Smooth Muscle Cell Mitochondrial Respiration – Effects of Aerobic Training and Type I Diabetes

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ABSTRACT

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Dana-Rae Reguis Yadao

Cardiovascular disease is the leading cause of death among diabetic patients, due mostly to mitochondrial dysfunction and resulting oxidative stress. Endurance exercise has been firmly established as a way to reduce diabetes and cardiovascular disease morbidity and mortality. The importance of mitochondria for both training performance and health underlies the significance of better understanding the factors that regulate exercise-induced adaptations in mitochondrial network dynamics. The effects on mitochondrial skeletal and cardiac muscle have already been well documented in the past. However, the specific impact of aerobic training on respiratory capacity in the vasculature remain to be established. Furthermore, the relationship between aerobic exercise and mitochondrial function in diabetic patients has yet to be investigated. We hypothesized that a long-term aerobic exercise intervention would enhance mitochondrial respiratory capacity in vascular smooth muscle cells. First, we investigated the effects of training in the vasculature of healthy mice (C57BL/6) and compared them to their sedentary counterparts. We found that exercise stimulates a rise in vascular mitochondrial content and respiratory capacity, especially complex I respiration ($p < 0.05$). However, when comparing diabetic trained mice to diabetic sedentary controls, it was found that aerobic training induced a downregulation in mitochondrial protein expression as well as a decrease in complex I respiration. In these experiments, our findings suggest that despite the demonstrated benefits of exercise, careful measures should be taken when exposing diabetic models that carry an inherently high risk of cardiovascular disease to prolonged exercise.

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I

Introduction

Pathophysiology of Diabetes Mellitus

Diabetes mellitus is a metabolic disease that is one of the leading causes of death worldwide [American Diabetes Association, 2010]. It is estimated that 193 million individuals globally remain undiagnosed, which consequently increases their risk of developing severe complications of untreated diabetes [American Diabetes Association, 2010]. Currently, 11 million Canadians are living with diabetes and the prevalence is expected to rise to more than one in three by the year 2020 [Diabetes Canada, 2017]. All diabetes-related complications cost the Canadian healthcare system $11.7 billion in 2010, an expenditure projected to rise [Diabetes Canada, 2017].

Diabetes mellitus (DM) is classified into different categories, with Types I and II being the most common. All types of diabetes, though different in etiology, are characterized by chronic hyperglycemia [American Diabetes Association, 2010]. Type I diabetes (T1DM) is most often caused by an autoimmune process or a trauma that destroys the insulin-producing ß-cells in the pancreas, resulting in a lack of insulin [Matough, 2012]. T1DM is regarded as the more severe form of diabetes, as the disease typically begins at a young age with intensive daily self-management and exogenous insulin therapy required for effective glucose control and better quality of life [Peasgood *et al.,* 2016]. Consequently, poorly regulated T1DM can lead to life-threatening acute metabolic complications such as hypoglycemia and diabetic ketoacidosis (DKA) [Ceriello, 2003; Peasgood *et al.,* 2016]. Type II diabetes mellitus (T2DM) is the more prevalent form of diabetes, representing up to 90% of all cases [Diabetes Canada, 2017]. T2DM is triggered by peripheral insulin resistance and impaired fasting glucose due to a combination of genetic and lifestyle factors, namely overnutrition and physical inactivity [Hu, 2001]. Common risk factors for the development of T2DM include hypertension, lipid concentrations and impaired fasting glucose [American Diabetes Association, 2010]. As such, T2DM is generally managed by patients through careful meal planning, regular physical activity and medications [Diabetes Canada, 2017].

Despite their differences in etiology, both T1DM and T2DM result in chronic hyperglycemia with subsequent abnormal changes in the metabolism of macronutrients [Matough, 2012]. Studies on the adverse effects of diabetes on specific tissues are extensive, especially in skeletal muscle given that it is the largest site for glucose uptake, namely through the action of transport proteins GLUT1 and GLUT4 [DeFronzo *et al.,* 1981]. In T1DM specifically, impaired translocation and activation

of glucose transporters, particularly GLUT4, was observed in the vastus lateralis muscle [Kahn *et al.,* 1992]. Moreover, insulin delivery to skeletal muscles are decreased in diabetes, with myopathy recognized as a severe complication [D'Souza, 2013; Hernández-Ochoa *et al.,* 2017]. With respect to cardiac muscle, myocardial performance is impaired via a reduced capacity for glucose transport, indicating a greater risk of diabetic cardiomyopathy and related cases of cardiovascular mortality [Rodrigues, 1998; Stanley, 1997]. Overall, the common co-morbidities of poorly regulated diabetes include neuropathy, nephropathy, vision disorders and cardiovascular disease [Ceriello, 2003]. T1DM, however, is considered the more severe form of diabetes as patients have a reduced life span due to hyperglycemia-induced cardiovascular disease and acute metabolic complications secondary to exogenous insulin therapy, specifically life-threatening ketoacidosis [Warnes *et al.,* 2018]. Moreover, patients with T1DM often endure irreversible changes leading to long-term health issues as the disease most often occurs in childhood or adolescence, a period where growth is critical [Mozdziak, 2000]. Despite advancements in the treatment of diabetes, cardiovascular disease is leading cause of mortality among all diabetic patients [Cai, 2001]. Consequently, 80% of Canadians with diabetes will die of myocardial infarction or stroke [Stehouwer, 1997; Diabetes Canada, 2017].

Diabetes and the Vasculature

Nearly all tissues depend on a blood supply, which in turn depends on endothelial cells. Endothelial cells make up the innermost lining of blood cells and have the ability to directly detect changes in the blood, such as O_2 availability, pH, shear stress and levels of inflammation [Alberts, 2002]. The endothelium produce and release a variety of vasoactive substances that act on surrounding layers of the blood vessel, namely vascular smooth muscle, in order to maintain regulation of vascular homeostasis [Hu *et al.,* 2018]. Vascular smooth muscle cells provide vasoactive control as well as structural integrity through the action of contractile proteins [Metz *et al.,* 2012]. Disturbances to endothelial properties may lead to alterations in permeability, vascular tone and cell-matrix interactions [Stehouwer, 1997]. When the endothelium loses its' capacity to properly detect changes in the vascular environment and relay signals to vascular smooth muscle cells, endothelial dysfunction ensues, leading to the development of cardiovascular complications [Hu *et al.,* 2018]. The impaired metabolism of glucose and insulin during the diabetic state is involved in endothelial

dysfunction, which is an early marker of damage to blood vessels. [Ceriello, 2003; Schalkwijk, 2005].

Diabetes-induced hyperglycemia is closely linked to the progression of endothelial dysfunction, resulting in coronary heart disease, peripheral arterial disease, diabetic nephropathy, neuropathy and retinopathy [Giacco, 2010; Hu *et al.,* 2018]. Changes in the microvasculature (e.g. capillaries) include the synthesis of extracellular matrix proteins and expansion of the capillary basement membrane, which are key features of diabetic microangiopathy [Scheede-Bergdahl, 2014; Chawla, 2016]. Microvascular complications also lead to retinopathy, nephropathy and peripheral neuropathy, with blindness and renal failure as major consequences [Cade, 2008; Stehouwer, 1997]. The progression of macrovascular (e.g. arteries and veins) complications is indicated by an accelerated form of atherosclerosis, thus increasing the risk of ischemic heart disease, stroke and myocardial infarction [Cade, 2008; Brownlee, 2005].

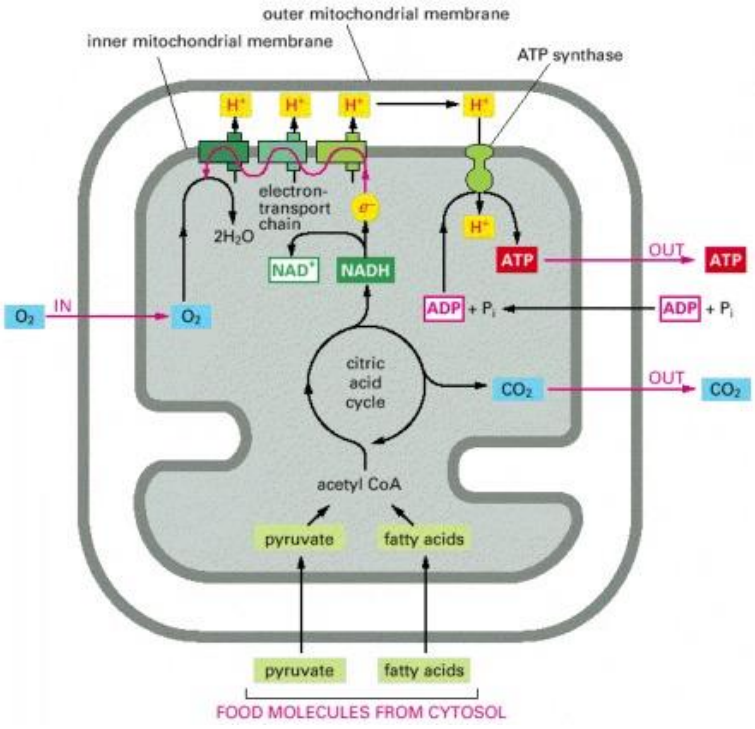
Various theories have been proposed to explain how endothelial cells exposed to high glucose can cause extensive structural and metabolic changes to the cardiovascular system. Many hypotheses focus on the dysregulation of intracellular metabolism of glucose [Brownlee, 2005]. Consequently, the function of vascular cells is significantly altered through various pathways, which will be elaborated in upcoming sections. In brief, all of these pathways are manifestations of a single phenomenon: oxidative stress [Cade, 2008]. Therefore, mitochondrial dysfunction, when derived from chronic hyperglycemia, is the primary mediator in the progression of vascular damage in diabetic patients [Ceriello, 2003].

Mitochondria

Mitochondria make up a significant portion of the cytoplasmic volume in nearly all living eukaryotic cells and is the primary site of ATP production. [Chiong *et al.,* 2014; Alberts *et al.,* 2002]. Mitochondria carry out several important tasks including the production of reactive oxygen species (ROS), contractile function, calcium signaling and apoptosis programming [Knaub *et al.,* 2014]. ATP is produced through oxidative phosphorylation, the process by which substrates derived from glucose and fatty acids via glycolysis and beta oxidation are metabolized, resulting in Acetyl-coenzyme A [Alberts *et al.,* 2002]. Next, a series of redox reafdctions in the tricarboxylic

acid cycle occurs to form high-energy molecules NADH and $FADH_2$, which then carry electrons to mitochondrial complexes I and II respectively, with parallel proton transfer outward from the intermembrane space to complex I, III and IV (Fig. 1) [Alberts *et al.*, 2006]. This is offset by proton transfer in the opposite direction, known as proton leak [Sivitz and Yorek, 2010]. The resulting potential difference ultimately drives ADP phosphorylation to ATP via complex V [Duchen, 2004].

Figure 1: Summary of oxidative phosphorylation for ATP production by substrate metabolism



Pyruvate and fatty acids derived from glycolysis and beta-oxidation enter the mitochondrion (bottom) and produce acetyl CoA, which enters the citric acid cycle and allows for the reduction of NAD^+ to NADH, a high-energy molecule that serves as an electron carrier. Electrons are transferred along the electron-transport chain to O_2, and generates a proton gradient across the inner membrane, thus driving the production of ATP via ATP synthase [Alberts et al., 2002].

ATP is essential to sustain growth, metabolism and reproduction [Duchen, 2004]. As such, nearly all living cells rely on ATP for their survival. Mitochondrial function can be affected by several factors, such as respiratory capacity, generation and detoxification of ROS as well as regulation of mitochondrial matrix calcium, with abnormalities in any of these linked to dysfunction. However, given that the predominant physiological function of mitochondria is the production of ATP, impaired respiratory capacity and oxygen consumption are generally considered determinants of dysfunction. Given the importance of these organelles in mediating nearly all cellular processes, strong emphasis is placed on understanding mitochondrial function and the pathogenic processes involved in disease and ageing. The centrality of mitochondrial dysfunction in a wide range of

major diseases is becoming the focus of many epidemiological studies, thus highlighting the prospect of novel therapeutic approaches to treat a multitude of highly prevalent diseases [Duchen, 2004].

Mitochondrial Dysfunction and Diabetes

Mitochondrial respiration is a predominant source of reactive oxygen species (ROS), which are toxic agents derived from molecular oxygen (O_2) [Addabbo, 2009; Liochev, 2013]. ROS generation is important because it contributes to mitochondrial dysfunction and subsequent oxidative damage seen in various pathologies [Murphy, 2009]. For instance, excessive ROS production is one of the driving forces of insulin resistance and increased lipid accumulation in skeletal muscle [Petersen *et al.,* 2003]. As a predominant site for energy production, it is not surprising that mitochondria are involved in many aspects of diabetes, including etiology, complications, management and prevention. Indeed, the relationship between insulin resistance and mitochondrial function is complex and is characterized by a decrease in mitochondrial ATP production, lowered mitochondria RNA and reduced oxidative phosphorylation [Petersen *et al.,* 2003]. While mitochondrial function is generally assessed by respiratory capacity, it is also important to note that evidence exists that the metabolic disease is also responsible for causing defects in mitochondrial biogenesis, number, morphology, fusion and fission [Sivitz and Yorek, 2010].

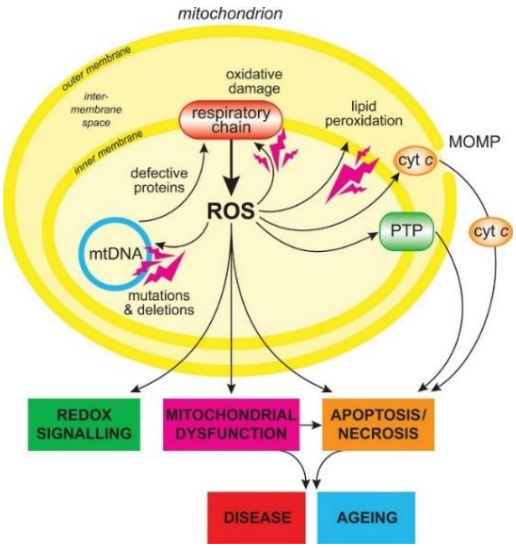


Figure 2: Mitochondrial dysfunction and disease as consequences of excessive ROS production

Mitochondrial dysfunction is linked to ROS production, which causes damage to proteins, DNA and activates cell death via the release of intermembrane space proteins such as cytochrome c (cyt c). While low levels of ROS can contribute to regulating biological and physiological processes via redox signalling, excessive ROS can lead to a variety of pathologies related to mitochondrial dysfunction and apoptosis/necrosis, including disease and ageing [Murphy, 2009].

Greater degrees of oxidative stress and respiratory incapacities occur in diabetes, which promotes abnormal gene expression, altered cell signalling and the activation of pathways leading to cell death [Cai, 2001]. While this can result from several mechanisms, abnormal mitochondrial respiration plays a key role [Panth, 2016]. In hyperglycemic states, endothelial cells are unable to efficiently control glucose transport compared to other cells, making them susceptible to oxidative-induced damage [Ceriello, 2003]. The mitochondria are consequently supplied by a greater flux of glycolysis-derived pyruvate to the tricarboxylic acid cycle [Giacco, 2010]. This creates an overload of electron donors, driving the mitochondrial membrane potential upwards, which subsequently increases ROS generation at the complex II level in the respiratory chain [Bajaj, 2012].

