage as opposed to ceramides, which act at the level of PKC ζ ⁴⁸. Our results align with other studies, showing that DAG, but not ceramide, is more readily altered following lipid infusion^{49,50}.

Given the elevation in DAG, we examined the expression of hormone sensitive lipase (HSL), an enzyme that displays a high DAG substrate specificity and is considered the major DAG hydrolase in several tissues⁵¹. Reduced HSL protein content could potentially account for the increased DAG content observed in muscle, and this metabolite has been shown to be decreased in skeletal muscle of obese, T2D individuals⁵². Our findings reveal no significant differences in HSL protein content in soleus muscle between groups. This finding is in accordance with others who have found no significant effects of exercise training on skeletal muscle HSL content^{53,54}. It is important to note that diabetes-related changes in HSL function may be due to posttranslational regulation, as endurance training increases phosphorylation but not total protein content⁵⁵. However, it has also

been shown that reductions in HSL phosphorylation in obese subjects is entirely due to lower HSL protein content, suggesting that muscle HSL content could be representative of enzyme activity ⁵⁶. Further work is required to ascertain the mechanisms involved in the accumulation of DAG in skeletal muscle of T1D animals and the molecular means by which exercise may reduce the accumulated levels of this lipid intermediate.

Possible limitations to this study should be addressed. First, due to poor fragmentation, we were unable to identify various ceramide species. In addition to DAG, ceramide may also play an important role in insulin signalling impairment, as has been well documented to occur in T2D ⁵⁷. Secondly, while Oil Red O staining provides a visual representation of neutral lipid content within the muscle, it is unable to detect polar lipids such as ceramides or phospholipids. This figure serves solely as a representative image, and cannot be used to determine if the differences in total intramyocellular lipid content between groups. Lastly, food intake was not measured in the current study. Considering the impact of dietary behavior may shed further light on the causative factors leading to changes in metabolite levels and metabolic rate in diabetic animals. While hyperphagia has been well documented in STZ-T1D animals, it has been reported that hyperphagia is restored and/or prevented in models of T1D following insulin treatment ^{58,59}.

In summary, our results demonstrate that moderately hyperglycemic T1D rats develop insulin resistance which is accompanied by significant alterations in skeletal muscle lipid metabolism. Ten weeks of aerobic exercise training improved insulin sensitivity and ameliorate the accumulation of harmful insulin desensitizing lipid intermediates in red oxidative skeletal muscle of T1D animals. The underlying

mechanisms by which exercise training improves skeletal muscle lipid metabolism and insulin receptor function in T1D need to be determined.

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CHAPTER 3

3 « Aerobic and Resistance Exercise Effect Versus Aerobic Only Training on Skeletal Muscle Lipid Metabolism in a Rodent Model of Type 1 Diabetes »

3.1 Introduction

Type 1 Diabetes (T1D) results from the autoimmune-mediated destruction of pancreatic beta cells, leading to insufficient insulin secretion and hyperglycemia. Therefore, exogenous insulin therapy is required to maintain normal blood glucose levels. A subset of patients with T1D are unable to maintain normal blood glucose levels despite persistent glucose monitoring and insulin adjustment ¹. Termed “double diabetes”, the combination of immune-related T1D and insulin resistance has been shown to heighten the risk for developing cardiovascular disease (CVD) ².

Hyperglycemia and abnormal lipid metabolism are believed to initiate the development of insulin resistance among individuals with T1D ³. Hyperglycemia increases skeletal muscle lipid accumulation via inhibition of carnitine palmitoyltransferase-1 (CPT-1), which in turn reduces mitochondrial beta-oxidation ⁴. Abnormal skeletal muscle lipid metabolism has been shown to stimulate inflammation and the development of insulin resistance, as excess lipid flux through oxidative pathways drives reactive oxygen species (ROS) production and depletes reducing enzymes such as glutathione ⁵. Further, harmful lipid species such as diacylglycerols (DAGs) and ceramides can exert inhibitory effects on the insulin signalling pathways ⁶.

We have previously reported 10 weeks of aerobic exercise training can ameliorate insulin resistance and improve cardiovascular function in a rodent model of T1D ^{7,8}.

Further, regular aerobic exercise training leads to a reduction in skeletal muscle lipid content including the accumulation of harmful insulin desensitizing lipid intermediates such as DAG ⁷. It is well established that aerobic exercise induces significant skeletal muscle adaptations including mitochondrial biogenesis, increased oxidative capacity (citrate synthase activity) and glucose uptake ⁹⁻¹². Aerobic exercise also elicits a robust increase in lipid oxidation and storage. AMPK activation during exercise alleviates CPT-1 inhibition permitting greater fatty acid transport into the mitochondria for oxidation ¹³.

Despite the known benefits to metabolic and cardiovascular health, aerobic training remains difficult for patients with T1D, as there is a significant risk of post-exercise hypoglycemia with this modality of exercise. We have previously observed a rapid and significant reduction in blood glucose following acute aerobic exercise in T1D rats, which persists following training ^{8,14}. The Canadian and American Diabetes Associations both recommend a combined approach to exercise, involving both aerobic and resistance training for individuals with T1D ¹⁵. Indeed, it has been shown that combining resistance with aerobic exercise can attenuate the decline in blood glucose levels evident following aerobic training alone ¹⁶. Sustained elevations in catecholamines and/or growth hormone as a result of resistance training is believed to enhance hepatic-mediated release of glucose during the subsequent aerobic session ^{17,18}. Resistance training elicits different adaptations than aerobic training, including skeletal muscle hypertrophy, fibre type conversion and a reduction in fat free mass ¹⁹. Further, resistance training increases mitochondrial function and oxidative capacity as well as insulin sensitivity ^{20,21}.

Little is known regarding the metabolic effects of combined modalities of exercise in T1D patients. In non-diabetic individuals, an “interference effect” has been reported

whereby simultaneously training for strength and endurance hinders strength development, reduces maximum voluntary contraction and running capacity^{22,23}. In obese individuals and patients with T2D, evidence would suggest that despite impairments to athletic performance, combined training may significantly improve oxidative capacity, glycemic control, insulin sensitivity, and body composition²⁴⁻²⁶. Moreover, evidence has shown that the combination of aerobic and resistance exercise is associated with greater improvements in mitochondrial capacity, demonstrated by elevations in citrate synthase activity to a greater extent than either aerobic or resistance training alone^{24,27,28}.

The combination of aerobic and resistance training has been suggested as an effective strategy to mitigate the risk of exercise-mediated hypoglycemia, and may provide substantial benefits for cardiovascular and metabolic health^{16,29,30}. Work is needed to better understand the effects of combined (aerobic and resistance) exercise on skeletal muscle metabolism among individuals with T1D, particularly as it pertains to the metabolism of intramyocellular lipids within skeletal muscle. The purpose of this study was to determine whether combined (aerobic and resistance) exercise is an effective means to improve skeletal muscle oxidative capacity and lipid metabolism. We hypothesized that combined exercise training would reduce intramyocellular lipid content and enhance oxidative capacity in skeletal muscle of rodents with T1D to an equal or greater extent than aerobic training alone.

3.2 Methods

Ethics Approval and Animals. Eight-week-old male Sprague-Dawley rats were obtained from Charles River Laboratories (St. Constant, QC, Canada), and housed two per

cage. Temperature and humidity were maintained constant with a 12-h dark/light cycle. Animals received standard chow (Prolab-RMH-3000; PMI Nutrition International) and water *ad libitum*. Ethics approval was obtained through the University of Western Ontario Research Ethics Board, in accordance with the Canadian Council on Animal Care guidelines.

Experimental Groups. Rats were randomly assigned to one of four groups: Control sedentary (C, n=12), Diabetic insulin-treated sedentary (CD, n=12), Diabetic insulin-treated high intensity aerobic exercise (DAE, n=12), and Diabetic insulin treated combined aerobic and resistance exercise (DARE, n=12).

Diabetes Induction. Rats were housed for one week after arrival to familiarize with their surroundings. T1D was induced with multiple low-dose streptozotocin (STZ) injections (week 1). STZ (20mg/kg; Sigma Aldrich, Oakville, ON, Canada) was injected into the intraperitoneal cavity for five consecutive days. Diabetes was confirmed with two non-fasting blood glucose readings of ≥ 18.0 mmol/L. Subsequently, insulin pellets (LinShin, Toronto, ON, Canada) were implanted subcutaneously, and dosages were monitored and adjusted throughout the experimental study to maintain blood glucose concentrations between 9-15 mmol/L. This range of blood glucose is representative of levels patients with T1D utilize while participating in a regular exercise program in order to avoid the risk of post-exercise hypoglycemia³¹.

Exercise Training. Upon the completion of diabetes induction and insulin pellet implantation, rats in the exercise training groups (DAE, DARE) underwent a familiarization protocol (experimental week 3) and exercised for the following 12 weeks (experimental weeks 4-15). Treadmill familiarization consisted of progressively increased

running up to 27 m/min on a six percent grade over five days. Rats were encouraged with small blasts of compressed air if they ceased running and broke a photoelectric beam at the rear of the treadmill. DARE rats were also familiarized to resistance training, and performed 10 vertical ladder climbs per day with weights attached to the base of the tail (5%, 15%, 20% and 35% of rat body mass). Training for the DAE group consisted of treadmill running 5 days per week at 27 m/min on a six percent grade for one hour. The DARE group alternated days of treadmill running (27 m/min on a six percent grade for one hour) with climbing sessions consisting of climbing with 50%, 75%, 90%, and 100% of maximum lifting capacity. Maximum capacity was determined after every fourth day of resistance training with the sequential addition of 30 grams of weight until exhaustion. The increase in maximal carrying capacity following the 12 weeks of combined exercise training is reported in Table 3.1.

Body Mass and Blood Glucose. Body mass and non-fasting blood glucose was measured weekly. Blood glucose was measured via blood collection from the saphenous vein, using a Freestyle Lite Blood Glucose Monitoring System (Abbott Diabetes Care, Mississauga, Ontario, Canada).

Tissue Collection. Animals were euthanized three days following the final bout of exercise (training week 12) by isoflurane anaesthesia and exsanguination via cardiac excision. Animals were euthanized three days following the final bout of exercise to avoid any residual effects of acute exercise in tissue analysis. Following sacrifice, serum was collected, centrifuged and flash frozen in liquid nitrogen. Red portions of the gastrocnemius (red gastrocnemius) muscle were collected and flash frozen, while the soleus

muscles were mounted on cork and frozen in isopentane cooled with liquid nitrogen. Tissues were stored at -70°C for later analysis.

Blood Lipids and Free Fatty Acids. Serum triglycerides, total cholesterol, high density lipoprotein (HDL), low density lipoprotein (calc) (LDL), and cholesterol:HDL analysis was performed at the University Hospital (London, ON, Canada). Serum free fatty acids were quantified using a commercially available colorimetric ELISA kit (ab65341, Abcam, Cambridge MA, USA).

Serum Insulin and HOMA-IR. Fasting serum insulin was quantified using a commercially available ELISA kit (Alpco, Salem NH, USA). Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated using the following equation:

$$= \frac{\left(\text{fasting serum insulin} \left(\frac{\mu}{\text{ml}} \right) \times \text{fasting blood glucose} \left(\frac{\text{mmol}}{\text{L}} \right) \right)}{22.5}$$

Histochemistry. Oil Red O (ORO) staining for neutral lipids was performed on 10 μm sections of frozen soleus muscle, as reported previously⁷. Briefly, following staining in 0.5% ORO (Sigma Aldrich, Canada) in propylene glycol overnight, slides were washed in distilled water, and allowed to dry and mounted in an aqueous mounting medium (10% PBS, 90% glycerol). Images were obtained at 10x magnification on an Olympus BX50 microscope, exposure 10.08 ms, gain 30.72%, offset 30.98%.

Citrate Synthase Activity. Citrate synthase (CS) activity was measured in the red gastrocnemius muscle. Muscle samples (100 mg) were homogenized 1:20 (w:v) in buffer containing 100mM KPO_4 , 5mM EDTA, 5mM EGTA, pH 7.4. Samples were then re-diluted 1:20 (v:v) and freeze fractured by freezing samples at -80°C and thawing to room temperature three times. On a 96-well plate, 2 μL sample, Tris buffer (100 mM Tris), acetyl

CoA (3mM), 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) and oxaloacetate (5mM) were combined and rate of absorbance change was read at 412 nm. Samples were run in triplicate.

Activity was calculated from the following equation:

$$Activity = \Delta A_{405} / \min \frac{\Delta A_{405} / \min \times V (ml) \times DF}{\epsilon^{mM} \times L (cm) \times V_{enz}(ml)}$$

Where $\Delta A_{405}/\min$ is the rate, $V(ml)$ is the total volume of the well, DF is the dilution factor, ϵ^{mM} is the DTNB extinction coefficient, $L (cm)$ is microplate path length, and $V_{enz}(ml)$ is the sample volume.

Western Blotting. A second portion of the red gastrocnemius muscle (approximately 100 mg) from each group was homogenized in a 1:10 (w:v) ratio with homogenizing buffer (100 mmol/L NaCl, 50 mmol/L Tris base, 0.1 mmol/L EDTA, 0.1mmol/L EGTA and 1% Tritonx100, pH 7.5). A Bradford assay was used for total protein content. Samples were run on polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in non-fat dry milk or BSA and Tris buffered saline with Tween20 (TTBS) and incubated overnight in primary antibody (CD36 1:2500, ab133625; Lipin-1 2ug/mL, ab181389; IRS-1, 1:750, ab52167, Abcam, Cambridge MA, USA; phospho-IRS-1 Ser1101, 1:750, #2385 Cell Signalling, Danvers MA, USA). Following secondary antibody incubation (BioRad goat-anti rabbit IgG (H+L)-HRP conjugated 1662408 and goat-anti-mouse IgG (H+L)-HRP conjugated 1721101 as per manufacturer instructions), membranes were washed and visualized using chemiluminescent substrate (BioRad Western C Enhanced Chemiluminescent Kit, 170-5070) on a BioRad Chemidoc XRS imager. Densities were determined using Quantity One software).

Data Analysis and Statistics. Body weight, blood glucose, blood lipids and free fatty acids, CS activity and Western Blot densities were compared via one-way ANOVA with Tukey's post hoc test and a p value set at <0.05 . Maximal carrying capacity was compared with a paired t-test with a p value set at <0.05 . For non-parametric data, a one-way ANOVA on ranks was performed. Statistical analysis was completed with GraphPad Prism.

3.3 Results

Animal Mass, Blood Glucose and Lifting Capacity. Mass (g) and blood glucose (mmol/L) values are presented for experimental week 14 (upon the completion of the 12 weeks of training) (Table 3.1). Following 12 weeks of training, mass is significantly reduced in DAE and DARE compared to C, while blood glucose levels in all diabetic groups is significantly higher than in non-diabetic sedentary controls (C) ($p<0.05$). Maximal lifting capacity in the DARE group is significantly increased at week 12 of training compared to week 1 ($p<0.001$) (Table 3.1).

Serum Lipids, Free Fatty Acids and HOMA-IR. Serum lipid concentrations (mmol/L) are presented in Table 3.2. There were no significant differences between groups for triglyceride, total cholesterol, HDL, LDL (calc), cholesterol:HDL, or serum free fatty acids. HOMA-IR was significantly reduced in DARE compared to CD ($p<0.05$) (Table 3.2). These results indicate that DARE had significant improvements in insulin sensitivity in comparison to CD animals.

Oil Red O Staining. Representative images of Oil Red O staining for neutral lipids revealed darker staining in CD and DARE groups indicative of elevated neutral lipid

accumulation. There appears to be minimal fibre staining in the DAE and C groups. (Fig.3.1).

Citrate Synthase Activity. Citrate synthase (CS) activity was measured in the red gastrocnemius muscle. Results of the activity assay revealed significantly lower CS activity in the CD group compared to C and DARE ($p < 0.05$). The DARE group showed significantly elevated CS activity compared to CD and DAE groups, indicative of improvements in oxidative capacity of red skeletal muscle ($p < 0.05$). (Fig. 3.2).

Western Blot Analysis. CD36 protein content was significantly higher in the CD group compared to C and DARE ($p < 0.05$). CD36 functions in long chain fatty acid uptake, suggesting increased uptake in the CD animals (Fig. 3.3). Lipin-1 facilitates the conversion of phosphatidic acid to diacylglycerol within skeletal muscle. Lipin-1 protein content was significantly elevated in DARE compared to C ($p < 0.05$), indicative of increased diacylglycerol and/or triacylglycerol storage in this group. (Fig. 3.4).

Given the role of diacylglycerol in the inhibition of the insulin signal, we examined IRS-1 phosphorylation of Ser1101. Quantification of the ratio of phosphorylated to non-phosphorylated IRS-1 revealed no significant differences between any of the groups ($p > 0.05$). (Fig. 3.5).

Week 14:	C (n=12)	CD (n=12)	DAE (n=8)	DARE (n=6)
Mass (g)	617.75 ± 18.61	552.00 ± 19.76	510.13 ± 15.30*	527.33 ± 20.45*
Glucose (mmol/L)	4.2 ± 0.13	17.26 ± 0.66 [#]	15.66 ± 0.46 [#]	16.65 ± 1.68 [#]
Maximal Lifting Capacity (g)	-	-	-	Week 1: 437.43 ± 25.35
				Week 12: 1689.14 ± 67.02 [°]

Table 3.1. Animal characteristics upon completion of the study. Animal mass (g): (*) Significantly different from control (p<0.05). Blood glucose (mmol/L): ([#]) Significantly different from control (p<0.001). Maximal lifting capacity (g): ([°]) Week 12 is significantly greater than week 1 in the DARE group (p<0.05). Values are presented as mean ± SE.

	C (n=12)	CD (n=12)	DAE (n=8)	DARE (n=6)
Cholesterol (mmol/l)	1.65 ± 0.08	1.67 ± 0.09	1.62 ± 0.10	1.71 ± 0.08
TRG (mmol/l)	1.47 ± 0.16	1.43 ± 0.23	1.34 ± 0.37	0.80 ± 0.08
HDL (mmol/l)	1.10 ± 0.08	1.14 ± 0.06	1.2 ± 0.07	1.36 ± 0.07
LDL (calc) (mmol/l)	-0.11 ± 0.44	-0.19 ± 0.08	-0.20 ± 0.12	0.01 ± 0.08
Cholesterol:HDL (mmol/l)	1.55 ± 0.08	1.52 ± 0.12	1.36 ± 0.65	1.27 ± 0.05
	C (n=8)	CD (n=5)	DAE (n=5)	DARE (n=5)
Serum	1184.92	± 2212.62	± 3061.82	± 1312.70
FFA (µM)	761.14	642.712	836.37	249.25
	CD (n=11)	DAE (n=7)	DARE (n=7)	
HOMA-IR	4.72	1.33	0.41*	

Table 3.2. Blood lipid analysis of terminal serum (week 14) and HOMA-IR. There were no significant differences between group. HOMA-IR: (*) significantly reduced in the DARE group compared to the CD group ($p < 0.05$). TRG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein; FFA, free fatty acid; HOMA-IR, homeostatic assessment of insulin resistance. Values are presented as mean ± SE, HOMA-IR data are presented as median.

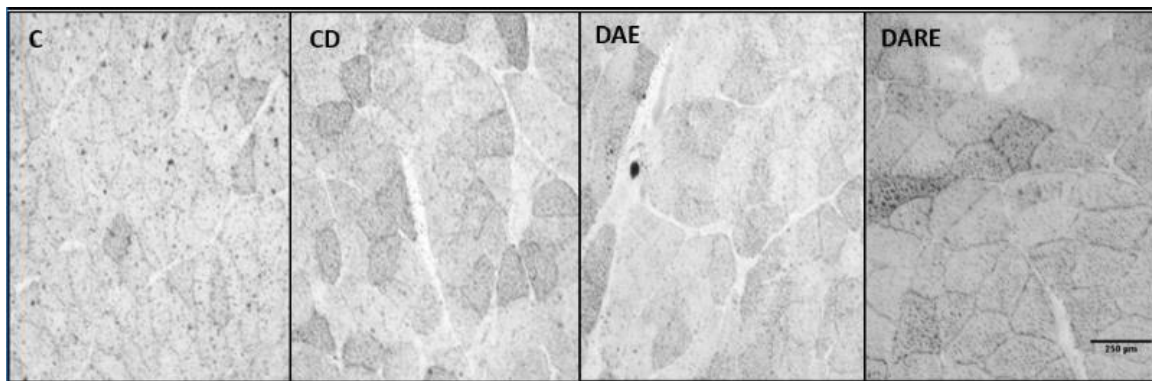


Figure 3.1. Representative images for Oil Red O neutral lipid staining.

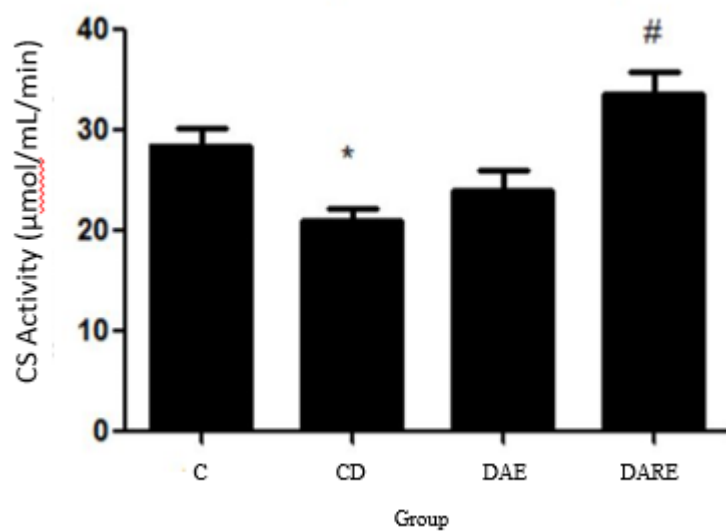


Figure 3.2. Citrate synthase enzyme activity in the red portion of the gastrocnemius. (*) Rate in CD is significantly lower than in DARE and C ($p < 0.05$). (#) Rate in DARE is significantly greater than CD and DAE ($p < 0.05$). Values are presented as mean \pm SE.

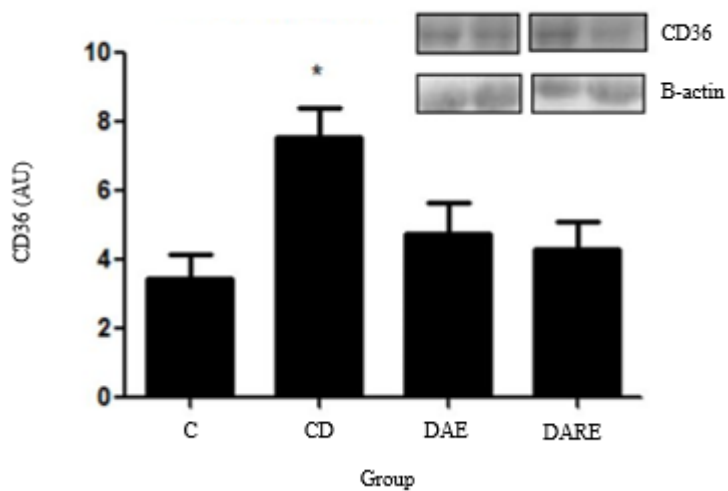


Figure 3.3. Fatty acid transporter CD36 protein content in the red portion of the gastrocnemius. (*) Significantly greater in CD compared to C and DARE ($p < 0.05$). Values are presented as mean \pm SE.

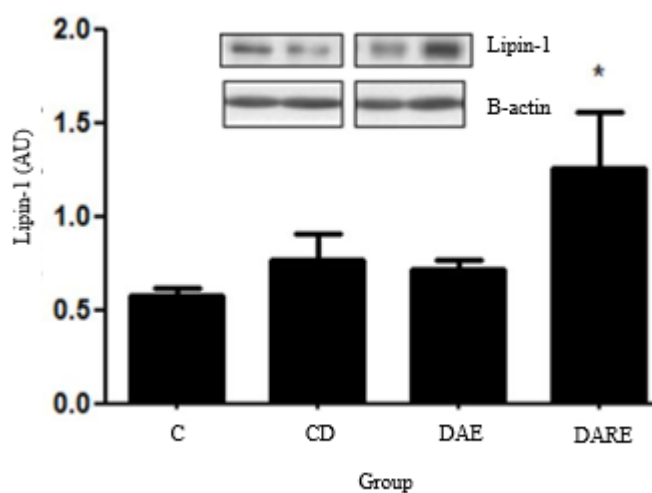


Figure 3.4. Lipin-1 protein content in the red portion of the gastrocnemius. (*) Significantly greater in DARE compared to C ($p < 0.05$). Values are presented as mean \pm SE.

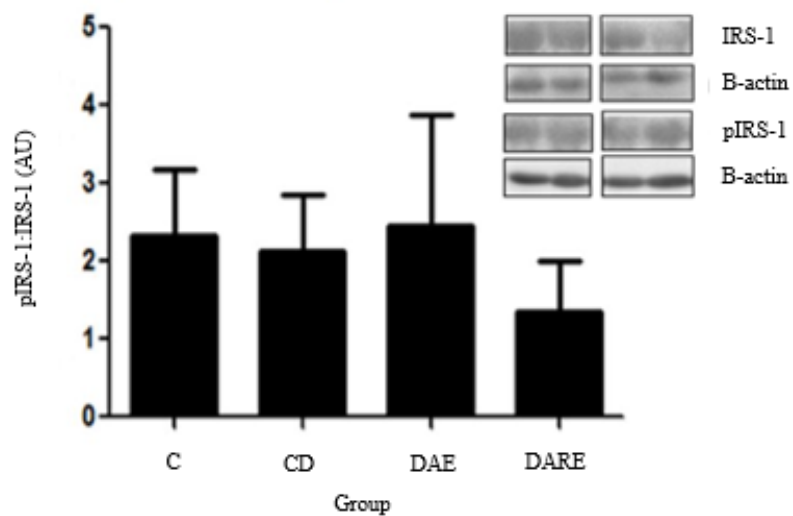


Figure 3.5. Total pIRS-1 Ser1101: IRS-1 protein content in the red portion of the gastrocnemius. Protein content is not significantly different between groups ($p=0.8735$). Values are presented as mean \pm SE.