Mitochondrial Pathways Leading to Vascular Complications

When endothelial cells are exposed to high glucose levels, the overproduction of ROS by the mitochondria triggers the activation of four specific pathways underlying the development of vascular complications: activation of protein kinase C (PKC); increased advanced glycation end-product (AGE) formation; and greater glucose flux both through both the polyol and hexosamine pathways [Giacco, 2010].

Firstly, the chronic activation of PKC leads to vascular disturbances such as increased permeability, contractility and cell death [Geraldes, 2011]. The changes caused by PKC activation are linked to the development of atherosclerosis and cardiomyopathy [Geraldes, 2011]. Secondly, AGE form as a result of a process known as protein glycation, where glucose molecules and plasma proteins form irreversible covalent bonds [Singh, 2014]. This process promotes the pro-inflammatory response as well as interferes with normal molecular conformation and receptor functioning, which in turn can lead to neuropathy, nephropathy and cardiomyopathy [Scheede-Bergdahl, 2004; Singh, 2011]. Increased formation of AGE leads to greater ROS generation and can deactivate local antioxidant systems [Sugimoto, 2008]. AGE's also induce microvascular disease through the expansion of the capillary basement membrane (CBM) [Scheede-Bergdahl, 2014]. Thirdly, an increased glucose flux into the polyol pathway results in the accumulation of ROS in the tissues, including the heart and vasculature [Tang 2012]. Finally, an overload of glucose into the hexosamine pathway inhibits endothelial nitric oxide synthase (eNOS), which is crucial in

maintaining vascular tone [Giacco, 2010]. Therefore, the continuous production of ROS in endothelial cells exposed to high glucose has deleterious effects on the vasculature and thus significantly increases the risk for cardiovascular disease.

Complications linked with abnormal mitochondrial respiration and oxidative stress are not limited to the vasculature. Skeletal and cardiac muscles are also negatively affected by the same pathways that damage the vasculature in diabetes. Much research has already been done to explore the topic of diabetes-induced cardiomyopathy, with leading theories based on altered energy metabolism resulting in mitochondrial dysfunction within the myocytes [Brahma, 2017]. One study concluded that mitochondrial dysfunction defined by a reduction in complex III activity in the electron transport chain resulted in increased ROS generation and damage to cardiomyocytes [Suematsu *et al.,* 2003]. Further, impaired muscle force output, greater fatigue rates and reduced local blood flow occur due to excessive ROS production [Kelley, 2002]. Another study found that decreased mitochondrial respiratory capacity, reduced ATP synthesis and greater ROS production lead to skeletal muscle insulin resistance and subsequent T2DM [Pagel-Langenickel *et al.,* 2010]. Thus, mitochondrial dysfunction underlies all the major pathogenic pathways leading to diabetic complications in the vasculature, skeletal and cardiac muscles.

Figure 3

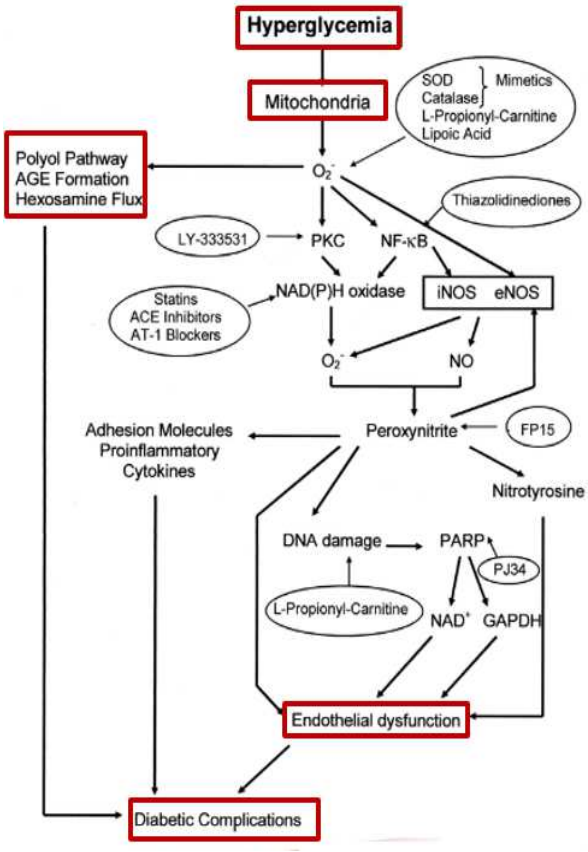


***Figure 3** : Hyperglycemia-induced diabetic complications begin at the mitochondrial level with the activation of four pathways: chronic activation of protein kinase C (PKC), AGE formation, and increased glucose flux into both the polyol and hexosamine pathways. This process causes endothelial dysfunction, ultimately leading to diabetes-specific vascular complications [Adapted from Ceriello, 2003].*

Exercise and Diabetes

Oxidative stress occurs continuously as part of metabolic processes, including the contraction of skeletal muscles during exercise, where a greater amount of oxygen is required to produce ATP [Lobo, 2010]. As mentioned previously, ROS triggers oxidative reactions with proteins, lipids and DNA, thus leading to detrimental effects. In contrast to the prolonged oxidative stress that occurs in diabetes, an acute and normal increase in free radical generation is observed during exercise [Urso, 2003]. Predominant sources of ROS in the muscle are produced by the mitochondria, specifically through a leak of single electrons leading to the generation of superoxide ($O_2^{\bullet-}$) [Steinbacher, 2015]. It is generally accepted that exercise promotes low-to-moderate levels of ROS, which can have some benefits, including regulation of cell signaling and skeletal muscle force production [Powers, 2008]. It has been reported that the human body can effectively regulate exercise-induced oxidative stress, with previous studies suggesting that a modest increase in ROS can facilitate muscle adaptation [Jackson, 1999]. This regulation is due in part to the activation of

endogenous antioxidant systems and their ability to adequately prevent oxidative stress [Powers, 2008].

Exercise has long been understood to provide many health-promoting benefits for people and without diabetes. Indeed, long-term physical activity is encouraged by therapists as a non-pharmacological approach to effectively regulate diabetes-related health issues. The goals of exercise therapy for patients with diabetes include the reduction of hyperglycemia, insulin resistance, dyslipidemia and hypertension, all of which translate into a lower risk of vascular disease in children, adolescents and adults [Sato *et al.,* 2007]. With regards to T1DM specifically, special considerations are made for the pediatric population, with difficulties adhering to a strict diabetes regimen as a common setback [Silverstein *et al.,* 2005]. Endurance exercise in particular has been firmly established as a way to reduce diabetic complications and cardiovascular disease morbidity and mortality. However, existing data regarding the effects of exercise training in T1DM patients are far less extensive than it is for T2DM patients. Strong evidence shows that both aerobic and resistance exercise can improve insulin action, and therefore promote glucose uptake into local tissues in T2DM [Colberg, 2010]. One study found that endurance training stimulated an increase in VO_{2max} in patients with T2DM, accompanied by an increase in both mitochondrial content and function [Phielix *et al.,* 2010]. This is essential as mitochondrial dysfunction is heavily implicated in the pathophysiology of disease such as diabetes. In cardiac muscle, increased substrate metabolism and enhanced mitochondrial dynamics within myocytes have been reported. However, significant increases in ROS during aging and/or disease can cause contractile impairments and muscle atrophy, which in turn can lead to muscle weakness and fatigue [Steinbacher, 2015].

Given the beneficial effects of exercise on the cardiovascular system and the evidence that T1DM patients present a higher risk of developing vascular complications, an exercise program specific to reducing the risk factors for cardiovascular disease at the level of vascular smooth muscle would be valuable. More importantly, there is a need to investigate the effects of aerobic exercise on mitochondrial function of the vasculature, considering that the mitochondria is deeply involved in the progression of diabetes.

Project Overview

Cardiovascular disease as the leading cause of mortality among the diabetic population. The benefits of regular physical activity on diabetes and cardiovascular disease are firmly established. However, the risk for mitochondria-mediated vascular diseases remains significant, even in the presence of rigorous glycemic control, including diet and exercise. It was recently reported that impaired mitochondrial function in the context of reduced ATP synthesis in T2DM patients also extends to metabolically well-controlled patients [Szendroedi *et al.,* 2007]. Indeed, mitochondrial dysfunction is considered to be critically involved in the pathophysiology of diabetes, and exercise has been regarded as a way to stimulate favourable adaptations such as mitochondrial biogenesis and enhanced respiratory capacity.

Despite evidence affirming the role of exercise in stimulating enhanced mitochondrial dynamics in skeletal and cardiac muscle, surprisingly little is known about the vasculature. The main objective of this thesis is to provide novel insight on the specific impact of aerobic training on mitochondrial respiratory capacity in both healthy and untreated T1DM vessels. Existing data on oxidative capacity of vascular smooth muscle cells in response to training is limited. In our first experiment, we hypothesized that exercise will promote mitochondrial respiration in the vasculature of healthy mice. Next, in a separate study, we examined the same relationship in streptozotocin-induced T1DM mice. We hypothesized that exercise would improve mitochondrial respiration of vascular smooth muscle cells in untreated T1DM mice in response to training. Following an aerobic exercise programme over four weeks, respiratory capacity will be assessed in permeabilized and intact aortic vessels from mice. Immunoblotting will determine mitochondrial content and relative protein levels. High-resolution respirometry will allow us to examine each mitochondrial complex as well as determine the efficiency of oxidative phosphorylation based on oxygen consumption of different respiration states.

Diabetes is quickly becoming one of the biggest health epidemics of the 21st century. The rise in the incidence of metabolic diseases worldwide has driven scientific research with the aim of identifying new adjunct therapy for both T1DM and T2DM. Together with the findings outlined in our review paper on the efficacy of selectively reducing branched-chain amino acid intake in reducing metabolic complications of obesity and T2DM, this thesis can contribute to current

research in identifying new molecular targets that may prove amenable to therapies addressing mitochondrial function to reduce the overall risk of cardiovascular disease in poorly regulated T1DM patients.

II

Aerobic Training Increases Respiratory Capacity in Vascular Mitochondria

Dana-Rae Reguis Yadao, Andreas Bergdahl and Stephanie MacKenzie

Contributions

Dana-Rae Reguis Yadao: preparation of manuscript, experimental techniques

Andreas Bergdahl: editing of manuscript, experimental techniques

Stephanie MacKenzie: experimental techniques

Abstract

Mitochondria are the primary site of ATP production, and its' function is heavily implicated in cardiovascular disease and metabolic disorders such as diabetes. Exercise-induced mitochondrial adaptations include enhanced network morphology, increased protein expression and augmented respiratory capacity, all of which are strongly correlated with greater substrate oxidation, energy metabolism and overall training performance. Thus, the opportunities for effective exercise therapy interventions are significant. To date, most studies examining the relationship between mitochondrial network dynamics and exercise have focused largely on skeletal and cardiac muscle. As a result, little has been reported on mitochondrial respiratory capacity in the vasculature, specifically in response to chronic exercise. We hypothesized that a 4-week aerobic training intervention in mice would elicit greater vascular oxidative capacity compared to sedentary counterparts. After performing high-resolution respirometry in aortic vessels, we found that the trained mice group exhibited a significant increase in the respiration of complex I-linked, glucose-based substrates ($p<0.05$). Additionally, immunoblotting demonstrated a trend towards upregulation in relative mitochondrial protein expression in all complexes (I-V) as well as in mitochondrial density. Understanding the importance of exercise-induced mitochondrial adaptations in the vasculature is vital to developing new therapeutic treatments of vascular diseases that stem from mitochondrial dysfunction.

Introduction

Mitochondria are important organelles that make up approximately 10% of the human body weight and carry out a variety of cellular functions necessary to sustain growth, metabolism and reproduction [Duchen, 2004; Park *et al.*, 2014]. Mitochondria are also involved in the generation of reactive oxygen species (ROS), contractile function, calcium signaling and apoptosis programming [Knaub *et al.,* 2014]. However, its' best-known role is the production of ATP via mitochondrial respiration. The importance of mitochondria in mediating nearly all cellular processes underlies the premise that mitochondrial function is central in understanding the pathogenic processes of various diseases. Indeed, poor oxidative capacity due to a decline in mitochondrial content and function is associated with aging, insulin resistance and diabetes [Menshikova, 2006].

While mitochondria are found in nearly all cells of the body, they have different implications in different tissues, and their capacities vary depending on certain metabolic demands. For instance, mitochondria found in cardiac muscle represent approximately 35% of the total tissue volume, compared to only 4-7% in skeletal muscle [Stride *et al.*, 2013]. Cardiomyocytes contain a particularly high concentration of mitochondria to continuously provide energy supplies needed for oxygen transport, even producing up to 90% of ATP by beta-oxidation at rest [Song *et al.*, 2014]. For skeletal muscle, the ATP requirement during exercise increases dramatically to allow for contraction and locomotion. Despite their differences in mitochondrial content, the increased metabolic demands of exercise require both the cardiovascular and musculoskeletal systems to work together, and the mitochondria within each distinct muscle respond similarly. The efficiency of cardiac, skeletal and vascular smooth muscle to perform cellular respiration determines performance during exercise [Park *et al.*, 2014].

Mitochondria undergo extensive biochemical and morphological adaptations in response to exercise training. These changes are correlated with improved oxidative capacity and metabolism, both of which are considered determinants for enhanced performance. Mitochondrial function can be assessed by measuring state 3 respiration (oxygen consumption activated by ADP) and the

respiratory control ratio (RCR), which is the ratio of state 3 to state 4 respiration and provides an index of mitochondrial coupling as well as the efficiency of ATP formation during complex I+II respiration [Madsen *et al*., 1996; Sivitz and Yorek, 2010]. Generally, a reduction in RCR is associated with mitochondrial dysfunction and inefficiency [Boudina *et al.,* 2007]. One study demonstrated an increase in RCR due to an increase in state 3 respiration in cardiac mitochondria following prolonged exercise [Madsen *et al.,* 1996]. Another study highlighted the importance of endurance exercise in the protection of the heart against cardiac injury based on the modulating effects of mitochondria [Ascensão *et al.,* 2006]. With regards to skeletal muscle, lower fatigue rates and greater substrate metabolism in trained individuals have been found to be associated with greater respiratory capacity [Hawley *et al.*, 2002]. In addition, it has been established that training stimulates an increase in mitochondrial content and function in skeletal muscle [Russel *et al.,* 2014]. To date, little is known about the effects of a long-term exercise programme on vascular mitochondria. Given the established relationship between exercise and augmented mitochondrial bioenergetics of cardiac and skeletal muscle, it is possible that similar benefits can also be observed in the vasculature. Thus, the present study used a novel approach to investigate how mitochondrial respiratory capacity of vascular smooth muscle cells in healthy subjects is affected by aerobic training.

Materials and Methods

Animal Care

Healthy, age-matched (2-4 months) male C57BL/6 mice were obtained from the Concordia University breeding colony. Mice were individually housed in a 22°C room with a constant 12-hour light/dark cycle and access to rodent chow and water. Mice were randomly assigned into two groups: Normal sedentary, which served as the controls (CON), and the Exercise group (EX). Measurements of body mass (g) as well as consumption of food (g) and water (ml) were obtained once a week. All procedures were approved by the Concordia University Animal Research Ethics Committee and certified by the Canadian Council on Animal Care.

Aerobic Exercise Intervention

The EX group performed aerobic exercise three days a week for four weeks, for a total of twelve days. Exercise was carried out using an in-house built, customized, graded rodent treadmill. The protocol consisted of two phases: acclimation and training. During acclimation (day one), mice were placed on the treadmill for three minutes to explore it [Bouganim and Bergdahl, 2017]. Next, the motor was turned on at zero speed for approximately two minutes to introduce the mice to the sounds of the treadmill [Bouganim and Bergdahl, 2017].

The training phase consisted of increasing the speed from 3, 5, 8 to 12 m/min at 0-2, 2-4, 4-6 and 6-15 minutes respectively (Table 1). Each day, the session increased by 5 minutes (starting at 15 minutes), until a total training time of 60 minutes was reached (day ten). For instance, mice were trained for 15 minutes on day one, followed by 20 minutes on day two, 25 minutes on day three, and so on. This pattern was maintained until the end of the protocol. To encourage mice to remain active on the treadmill, brushes were placed at the back of each lane to tickle their tails. When mice were unable to keep up with the treadmill, the exercise session was terminated.

Table 1: *Days 1 and 2 of Aerobic Exercise Protocol for EX Group*

	Time (min)	Speed (m/min)
Day one	0-2	3
	2-4	5
	4-6	8
	6-15	12
Day two	0-2	3
	2-4	5
	4-6	8
	6-20	12

Tissue permeabilization and preparation

Mice were euthanized by CO_2, after which aortic vessels were harvested and immediately stored in an ice-cold buffer solution (BIOPS) containing the following in mmol/L: CaK_2EGTA 2.77, K_2EGTA 7.23, Na_2ATP 5.77, $MgCl_2 \cdot 6H_2O$ 6.56, taurine 20, phosphocreatine 15, imidazole 20, dithiothreitol 0.5, MES 50, pH 7.1 After removal of adventitial fat tissue, vessels were opened longitudinally and denuded of endothelium in ice-cold BIOPS buffer with a sponge. Tissues were then broken into smaller pieces and permeabilized in 2 ml BIOPS buffer containing 50µg/ml of saponin for 30 minutes, followed by two washes in 2 ml of ice-cold Mir05 buffer for 10 minutes each [Larsen *et al.,* 2015]. Mir05 buffer contains (in mmol/L): EGTA 0.5, $MgCl_2 \cdot 6H_2O$ 3.0, K-lactobionate 60, Taurine 20, KH_2PO_4 10, HEPES 20, sucrose 110, BSA 1g/l, pH 7.1.

Measurements of mitochondrial respiration

High-resolution respirometry was performed to quantify mitochondrial function using a polarographic oxygen sensor (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). Permeabilized tissue (3-5 mg wet wt) were incubated in the Oxygraph chambers with 2 ml of Mir05 buffer each and stirred continuously at 37 °C for 15-20 minutes to establish a baseline in the absence of respiratory substrates. Hyper-oxygenated conditions were maintained to prevent potential O_2 diffusion limitations. Next, for each mitochondrial complex to be studied independently, the following substrates were injected sequentially in each chamber in accordance with the protocol described by Larsen *et al.* [2015]: malate (2 mmol/L); pyruvate (2 mmol/L); glutamate (10 mmol/L); ADP (5 mmol/L); 20 µl succinate (10 mmol/L) ; oligomycin (2 µg/ml) and antimycin A (2.5 µM) . The presence of malate + pyruvate + glutamate allowed for complex I, state 2 respiration to be examined, which represents the ADP-restricted, non-phosphorylating basal resting state. The addition of ADP allowed for complex I, state 3 to be analyzed, which provided an index of oxidative phosphorylation (OXPHOS). Succinate allowed for maximal state 3 respiration to be achieved, with simultaneous activation of Complex I and Complex II. Finally, inhibitors oligomycin and antimycin A were injected to block Complexes V (LEAK) and III respectively. Respiration rates and O2 consumption were recorded and analyzed using the Oroboros DatLab software. In each condition, respiration rates were recorded for 3-5 minutes, with the average of the last minute used for data analysis.

Immunoblotting and immunofluorescence

Protein levels were assessed following the protocol described by Rocha *et al.* [2014]. Cell lysis of aortic vessels was performed in a lysis buffer containing (in mmol/L): NaCL 250, HEPES 50, glycerol 10%, Triton X-100 1%, $MgCl_2$ 1.5, EGTA 1, $Na_4P_2O_7$ 10, NaF 1, Na_3VO_4 800 μmol/L, pH 5. The solution was centrifuged for 10 minutes at 10,000 *g,* after which the supernatant was extracted. The Pierce BCA Protein Assay Kit (Thermo Scientific, Mississauga, Ontario, Canada) was used to assess protein levels with 10 μg of protein samples first separated on a 12.5% SDS-PAGE and then transferred to a nitrocellulose membrane (0.45 μm, 162-0115, Bio-Rad) with 10 mmol/L sodium tetraborate buffer. 5% BSA in TBS-T buffer (10 mmol/L Tris-HCL, pH 7.5, 150 mmol/L NaCl, 0.05% Tween 20) was used to block the membranes for 1 hour at room temperature, followed by an overnight incubation period at 4 ºC with the antibody for voltage-dependent anion channel (VDAC; 1:1000, ab14734 Abcam) as well as total OXPHOS rodent antibody cocktail (1:500, MS604 MitoSciences). The resulting blots were washed and incubated with horseradish-peroxidase-conjugated secondary antibodies (anti-mouse, ab6728 Abcam). Relative protein expression was visualized and studied using a chemiluminescence system (Immun-Star Chemiluminiscent, 1705070; Bio-Rad, Mississauga, Ontario, Canada) and bands analyzed with ImageJ software.

Statistics

Given the comparison between the DE and CON groups, a two-tailed Student's *t* test was used for data analysis, where $P < 0.05$ was considered significant. Immunoblotting data and all other figures were presented as means ± SE.

Results

Mitochondrial density

Immunoblotting using an antibody specific to the voltage-dependent anion channel (VDAC) was performed to control for differences in mitochondrial content between the EX and CON groups. The EX group displayed a non-significant increase in mitochondrial protein density compared to CON, indicating that mitochondrial content was increased in the EX group in response to aerobic training.

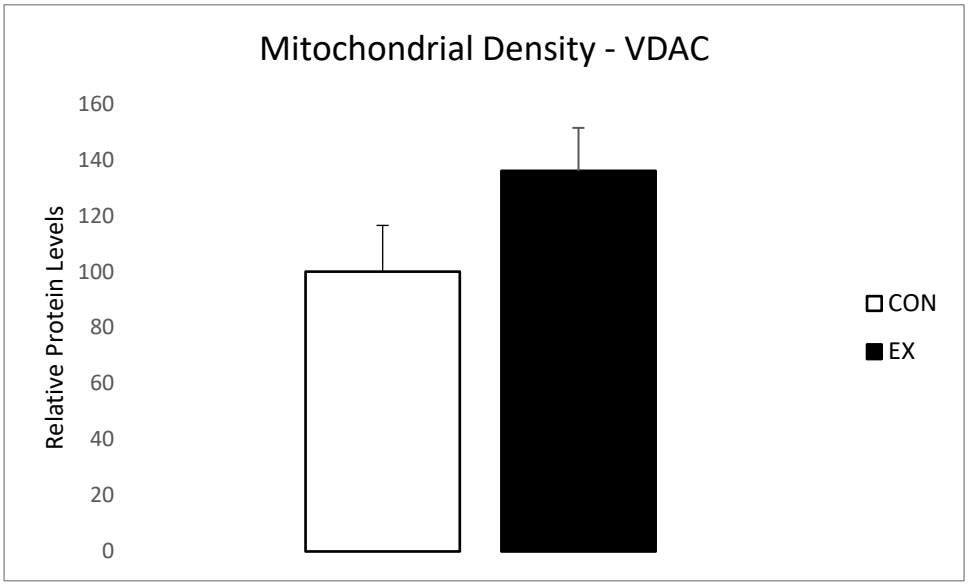


Figure 1: *Immunoblots reveal that the EX group (n=10) exhibited an increase in mitochondrial density compared to CON (n=7) in response to a long-term, 4-week aerobic training programme (EX:135.99% ± 15.38% versus CON: 100% ± 16.52%).*

Mitochondrial respiration

To examine mitochondrial function and respiratory capacities, oxygen consumption rates of permeabilized tissues were measured via high resolution respirometry. OXPHOS capacity in the EX group were all significantly higher compared to CON following the addition of three different Complex I-linked, glucose-based substrates. Respiration rates after the injection of malate was higher in the EX group compared to CON (EX: 1.39 ± 0.23 versus CON: 0.80 ± 0.12, $p<0.05$ with $n_{ex} = 10$ and $n_{con} = 7$), as shown in Fig. 2a. This indicated an increase in basal, ADP-restricted

respiration. Additionally, Fig. 2b displays a similar significant increase in respiration in the EX group following the addition of pyruvate (EX: 2.34 ± 0.27 versus CON: 1.35 ± 0.21, $p < 0.05$). Finally, the addition of glutamate (Fig. 2c) also stimulated a significant increase in respiration in the EX group compared to CON (EX: 2.29 ± 0.26 versus CON: 1.35 ± 0.28, $p < 0.05$).

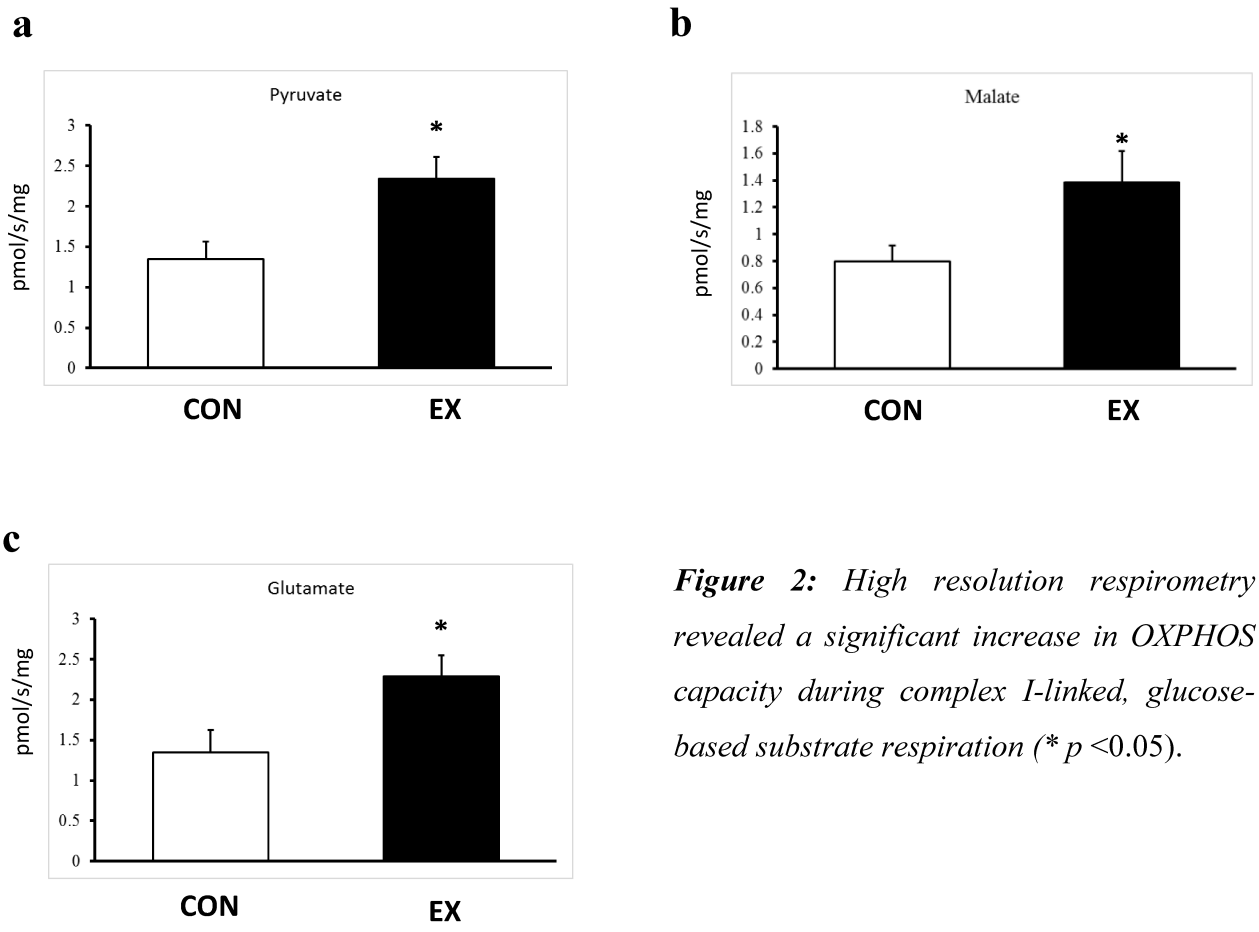


Figure 2: *High resolution respirometry revealed a significant increase in OXPHOS capacity during complex I-linked, glucose-based substrate respiration (* p <0.05).*

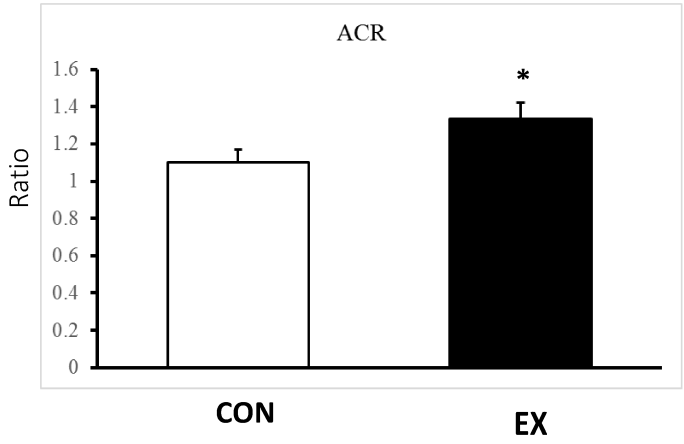


Figure 3: *The acceptor control ratio (ACR) was significantly higher in the EX group compared to CON (* $p<0.05$), signifying enhanced coupling between oxidation and phosphorylation during complex I activation in trained subjects.*

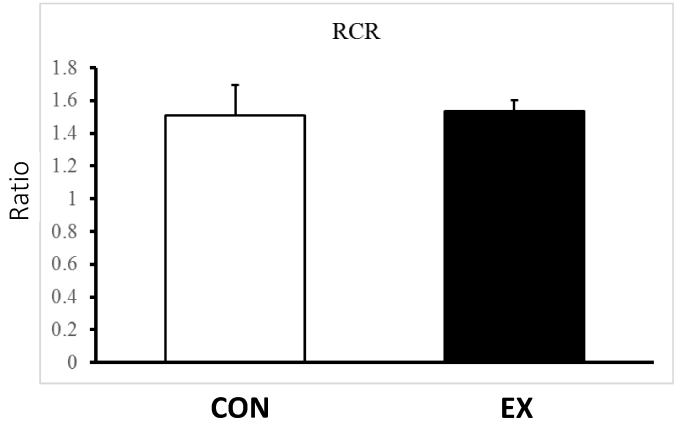


Figure 4: *No detectable differences were observed in the respiratory control ratio (RCR) between both groups, indicating that the coupling between O_2 consumption and oxidative phosphorylation during Complex I+II activation was unaffected in response to aerobic training.*

The acceptor control ratio (Fig. 3), representing the coupling between oxidation and phosphorylation when Complex I is activated, was significantly upregulated in the EX group when compared to CON (EX: 1.34 ± 0.09 versus CON: 1.10 ± 0.07, $p<0.05$). However, the respiratory control ratio (Fig. 4), which is an index of the coupling between O_2 consumption and oxidative phosphorylation in Complex I+II only, was unaffected in the EX vessels when compared to CON (EX: 1.53 ± 0.07 versus CON: 1.51 ± 0.19), indicating no change in substrate oxidation capacity and ATP turnover with regards to Complex II. Fig. 5 reveals that Complex I+II, state 3 respiration was significantly increased in the EX group, as assessed by the addition of the substrate succinate.