3.4 Discussion

The purpose of this study was to examine the effects of combined exercise training versus aerobic only exercise training on skeletal muscle oxidative capacity and intramyocellular lipid content in T1D rodents. We hypothesized that combined training would result in reduced skeletal muscle lipid accumulation and enhance oxidative capacity to a similar or greater extent than aerobic exercise alone. Results from this study indicate that combined exercise training offers a significant improvement to skeletal muscle oxidative capacity as well as insulin sensitivity measures, beyond that evident following aerobic exercise. Contrary to our original hypothesis, unlike aerobic only exercise training, combined exercise did not reduce skeletal muscle lipid content. However, a reduction in CD36 and increased levels of a triglyceride converting factor Lipin-1 would suggest that the skeletal muscle of combined exercise trained rats demonstrates increased lipid storage capacity. This, coupled to significantly enhanced citrate synthase enzyme activity indicates combined exercise confers a greater capacity to channel substrate toward neutral lipid stores or oxidation, preventing the accumulation of insulin-desensitizing lipid metabolites.

The measurement of CS activity is valuable marker to evaluate skeletal muscle oxidative capacity and mitochondrial density³². In the current study, we observe a significant reduction in CS activity in CD animals compared to C, indicative of reduced muscle oxidative capacity in our rat model of T1D. This may be the result of lipid accumulation and uncoupling of oxidative phosphorylation in the slow-twitch muscle³³. Our findings are consistent with other animal models of diabetes, as STZ-induced diabetic mice and Zucker diabetic fatty rats demonstrate reduced CS activity and

oxidative capacity^{33,34}. Further, following acute exercise, patients with T1D demonstrate a reduction in blood citrate levels and attenuated lipolysis, which may stem from the effects of exogenous insulin treatment³⁵.

Aerobic only exercise training restored, or at least, prevented the decline in oxidative capacity evident in the CD group. It is well-established that aerobic exercise training substantially improves CS activity through a variety of mechanisms, including factors such as AMPK-induced mitochondrial biogenesis³⁶. Interestingly, we observed a substantial increase in CS activity in the DARE group compared to DAE despite fewer total bouts of treadmill running (aerobic exercise). This finding suggests that aerobic and resistance exercise training may produce a synergistic effect on muscle oxidative capacity. Indeed, others have observed that combined exercise training can increase oxidative capacity to a greater extent than aerobic exercise alone²⁴. Aerobic training and combined exercise training (aerobic/resistance) both lead to significantly increased mitochondrial oxidative phosphorylation capacity (measured by high-resolution respirometry); however, phosphorylation capacity, mRNA levels of mitochondrial transcription factors and mitochondrial proteins were elevated to a greater extent following combined exercise training²⁴.

It has been reported that the integration of aerobic exercise may potentiate the skeletal muscle hypertrophic response to resistance exercise through a net increase in protein turnover²⁸. A recent study compared the skeletal muscle adaptations following combined (aerobic/resistance) versus resistance only exercise²⁷. Subjects were asked to perform both cycling and maximal knee extensions exercising with one leg while performing only maximal knee extensions with the other leg²⁷. In the leg that performed

both exercises, vascular endothelial growth factor (VEGF), cAMP-response element-binding protein (CREB) and mechanistic target of rapamycin (mTOR) were activated, while these factors were unchanged in the leg that completed resistance only exercise ²⁷. These genes are involved in myofibre growth and oxidative capacity, and their activation following combined exercise suggests this approach may elicit a greater adaptation than one exercise modality in isolation. Lastly, improvements in muscle oxidative capacity associated with combined exercise may be due to the progressive nature of resistance-type exercise. This is particularly relevant to the current data set as running intensity in the DAE and DARE groups was not increased over the 12 weeks of training. Resistance training resulted in a progressive increase in carrying capacity over the course of the training program, indicative of improved muscular strength (see Table 3.1).

In the CD group, the increased intensity of ORO staining is coupled to increased CD36 protein content and reduced CS activity. CD36 is a membrane protein involved in long-chain fatty acid transport into skeletal muscle, and is associated with negative effects on insulin responsiveness ³⁷. The increased CD36 content in the CD group may account for increased skeletal muscle lipid uptake. Circulating fatty acids (as well as triglycerides and cholesterol) did not differ between groups, suggesting that increased lipid accumulation was due to increased fatty acid uptake (via increased CD36) by skeletal muscle rather than increased over supply of circulating free fatty acids from adipose stores. While it is not clear as to the mechanisms leading to increased CD36 expression in T1D rats, the membrane transporter protein has been shown to be upregulated following hyperglycemia ³⁸. To this point, it is interesting that DAE and DARE groups demonstrated a reduced expression of CD36 despite being maintained at similar resting blood glucose levels (9-

15mM). We have previously shown that at this intensity of aerobic exercise utilized in the DAE and DARE groups, a significant and consistent drop in blood glucose is evident during and following exercise³⁹. It is plausible that the acute reductions in blood glucose resulting from each bout of aerobic exercise may have mitigated the increased expression of CD36 that was evident in the CD animals⁴⁰.

Contrary to our original hypothesis, we observed increased lipid staining in the DARE group in comparison to DAE. It is important to note that ORO staining identifies neutral lipid stores which would include both DAG and triacylglycerol (TAG) molecules. While both the CD and DARE group demonstrate increased lipid staining, the composition of neutral lipid stores in skeletal muscle may differ between these two groups. For instance, the greater neutral lipid stores in DARE animals may be comprised of energy rich TAG molecules rather than the insulin desensitizing DAG molecules⁴¹. TAGs do not exert negative effects on the insulin signal, while saturated DAGs (i.e., di-C18:0) are tied to insulin resistance as are membrane species of DAG⁴². Indeed, previous work from our laboratory has shown that sedentary T1D (CD) animals demonstrate significant elevations in skeletal muscle DAG content that are coupled to reductions in insulin sensitivity measures⁷. These observations in DARE animals are likely to symbolize the reported “athlete’s paradox” evident in highly trained individuals⁴³. Indeed, results from HOMA-IR in the present study indicate the DARE group maintains insulin sensitivity compared to the CD group, despite both showing similar intensities of skeletal muscle lipid staining. While we attempted to quantify the phosphorylation status of IRS-1 on ser1101 to examine the potential mechanism by which DAG may impede insulin signalling in CD animals, we were unable to see discernible differences IRS-1 phosphorylation between CD and DARE

animals. The lack of significant differences may be due to tissue status, as animals were sacrificed three days post-exercise and were not fasted. It may also be that the skeletal muscle lipid accumulation we observed here does not consist of saturated DAG species, as saturated fatty acid incorporation into DAG is associated with insulin resistance in skeletal muscle cells^{7,42,44}.

Lipin-1 is a phosphatidate phosphatase that regulates the balance between phosphatidate and DAG in de novo synthesis of DAG and TAG from glyceraldehyde 3-phosphate (G3P)⁴⁵. It is also a transcriptional coactivator of fatty acid oxidation genes and has been linked to greater insulin sensitivity improvements⁴⁵. Lipin-1 mRNA increases in skeletal muscle following exercise in rats⁴⁶. In the present study, DARE animals demonstrate an exercise training-induced increase in Lipin-1 protein, without an increase in CD36 protein. This uncoupling of Lipin-1 and CD36 may be indicative of greater de novo DAG and TAG synthesis, which in turn, is associated with greater oxidative capacity⁴⁷. In the DAE group, Lipin-1 protein content was not increased. It has been shown that exercise-mediated increases in skeletal muscle Lipin-1 mRNA are regulated by catecholamine-induced activation of the AMPK- β_2 -adrenergic receptor pathway⁴⁶. Resistance exercise is well documented to elicit a greater catecholamine response to exercise than aerobic type exercise in patients with T1D⁴⁸. Further, it has been shown that both aerobic and resistance training activate mTOR, with more robust effects observed following resistance training⁴⁹. mTOR and its regulatory protein complex mTORC1 has been shown to promote Lipin-1 translocation into the nucleus, where it regulates genes for fatty acid oxidation⁵⁰. Therefore, it is plausible that alterations in catecholamine levels

and mTORC1 in the DARE group drives Lipin-1 expression and translocation may promote a greater capacity for fatty acid storage and oxidation ²⁷.

Combined exercise training results in greater oxidative capacity in skeletal muscle of T1D rats. The integration of aerobic and resistance exercise may exert a synergistic effect, producing greater adaptations than aerobic training alone. While combined exercise training led to an increase in skeletal muscle lipid storage, this response is believed to be characteristic of the “athlete paradox” whereby changes in lipid oxidation capacity lead to greater lipid storage. Elevated levels of lipid storage mediator Lipin-1 and improvements in insulin sensitivity that accompanied enhanced citrate synthase activity would support this training specific adaptation. Given that combined resistance and aerobic exercise is a useful strategy to reduce the risk of post-exercise hypoglycemia among T1D patients, the integration of resistance and aerobic exercise may result in a safer exercise program while potentiating metabolic adaptations to exercise in skeletal muscle to a greater extent than aerobic training alone.

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CHAPTER 4

4 The Influence of Combined Aerobic and Resistance Exercise Training versus Standard Intensive Insulin Therapy on Insulin Resistance Development in a Rodent Model of Type 1 Diabetes »

4.1 Introduction

Insulin resistance is characterized by an insufficient biological response to circulating insulin and is critical in the pathogenesis of Type 2 Diabetes (T2D). More recently, insulin resistance has been shown to occur in the presence of Type 1 Diabetes (T1D), referred to as “double diabetes”¹. Individuals with both T1D and insulin resistance are at a significantly higher risk of cardiovascular complications, as insulin resistance contributes to the development of atherosclerosis via hyperglycemia, hyperinsulinemia, and dyslipidemia^{1,2}.

The pathogenesis of insulin resistance in T1D is not fully understood. Several lines of evidence would suggest that hyperglycemia or glucotoxicity is the primary driving force underlying the development of insulin resistance. Hyperglycemia results in increased hexosamine pathway activity, and activates stress-regulated pathways and the formation of advanced glycation end products (AGEs)³. On the contrary, others have demonstrated that hyperglycemia is not the sole aspect of metabolic dysfunction contributing to insulin resistance in T1D. Increased accumulation of intramyocellular lipid content leading to the increased production of harmful lipid metabolites is believed to be a major contributor to the pathogenesis of insulin resistance^{4,5}. Termed glucolipotoxicity, it is likely that these mechanisms are interrelated, as excess glucose can promote the accumulation of malonyl-CoA and diacylglycerol (DAG) in tissues including skeletal muscle³.

Regular exercise is a valuable tool for the maintenance of insulin sensitivity and cardiovascular and metabolic health. We have shown previously that regular aerobic exercise restores insulin sensitivity in our rodent model of T1D, improves bone health, neurovascular and endothelial function, and contributes to protection from ischemia-reperfusion injury⁶⁻⁹. However, the primary limitation to participation in regular exercise among individuals with T1D is fear of hypoglycemia¹⁰. Strategies to avoid post-exercise hypoglycemia include reduction of insulin dose, ingestion of carbohydrates and brief, high intensity efforts such as sprints at the initiation of an exercise bout^{10,11}. The necessity of carbohydrate ingestion has led many to question the benefit of exercise for patients with T1D, as overcompensation with carbohydrate can compromise glycemic control¹². However, we have observed previously combined exercise in T1D rats with moderate hyperglycemia (blood glucose 9-15 mM) produced significant improvements to oxidative capacity, beyond that of aerobic exercise alone (Chapter 3). Further, combined (aerobic and resistance) exercise training was associated with greater Lipin-1 protein content, indicative of enhanced capacity to channel substrate toward neutral lipid storage (Chapter 3). Others have reported improved glycemic control, insulin sensitivity and body composition among individuals with obesity and T2D following combined exercise¹³⁻¹⁵.

Despite the known benefits of exercise, findings from large Diabetes Trials (such as the DCCT (Diabetes Control and Complications Trial)) are the basis for recommending intensive insulin therapy for the management of T1D. Findings from the DCCT include a 42% decrease in the risk of cardiovascular disease development in the intensively treated group.^{16,17} In further support for the prescription of intensive insulin therapy, a retrospective analysis of the DCCT has revealed exercise does not improve microvascular

outcomes in T1D ¹⁸. However, more recent analyses of DCCT results has revealed methodological considerations and potential side effects associated with intensive insulin therapy. Based on stringent exclusion criteria, patients were otherwise healthy and not considered representative of the population of T1D patients ¹⁹. Further, 33% of intensively treated patients developed obesity compared to only 19.1% of conventionally treated patients, and intensively treated patients gained nearly twice as much weight as conventionally treated patients across all quartiles ²⁰. This weight gain was accompanied by increased blood pressure, triglycerides, total cholesterol and reduced high density lipoprotein (HDL) cholesterol despite stable glycemic control in the intensively treated group ²⁰. It also has been suggested that insulin resistance serves as a better predictor for coronary risk than HbA_{1C} ¹⁹. Intensive treatment is problematic as it is associated with a significantly increased risk of hypoglycemia, therefore limiting exercise participation ²¹. However, less stringent blood glucose control and combined exercise training is associated with reduced post-exercise hypoglycemia risk, and induces significant metabolic adaptations in skeletal muscle (Chapter 3).

We have shown previously combined exercise training induces similar, and potentially even greater benefits to skeletal muscle lipid metabolism and insulin resistance measures compared to aerobic exercise training alone. Given that combined training is associated with reduced risk of post-exercise hypoglycemia, it may be considered a safer modality for patients with T1D. Despite this, intensive insulin therapy is currently recommended to T1D patients. Therefore, the purpose of this study was to compare the effects of intensive insulin therapy versus combined (aerobic + resistance) exercise on insulin resistance and the distribution of lipid content (diacylglycerol vs. triacylglycerol) in

skeletal muscle in our rodent model of T1D. We hypothesized that combined exercise would be associated with enhanced insulin sensitivity in comparison to intensive insulin therapy alone. Differences in insulin responsiveness would be consistent with the changes in the type of fat storage whereby skeletal muscle of intensive insulin therapy would demonstrate increased harmful DAG accumulation and combined exercise would favour the accumulation of neutral, energy rich TAG.