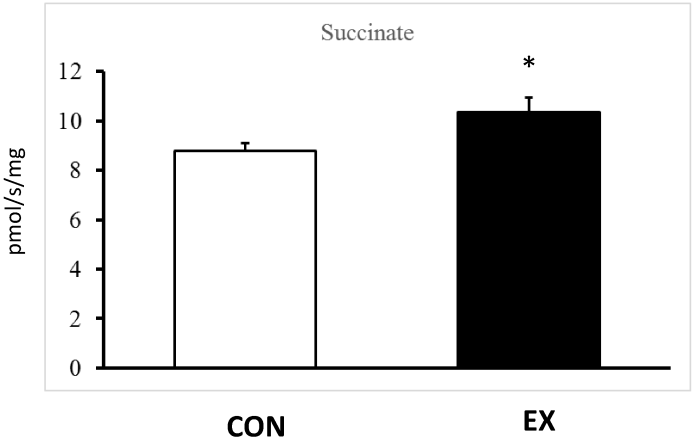


Figure 5: *The addition of the substrate succinate activates complex I+II, state 3 respiration. EX vessels demonstrated a significant increase in respiratory capacity in the presence of succinate, which is representative of maximal oxygen utilization (EX: 10.35 ± 0.61 versus CON: 8.77 versus 0.34, * p<0.05).*

Mitochondrial Proteins

Immunoblotting results demonstrate that there was an upregulation of relative mitochondrial protein expression in all complexes (I-V) in the EX vessels compared to CON (Fig. 6). However, the differences in Complex I were less evident (EX: 113% ± 25.5% versus CON: 100% ± 34.9%) compared to the amounts expressed in complex II (EX: 138.5% ± 30.1% versus CON: 100% ± 19.7%), complex III (EX: 167.8% ± 62.1% versus CON: 100% ± 37.4%), complex IV (EX: 152.6% ± 25.5% versus CON: 100% ± 35.6%) and complex V (EX: 183.8% ± 48.9% versus CON: 100% ± 18.9%).

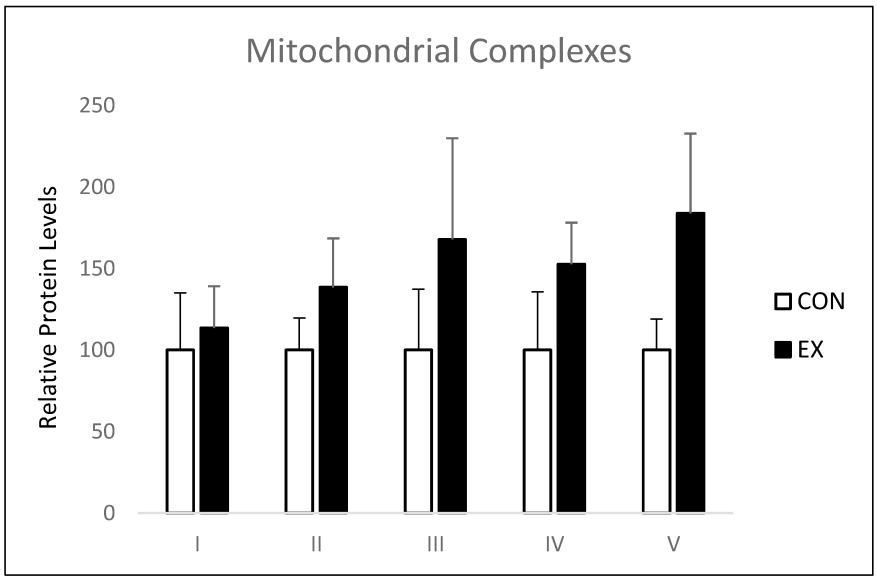


Figure 6: *Immunoblotting of mitochondrial complexes I-V with total OXPHOS rodent antibody cocktail from MitoSciences (MS604). All complexes demonstrate a trend towards upregulation in the expression of mitochondrial subunits in the EX vessels compared to CON.*

Weight, Food and Water Consumption

Average consumption (Fig. 7) was calculated by subtracting weekly measurements from the previous week. Both EX and CON groups appeared to gain weight overall throughout the intervention, with the EX group gaining approximately 5.64% ± 1.88% of its' baseline mass compared to 4.42% ± 2.06% in CON at the final weigh-in. Results indicate that CON group gained more weight overall when compared to EX, which is expected in sedentary models, except at week 2 (EX 101.12% ± 1.67% versus CON: 98.33% ± 1.76%). CON group exhibited a weight increase at week 3, weighing approximately 104.34% compared to 100% at baseline, and continued to maintain that weight until the end of the intervention.

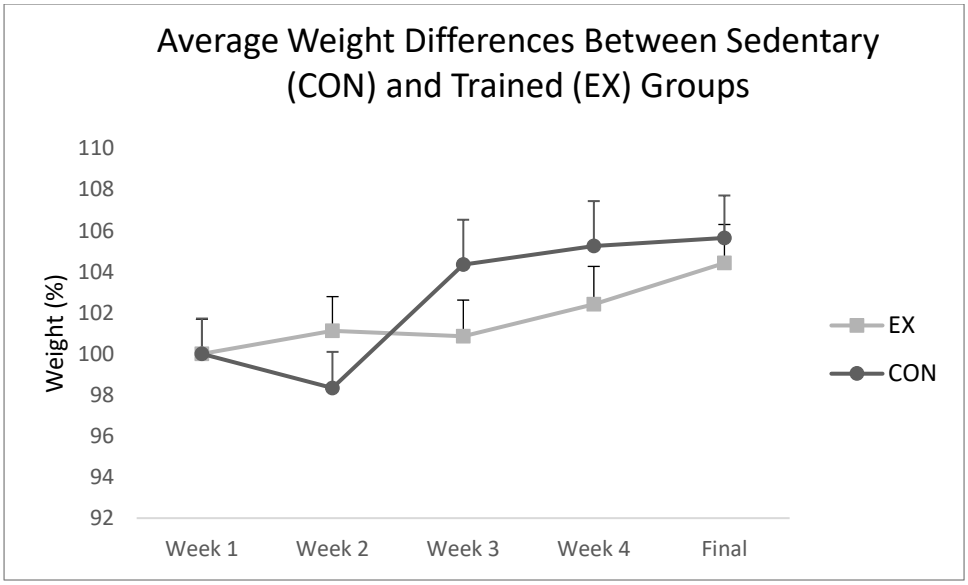


Figure 7: *Average weekly weight differences between EX and CON groups. Sedentary, CON animals gained more weight overall (except at week 2), signifying the positive correlation between physical inactivity and body mass. However, exercise also stimulated an increase in body mass, but was consistently lower compared to CON group (except at week 2).*

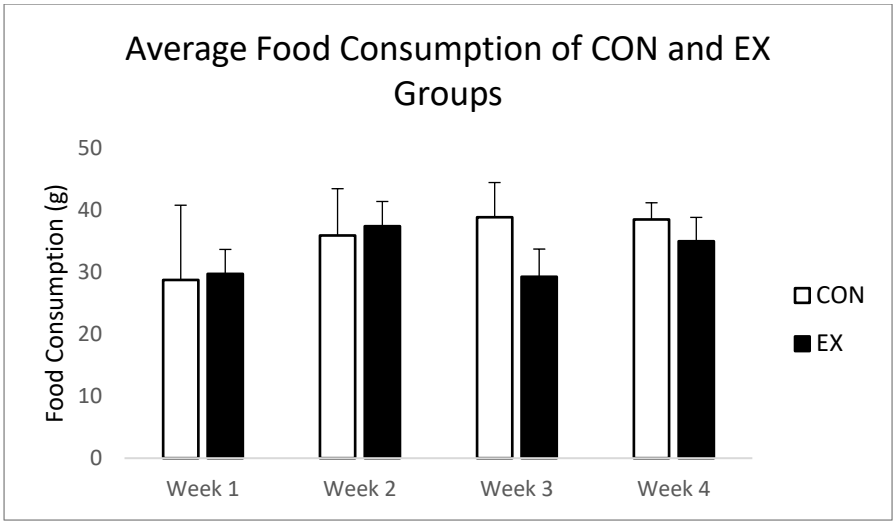


Figure 9: *Food consumption (g) was calculated by subtracting weekly measurements from previous week. No detectable differences in consumption between EX and CON were observed during weeks 1 and 2 (EX: 29.73 g ± 3.97 versus CON: 28.76 g ± 12.1 at during week 1 and EX: 37.47 g ± 3.98 versus CON: 35.97 g ± 7.5 during week 2). However, CON appeared to have consumed the most food starting at week 3 until the end of the intervention with EX: 29.29 g ± 4.5 versus CON: 38.92 g ± 5.6 during week 3 and EX: 35.03 g ± 3.85 versus CON: 38.55 g ± 2.7 during week 4.*

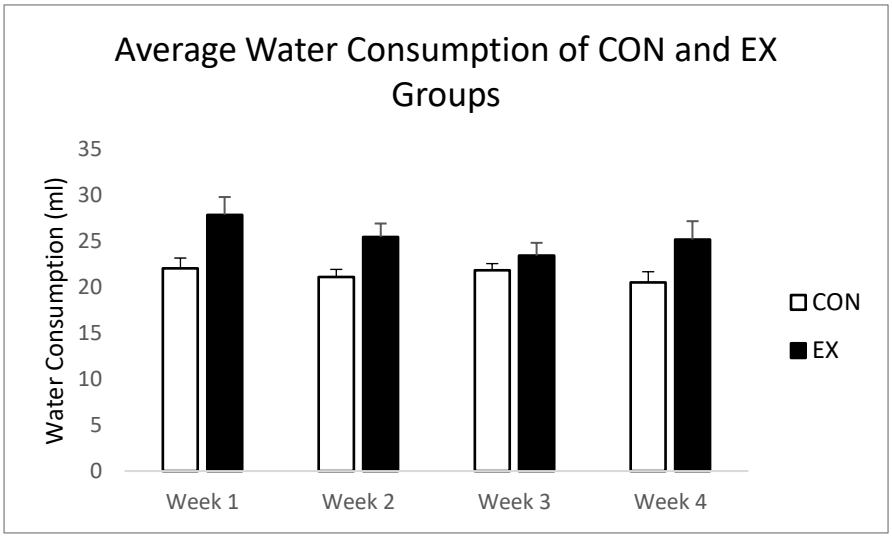


Figure 10: *Average water consumption was calculated by subtracting weekly measurements from the previous week. Results reveal that EX group consistently consumed more water throughout the 4-week intervention when compared to CON. The largest difference occurred at the beginning of the intervention at week 1 (EX: 27.88 ml ± 1.93 versus CON: 22.05 ml ± 1.14).*

Discussion

Exercise-induced mitochondrial adaptations include increased expression of mitochondrial subunits as well as improved oxidative/respiratory capacity that facilitate aerobic metabolism [Lundby and Jacobs, 2016]. Enhanced training performance is also determined by lower fatigue rates and greater substrate metabolism, which can be explained by measurements of mitochondrial density and relative protein levels. Evidence on the physiological adaptations of mitochondria in response to endurance training in skeletal and cardiac muscle are robust. Endothelial cells of the vasculature have an inherently low respiratory function, and vascular smooth muscle cells are ideal targets in studying mitochondrial respiration in the vasculature. To date, little is known about the exact impact of exercise on mitochondrial function and respiratory capacity of major blood vessels. Engaging in regular physical activity is a strong component for reducing the severity of cardiovascular risk factors, including obesity, dyslipidemia, hypertension, metabolic complications and atherosclerosis [Vanhees *et al.*, 2011]. The purpose of this study was to examine oxidative capacity in healthy vascular tissue following a 4-week aerobic exercise programme.

Relative increases in mitochondrial density and subsequent hypertrophy have been observed in skeletal muscle as a result of endurance exercise training, with a reported ~40% increase in skeletal muscle [Montero *et al.,* 2015]. Immunoblotting specific to VDAC in our study revealed that exercise training induced an increase in mitochondrial density in the EX group compared to CON, which is consistent with the trend reported in previous studies of skeletal and cardiac muscle. Exercise has also been found to stimulate important morphological changes such as an increase in mitochondrial protein expression [Hawley *et al.*, 2002]. Our findings reflect this phenomenon where an overall upregulation of mitochondrial proteins in each complex (I-V) was observed in EX vessels compared to CON after training for 3 days/week for 4 weeks. Thus, these findings are in line with the premise that describes chronic adaptations as a result of the cumulative effects of repeated training sessions [Hawley *et al.*, 2002].

An increase in mitochondrial respiration was also an important finding of this study, with significance during Complex I specific respiration. The addition of malate lead to an increase in oxidative capacity in the EX compared to CON, signifying enhanced basal, ADP-restricted state 2 respiration. Furthermore, significant increases in response to the addition of pyruvate and glutamate were also reported in EX vessels compared CON, which demonstrates augmented efficiency in

glucose metabolism during Complex I respiration as whole. This is further supported by the significant increase in the coupling of oxidation and phosphorylation during Complex I respiration observed in the EX group, as determined by the ACR. Recently, impairments in complex I activity has been strongly linked to Parkinson's disease, type II diabetes and cardiovascular disease [Taylor *et al.* 2005]. Complex I is considered to be the most physiologically relevant complex in the electron transport chain as it is predominantly involved in energy metabolism. As a consequence, complex I directly contributes to excessive ROS production and oxidative stress, which are hallmark features of cardiovascular and metabolic disorders [Brownlee, 2005; Sharma *et al.* 2009]. Furthermore, maximal respiratory capacity was significantly increased in the EX vessels, as detected by the activation of succinate-stimulated complex I+II, state 3 respiration. This parameter can be compared to an athlete testing for VO_{2max} and reflects the potential of the mitochondria to consume O_2 during maximal Complex I+II respiration. The augmented OXPHOS capacity detected in the EX vessels following the addition of succinate implies that training had increased the maximal threshold of mitochondrial respiration when compared to CON, which can contribute to improved exercise performance.

As illustrated in this study, long-term exercise has a clear and positive impact in Complex I respiration of EX vessels compared to CON, which is consistent with our hypothesis. This is important as deficiency of complex I is heavily implicated in mitochondrial dysfunction, ROS production and apoptosis programming [Brownlee, 2005; Sharma *et al.*, 2009]. The ACR, as measured by the addition of the substrate ADP, was also significantly augmented in the EX group compared to CON. This finding indicates that the EX vessels has a higher oxidation:phosphorylation ratio, which compares maximal O_2 consumption stimulated by ADP to basal respiration in the absence of ADP during Complex I activation. Indeed, as an adaptation to long-term endurance exercise, the EX vessels displayed enhanced ability to consume O_2 based on ADP availability as a substrate for phosphorylation, and thus reflects the efficiency of ATP production. However, no detectable differences were observed in the respiratory control ratio (RCR) between both groups, indicating that the coupling between O_2 consumption and oxidative phosphorylation for both complex I and II was unaffected in response to aerobic training. The RCR is described as the respiration rate in state 3 versus state 4, with higher values signifying greater mitochondrial capacity for substrate oxidation and subsequent ATP turnover as well as reduced proton leak [Brand, 2011]. Despite RCR representing a major part of mitochondria, it is not an

absolute method that is diagnostic of mitochondrial dysfunction. Therefore, with minimal differences in the RCR between EX and CON vessels, we can assume that Complex II may not be a limiting factor with regards to changes in mitochondrial respiration in response to exercise. Additionally, it could also indicate that vascular smooth muscle cell mitochondria do not inherently respond to exercise by increasing maximal O_2 consumption (state 3 respiration), considering that blood vessels are involved mostly in mediating relatively minor increases in pulse pressure. As a comparison, the RCR in skeletal muscle mitochondria is generally expected to be higher in trained individuals compared to sedentary subjects, where an increase in state 3 to state 4 respiration would translate into an increase in maximal OXPHOS capacity to meet the greater metabolic demands of sustained skeletal muscle contractions during exercise.

The relationship between food intake and exercise is complex and has been the focus of many research studies investigating lifestyle behaviours. In our project, EX mice did not exhibit significant changes in food intake throughout the intervention, with a decrease observed at week 3. This finding is consistent with previous reports of decreased energy intake in male laboratory rats forced to run on treadmill [Titchenal, 1988]. Similarly, weekly food intake in the CON group did not change significantly, which was expected. The major difference is that sedentary CON mice, on average, consumed more food compared to EX mice over the course of 4 weeks. A possible explanation to this is a lack of stimuli, with feeding being the sole activity available to sedentary mice. Moreover, the CON group gained more weight overall (with the exception of week 2) compared to EX, representing the positive correlation between physical inactivity and body mass. Finally, water consumption increased in the EX group compared to CON, which was expected given the higher water requirements following exercise exposure.

Conclusion

Mitochondrial dysfunction underlies the hallmark features of diabetes, obesity and vascular disease. The benefits of exercise in maintaining cardiovascular and metabolic health begin at the molecular level and stem from favourable physiological adaptations in the mitochondria. Increased mitochondrial network dynamics, electron transport activity, protein expression and energy metabolism in skeletal and cardiac muscle have all been previously reported to be determinants for enhanced training performance. Our study demonstrates that these adaptations extend to the vasculature, with a trend towards upregulation in mitochondrial subunits in complexes I-V as well

as mitochondrial density, with a significant increase in OXPHOS capacity during complex-I respiration of glucose-based substrates. Most notably, an increase in basal, ADP-restricted respiration was observed, as measured by the addition of malate, signifying augmented mitochondrial function during resting states. Indeed, the therapeutic implications of physical activity are substantial and treatments that target mitochondrial function can provide novel ways to minimize complications associated with cardiovascular and metabolic disorders.

III

Aerobic Training is Linked to Reduced Vascular Mitochondrial Respiratory Capacity in Type I Diabetes

Dana-Rae Reguis Yadao, Andreas Bergdahl, Stephanie MacKenzie

Contributions

Dana-Rae Reguis Yadao: preparation of manuscript, experimental techniques

Andreas Bergdahl: editing of manuscript, experimental techniques

Stephanie MacKenzie: experimental techniques

Abstract

Cardiovascular disease continues to be the leading cause of morbidity and mortality in the diabetic population, with impaired mitochondrial function as a primary contributor to vascular complications. Lifestyle modifications such as rigorous diet and exercise are often the first steps to promote insulin sensitivity and cardiovascular health. Benefits of exercise have been heavily studied in both skeletal and cardiac muscle, with the effects on type II diabetes extended to type I. To date, little is known about the impact of aerobic training on mitochondrial oxidative capacity and respiration in the diabetic vasculature. The goal of this study was to examine the effects of a long-term aerobic exercise programme on mitochondrial function in the vessels of T1DM subjects. After induction of diabetes via peritoneal streptozotocin injection, male C57BL/6 mice were randomly assigned to either the diabetes control group (CON) or the diabetes and exercise group (DE) for 4 weeks. Aortic vessels were harvested and used for immunoblotting and high-resolution respirometry to study oxidative phosphorylation (OXPHOS) capacity. Our findings suggest that prolonged exercise induced a reduction in mitochondrial protein expression along all five complexes of the mitochondrial electron transport chain in the exercise group compared to sedentary counterparts. Moreover, non-significant changes in glucose-based substrate efficiency were observed, indicating that exercise did not stimulate enhanced mitochondrial dynamics in the vasculature with regards to Complex I. These findings are not consistent with the previously demonstrated advantages of exercise in healthy subjects, where mitochondrial biogenesis and increased efficiency are considered determinants of enhanced performance. As such, the precise mechanisms behind exercise training as a contributor to diabetes-related cardiovascular insults merits further investigation.

Introduction

The global epidemic of diabetes is rapidly growing, with the prevalence expected to change the lives of approximately 439 million adults (aged 20-79) by 2030 [Shaw, 2009]. Of even greater concern is that this estimate does not consider that nearly 193 million individuals worldwide currently remain undiagnosed, consequently increasing their risk of developing severe complications related to untreated diabetes [American Diabetes Association, 2018]. Diabetes mellitus (DM) exists largely in two forms, Type I diabetes mellitus (T1DM) and Type II diabetes mellitus (T2DM). The former is characterized by an autoimmune process or a trauma leading to the destruction of pancreatic β-cells, and the latter, which represents most cases (90-95%), is caused by over-nutrition and a sedentary lifestyle [Sivitz and Yorek, 2009; Matough, 2012]. Despite differences in etiology, the ultimate consequences are chronic hyperglycemia [American Diabetes Association, 2010]. T1DM, however, is considered a greater health risk given that the disease develops early in life and is associated with long-term complications [Silverstein *et al.,* 2005]. The emerging epidemic of diabetes largely contributes to the development of cardiovascular disease worldwide, with heart disease as the leading cause of death for both men and women [Pop *et al.*, 2015]. Diabetes is also closely related to the pathophysiology of dyslipidemia and obesity [Pop *et al.,* 2015]. Additional pathological consequences include the failure of several organs, especially the eyes, kidneys, nerves, heart and blood vessels [Ceriello, 2003]. Pathways leading to the development of diabetes-specific complications of both the neuronal and vascular systems appear to be manifestations of mitochondrial dysfunction and subsequent oxidative damage [Brownlee, 2005; Murphy *et al.*, 2009].

Mitochondria are important organelles that carry out several important tasks such as ROS generation, contractile function, calcium signaling and apoptosis programming [Knaub *et al.,* 2014]. However, its' best-known role is the production of ATP, which is the energy required to sustain growth, metabolism and reproduction [Duchen, 2004]. ATP is produced through mitochondrial respiration, a process by which substrates, namely glucose-derived pyruvate, undergo a series of redox reactions in the tricarboxylic acid cycle to be reduced to NADH and $FADH_2$, which then carry electrons to the respiratory chain through various protein complexes. [Duchen, 2004]. This process is accompanied by the simultaneous transfer of protons across the inner membrane outward, resulting in a potential difference, ultimately driving the synthesis of

ATP [Duchen, 2004]. Given the importance of these organelles in mediating nearly all cellular processes, strong emphasis is placed on understanding mitochondrial function and the pathogenic processes involved in various diseases, especially diabetes. Much work has already addressed the deleterious effects of diabetes on mitochondrial function in skeletal and cardiac muscle. For instance, oxidative stress is linked to mitochondrial alterations in skeletal muscle of both diet-induced and streptozotocin (STZ)-treated diabetic mice, leading to impaired muscle force output, greater fatigue rates and reduced local blood flow [Kelley *et al.*, 2002; Bergdahl *et al.,* 2015]. STZ is commonly used in classical diabetes research as a drug to destroy pancreatic β-cells, thus resulting in an absolute deficiency in insulin and the development T1DM. Mitochondrial dysfunction and concomitant excessive ROS generation within cardiomyocytes were also found to be associated with structural changes of cardiac muscle, resulting in the progression of heart failure in diabetic patients [Boudina *et al.*, 2010]. Regarding the vasculature, research has found that endothelial cells are particularly susceptible to hyperglycaemia-induced damage, with endothelial dysfunction considered as an early sign of impaired mitochondrial function [Tang, 2014].

Significant reduction in the risk for cardiovascular disease is strongly associated with physical fitness, with exercise considered to be an effective prescription to regulate diabetes-related health issues. The benefits of regular physical activity on T2DM are firmly established, with significant improvements in insulin action as a major outcome [Colberg, 2010]. However, the risk for mitochondria-mediated vascular diseases remains significant, even in the presence of rigorous glycemic control, including diet and exercise.

Studies examining mitochondrial bioenergetics in the vasculature of diabetic targets following physical activity have been challenging. As a consequence, the precise mechanisms underlying this process remain relatively unknown and cardiovascular disease continues to be the leading cause of mortality among diabetic patients. More importantly, past work has focused largely on T2DM, and thus little has been produced about the mitochondria in the vasculature of T1DM subjects. Given the demonstrated benefits of exercise, and that diabetes elicits serious vascular complications, we hypothesized that long-term aerobic training would elicit an increase in mitochondrial function in major blood vessels of T1DM rodent models.

Methods

Animal Care

Age-matched (2-4 months) male C57BL/6 mice were obtained from the Concordia University breeding colony. Mice were individually housed in a 22°C room with a constant 12-hour light/dark cycle and access to rodent chow and water. Mice were randomly assigned into two groups for 4 weeks: Sedentary Diabetic (CON) and Diabetes with Exercise (DE). Diabetes was induced with a single injection (150 mg/kg) of streptozotocin (STZ) peritoneally. 1-2 days post-injection, blood glucose concentration was measured from the mouse-tail-vein using a glucometer (One Touch Verio Flex). A reading that exceeded 14 mmol/L was considered diabetic. Measurements of body mass (g) as well as consumption of food (g) and water (ml) were obtained once a week. All procedures were approved by the Concordia University Animal Research Ethics Committee and certified by the Canadian Council on Animal Care.

Aerobic Exercise Intervention

The DE group performed aerobic exercise three days a week for four weeks, for a total of twelve days. Exercise was carried out using an in-house built, customized, graded rodent treadmill. The protocol consisted of two phases: acclimation and training. During acclimation (day one), mice were placed on the treadmill for three minutes to explore it [Bouganim and Bergdahl, 2017]. Next, the motor was turned on at zero speed for approximately two minutes to introduce the mice to the sounds of the treadmill [Bouganim and Bergdahl, 2017].

The training phase consisted of increasing the speed from 3, 5, 8 to 10 m/min at 0-4, 4-8, 8-12 and 12-15 minutes respectively (Table 1). Each day, the session increased by 5 minutes (starting at 15 minutes), until a total training time of 60 minutes was reached (day ten). For instance, mice were trained for 15 minutes on day one, followed by 20 minutes on day two, 25 minutes on day three, and so on. This intensity was maintained until the end of the protocol (day twelve). To encourage mice to remain active on the treadmill, brushes were placed at the back of each lane to tickle their tails. When mice were unable to keep up with the treadmill, the exercise session was terminated.

Table 1. *Days 1 and 2 of Aerobic Exercise Protocol for DE Group*

	Time (min)	Speed (m/min)
Day one	0 – 4	3
	4 – 8	5
	8 – 12	8
	12 – 15	10
Day two	0 – 4	3
	4 – 8	5
	8 – 12	8
	12 – 15	12
	15 – 20	12

Tissue permeabilization and preparation

Mice were euthanized by CO_2, after which aortic vessels were harvested and immediately stored in an ice-cold buffer solution (BIOPS) containing the following in mmol/L: CaK_2EGTA 2.77, K_2EGTA 7.23, Na_2ATP 5.77, $MgCl_2 \cdot 6H_2O$ 6.56, taurine 20, phosphocreatine 15, imidazole 20, dithiothreitol 0.5, MES 50, pH 7.1 After removal of adventitial fat tissue, vessels were opened longitudinally and denuded of endothelium in ice-cold BIOPS buffer with a sponge. Tissues were then broken into smaller pieces and permeabilized in 2 ml BIOPS buffer containing 50μg/ml of saponin for 30 minutes, followed by two washes in 2 ml of ice-cold Mir05 buffer for 10 minutes each [Larsen *et al.* 2015]. Mir05 buffer contains (in mmol/L): EGTA 0.5, $MgCl_2 \cdot 6H_2O$ 3.0, K-lactobionate 60, Taurine 20, KH_2PO_4 10, HEPES 20, sucrose 110, BSA 1g/l, pH 7.1.

Measurements of mitochondrial respiration

High-resolution respirometry was performed to quantify mitochondrial function using a polarographic oxygen sensor (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). Permeabilized tissue (2-5 mg wet wt) were incubated in the Oxygraph chambers with 2 ml of Mir05 buffer each and stirred continuously at 37 °C for 15-20 minutes to establish a baseline in the absence of respiratory substrates. Hyperoxygenated conditions were maintained to prevent potential O_2 diffusion limitations. Next, for each mitochondrial complex to be studied independently, the following substrates were injected sequentially in each chamber in accordance with the protocol described by Larsen *et al.* [2015]: malate (2 mmol/L); pyruvate; glutamate (10 mmol/L); ADP (5 mmol/L); 20 µl succinate (10 mmol/L) ; oligomycin (2 µg/ml) and antimycin A (2.5 µM) . The presence of malate + pyruvate + glutamate allowed for complex I, state 2 respiration to be examined, which represents the ADP-restricted, non-phosphorylating basal resting state. The addition of ADP allowed for complex I, state 3 to be analyzed, which provided an index of oxidative phosphorylation (OXPHOS). Succinate allowed for maximal state 3 respiration to be achieved, with simultaneous activation of Complex I and Complex II. Finally, inhibitors oligomycin and antimycin A were injected to block Complexes V (LEAK) and III respectively. Respiration rates and O2 consumption were recorded and analyzed using the Oroboros DatLab software. In each condition, respiration rates were recorded for 3-5 minutes, with the average of the last minute used for data analysis.