4.2 Methods

Ethics Approval and Animals. Eight-week-old male Sprague-Dawley rats (Charles River Laboratories) were used in this study. Animals were housed two per cage at constant temperature and humidity, with a 12-h dark/light cycle, with access to water and standard chow *ad libitum*. The experimental protocol followed the Principles of Laboratory Animal Care (US NH publication No. 83-85, revised 1985). Ethics approval was obtained through the University of Western Ontario Research Ethics Board, in accordance with Canadian Council on Animal Care guidelines.

Experimental Groups. Animals were randomly divided into sedentary control (C, n=20), sedentary control diabetic (CD, n=20), sedentary intensive insulin-treated diabetic (DIT, n=20), and diabetic combined aerobic and resistance exercise (DARE, n=16). In each of these groups animals were further divided into two subgroups. The first subgroup (n=8/group) of animals underwent the hyperinsulinemic-euglycemic clamp experiment three days following their last bout of exercise (Experimental week #15) and were sacrificed immediately after the clamp procedure. The remaining animals in each subgroup (C, n=12; CD, n=12; DIT, n=12; DARE, n=8) did not undergo a hyperinsulinemic-

euglycemic clamp and were sacrificed three days following their last bout of exercise (Experimental week #15) in order to assess baseline measures.

Diabetes Induction. Rats were housed for one week to familiarize with their surroundings. After one week (Experimental week #1), T1D was induced with multiple low-dose STZ injections (Experimental week #2). For five consecutive days, STZ (20mg/kg; Sigma Aldrich, Oakville, ON, Canada) was injected into the intraperitoneal cavity²². Insulin pellets (LinShin, Toronto, ON, Canada) were implanted following two consecutive non-fasting blood glucose readings of ≥ 18.0 mmol/L). Blood glucose was monitored throughout the duration of the study and insulin pellets/dosages were adjusted accordingly. Blood glucose in the CD and DARE groups was maintained between 9-15 mmol/L to mimic poorly controlled T1D, while blood glucose in the DIT group was maintained between 5-9 mmol/L, reflective of intensive insulin therapy.

Exercise Training. Following diabetes induction, rats in the DARE group underwent a familiarization protocol followed by 12 weeks of exercise training (Experimental weeks #4-15). Familiarization was performed over five days, alternating between one day of aerobic exercise (treadmill running) and one day of resistance exercise (weighted ladder climbing). Treadmill familiarization consisted of progressively increasing running up to 27 m/min on a six percent grade daily for five days. Small blasts of compressed air were used to encourage rats should they cease running and break a photoelectric beam at the rear of the treadmill. Rats were also familiarized to resistance training, and performed 10 vertical ladder climbs per day with weights attached to the base of the tail, corresponding to 5%, 15%, 20%, and 35% of body mass. Following the familiarization week (Experimental week #3), training consisted of alternating days of one

hour treadmill running at 27 m/min on a six percent grade, and climbing sessions consisting of climbing with 50%, 75%, 90% and 100% of maximum lifting capacity. Maximum lifting capacity was re-tested every fourth day of resistance training using the sequential addition of 30 grams of weight until exhaustion.

Hyperinsulinemic-Euglycemic Clamp. Three days after the completion of the final exercise bout (Experimental week #15), the first set of animals underwent a hyperinsulinemic-euglycemic clamp to assess insulin resistance. Animals were overnight fasted for twelve hours and anaesthetized using isoflurane and an intraperitoneal injection of urethane (25 mg/kg)/ α -chloralose (4 mg/kg)^{23,24}. Rats were removed from isoflurane and the anesthetic injection maintained anaesthesia. A catheter was surgically inserted into the right jugular vein for insulin (Novolin ge Toronto, Novo Nordisk, ON, Canada) and glucose infusion. A second catheter was surgically inserted into the right carotid artery for arterial blood glucose sampling. Insulin was infused at 10 mU/kg/min; 0.4 μ IU/mL, and glucose was infused at 20 mg/kg/min (0.2 g/mL). Arterial blood glucose was sampled every 5 minutes until 20 minutes and every 10 minutes thereafter. Glucose infusion rate (GIR) was adjusted to maintain blood glucose at the “clamped” value of 4-6 mmol/L. Blood glucose values and GIR was recorded for 90 minutes and reported for the final 20 minutes of the clamp .

Tissue Collection. The first set of animals was sacrificed upon completion of the hyperinsulinemic-euglycemic clamp, which occurred three days after the last bout of exercise (Experimental week #15). The second set of animals did not undergo the clamp procedure and were sacrificed three days following the last bout of exercise (Experimental week #15). Animals were sacrificed via gas anaesthesia followed by exsanguination and

cardiac excision. Soleus muscles were removed and immediately flash frozen in liquid nitrogen and stored at -80°C for later analysis.

Thin Layer Chromatography. Lipid was extracted from 200-300 mg of minced soleus muscle. Tissue was submerged in chloroform: methanol (2:1, v:v) and placed in the dark for one hour. The extract was poured over Whatman filter paper into a glass test tube. The extraction vessel was rinsed and vortexed with 1 mL chloroform:methanol solution, filtered, and added to the original extract. Samples were dried under air stream in a 30-40 $^{\circ}\text{C}$ water bath. The extract was weighed and diluted in chloroform:methanol (2:1). Lipid extracts, diacylglycerol (1-Palmitoyl-3-Oleoyl-sn-Glycerol) and triolein (Avanti Polar Lipids Inc. Alabama USA) were spotted on glass plates with a silica gel matrix (Analtech TLC uniplates, Sigma Aldrich, Canada). Plates were immersed in a few centimeters of mobile phase consisting of toluene:methanol (7:3, v:v). Samples and standards were visualized using Iodine ACS Reagent (Sigma Aldrich, Canada) in a glass chamber and images were obtained with a flatbed scanner for analysis using ImageJ.

Data Analysis and Statistics. Animal mass, blood glucose, food intake, and glucose infusion rate during hyperinsulinemic-euglycemic clamp were assessed via one-way ANOVA with Tukey's post hoc test. Diacylglycerol and Triacylglycerol content was assessed via two-way ANOVA with Tukey's post hoc test. Statistical analyses were performed with SigmaPlot, graphs were generated using GraphPad Prism.

4.3 Results

Animal Characteristics and Food Intake. All diabetic rats (CD, DIT, DARE) weighed significantly less than non-diabetic control animals ($p < 0.05$) (Fig.4.1). Intensively insulin treated animals (DIT) weighed significantly more than DARE animals ($p < 0.05$) (Fig.4.1). Blood glucose in all diabetic groups (CD, DIT, DARE) was significantly higher than C ($p < 0.05$) (Fig.4.2.). DIT blood glucose was significantly lower than CD and DARE ($p < 0.05$) (Fig.4.2.). The number of pellets implanted for blood glucose management throughout the course of the study was significantly higher in the DIT group compared to CD and DARE ($p < 0.05$) (Table 4.1). Further, food intake was assessed at week 3 of training (Experimental week #6) in the hyperinsulinemic-euglycemic clamp subgroup, and was significantly higher in CD compared to C ($p < 0.05$) (Table 4.1).

Insulin Resistance Measures. Glucose infusion rate (GIR) during the hyperinsulinemic-euglycemic clamp was significantly lower in CD compared to C, DIT and DARE ($p < 0.05$), indicative of insulin resistance. GIR in the DARE group was significantly higher than in C, CD and DIT, indicative of enhanced insulin sensitivity among the exercise trained animals ($p < 0.05$). (Fig.4.3).

Soleus Muscle Lipid Content. Soleus Diacylglycerol content was significantly increased in DIT following hyperinsulinemic-euglycemic clamp ($p < 0.05$) (Fig.4.4.A). The increase in DAG content in the CD group approached significance ($p = 0.09$) (Fig.4.4.A). Triacylglycerol content was significantly increased in CD and DARE following hyperinsulinemic-euglycemic clamp ($p < 0.05$) (Fig.4.4.B).

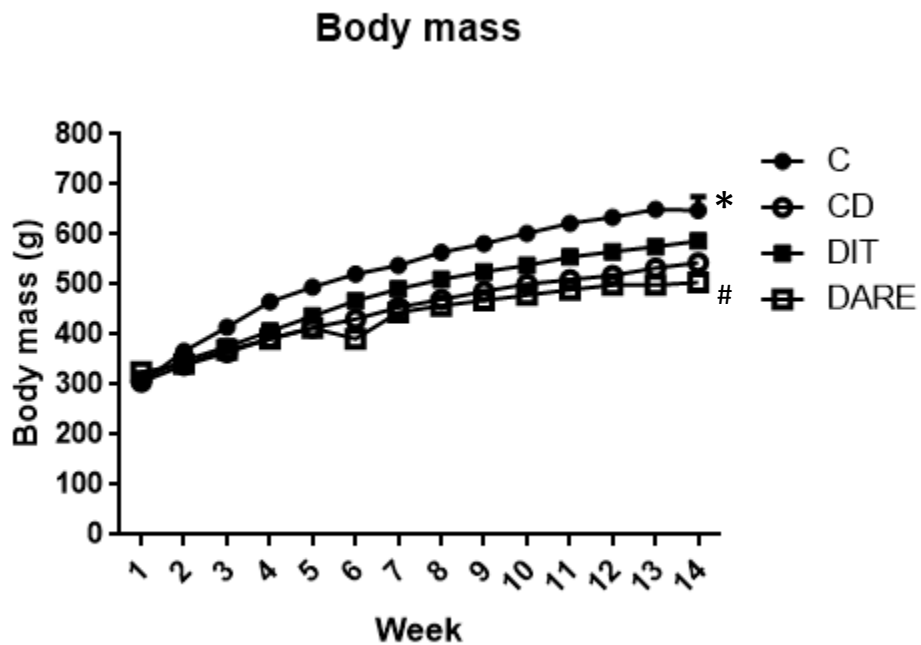


Figure. 4.1. Animal mass (g) for the duration of the study. (*) C rats weighed significantly more than CD, DIT and DARE rats ($p < 0.05$). (#) DARE rats weighed significantly less than DIT rats ($p < 0.05$).

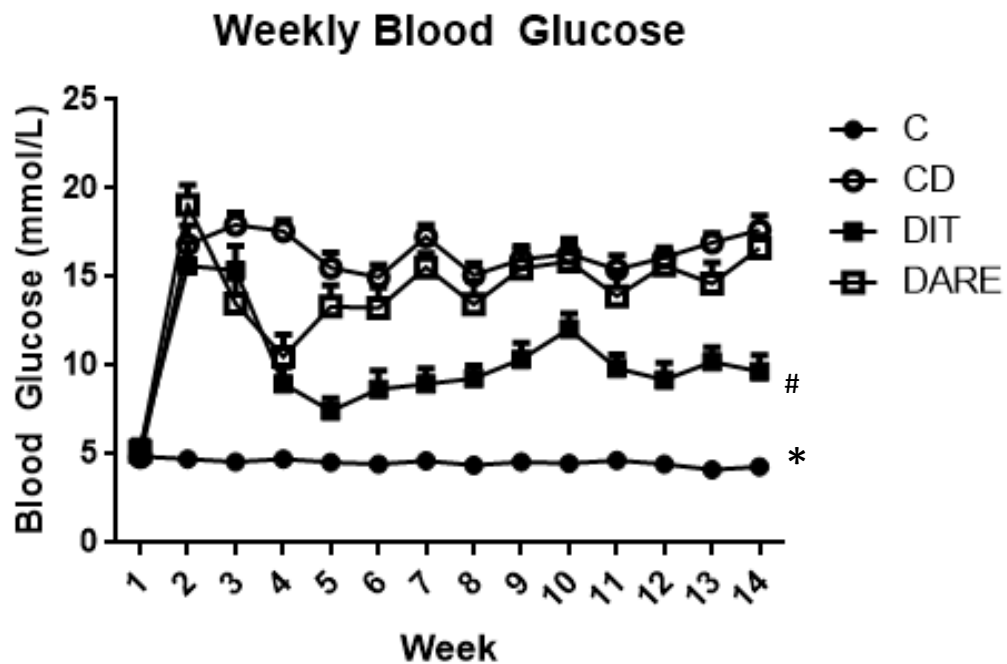


Figure. 4.2. Weekly blood glucose (mmol/L) for the duration of the study. (*) C blood glucose was significantly lower than CD, DIT and DARE ($p < 0.05$). (#) DIT blood glucose was significantly lower than CD and DARE ($p < 0.05$).

	C	CD	DIT	DARE
Food Intake (g)	203.83 ± 6.42	411.53 ± 61.89 °	304.48 ± 56.74	313.33 ± 7.94
#Pellets	-	2.57 ± 0.40	5.0 ± 0.59 *	1.81 ± 0.33

Table 4.1. Food intake and number of insulin pellets. (°) Food intake in the hyperinsulinemic-euglycemic clamp subgroup was significantly higher in CD compared to C ($p < 0.05$). (*) Total number of pellets implanted over the duration of the study was significantly greater in DIT compared to CD and DARE ($p < 0.05$).