Immunoblotting and immunofluorescence

Protein levels were assessed following the protocol described by Rocha *et al.* [2014]. Cell lysis of aortic vessels was performed in a lysis buffer containing (in mmol/L): NaCL 250, HEPES 50, glycerol 10%, Triton X-100 1%, $MgCl_2$ 1.5, EGTA 1, $Na_4P_2O_7$ 10, NaF 1, Na_3VO_4 800 µmol/L, pH 5. The solution was centrifuged for 10 minutes at 10,000 *g,* after which the supernatant was extracted. The Pierce BCA Protein Assay Kit (Thermo Scientific, Mississauga, Ontario, Canada) was used to assess protein levels with 10 µg of protein samples first separated on a 12.5% SDS-PAGE and then transferred to a nitrocellulose membrane (0.45 µm, 162-0115, Bio-Rad) with 10 mmol/L sodium tetraborate buffer. 5% BSA in TBS-T buffer (10 mmol/L Tris-HCL, pH 7.5, 150 mmol/L NaCl, 0.05% Tween 20) was used to block the membranes for 1 hour at room temperature, followed by an overnight incubation period at 4 ºC with the antibody for voltage-dependent anion

channel (VDAC; 1:1000, ab14734 Abcam) as well as total OXPHOS rodent antibody cocktail (1:500, MS604 MitoSciences). The resulting blots were washed and incubated with horseradish-peroxidase-conjugated secondary antibodies (anti-mouse, ab6728 Abcam). Relative protein expression was visualized and studied using a chemiluminescence system (Immun-Star Chemiluminiscent, 1705070; Bio-Rad, Mississauga, Ontario, Canada) and bands analyzed with ImageJ software.

Statistics

Given the comparison between the DE and CON groups, a two-tailed Student's *t* test was used for data analysis, where $P < 0.05$ was considered significant. Immunoblotting data and all other figures were presented as means ± SE.

Results

Mitochondrial density

Immunoblotting using an antibody that targets the voltage-dependent anion channel (VDAC) was performed to control for differences in mitochondrial content between the DE and CON groups (Fig. 1). There were no significance differences in mitochondrial protein density indicating that mitochondrial content was similar between DE and CON groups after a 4-week intervention.

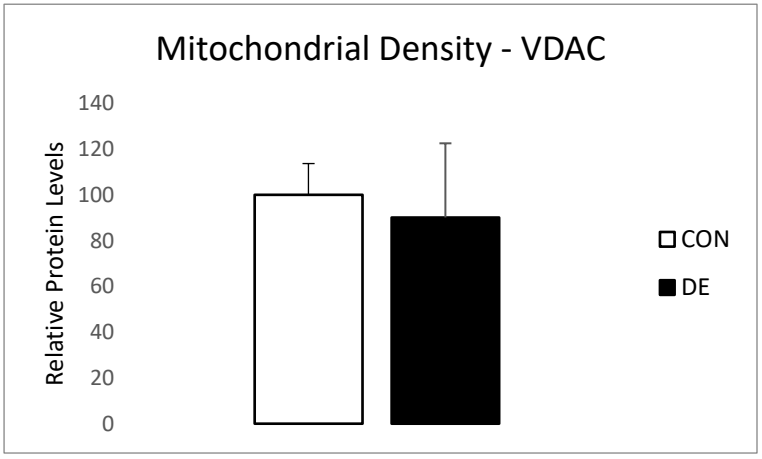


Figure 1: *A downregulation in mitochondrial density was observed in the DE group compared to CON after the aerobic exercise intervention (DE: 90.1% ± 32.4% versus CON: 100% ± 13.6%.)*

Mitochondrial respiration

To examine mitochondrial function and respiratory capacities, oxygen consumption rates of permeabilized tissues were measured. OXPHOS capacity following the addition of complex I-linked substrate malate (basal, ADP-restricted). A non-significant trend towards downregulation in the DE compared to CON was found (DE: 0.82 ± 0.12 versus CON: 0.61 ± 0.13), as shown in Fig 2a. Additionally, no significant change was shown following the addition of complex I-linked pyruvate (DE: 2.34 ± 0.29 versus CON: 2.34 ± 0.29), as demonstrated in Fig. 2b. The addition of complex I-linked glutamate (Fig. 2c) demonstrated a similar non-significant downward trend (DE: 2.34 ± 0.33 versus CON: 2.20 ± 0.31), suggesting that aerobic exercise has minimal effects during complex I respiration.

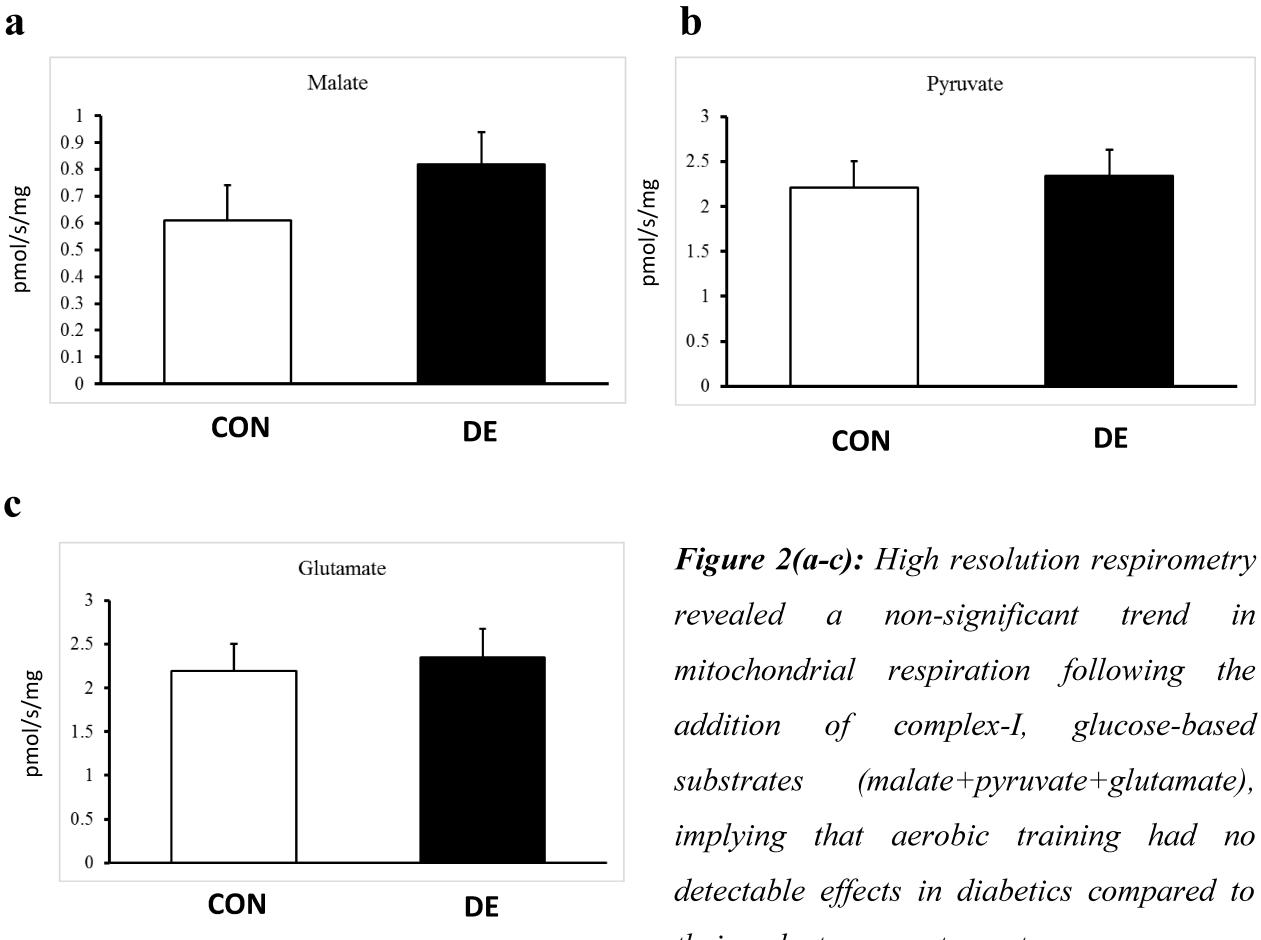


Figure 2(a-c): *High resolution respirometry revealed a non-significant trend in mitochondrial respiration following the addition of complex-I, glucose-based substrates (malate+pyruvate+glutamate), implying that aerobic training had no detectable effects in diabetics compared to their sedentary counterparts.*

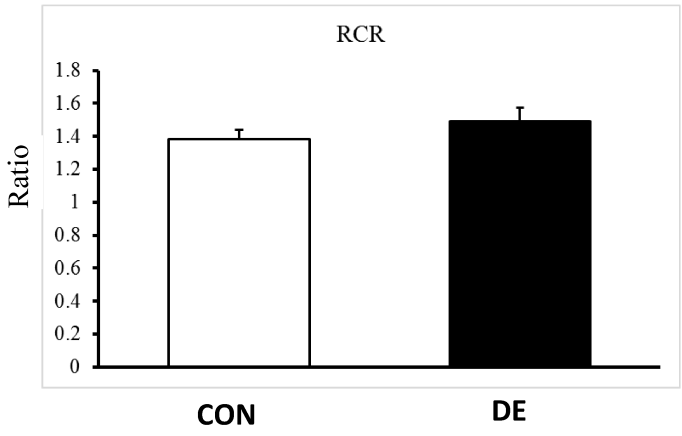


Figure 3: *A calculation of the respiratory control ratio (RCR) between DE and CON demonstrate that training had no significant effect compared to CON (DE: 1.49 ± 0.09 versus CON: 1.38 ± 0.06). The RCR is an important index of respirometry that represents the coupling between O_2 consumption and oxidative phosphorylation during Complex I+II respiration.*

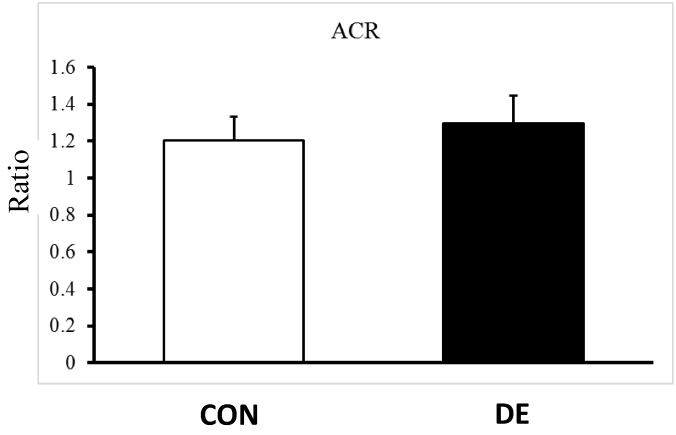


Figure 4: *The acceptor control ratio (ACR) signifies the tightness in the coupling of oxidation and phosphorylation during complex I activation. ACR did not differ significantly between DE and CON groups, signifying that Complex I respiration was minimally affected in the DE group in response to aerobic training (DE: 1.30 ± 0.15 versus CON: 1.20 ± 0.13).*

Mitochondrial Proteins

Immunoblotting (Fig. 5) revealed that training had induced a non-significant trend towards downregulation in the expression of mitochondrial proteins in DE vessels compared to CON, where the largest difference in respiratory capacity occurred in Complex I (EX: 74.9 % ± 13.4% versus CON: 100% ± 17.5%). Aerobic training also lead to a downregulation in the expression of mitochondrial subunits in Complex II (DE: 79.3% ± 9.6% versus CON: 100% ± 17.03%); Complex III (DE: 64.54% ± 18.3% versus CON: 100% ± 25.3%); Complex IV (DE: 52.9% ± 4.6% versus CON: 100% ± 18.2%) and Complex V (DE: 41.6% ± 15.9% versus CON: 100% ± 35.6%), indicating that training had caused a decrease in vascular mitochondrial protein expression compared to diabetic, sedentary mice.

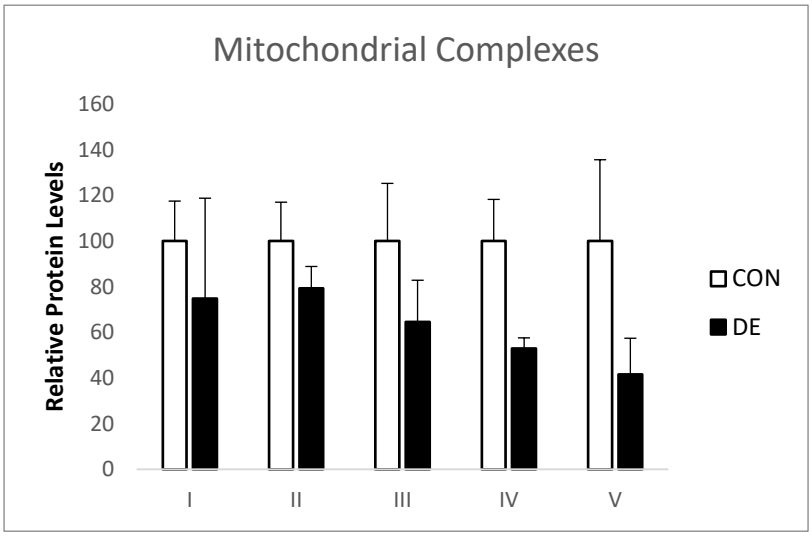


Figure 5: *Immunoblotting revealed a non-significant downward trend in the expression of mitochondrial subunits of DE vessels along all mitochondrial complexes (I-V), relative to the control, where n=9 in the CON group and n=5 in the EX group.*

Weight, Food and Water Consumption

Fig. 6 depicts weight measurements taken at each week. Mice belonging to the exercise group (DE) demonstrated, on average, a decrease in body mass throughout the intervention, dropping down dramatically to 90% compared to their baseline values at the final weigh-in. Diabetic controls appeared to gain weight until week 2, having achieved a 5% increase in mass. Thereafter, weight loss was observed in both groups at week 4, with DE weighing approximately 10% less than baseline and CON dropping down to near baseline.