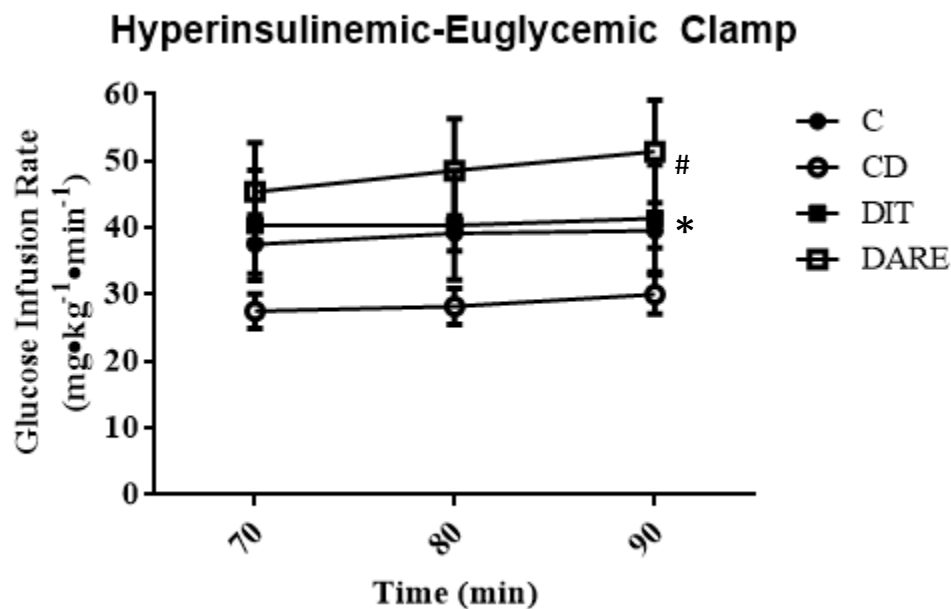


Figure. 4.3. Hyperinsulinemic-Euglycemic Clamp for assessment of insulin resistance. Groups were compared during the final 20 minutes of the clamp when animals were at or near steady-state GIR. (*)GIR in CD was significantly lower than in C, DIT and DARE ($p<0.05$). (#) GIR in DARE was significantly higher than in C, CD and DIT ($p<0.05$).

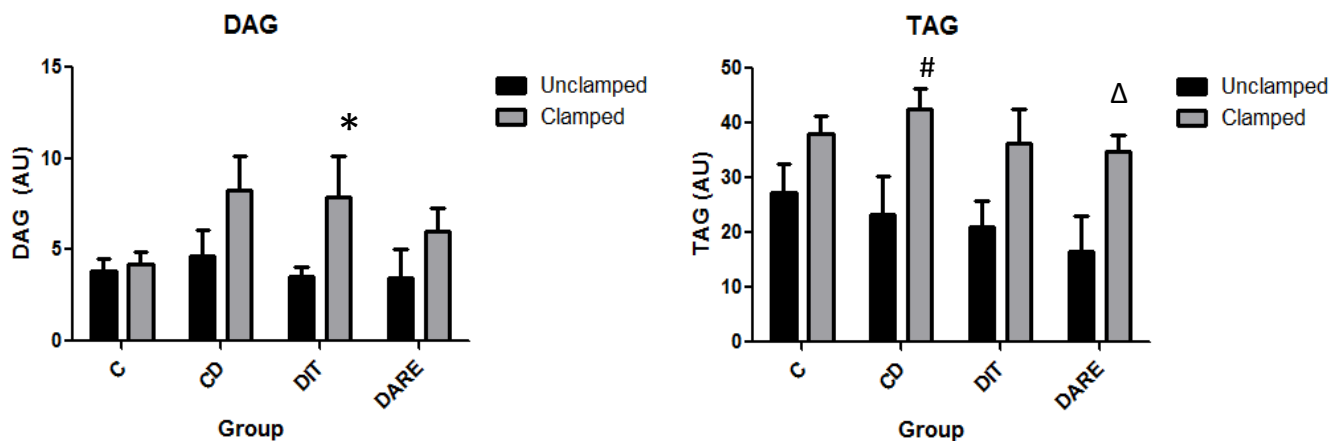


Figure. 4.4. Soleus lipid content. A. Diacylglycerol (DAG) Content. (*) There was a significant increase in DAG in DIT following hyperinsulinemic-euglycemic clamp ($p < 0.05$). B. Triacylglycerol (TAG) Content. There was a significant increase in TAG in CD (#) and DARE (Δ) following hyperinsulinemic-euglycemic clamp ($p < 0.05$).

4.4 Discussion

The purpose of this study was to compare the effects of combined exercise training versus standard intensive insulin therapy on skeletal muscle lipid content and insulin resistance development in a rodent model of T1D. Consistent with our hypothesis, we observed enhanced insulin sensitivity following combined exercise training compared to intensive insulin therapy. Further, we observed increased skeletal muscle DAG accumulation in intensive insulin therapy-treated animals following a hyperinsulinemic clamp, which did not reach significance in the combined exercise trained group (with conventional insulin therapy). Combined exercise training appeared to favour significant increases in the energy-rich TAG accumulation following the hyperinsulinemic clamp. Analysis of two subgroups (hyperinsulinemic-euglycemic clamp animals and unclamped animals) provided insight into the effects of acute glucose and insulin stimulation on skeletal muscle lipid content. Excess glucose uptake into skeletal muscle may drive malonyl-CoA formation, resulting in inhibition of CPT-1 and increasing the pool of fatty acyl-CoA and DAG ²⁵.

The gold standard for assessing insulin resistance development in humans is the hyperinsulinemic-euglycemic clamp ²⁶. Here, we report a significant reduction in glucose infusion rate in the CD group compared to C and DIT. This would support previous work demonstrating the negative impact of hyperglycemia on insulin sensitivity ^{3,27}. It has been reported that the onset of T1D is associated with skeletal muscle DAG accumulation resulting from increased adipose tissue lipolysis, and an increase in PKC θ activity, which directly interferes with the insulin signalling pathway ²⁸. Several lines of evidence have shown that impaired insulin signalling is further potentiated as T1D progresses, leading to

decreases in mitochondrial oxidative capacity and impaired lipid oxidation ²⁹. Indeed, our laboratory has reported a reduction in citrate synthase activity and increased DAG in skeletal muscle of conventionally treated T1D rodents (Chapter 2,3).

Although hyperglycemia and/or glucotoxicity may in part drive the development of insulin resistance ³, findings from the present study reveal that hyperglycemia itself may not be the primary cause of insulin resistance. Rather, insulin resistance may develop from an interaction between hyperglycemia and abnormal lipid accumulation in skeletal muscle referred to as glucolipotoxicity. Hyperglycemia contributes to insulin resistance via increased hexosamine pathway activity, producing UDP-N-acetylgalactosamine (UDP-GalNAc) which O-GINAcylates IRS-1 and GLUT-4 ^{3,30}. An important finding of the current study is that the DARE group exhibited a significant enhancement of insulin sensitivity, despite the maintenance of blood glucose between 9-15 mmol/L in both the DARE and the CD groups. We have previously shown increased DAG as well as insulin-desensitizing lipid metabolites in a conventionally treated group that were ameliorated with aerobic training ⁶. These findings support the concept of glucolipotoxicity, whereby hyperglycemia drives the accumulation of diacylglycerol, which directly inhibits PKC θ to impair insulin signalling ³.

In the DIT group, intensive insulin therapy was used to maintain blood glucose between 5-9 mmol/L for the duration of the study, and reduced the severity of insulin resistance in this group compared to the conventionally treated animals. However, insulin sensitivity in the DARE group was significantly higher than the DIT group, suggesting that reducing hyperglycemia alone is not sufficient to enhance insulin sensitivity. This phenomenon has also been observed in a clinical setting ³¹. A three-stage hyperinsulinemic-

euglycemic clamp performed on T1D patients revealed significant whole-body insulin resistance compared to non-diabetic subjects ³¹. Among the T1D patients, HbA1c was 7.7%, indicative of good glycemic control. Three days prior to the clamp procedure, patients wore a continuous glucose monitoring device, and glucose was normalized with variable insulin infusion overnight. Whole-body insulin resistance was therefore not entirely attributable to hyperglycemia, and these findings suggest other factors may contribute to insulin resistance among individuals with T1D.

Combined exercise resulted in significantly enhanced insulin sensitivity compared to C, CD and DIT. This was accompanied by increased skeletal muscle TAG content following the hyperinsulinemic-euglycemic clamp. We have previously shown aerobic exercise training effectively restores insulin sensitivity in T1D rodents, however, these findings are the first from our lab to show enhanced insulin sensitivity with combined exercise training ⁶. It is possible that an increase in neutral lipid stores (TAG) is coupled to increased oxidative capacity (as we have previously shown (Chapter 3)) effectively reducing the accumulation of harmful lipid intermediates such as DAG despite a lack of stringent blood glucose control with exogenous insulin.

Increased soleus TAG in the DARE group is likely mediated by increased diacylglycerol acyltransferase 1 (DGAT1), which aids in channeling fatty acid substrate into TAG, reducing DAG and ceramide accumulation ³². Transgenic overexpression of DGAT1 in type 1 muscle fibres protects mice from high fat diet induced insulin resistance ^{32,33}. This exercise-mediated shift in fatty acid channeling may result in a metabolic state similar to the “athlete’s paradox”, in which skeletal muscle maintains insulin sensitivity despite increased TAG content ^{32,33}. Maintenance of insulin sensitivity in these

circumstances is likely the result of reduced lipid (DAG)-induced activation of PKC isoforms and JNK, as fatty acids are directed toward TAG as opposed to DAG³³.

Interestingly, TAG was also increased in skeletal muscle of the CD group, despite a significant reduction in insulin sensitivity. Hyperglycemia and exogenous insulin administration may account for some of this accumulation, as they have an additive effect on DGAT1 activity³⁴. Further, insufficient oxidative capacity may contribute to elevated TAG content in CD animals. Our previous findings align with others who have shown T1D is associated with reduced oxidative capacity, potentially mediated by lipid peroxidation, and inhibition of mitochondrial biogenesis with insulin treatment (Chapter 3)²⁹. Taken together, increased TAG in the CD group suggests lipid accumulation is pathological in nature in sedentary T1D, stemming from reduced oxidative capacity, and is associated with insulin resistance. Conversely, increased TAG in the DARE group is an adaptation to exercise, accompanied with increased oxidative capacity and is not associated with impaired insulin signalling.

Soleus muscle DAG content was significantly increased in the DIT animals following the hyperinsulinemic clamp. Insulin has been shown to increase DAG via *de novo* phosphatidic acid synthesis³⁵. Insulin stimulates increased activity of glycerol-3-phosphate acyltransferase, resulting in phosphatidic acid synthesis and conversion into DAG³⁵. The use of insulin pellets to maintain stringent blood glucose control may account for this finding in the DIT group. Interestingly, Lipin-1 protein converts phosphatidic acid into DAG, and we have previously observed increased Lipin-1 protein content in skeletal muscle of conventionally treated T1D rodents (Chapter 3). The CD group in the present

study did not demonstrate a significant increase in DAG following the clamp, however the differences approached statistical significance ($p=0.09$) perhaps indicative of a trend.

It is important to note that the DAG-PKC interaction differs according to the DAG species involved, as saturated DAG are associated with insulin resistance and unsaturated DAG are not ³⁶. DAG containing C16:0, C18:0, C18:1, C18:2 and C20:4 have a strong association with PKC θ activation among obese and T2D patients ⁴. The subcellular location of DAG may also contribute to its involvement in insulin signalling, as well as the training status of individuals tested, as insulin signalling is preserved in endurance athletes ³⁷. Further work should investigate whether DAG composition (ie. saturation) differs between sedentary and exercise trained T1D rats and its relationship to insulin sensitivity.

Combined aerobic and resistance exercise is effective for enhancing insulin sensitivity in a rodent model of T1D, even in the context of moderate hyperglycemia. Intensive insulin therapy was sufficient to attenuate the drastic decline in insulin sensitivity observed with conventional insulin therapy. Altered insulin sensitivity was observed in parallel with changes in skeletal muscle lipid content, further supporting the role of lipotoxicity in the development of insulin resistance.

In conclusion, combined exercise training and maintenance of moderate glycemic control is significantly more effective for the enhancement of insulin sensitivity compared to intensive insulin therapy. These findings indicate exercise-induced alterations in skeletal muscle lipid metabolism, including enhanced oxidative capacity (Chapter 3) and greater sequestration of fatty acids as neutral TAG stores underlie these effects.

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CHAPTER 5

5 «General Discussion»

5.1 Summary

The principal objective of this dissertation was to better understand the relationship between skeletal muscle lipid metabolism and insulin resistance in a rodent model of T1D. The second objective was to examine the effect of both exercise modality and insulin therapy to enhance muscle lipid metabolism and whole body insulin sensitivity. In the context of T2D and metabolic syndrome, insulin resistance manifests due to increased skeletal muscle lipid metabolites which directly interact with the insulin signalling cascade¹. The rise of insulin resistance among individuals with T1D is associated with an increased risk of cardiovascular complications, and emerging evidence suggests a potential role for skeletal muscle lipids in the pathogenesis of insulin resistance in this population². Trials such as the Diabetes Control and Complications Trial (DCCT) have led to the promotion of intensive insulin therapy and stringent glycemic control as the optimal management strategy for T1D. However, evidence suggests that hyperglycemia itself is not the primary factor contributing to insulin resistance, and that disturbances in lipid metabolism may also be an important factor³⁻⁵. It is well documented that engaging in regular exercise significantly improves skeletal muscle lipid accumulation and insulin sensitivity among T2D and metabolic syndrome populations, and is recommended for patients with T1D to mitigate the development of several diabetic-related complications^{6,7}.

The main findings of this thesis were as follows; (1) sedentary, moderately hyperglycemic T1D rats develop insulin resistance and have increased skeletal muscle lipid metabolites, which is ameliorated with aerobic exercise training (*Chapter 2*), (2) sedentary

T1D rats have reduced skeletal muscle oxidative capacity coupled to increased fatty acid transport capacity. While aerobic exercise alone restores oxidative capacity, combined exercise training (resistance and aerobic) leads to greater improvements, and is coupled to a greater capacity to sequester lipid within skeletal muscle (*Chapter 3*), and (3) combined exercise training in the context of moderate hyperglycemia significantly enhances insulin sensitivity, oxidative capacity and neutral lipid storage in T1D rats. While intensive insulin therapy is sufficient to prevent the dramatic decline in insulin sensitivity observed in sedentary T1D rodents, combined exercise training induces significantly greater improvements to insulin sensitivity in T1D rats (*Chapter 3,4*).

To fulfil the objectives of this dissertation, it was necessary to determine whether insulin resistance and exercise training were associated with alterations in skeletal muscle lipid metabolites in our model of T1D. We employed an aerobic exercise training protocol as this modality is associated with a variety of metabolic and cardiovascular improvements in our model of T1D⁸⁻¹¹. In skeletal muscle of obese and T2D individuals, increased skeletal muscle triglyceride is linked to insulin resistance¹². Evidence suggests this is also a characteristic of T1D, as intramyocellular lipid content is associated with insulin resistance in patients with T1D¹³. Further, STZ-induced diabetic rats have increased neutral lipid staining and fatty acid transporter (FAT/CD36) protein content¹⁴. These metabolic alterations have been proposed to stem from hormonal changes coupled to hyperglycemia, and also include reduced oxidative enzyme capacity¹⁵. Consistent with these findings, metabolomic analysis of the red portion of the gastrocnemius muscle revealed several lipid metabolites differentiated muscle of sedentary, insulin resistant T1D rats from nondiabetic rats and aerobically trained rats (*Chapter 2*). Muscle from sedentary, insulin resistant rodents was unique from that of nondiabetic and exercise trained T1D

animals as it was characterized by octadecenoic acid, linoleic acid, arachidonic acid, docosahexaenoic acid and palmitic acid. These fatty acids have been linked to insulin resistance through the formation of inflammatory cytokines, direct interaction with proteins in the insulin signalling cascade and reduction of GLUT4 expression *in vitro*¹⁶⁻¹⁸. Further, diacylglycerol, a lipid species well-known to contribute to insulin resistance due to its interaction with PKC θ , was significantly increased in muscle of sedentary T1D rats^{19,20}.

Interestingly, while aerobic exercise was sufficient to restore insulin sensitivity in T1D rodents, it was not sufficient to enhance insulin sensitivity beyond that of a sedentary nondiabetic animal. Previous observations from our laboratory show aerobic exercise significantly enhances insulin sensitivity in nondiabetic animals, however we did not observe the same robust effect in T1D rodents²¹. In patients with T1D, metabolomics analysis indicates altered muscle metabolism following exercise in comparison to nondiabetic individuals, evidenced by attenuated Krebs cycle intermediates in circulation post-exercise²². These findings, coupled to our observations following metabolomic analysis of skeletal muscle, suggests altered muscle metabolism in our rat model of T1D, which may necessitate a unique or increased stimulus to elicit similar adaptations to exercise.

Hypoglycemia is a major barrier to engaging in regular exercise among patients with T1D²³. Aerobic exercise, despite eliciting several metabolic and cardiovascular adaptations and improving overall health and well-being, is associated with substantial, acute, blood glucose reductions²⁴. Resistance training is emerging as an exercise modality associated with a reduced risk of hypoglycemia and as a strategy to prevent aerobic-exercise-induced hypoglycemia²⁵. Among trained T1D patients, blood glucose during and after resistance exercise is more stable, compared to aerobic exercise²⁶. Further, performing a bout of resistance exercise prior to aerobic exercise is effective in reducing

the severity of post-exercise hypoglycemia among T1D patients ²⁷. It is believed that the increase in epinephrine and norepinephrine may account for the blood glucose stabilizing effects of this exercise modality ²⁵. We have previously shown increased GLUT4 protein in the white portion of the gastrocnemius following resistance training, as well as an improvement in insulin sensitivity ⁸. There is also increasing evidence to suggest that combined exercise training, consisting of aerobic and resistance modalities produces greater fitness adaptations than each modality in isolation ²⁸. Aerobic exercise performed prior to resistance exercise has also been shown to significantly enhance the muscle transcriptome associated with resistance exercise, and is effective in reducing HbA1c and cholesterol in patients with T2D ^{6,29}. Accordingly, the second objective of this thesis was to establish the efficacy of combined exercise training for improving skeletal muscle lipid metabolism and oxidative capacity in our model of T1D. We began by quantifying citrate synthase enzyme activity as a marker of oxidative capacity (*Chapter 3*). Consistent with the literature, we observed a significant reduction in citrate synthase enzyme activity in sedentary T1D animals ¹⁵. This was also coupled to an increased fatty acid transporter protein content, suggesting an increase in fatty acid entry into the muscle without an accompanying increase in oxidative capacity. Aerobic exercise effectively restored oxidative capacity in T1D animals, however, combined exercise exerted a significant increase in oxidative capacity. Combined exercise was also associated with increased protein content of Lipin-1, an essential enzyme to allow the esterification and storage of free fatty acids. Taken together, we proposed that combined exercise elicits significant improvements to skeletal muscle lipid oxidation and storage. Given the role of insulin resistance in the development of cardiovascular disease, it was of importance to ensure that the improvements in insulin sensitivity we observed with aerobic exercise (*Chapter 2*) were

not lost when combined with resistance exercise. Therefore, *Chapter 4* examined the effects of combined exercise on insulin sensitivity in T1D rats.

Stemming from the findings of the DCCT, the current recommended insulin treatment regime for T1D patients is intensive insulin therapy with the target of maintaining normal HbA1c (approximately 6.5%) through multiple daily insulin injections or an insulin pump device ³⁰. Intensive insulin therapy is still considered the most highly effective treatment strategy for patients with T1D, as a 42% reduction in the risk of cardiovascular disease development has been reported in these patients ^{3,31}. However, recent follow up studies of DCCT participants have revealed a significantly higher rate of obesity in the intensively insulin treated patients, as well as an increased levels of circulating lipids ³². Some also suggest the exclusion criteria in this cohort study was problematic, excluding T1D patients with a history of cardiovascular disease, hypertension, dyslipidemia, and neuropathy requiring treatment ³⁰. This resulted in the inclusion of an otherwise healthy population of T1D patients, not considered representative of the average population with T1D ⁴. It also remains unclear as to whether intensive insulin therapy is associated with increased insulin resistance, although emerging findings indicate an association between intensive insulin therapy and the development of obesity, metabolic syndrome and insulin resistance ^{3,32}. Insulin resistance is a critical component in the pathogenesis of cardiovascular disease, and is often improved with exercise training ³³⁻³⁵. However, intensive insulin therapy is the current recommended therapy in the management of T1D, and participation in exercise training, especially aerobic exercise, is problematic for this population ²³.

The third objective of this thesis was to compare the effects of intensive insulin therapy versus combined exercise training on insulin sensitivity and lipid metabolism

(*Chapter 4*). We also sought to examine whether the composition of skeletal muscle lipids (diacylglycerol and triacylglycerol) differed between these treatment strategies (exercise vs. intensive insulin therapy). What may be the most critical finding from this experiment is that the pathophysiology of insulin resistance is not in accordance with the “glucose hypothesis”, whereby hyperglycemia is the causative factor underlying the onset of complications in T1D³⁰. Consistent with previous reports²¹, glucose infusion rate (GIR) during the hyperinsulinemic-euglycemic clamp was significantly decreased in sedentary conventionally treated T1D rats (CD), the moderately hyperglycemic group with glucose maintained between 9-15 mM for the duration of the study. GIR in the intensively treated T1D group (DIT), with glucose maintained between 5-9 mM for the duration of the study, was restored to that of nondiabetic control animals. These findings demonstrate that in the sedentary state hyperglycemia drives insulin resistance, supporting the importance that stringent blood glucose control through exogenous insulin administration is efficacious in the preservation of insulin sensitivity. However, the combined exercise treated (DARE group) was maintained in the same glycemic range as the CD group for the duration of the study, with glucose between 9-15 mM, and GIR was substantially increased compared to all other groups. Therefore, despite moderate hyperglycemia, combined exercise elicited a significant improvement in insulin sensitivity, implicating factors other than hyperglycemia in the pathogenesis of insulin resistance. Glucolipotoxicity occurs when hyperglycemia drives lipid storage and the production of insulin-desensitizing lipid metabolites³⁶. In the DARE group, increased oxidative capacity and lipin-1 protein content (*Chapter 3*) may, at least in part, be a potential mechanism by which exercise-trained animals drive high circulating blood glucose toward oxidation or storage as neutral TAG stores, which are also increased in this group (*Chapter 4*). By channeling substrate toward oxidation or storage

as neutral TAG, combined exercise training may induce favourable adaptations in skeletal muscle and protect against the glucolipotoxic effects of hyperglycemia on insulin signalling.

5.2 Conclusion

Upon completion of this dissertation, there are two significant findings that contribute to our understanding of insulin resistance in T1D. 1) The pathogenesis of insulin resistance is attributable to abnormal skeletal muscle lipid accumulation and potential inability to effectively metabolically channel excess substrate. This may be amplified by hyperglycemia, manifesting as glucolipotoxicity, and 2) Combined exercise training is a more effective treatment strategy than intensive insulin therapy for the enhancement of insulin sensitivity in T1D.

We consistently observed that sedentary lifestyle in T1D was associated with skeletal muscle lipid accumulation and insulin resistance. However, the increase in insulin-desensitizing lipid metabolites in skeletal muscle with both moderate hyperglycemia and intensive insulin treatment suggests that hyperglycemia alone does not account for insulin resistance, and that glucolipotoxicity is a significant driver of insulin resistance. While insulin sensitivity is restored with stringent glucose lowering, combined exercise training significantly enhances insulin sensitivity. In addition to enhanced insulin sensitivity, combined exercise training significantly increases oxidative capacity and neutral triglyceride content. These findings are perhaps best described as “*running water carries no poison*”³⁷, whereby the proper oxidation, storage and channeling of lipids in the skeletal muscle cell (as an adaptation to combined exercise training) prevents the accumulation of insulin-desensitizing metabolites. This can be achieved through the implementation of combined exercise training while maintaining moderate hyperglycemia as opposed to strict

glycemic control with intensive insulin therapy alone. Further, exercising while in a moderately hyperglycemic state substantially reduces the risk of hypoglycemia. Initiating exercise at an elevated blood glucose concentration can help ensure that post-exercise concentrations would not fall into a hypoglycemic range. Collectively, this work suggests that combined exercise training may be a superior exercise modality for patients with T1D, as it induces significant adaptations in skeletal muscle. Most notably, combined exercise training may reduce the risk of cardiovascular complications in T1D by enhancing insulin sensitivity and preventing the onset of “Double Diabetes”.

5.3 References

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Appendices

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

PURPOSE:

Diabetes induction in rats.

PROCEDURE:

Preparing 5X Citric Acid/Citrate Buffer

1. For a pH 4.6 buffer at 765 mM (5X stock solution), in a beaker, Add
 - i. 13.8g Anhydrous Citric Acid (Sigma) or 15.1g Citric Acid Monohydrate
 - ii. 23.8g Sodium Citrate Dihydrate (Sigma)
 Mix into
 - iii. 175mL of MilliQ water

The pH should be at 4.6, Add HCl or NaOH to adjust (do not over-shoot pH)

2. Once the proper pH is obtained, add MilliQ water until you are close to the 200 ml mark (pH will move slightly). If satisfied with the pH, adjust volume in a 250 ml graduated cylinder and filter in a 0.2µm filter.
3. Store at room temperature. This is your 5X stock solution.

Making up Streptozotocin (STZ) for Injection

1. Place 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). Check pH again.
4. STZ must be injected within 15 minutes of preparation. Weigh STZ based on calculations below:
Inject 20mg/kg of STZ per animal. If the average weight of 10 animals is 200g, then 20mg/kg X 0.2kg = 4 mg/animal. To account for solution lost in filtering, weigh extra. Ie. (4mg (per animal) X 12 rats = 48 mg total (0.048 g).
5. Dissolve the STZ into buffer by vortexing then sterile filter using a 0.2µm syringe filter.
Ex. 48mg STZ ÷ 3 mL buffer = 16mg/mL solution
$$4\text{mg} \div 16\text{mg/mL solution} = 0.25\text{mL}$$

Injecting and Follow-Up of the Animals

1. Inject each rat intraperitoneally with a dose of 20mg/mL within 15 minutes of solution preparation.
2. Dispose containers in a biohazardous waste receptacle and needles in a sharps container.
3. 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, which is usually achieved after 5-8 injections (i.p. 20mg/kg).

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Appendix B. Insulin Pellet Implantation (rat)

PURPOSE:

Exogenous insulin administration in diabetic rats.