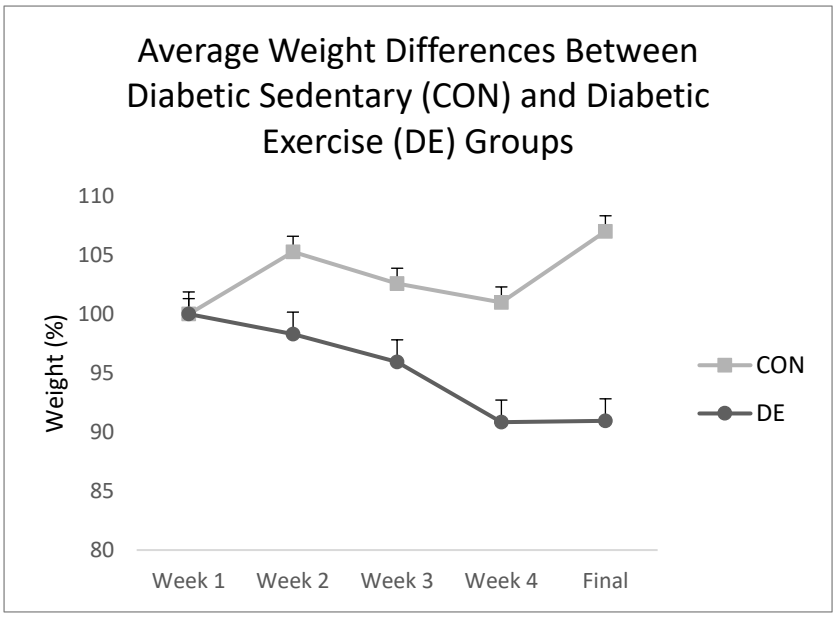


Figure 6: *Weight measurements (g) throughout the 4-week aerobic exercise intervention showed that the DE group consistently lost body mass. CON displayed a significant increase at week 2, a decrease towards baseline values at weeks 3 and 4 and finally weighing approximately 7% more at the final weigh-in compared to baseline. (n = 5 in DE and n = 9 in CON).*

Fig. 7 illustrates food quantities (g) that were measured once a week, which allowed us to calculate weekly consumption by subtracting weekly values from those obtained in the previous week. An average increase in rodent chow consumption was observed in the DE group up until week 3, at which point consumption appeared to decrease slightly until the end of the intervention. CON mice also displayed an increase in food intake, with a dramatic reduction observed at week 3 and was maintained until the end of the intervention. Food intake in the DE group were consistently higher compared to CON, with significance observed during week 3 (DE: 63.05 g ± 4.73 versus CON: 42.25 g ± 14.23).

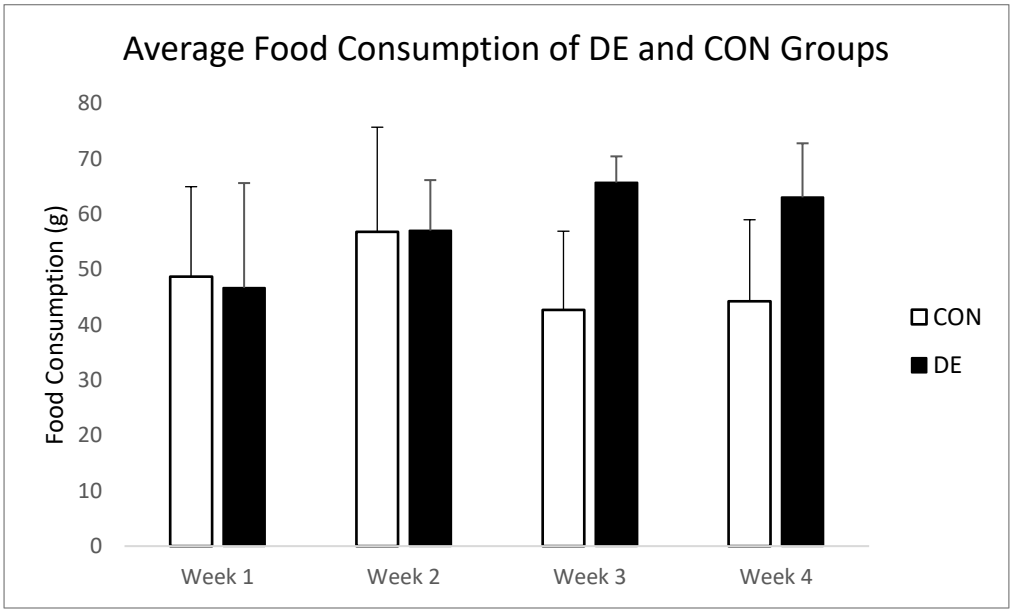


Figure 7 : *Average rodent chow consumption (g) on a weekly basis was calculated. An overall increase in food intake in the DE group was observed compared to CON. Similar intake patterns of the groups were displayed in weeks 1 and 2, with notable differences observed at weeks 3 and 4 between DE and controls.*

Water intake measurements revealed that the DE group consumed more water than controls, which was expected given the nature of the disease (i.e. polydipsia) and exercise exposure. No significant differences were reported between the groups, and Fig. 8 illustrates that both DE and controls had similar water intake patterns week-by-week, with higher amounts of water consumed at weeks 1 and 2 (DE: 73.3 ml ± 4.9 versus CON: 59.1 ml ± 2.6 and DE: 71.9 ± 5.2 versus CON: 56.7 ± 3.5) and lower amounts consumed beginning at week 3 until week 4 (DE: 57.6 ± 10.9 versus CON: 48.4 ± 3.8 and DE: 53.6 ± 11.4 versus CON: 42.8 ± 4.4).

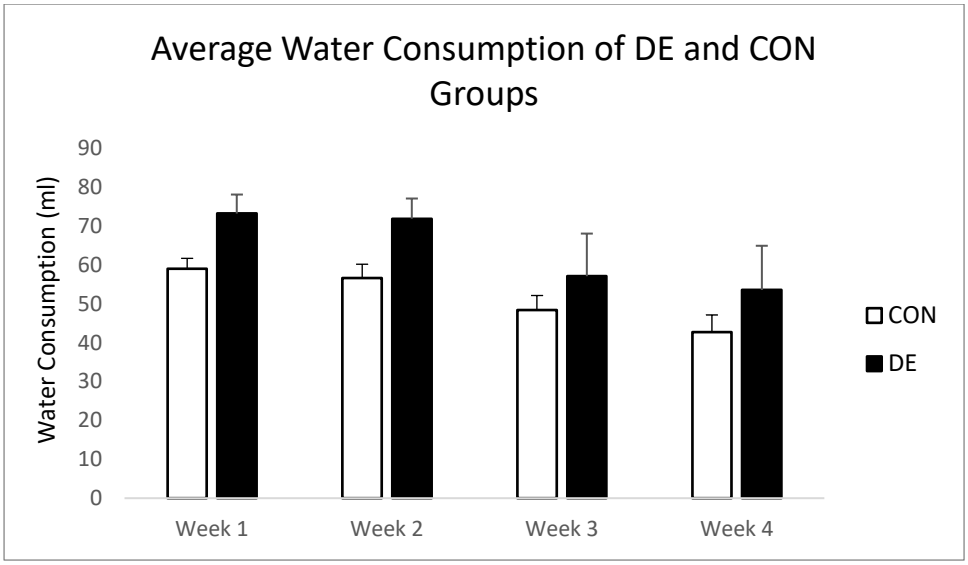


Figure 8: *Average water consumption (ml). An overall decrease in water consumption was observed throughout the course of the intervention in both groups compared to week 1. However, this figure illustrates that there was a similar drop in water intake at week 3 (DE: 57.1 ± 10.9 and CON: 48.4 ± 3.8) compared to previous weeks. DE g appeared to consistently consume more water each week compared to CON, with the largest difference in intake observed at week 2 (DE: 71. 9 ± 5.2 versus CON: 56.7 ± 3.5).*

Mortality Rates

Over the course of the research, the total number of mice randomly chosen for the DE group was n=45 and n=40 for CON. The number of deaths were reported each week in each group, which allowed us to calculate mortality rate, as illustrated in Fig. 9. Both groups followed a similar trend, in which mortality rates increased at weeks 2 and 3 and dropped down at week 4. However, the DE group experienced an overall greater morality rate, indicating a positive correlation between endurance training in diabetic mice and mortality compared to CON.

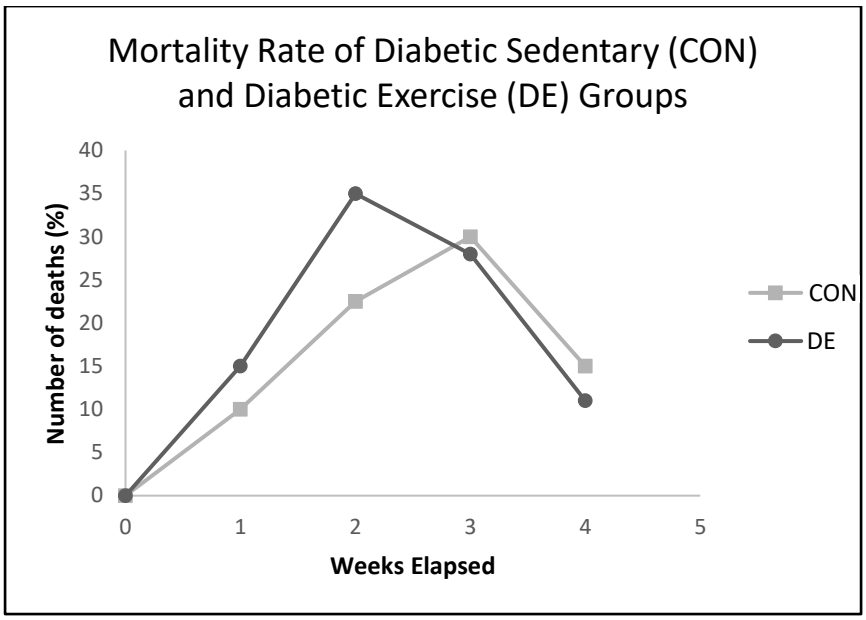


Figure 9: *High mortality rates were observed in both groups, however the DE group appeared to have a higher mortality rate overall, especially at week 2 in which 35% of the group died. The upward trend follows the increasing intensity of the exercise protocol up until week 3, after which both groups displayed a decrease in mortality rate. At the end of the research project, only n=5 in the DE group as well as n=9 in the CON group had survived for vascular tissue analysis of mitochondrial respiratory capacity, representing 11% of the total DE mice and 22.5% of the total CON mice from the beginning of the research.*

Discussion

Physical activity is considered an effective prescription to treat and prevent a variety of illnesses including diabetes and cardiovascular disease. Indeed, exercise training has been shown to stimulate mitochondrial hypertrophy and biogenesis, which are key features of enhanced muscle oxidative capacity and performance [Taivassalo and Haller, 2004]. In the case of diabetes, where mitochondrial dysfunction is associated with reduced mitochondrial abundance in skeletal muscle, exercise has been established as a formidable non-pharmacological approach to help maintain optimal glycemic control by improving insulin sensitivity [Dela *et al.,* 1995; Patti *et al.,* 2003]. Exercise-induced glucose uptake has been shown to be maintained in insulin-resistant muscle and promotes glucose uptake up to 50-fold via three major stages: delivery to tissues, transport across

muscle cell membrane and augmented glycolysis [Sylow *et al.* 2017]. To date, little work has been done to examine the effects of long-term endurance exercise on vascular mitochondrial function in T1DM models. In this study, we investigated the effects of a 4-week progressive aerobic exercise programme on vascular mitochondria. Specifically, we evaluated whether mitochondrial respiratory capacity was enhanced in T1DM mice following endurance exercise compared to their sedentary counterparts.

Mitochondria are highly dynamic organelles that can adapt to various physiological stimuli, such as exercise [Bo *et al.* 2010]. The present study found that endurance training had no significant effect on mitochondrial density in the vasculature of the DE group compared to CON, as measured by VDAC-specific immunoblotting. This finding indicates that, despite the benefits demonstrated in our previous study, exercise induces a downregulation in mitochondrial protein expression in vascular smooth muscle cells. In addition, a reduction in the respiration of Complex I-linked, glucose-based substrates in the DE group was observed, which suggests that exercise did not promote an increase in glucose uptake in the vasculature. Glucose transport across the cell membrane is facilitated by proteins GLUT1 and GLUT4, with GLUT1 responsible for basal glucose transport [Bell *et al.,* 1990]. Moreover, GLUT4 increases intracellular glucose levels in response to insulin and contractions [Marshall *et al.,* 1993]. A previous study reported that high blood glucose levels lead to a decrease in GLUT1 protein expression in vascular smooth muscle cells [Giovanni *et al.,* 2003]. This is consistent with our results in the DE vessels, and could signify that the decrease in the respiration of glucose-based substrates could be attributed to hyperglycemia-induced impairments of GLUT1 transport proteins. Moreover, while it is possible that training in the DE group stimulated GLUT4 activation via skeletal muscle contractions, the downregulation of GLUT1 transport proteins could have caused the overall decrease in Complex I respiration. However, the precise underpinnings of the relationship between glucose transport regulation and Complex I respiration in diabetic vessels should be studied further.

Complex I is considered to be the centre of electron transport chain activity as it is predominantly involved in energy metabolism. As a consequence, a deficiency in complex I activity directly contributes to excessive ROS production and oxidative stress, which are hallmark features of cardiovascular and metabolic disorders [Brownlee, 2005; Sharma *et al.,* 2009]. A shift in free fatty

acid oxidation to meet the greater metabolic demands of aerobic exercise could also be attributed to the decrease in Complex I respiration observed in our study. Indeed, the major finding in our experiment is that the exercise training administered to diabetic mice with already compromised mitochondrial function facilitated an even greater degree of impaired respiratory capacity with regards to complex I activity. It is important to note that this unexpected outcome is based on subjects with poorly regulated T1DM. As a consequence, a recommendation for future work on this experiment would be to investigate the effects of aerobic training in conjunction with insulin therapy on mitochondrial function of vascular smooth muscle cells. The addition of a diabetic exercise group undergoing insulin therapy could greatly change this outcome to a favorable one, where Complex I respiration of glucose-based substrates could potentially be enhanced.

The RCR, which is a measurement of the coupling between O_2 consumption and oxidative phosphorylation (state 3 over state 4 respiration), is an important index of respirometry. Generally, a higher RCR is indicative of normal mitochondrial respiratory function [Fernstrom *et al.,* 2004]. One study suggested that a low RCR in trained mice is associated with cardiac mitochondrial damage, however, the reason for this remains to be elucidated. The present study demonstrated no change in RCR in the DE group compared to CON, suggesting that the coupling between O_2 and oxidative phosphorylation was not a limiting factor in the compromised respiratory function observed in complex I. This outcome was similar to that found in our first study, and the same rationale could be used to explain why the RCR between CON and DE remained unchanged post-intervention. Despite RCR representing a major part of mitochondria, it is not an absolute method that is diagnostic of mitochondrial dysfunction. Therefore, with minimal differences in the RCR between EX and CON vessels, we can assume that Complex II may not be heavily involved with regards to changes in mitochondrial respiration in response to exercise. Additionally, it could also indicate that vascular smooth muscle cell mitochondria do not inherently respond to exercise by increasing maximal O_2 consumption (state 3 respiration), considering that blood vessels are involved mostly in mediating relatively minor increases in pulse pressure. Indeed, more work needs to be done to investigate the association between a reduction in RCR in the vasculature of trained T1DM subjects.