PROCEDURE:

1. Anesthetize the animal with isoflurane machine set to 4-5% with an O₂ rate of 1 L/min. Once animal is unconscious, remove it from the chamber and place in the nose cone. Reduce isoflurane to 3% to maintain the plane of anesthesia.
2. Shave a small area on the abdomen where the pellet will be implanted.
3. Apply 10% providone-iodine solution to the skin, followed by 70% ethanol, to disinfect the site of insertion.
4. Make a small subcutaneous incision.
5. Using forceps, briefly immerse the pellet in 10% providone-iodine solution, rinse with saline and insert into subcutaneous region.
6. Use 1 pellet for the first 350g of body weight.
7. Pinch the skin closed after the last pellet is inserted. Place a drop of 10 % providone-iodine solution over the opening.
8. Close the incision by suturing.
9. Place the animal under a heat lamp and monitor until it recovers from anesthesia.
10. Record on the cage card that insulin pellets have been implanted.

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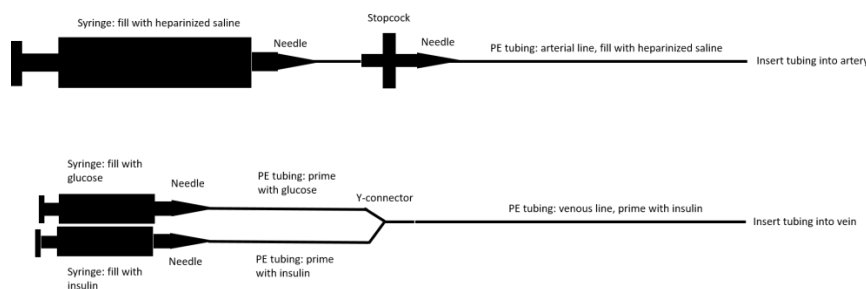
Appendix C. Hyperinsulinemic-Euglycemic Clamp (rat)

PURPOSE:

To assess insulin sensitivity in an anesthetized animal.

CATHETERIZATION PROCEDURE:

1. Turn on heating pad, prepare anesthetic in a clean beaker on a heated stir platform:
Urethane 1.0g/10 ml ddH₂O
 α -Chlorolose 160mg/10 ml ddH₂O
Let the solution cool before preparing syringes for injection.
2. Prepare glucose and insulin:
2.0g dextrose/4 ml saline (500 mg/ml solution)
50 μ l of 100 U/ml Toronto insulin in 12.5 ml saline + 1% bovine serum albumin (0.4 U/ml or 400 mU/ml solution)
3. Prepare saline with heparin:
Combine heparin (Sigma Aldrich, Canada, 210-6) with saline for a final concentration of (27 U/ml). (This should work out to approximately 6 vials of heparin per bag of saline but be sure to check the heparin concentration and adjust accordingly).
4. Prepare two PE50 or PE90 tubing catheters as follows:



5. Prepare surgical field with neck support, hooks, suture silk, tape and anesthetic syringe. For intraperitoneal injection of anesthetic: $(\text{mass (g)} / 4) / 100 = \text{ml for injection}$
ie. for a 300g rat injection volume = $(300/4) / 100 = 0.75 \text{ ml anesthetic}$
6. Anesthetize the animal with isoflurane machine set to 4-5% with an O₂ rate of 1 L/min. Once animal is unconscious, remove it from the chamber and place in the nose cone. Reduce isoflurane to 3% to maintain the plane of anesthesia. Inject approximately $\frac{1}{2}$ the anesthetic dose, and allow animal to stabilize for 10-20 min. Gradually wean the animal off isoflurane and maintain anesthesia with the urethane/ α -chlorolose injection.

7. Make a central incision with scissors from along the esophagus and blunt dissect to the right of the esophagus.
8. Expose and clean the jugular vein:
 - using #7 tweezers, slip a piece of suture silk under the vein and tie the cranial portion of the vein.
 - using #7 tweezers, slip a piece of suture silk under the vein and throw a knot at the cardiac portion of the vein but do not cinch down the suture.
 - make a small incision in the vein, insert and advance the venous catheter toward the cardiac suture. Cinch down the cardiac suture over the catheter and pull back on the syringe to ensure blood flow. Tie the catheter to the cranial suture to ensure it is secure in the vein.
9. Expose and clean the carotid artery:
 - blunt dissect between the trachea and jugular vein.
 - expose and clean the carotid artery.
 - set three sutures: one on the cranial portion of the artery (cinch), one at the cardiac portion of the artery (cinch) and throw one suture between the cranial and cardiac portions but do not cinch this down.
 - make a small incision in the artery, insert and advance the arterial catheter toward the cardiac suture.
 - tie down the middle suture around the catheter in the artery.
 - loosen the cardiac suture, advance the catheter and cinch down the cardiac suture over the artery and catheter.
 - Tie the catheter to the cranial suture to ensure it is secure within the artery.
 - open the stopcock and check for blood flow in the catheter. Immediately flush the line with heparinized saline to prevent clotting.
10. Inject the remaining $\frac{1}{2}$ dose of anesthetic intraperitoneally and allow the animal to stabilize for approximately 20 minutes.

HYPERINSULINEMIC-EUGLYCEMIC CLAMP PROCEDURE:

1. Connect syringes to infusion pumps.
2. Begin glucose and insulin infusion. Start glucose infusion at 20 $\mu\text{l}/\text{min}$ and adjust accordingly based on blood glucose values. Insulin infusion remains constant at 10 $\mu\text{l}/\text{min}$.
3. Measure blood glucose every 5 minutes until 20 minutes and every 10 minutes thereafter from the arterial line using a Freestyle Lite blood glucose monitor. Record blood glucose values and adjust and record the glucose infusion rate.
4. Continue the clamp for 90 minutes, “clamping” the animal’s blood glucose between 4-7 mmol/L.
5. Record the total volume of insulin and glucose infused during the clamp.

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Appendix D. Metabolomics: High Performance Liquid Chromatography coupled to Quadrupole Time-of-Flight Mass Spectrometry (HPLC-QTOF-MS)

PURPOSE:

To identify metabolites in rat skeletal muscle samples.

SAMPLE PREPARATION:

1. Weigh and cut 100 ± 3 mg of tissue.
2. Homogenize tissue in 250 μ l ice cold HPLC grade acetonitrile containing isatin (5 μ g/ml) and flurazepam (25 ng/ml) as internal standards for 2 minutes on an ice bath.
3. Vortex samples, centrifuge at 14 000 rpm at 4°C for 5 minutes.
4. Remove 120 μ l of supernatant and dilute with 30 μ l ultrapure water. The final sample should contain 80% acetonitrile.
5. Generate a control injection consisting of a pooled sample of an equal volume of all injected samples.
6. Transfer samples to vials and inject 1 μ l in triplicate from each vial.

CHROMATOGRAPHIC SEPARATION:

1. Separate using a Waters Acquity Ultra Performance Liquid Chromatography system, with an Acquity UPLC HSS T3 column (1.8 μ m particle size, 100 mm x 2.1 mm).
2. Set column temperature to 45°C in Waters ACQUITY UPLC I-Class System (Waters, Milford, MA).
3. Set the mobile flow: 0.45 ml/min, consisting of water (A) and acetonitrile (B) each with 0.1% formic acid. Set the following UPLC conditions:
0-2 min: 1-60% B
2-6 min: 60-85% B
6-8 min: 85-99% B
8-10 min: 99-1% B
4. For mass spectrometry, use a Waters Xevo™ G2S-QToFMS, and measure metabolites in positive and negative electrospray ionization mode.

5. Set capillary voltage to 2 kV and cone voltage to 40 V, set the source temperature to 150°C.
6. Set desolvation gas flow to 1200 L/h at 600°C and cone gas flow to 50 L/h.
7. Set the lockmass at 500 ng/ml leucine-enkephalin at a flow rate of 10 μ l/min, measuring every 10 seconds and averaged over 3 scans.
8. Data acquisition: centroid mode using MSE method with m/z range of 50-1200.

Appendix E. Homogenizing Protocol

PURPOSE:

To prepare skeletal muscle samples for Western Blotting.

PROCEDURE:

1. Prepare homogenizing buffer:
100 mmol/L NaCl
50 mmol/L Tris base
0.1 mmol/L EDTA (ethylenediaminetetraacetic acid)
0.1 mmol/L EGTA (ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid)
1% Tritonx100
pH 7.5
2. Cut and weigh 0.1 g of muscle sample.
3. Place in Eppendorf tube or glass test tube.
4. Add homogenizing buffer for a 1:10 ratio. Ie. 0.0957 g tissue to 957 μ l homogenizing buffer.
5. Repeat for each sample.
6. Homogenize sample and centrifuge homogenates.
7. Pipette homogenate into Eppendorf tube and freeze at -70°C .
8. Determine protein content of sample using Bradford Protein Assay (See Appendix F) prior to use.

Appendix F. Bradford Protein Assay

PURPOSE:

To determine protein content of sample homogenate.

PROCEDURE:

1. Dilute 1 part dye (Bio-Rad 500-0006) with 4 parts ddH₂O.
2. Filter the solution with Whatman 1 filter paper
3. Label a microplate template sheet with sample ID and standard.
4. Label a second set of Eppendorf tubes with each sample ID and standard.
5. In the second set of tubes, add 5 μ l of sample and 95 μ l of ddH₂O.
6. Pipette standards and samples into microplate as follows:

	BSA : Water + 200 μ l reagent			Add 10 μ l of mixed sample + 200 μ l reagent (everything done in 3's)								
	1	2	3	4	5	6	7	8	9	10	11	12
0	0 + 10											
1	1 + 9											
2	2 + 8											
3	3 + 7											
4	4 + 6											
5	5 + 5											

7. Shake microplate and Incubate at room temperature 5 to 60 minutes. Read at 595 nm.

Appendix G. Western Blotting Protocol

SAMPLE PREPARATION:

1. Thaw homogenized samples on ice.
2. Assess the amount of protein to be loaded in each well. Running a loading curve may be necessary to visualize the ideal amount for a given protein of interest.
3. Label a new set of Eppendorf tubes with sample IDs and dilute the sample (determined from the Bradford Assay) in sample buffer (see Western Blotting solutions #4) in a 1:1 ratio and vortex thoroughly.
4. Boil the samples for up to 5 minutes (depending on protein of interest).
5. Bring samples back to room temperature before loading in gel.

POLYACRYLAMIDE GEL PREPARATION:

1. Clean mini glass plates with 70% ethanol and assemble gel cassette.
2. Prepare separating gel according to chart below:
(% of acrylamide in the gel depends on the size of the protein of interest, example below is for a 10% gel).

10% Separating Gel					
	1 Gel	2 Gel	4 Gel	6 Gel	8 Gels
ddH ₂ O	4 ml	8 ml	16 ml	24 ml	32 ml
30% Acrylamide (in fridge)	3.33 ml	6.67 ml	13.3 ml	20 ml	26.7 ml
Separating Buffer	2.5 ml	5 ml	10 ml	15 ml	20 ml
10% SDS	100 μ l	200 μ l	400 μ l	600 μ l	800 μ l
10% APS (in fridge: 0.1g in 1ml DDH ₂ O)	62.5 μ l	125 μ l	250 μ l	375 μ l	500 μ l
TEMED (in fumehood)	6.25 μ l	12.5 μ l	25 μ l	37.5 μ l	50 μ l

3. Pour separating gel using pipette and be careful to avoid bubbles. Overlay the gel with water saturated isobutanol. This will prevent bubbles from forming and will ensure a continuous charge from separating to stacking gel.

- Wait 30-60 minutes for the gel to polymerize and rinse the overlay solution with ddH₂O and dry with filter paper.
- Prepare stacking gel according to chart below and pour over separating gel:

	1 Gel	2 Gel	4 Gel	6 Gel	8 Gels
ddH ₂ O	3.05 ml	6.1 ml	12.2 ml	18.3 ml	24.4 ml
30% Acrylamide (in fridge)	0.65 ml	1.3 ml	2.6 ml	3.9 ml	5.2 ml
Stacking Buffer	1.25 ml	2.5 ml	5 ml	7.5 ml	10 ml
10% SDS	50 µl	100 µl	200 µl	300 µl	400 µl
10% APS (in fridge)	31.3 µl	62.6 µl	125 µl	188 µl	250 µl
TEMED (in fumehood)	6.25 µl	12.5 µl	25 µl	37.5 µl	50 µl

- Place the lane comb in the stacking gel between the two glass plates. Allow to polymerize for 30-60 minutes.
- Prepare 1L of 1x running buffer (see Western Blotting Solutions #6) for every 2 gels and keep in refrigerator.
- Gently remove lane comb from stacking gel and fill wells with 1x running buffer.

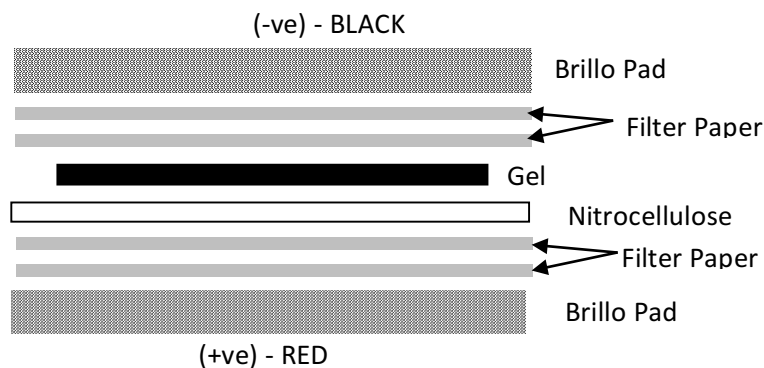
GEL ELECTROPHORESIS:

- Load the sample and ladder into the wells using a micropipette with a loading tip.
- Once loaded, place gels in running unit, fill with cold 1x running buffer, and be sure to fill the space between the gels as well.
- Run gels between 50-70 V until the samples have moved through the stacking gel (approximately 30 minutes) and then 120 V until samples travel completely through the gel.
- While the gels are running, prepare the transfer buffer (see Western Blot Solutions #7) and store in refrigerator.

TRANSFER TO NITROCELLULOSE MEMBRANE:

- Cut filter paper and nitrocellulose membranes to the same size as the gel. Soak with Brillo pads in cold transfer buffer.
- Gently separate gel from the glass plate and place in transfer buffer. Assemble the transfer apparatus “sandwich” as shown below:

15. Place “sandwich” in the transfer tank, add ice packs and fill with cold transfer buffer.
16. Run the transfer at 70 V for 90 minutes and keep cold on ice. Note, the transfer voltage and time may differ depending on the size of the protein of interest.



BLOCK AND INCUBATE MEMBRANES:

17. Prepare 1L of 1x TBS (see Western Blot Solutions #7 “10X TBS”) for every 2 membranes.
18. When the transfer is complete, remove the membrane from the “sandwich” and place in a container with 5% blocking solution (see Western Blot Solutions #9). Incubate for 1 hour on shaker at room temperature.
19. Prepare primary antibody.
20. Incubate membranes in primary antibody solution overnight at 4°C (incubation time may vary depending on antibody manufacturer instructions).
21. Wash the membranes 3 times in TTBS (see Western Blot Solutions #8) on a shaker for 5-10 minutes.
22. Prepare secondary antibody (HRP conjugated) solution.
23. Incubates membranes for 1 hour on shaker at room temperature.
24. Wash the membranes 3 times in TTBS (see Western Blot Solutions #8) on a shaker for 5-10 minutes.

CHEMILUMINESCENT IMAGING:

25. Prepare the substrate (BioRad ImmunStar Western C chemiluminescence kit (#1705070)) by combining 500 μ l of the Luminol/enhancer solution and 500 μ l of the peroxide solution into a 1.5 ml Eppendorf tube.
26. Place the membrane on a transparency and pipette 1 ml of the mixed solution over the blot.
27. Close the chemidoc door and proceed to image the membrane.

WESTERN BLOTTING SOLUTIONS:

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

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

SEPARATING GEL:

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

12 % Gel				
	2 gels	4 gels	6 gels	8 gels
ddH₂O	6.663 mL	13.325 mL	19.988 mL	26.65 mL
Acrylamide solution¹	8 mL	16 mL	24 mL	32 mL
Separating gel buffer²	5 mL	10 mL	15 mL	20 mL
SDS solution³	200 µL	400 µL	600 µL	800 µL
10% APS solution	125 µL	250 µL	375 µL	500 µL
TEMED	12.5 µL	25 µL	37.5 µL	50 µL
Total Volume	20 mL	40 mL	60 mL	80 mL

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

7.5 % Gel				
	2 gels	4 gels	6 gels	8 gels
ddH₂O	9.663 mL	19.325 mL	28.988 mL	38.65 mL
Acrylamide solution¹	5 mL	10 mL	15 mL	20 mL
Separating gel buffer²	5 mL	10 mL	15 mL	20 mL
SDS solution³	200 µL	400 µL	600 µL	800 µL
10% APS solution	125 µL	250 µL	375 µL	500 µL
TEMED	12.5 µL	25 µL	37.5 µL	50 µL
Total Volume	20 mL	40 mL	60 mL	80 mL

6 % Gel				
	2 gels	4 gels	6 gels	8 gels
ddH₂O	10.663 mL	21.325 mL	31.988 mL	42.65 mL
Acrylamide solution¹	4 mL	8 mL	12 mL	16 mL
Separating gel buffer²	5 mL	10 mL	15 mL	20 mL
SDS solution³	200 µL	400 µL	600 µL	800 µL
10% APS solution	125 µL	250 µL	375 µL	500 µL
TEMED	12.5 µL	25 µL	37.5 µL	50 µL
Total Volume	20 mL	40 mL	60 mL	80 mL

STACKING GEL:

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

Appendix H: Thin Layer Chromatography

PROCEDURE: All sample preparation and chromatography must be done in a fume hood.

1. Weigh and cut 200-300 mg of muscle. Mince well using a blade and place in a 14 ml falcon tube.
2. Add 8 ml of chloroform:methanol (2:1, v:v). Cap and keep dark in fume hood for 1 hour.
3. Weigh a set of glass test tubes and record the weight for each tube.
4. Pour the extract from the falcon tube over Whatman 2 filter paper into the glass test tube. Add 3 ml of chloroform:methanol (2:1) to the falcon tube, vortex, and pour into the test tube.
5. Repeat for all samples.
6. Dry the sample in the glass tube under a steady air stream in a 30-40°C water bath for approximately 1 hour or until all the chloroform:methanol has evaporated.
7. Obtain the weight of the sample by weighing the tube and subtracting the weight of the empty tube previously recorded.
8. Reconstitute the sample to 0.5 ml/g⁻¹ wet weight.
9. Line a glass dish with filter paper, and pour in approximately 1 inch of toluene:methanol (7:3, v:v).
10. Spot 5 µl of sample along a line approximately 1-1.5 inches from the bottom of the plate. Spot standards on the left and right of the plate.
11. Cover the plate in the toluene dish with a lid or wrap with foil to minimize evaporation.
12. Let sit in the fume hood and run the solution to the top of the plate.
13. In a separate dish, add a few grams (5-10) of Iodine ACS reagent. Cover the dish to keep the vapors from evaporating.
14. Remove the plate from the toluene:methanol container and let dry briefly.
15. Place the plate in the chamber with the Iodine ACS reagent but do not place the plate directly on the crystals.
16. Cover the dish and let it sit for 1-2 hours until desired intensity of spots is reached.
17. To quantify the plate, place it in a clear plastic sleeve and scan on a flatbed scanner. The sample spots may be quantified with ImageJ.

Appendix I: Citrate Synthase Enzyme Activity Assay

SAMPLE PREPARATION:

1. Prepare homogenizing buffer:
3.404 g KH_2PO_4 (monobasic)
4.355 g K_2HPO_4 (dibasic)
0.9510 g EDTA
0.9510 g EGTA
add to 400 ml ddH_2O
pH to 7.4 and top up to 500 ml with ddH_2O
2. Weigh and cut 0.1 g of skeletal muscle and add to sample buffer (1:20) in a glass test tube.
3. Homogenize.
4. Re-dilute homogenate: 50 μl homogenate + 950 μl buffer.
5. Freeze-fracture samples: freeze in -70°C freezer, remove, let thaw completely, and freeze again. Repeat this process three times.
6. Prepare Tris buffer:
100mM Tris, pH 8.3

CITRATE SYNTHASE ASSAY:

7. Turn on the plate reader, choose a kinetic protocol and set it to read for 2 minutes (6 readings, 20 seconds apart at 405 nm)
8. Prepare Oxaloacetate (OAA) (5mM):
1.321 mg dissolved in 1 ml Tris buffer

Prepare Acetyl CoA (aCoA) (3mM):
3.1 mg dissolved in 1 ml ddH_2O

Prepare 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) (5mM):
3.96 mg dissolved in 10 ml Tris buffer
9. Label a 96-well plate with sample IDs (only run a few samples at a time, as the reaction starts immediately).

10. Pipette samples in triplicate as follows:
2 μ l sample + 184 μ l Tris buffer + 2 μ l DTNB
11. Immediately before reading, add 2 μ l CoA and 10 μ l OAA
12. Record the mOD/min value (be sure the reaction is linear and reaches a plateau. Activity can be calculated with the following equation:
Where A_{405}/min is the rate, $V(\text{ml})$ is the total volume of the well, DF is the dilution factor, ϵ^{mM} is the DTNB extinction coefficient, $L(\text{cm})$ is the microplate path length, and $V_{\text{enz}}(\text{ml})$ is the sample volume.

$$\text{Activity} = \Delta A_{405} / \text{min} \frac{\Delta A_{405} / \text{min} \times V (\text{ml}) \times DF}{\epsilon^{\text{mM}} \times L (\text{cm}) \times V_{\text{enz}} (\text{ml})}$$

Appendix J: Histochemistry: Oil Red O Stain for Neutral Lipids


PROCEDURE A:

1. Obtain frozen skeletal muscle sections 10-12 μm in thickness.
2. Prepare ORO stock solution:
2.5 g Oil Red O (Sigma Aldrich, Canada)
400 ml isopropyl alcohol (99%)
stir for 2 hours
3. Prepare ORO working solution:
Mix 1.5 parts stock solution to 1 part ddH₂O
Cool in fridge
Filter through Whatman filter paper
4. Remove slides from freezer and allow to thaw to room temperature.
5. Place slides in coplin jar with working solution for 10 min to 1 hour.
6. Rinse with ddH₂O for 2-5 min.
7. Mount slides with aqueous mounting media (10% phosphate buffered saline and 90% glycerol). Aqueous mounting media is essential, using another mount may result in stain being extracted from the lipid in the sample.

PROCEDURE B:

1. Obtain frozen skeletal muscle sections 10-12 μm in thickness.
2. Circle sections with liquid blocking pen and pipette 500 μl (or enough to cover without evaporating) 0.5% ORO in propylene glycol (Sigma Aldrich, Canada).
3. Leave slides in fume hood for approximately 12 hours.
4. Rinse with ddH₂O for 2-5 min.
5. Mount slides with aqueous mounting media (10% phosphate buffered saline and 90% glycerol). Aqueous mounting media is essential, using another mount may result in stain being extracted from the lipid in the sample.

Appendix K: 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 L: Permission to Reproduce Published Materials

Published Dissertation Chapters:

Chapter 2: Metabolomic Response of Skeletal Muscle to Aerobic Exercise Training in Insulin Resistant Type 1 Diabetic Rats

Dotzert, MS; Murray MR; McDonald, MW; Olver, TD; Velenosi, TJ; Hennop A; Noble, EG; Urquhard, BL; Melling, CWJ. Metabolomic response of skeletal muscle to aerobic exercise training in insulin resistant Type 1 diabetic rats. *Scientific Reports* 6: 2016.

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From: <http://www.nature.com/srep/about/faq>

Curriculum Vitae

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Publications

- Submitted- American Journal of Physiology- Heart and Circulatory Physiology H-00371-2017: McDonald M, Olver T, **Dotzert M**, Jurrissen T, Noble E, Padilla J, Melling C. “High Intensity Aerobic Exercise Training Improves Insulin-Induced Vasorelaxation in a Vessel-Specific Manner”
- Submitted- Journal of Nanobiotechnology: Zhan D, Chen L, **Dotzert M**, Tse W, Chen L, Balaji A, Melling J, Zhang J. “Bioluminescence resonance energy transfer (BRET) based nanostructured biosensor for tear glucose detection”
- Submitted- Canadian Journal of Diabetes CJD_2017_188: **Dotzert M**, McDonald M, Murray M, Nickels J, Noble E, Melling C. “Effect of Combined Exercise Versus Aerobic Only Training on Skeletal Muscle Lipid Metabolism in a Rodent Model of Type 1 Diabetes”
- **Dotzert M**, Murray M, McDonald M, Olver T, Velenosi, T, Hennop A, Noble E, Urquhart, B, Melling, J. “Metabolomic Response of Skeletal Muscle to Aerobic Exercise Training in Type 1 Diabetic Rats”, Sci Rep 6: DOI:10.1038/srep26379, 2016.
- Abstract: **Dotzert M**, Murray M, McDonald M, Noble E, Urquhart B, Melling, J. "Metabolomic response of skeletal muscle to aerobic exercise in a model of Type 1 Diabetes", "American Diabetes Association 75th Scientific Sessions", Boston, Massachusetts, June 2015.
- Abstract: McDonald M, **Dotzert M**, Murray M, Nickels J, Noble E, Melling J. “Resistance exercise induces changes in vasorelaxation responses in insulin-treated T1D rats”, “American Diabetes Association 75th Scientific Sessions”, Boston, Massachusetts, June 2015.

Abstracts

- **Dotzert M**, Murray M, McDonald M, Noble E, Urquhart B, Melling J. “Metabolomic response of skeletal muscle to aerobic exercise in a model of Type 1 Diabetes”, American Diabetes Association 75th Scientific Sessions, Boston, Massachusetts, June 2015.
- McDonald M, **Dotzert M**, Murray M, Nickels J, Noble E, Melling J. “Resistance exercise induces changes in vasorelaxation responses in insulin-treated T1D rats”, American Diabetes Association 75th Scientific Sessions, Boston, Massachusetts, June 2015.
- **Dotzert M**, Gawinski K, Gurd B, Kenno K, and Milne K. “The effects of interval bout intensity on memory consolidation in young adult men and women”, “Canadian Society for Exercise Physiology”, Quebec QC, October 2011, Appl. Physiol. Nutr. Metab. Vol. 36, 2011, S313
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Scholarships

- Ontario Graduate Scholarship, Western University, May 2016-April 2017
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Work Experience

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