Mitochondrial adaptations in response to exercise depend on frequency, intensity, duration and type of exercise [Bo *et al.,* 2010]. The present study elected to administer a progressive aerobic exercise programme to avoid the plateau phenomenon, and to facilitate optimal exercise-induced changes in mitochondrial network dynamics. Initially, the dose of exercise given to the DE group was identical to the programme created by Bouganim and Bergdahl, which was created for normal, non-diseased mice [2016]. However, it was found that the intensity and duration were too high for T1DM mice to follow as well as recover from, with early onset fatigue and cessation of participation on the treadmill as indicators of fatigue and lowered aerobic capacity. As such, we adjusted the exercise protocol to a lower intensity. Despite these modifications, mortality rate of the DE group was significantly higher than CON. The majority of DE mice (~89%) eventually succumbed to the metabolic complications associated with diabetes at approximately weeks 2 and 3, thus explaining the difference in sample sizes between the groups (n = 5 in DE and n= 9 in CON). These findings are consistent with results of weekly body mass and food/water intake, with dramatic weight loss as well as lowered food/water consumption observed at weeks 3 and 4 respectively. However, the DE group consumed more water overall compared to CON, demonstrating additional water requirements in response to exercise combined with polydipsia as a symptom of increased blood sugar concentration in the diabetic population [Siddiqui *et al.,* 2011]. These outcomes are a clear indication of the severe clinical characteristics of diabetes, and in the context of our experiment, implies that exercise appeared to exacerbate diabetic symptoms.

Fatigue is recognized as a hallmark characteristic of mitochondrial dysfunction. Indications of fatigue include a lack of energy and a greater recovery time following physical activity [Rosenthal *et al.,* 2008]. However, the proper clinical definition of fatigue with regards to mitochondrial function and diabetes are unclear. As such, fatigue in our study was deemed significant based on three different qualitative behavioural observations in both DE and CON groups: (1) inability to keep pace on the treadmill (DE only); (2) reduced self-grooming activity and (3) decreased nesting patterns. DE mice unable to maintain treadmill activity during the exercise session signified exhaustion and diminished endurance, which appeared to extend to resting states. Self-grooming in rodents is an inherent, often compulsive behaviour that involves a patterned, sequential organization important for hygiene maintenance [Spruijt *et al.,* 1988]. During our experiment, diabetic mice exhibited abnormal self-grooming habits in the form of unkempt and sparse fur, compared to normal disease-free mice, who typically had flat and shiny coats. Furthermore, we

observed a discontinuation of nest building in diabetic mice, beginning at week 2 until the end of the 4-week intervention. This aberrant behaviour was important because nesting is strongly genetically determined in mice and allows for the maintenance of cage micro-climate factors such as temperature, light intensity and the ability to hide [Van Oortmerssen, 1971]. These observations could signify a compensatory mechanism in diabetic mice to conserve energy for more physiologically relevant processes such as homeostasis during metabolically compromised states and are consistent with our results of mortality rates.

While high exhaustion and mortality rates created setbacks in the progression of our experiments, it ultimately provided qualitative evidence to support our findings that exercise has the ability to negatively affect vascular mitochondria during poorly regulated T1DM, and perhaps lead to the accelerated pathogenesis of cardiovascular complications. This unexpected observation is important as it negates all previously demonstrated benefits of exercise, such as those reported in our initial study, even when the intensity is significantly reduced. The cause of death could possibly be explained by mitochondrial dysfunction-mediated characteristics of heart disease, including atherosclerosis, hypertension and subsequent risk for aneurisms and heart attacks [Cerellio, 2003]. Furthermore, the use of streptozotocin as a means to induce diabetes could have led to multi-organ damage, secondary to the pancreas. While the drug is known to specifically target the pancreatic β-cells, its' potential toxic effects on other tissues should also be examined. As such, more work needs to be done to explore the precise mechanisms behind the high mortality rate and whether cardiovascular insults or the administration of streptozotocin were directly associated with the deaths. More importantly, further investigation is required to determine the intensity and duration of exercise best suited for untreated T1DM patients.

Conclusion

The relationship between regular exercise, unregulated TIDM and cardiovascular risk should be interpreted carefully. While positive physiological adaptations in the mitochondria have been reported in T2DM cases, the present study provides new evidence that aerobic training elicits no significantly positive changes in the vasculature of T1DM models and could potentially pose a risk to cardiovascular health. Furthermore, the role of streptozotocin could have played a major role in the high mortality rate, with evidence supporting its' involvement in the damage of multiple organs. Indeed, our results indicate that aerobic training is linked to a decrease in vascular mitochondrial function, seen in the reduction in complex I respiration, as well as a trend towards downregulation of mitochondrial proteins along all complexes (I-V). To gain a more comprehensive understanding of the relationship between T1DM and aerobic exercise on mitochondrial function, future studies would benefit in including a group of T1DM models that receive insulin therapy in conjunction with an exercise programme. With cardiovascular disease as the leading cause of death among diabetic patients, there remains the urgency to identify mitochondria-specific therapeutic targets.

IV

Reducing branched-chain amino acid intake to reduce metabolic complications in obesity and type 2 diabetes

Dana-Rae Reguis Yadao, Stephanie MacKenzie and Andreas Bergdahl

Contributions

Dana-Rae Reguis Yadao : preparation of manuscript

Stephanie MacKenzie: preparation of manuscript

Andreas Bergdahl: editing of manuscript

JOURNAL CLUB

Reducing branched-chain amino acid intake to reverse metabolic complications in obesity and type 2 diabetes

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Edited by: Kim Barrett and Bettina Mittendorfer

The Western diet (WD) is defined as the high intake of calories, saturated fats and sugars, and has been linked with the alarming increase of obesity and Type 2 diabetes (T2D) in North America and Europe. Chronic consumption of the Western diet induces obesity, which originates as a positive energy balance and subsequent disruption of regulatory and metabolic processes leading to excess fat mass. T2D is characterized by insulin resistance, which results in the abnormal cellular uptake of carbohydrates and lipids. Ramifications of insulin resistance include hyperglycaemia, dyslipidaemia and oxidative stress. While caloric restriction has been considered an effective strategy for shedding excess weight and restoring metabolic function, many patients find it difficult to implement as a long-term solution. Recent studies have highlighted a different approach towards weight loss and metabolic health, one exclusively through the modification of protein composition.

Dietary proteins generally have an insulinotropic effect, consequently leading to the increased uptake of glucose and subsequent glycaemic control (Rietman *et al.* 2014). However, long-term consumption of a high-protein (HP) diet is correlated with a greater risk of developing conditions related to metabolic dysfunction. Recent observational and experimental evidence suggest that excessive consumption of a prominent group of amino acids (AAs), the branched-chain AAs (BCAAs), may elicit insulin resistance and glucose intolerance (Chen &Yang, 2015). Proteins are composed of both essential and non-essential AAs, with the three BCAAs – leucine, isoleucine and valine – constituting up to 20% of total dietary protein intake (Chen & Yang, 2015). Augmented levels of BCAAs have garnered much research attention as potential biomarkers of T2D and obesity (Rietman *et al.* 2014; Chen & Yang, 2015). Greater BCAA serum levels obtained from a HP diet result in poor insulin action and increased mortality in both rodents and humans, whereas a low-protein (LP) diet promotes metabolic health via enhanced insulin sensitivity as well as lower white adipose tissue (WAT) mass (Fontana *et al.* 2016). Additionally, it was found that a reduction in BCAA content can elicit positive metabolic effects similar to a normal LP diet (Fontana *et al.* 2016). Consequently, the potential to alter the pathophysiology of metabolic conditions through the specific modification of dietary proteins merits further investigation.

In a recent article published in *The Journal of Physiology*, Cummings *et al.* (2018) effectively filled this gap and helped to further our overall understanding of the positive correlation between augmented BCAA serum levels and metabolic dysfunction. Specifically, the authors challenged this relationship by determining whether mice already conditioned with obesity could achieve restored metabolic health in less than 4 weeks after switching from a high-fat Western diet to either a LP or a low-BCAA diet. A reduction in adiposity, lowered insulin resistance, adequate glycaemic control and greater energy expenditure (EE) served as indicators for overall metabolic health. Mice underwent diet-induced obesity (DIO) through the consumption of a WD for 12 weeks and were then randomized into one of five experimental groups: Control AA, ExLow AA (contains a formulation of AAs that are all significantly lower than the Control AA diet), ExLow BCAA (specifically has lowered amounts of L-Isoleucine, L-Leucine and L-Valine compared to the Control AA diet (Table 1, Cummings *et al.* 2018)), WD supplemented with BCAAs, or WD. All diets contained the same energy density and macronutrient composition as typical rodent chow. Body composition, EE, activity levels and thickness of dermal white adipose tissue (dWAT) were noted over a period of 15 weeks. The two WD groups continued to gain weight throughout the intervention while the ExLow AA and ExLow BCAA groups sustained rapid weight loss. Moreover, the ExLow AA group lost 25% of their body weight in 2 weeks and later settled at a lower weight compared to non-WD mice. Control AA mice normalized their weight but at a much slower pace, which required two additional months. Glucose tolerance and insulin sensitivity improved in all normal calorie diets compared to the WD group. However, ExLow BCAA and ExLow AA diets exhibited better glucose tolerance relative to all groups, and ExLow AA displayed the greatest correction with regards to insulin sensitivity. Interestingly, the experienced weight loss was not due to calorie restriction, but rather from a LP and low AA diet which subsequently increased EE, as confirmed via indirect calorimetry ($P < 0.05$). All DIO mice that were switched to a non-WD had thinner dWAT measurements (ExLow BCAA specifically), whereas DIO mice on the WD or WD + BCAAs demonstrated larger fat droplets.

To further verify the relationship between decreased BCAAs and energy balance in DIO mice, Cummings *et al.* created an additional series of WDs, each with varying levels of BCAAs. DIO mice were placed into one of four groups: WD Control AA, WD High BCAAs, WD Low BCAAs and WD Low AAs. Similar results were found wherein both WD Low BCAA and WD Low AA groups lost weight progressively for 3 weeks and restored their glycaemic control. No difference in spontaneous activity among all groups was noted, which indicates that the weight loss was due to augmented EE ($P < 0.05$). Further examination of the increased EE following the WD Low AA and WD Low BCAA diets revealed that fibroblast growth factor 21 (FGF21) blood levels were transiently elevated in the 12 days post-diet switch from DIO. FGF21 is a hormone that assists with energy balance regulation and appears to be responsible for the boost in EE. The mechanism involves the uncoupling of UCP1 through the sympathetic nervous system, thus establishing the connection between FGF21 and the browning of WAT, as well as the increased activation of brown adipose tissue (BAT), which causes the expression of mitochondrial-dense multicellular adipocytes within the WAT (Douris *et al.* 2015). These 'beige' spots are

 DOI: 10.1113/JP276274

very metabolically active, and thus allow for WAT to act in the same capacity as BAT (Douris *et al.* 2015). Consequently, a rise in EE is coupled with the loss of adipose mass – consistent with the weight loss seen in the WD Low BCAA and WD Low AA mice.

Previous work on this phenomenon has already suggested that reducing the protein:carbohydrate ratio mediates the augmentation of FGF21 levels (Solon-Biet *et al.* 2016). Indeed, Cummings *et al.* found that a WD containing a physiologically relevant 67% less BCAAs can temporarily induce FGF21, which consequently lead to the sustained increase in EE observed in DIO mice. As such, this novel finding demonstrates that selective AA reduction can directly promote metabolic health through an enhanced energy balance. Therefore, in conducting a secondary analysis of EE in groups fed with WDs of varying BCAA levels, and implementing precise physiological measures to test the hypothesis, the methods employed by Cummings *et al.* represent a complex and comprehensive *in vivo* study. Taking this into consideration, it is possible that these findings can be applied to humans, especially given that the relationship between serum BCAAs and obesity has already been established.

In conclusion, existing metabolic dysfunctions resulting from a high-calorie, high-fat and high-sugar WD can potentially be reversed without caloric restriction. Additionally, given that greater BCAA serum levels have the potential to serve as a biomarker for metabolic dysfunctions, its detection in clinical settings should be considered as it may contribute to lowering the prevalence of obesity and T2D. The Cummings *et al.* study also highlights that both significant weight loss and improved glycaemic control observed in mice belonging to the WD low BCAA group are likely a result of an increase in EE. However, while the findings are promising, more work needs to be done to determine at what level of BCAA manipulation and overall change in protein quality can best elicit positive metabolic effects and optimize EE in humans, and whether this can be considered as a viable, long-term solution for individuals suffering from metabolic disorders. Moreover, future research should address the FGF21 mechanism seen following BCAA restricted diets in order to shed light on this specific pathway, which includes how weight is lost through the beiging of WAT and how UCP1 expression affects the process. To ascertain what is precisely behind the shift in EE will allow for a more realistic, translatable weight loss alternative in humans. Thus, by selectively moderating BCAA content, whether through individualized diet plans or eliciting AA-specific catabolism via pharmaceutical measures, Cummings *et al.* have revealed that it is possible to reduce the complications associated with highly prevalent metabolic diseases, while simultaneously maintaining adequate nutrition.

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Additional information

Competing interests

None declared.

Author contributions

All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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V

Concluding Remarks

Conclusion

The objective of this thesis was to examine the effects of aerobic training on vascular mitochondrial respiration, with a follow-up investigation focusing on T1DM. Our research provides novel insight on how vascular mitochondrial respiratory capacity is affected by aerobic exercise and whether mitochondria-specific therapy could be used in the future to help reduce the risk of cardiovascular disease in T1DM patients.

Mitochondria are dynamic organelles and highly adaptable to various physiological stimuli, including physical activity. Our results derived from healthy aortic vessels support this notion, with significant increases in complex I-linked, glucose-based substrate respiration and capacity as major findings. However, in the context of diabetes, the opposite was observed in which aerobic exercise appeared to induce a decrease in vascular mitochondrial complex I respiration, indicating that training has the potential to exacerbate mitochondria-mediated diabetic complications in the vasculature.

Given the significant implications of mitochondrial dysfunction in the pathophysiology of diabetes, mitochondria-specific therapy may provide novel ways to treat the disease, or at least reduce the severity of complications associated with it. This project demonstrated that aerobic training led to a decrease in vascular mitochondrial content and function in poorly regulated T1DM models, and thus negated all previously demonstrated benefits of exercise. However, it is important to note that exercise is still important in maintaining a healthy lifestyle, and so more work needs to be done to investigate the exact dosage of aerobic training required to induce benefits in the vasculature of T1DM patients as a way to help reduce the risk of cardiovascular disease. Furthermore, it is important to determine the overall effects of the drug streptozotocin and its' potential involvement in multi-system organ damage which could also explain the high mortality rate in the T1DM subjects of our study. In order to refine our understanding of the role of aerobic exercise and untreated T1DM on mitochondrial function, future long-term adjuvant studies should focus on investigating insulin therapy as well as different exercise parameters that best elicit positive mitochondrial adaptations. Consequently, new treatment strategies for diabetes are required to address both mitochondrial function and oxidative stress. Pharmacological interventions that target mitochondrial biogenesis and respiration have potential therapeutic importance, with antioxidant

molecules already garnering much research attention to effectively control ROS production and protect against oxidative damage in diabetes.

Overall, this thesis contributes to potentially identifying novel methods to treat the metabolic and vascular complications of diabetes: from the specific modification of branched-chain amino acid intake in reducing the severity of T2DM to investigating the roles of aerobic exercise on mitochondrial respiration of vascular smooth muscle cells in T1DM patients.

VI

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