The relationship between elevations in glucocorticoids and diabetes development in rats on skeletal muscle insulin resistance and the microvasculature.

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ABSTRACT

Elevations in GC concentrations are well-known to cause a variety of negative systemic side effects, and subtle alterations in GC secretion and tissue-specific actions are a possible link between insulin resistance, the metabolic syndrome and T2DM development. These pathological conditions impair insulin-stimulated skeletal muscle blood flow and glucose uptake, in addition to causing profound myopathy, all of which could be significant contributors to the metabolic complications associated with insulin resistance. The focus of this dissertation was to understand how elevations in GC concentrations can be associated with skeletal muscle insulin resistance and diabetes development and whether augmentation to the muscle microvasculature is beneficial or even possible during conditions that generate significant exposure to elevations in GCs. To examine these situations, two rodent models of diabetes were used; exogenous elevations in GCs either combined with a HFD or provided a standard chow diet, to reproduce T2DM, and STZ-treated rats to induce β-cell destruction, simulating T1DM. As both regular exercise and circulating insulin levels have been well documented in the regulation of microvascular augmentation, we assessed the therapeutic potential of voluntary exercise and pharmacologically enhancing hyperemia in both GC-induced skeletal muscle insulin resistance and STZ-induced diabetes.

We revealed that the skeletal muscle is severely altered (decreased glycolytic muscle insulin-stimulated glucose transport and considerable glycolytic fibre atrophy) in the presence of hypercortisolemia coupled with HFD, but these alterations could be attenuated with the administration of voluntary aerobic exercise. The hypercortisolemia also resulted in alterations to the microvasculature (capillary rarefaction), which, when attenuated through α_1-antagonism, produced a correlative enhancement in insulin sensitivity. Finally, the combination of voluntary exercise and α_1-antagonism, in a model of chemically-induced (STZ) diabetes, demonstrated synergistic qualities through the increase in capillary growth within both glycolytic and oxidative skeletal muscle and enhanced glycemic control. This thesis provides considerable evidence proving that manipulation of the skeletal muscle

microvasculature, either through voluntary exercise or pharmacologically enhanced hyperemia, can attenuate the hypercortisolemia-induced skeletal muscle capillary rarefaction and improve glucose metabolism through enhanced glucose uptake and insulin sensitivity.

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

LIST OF TABLES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

LIST OF ABBREVIATIONS

T1DM – type 1 diabetes mellitus
T2DM – type 2 diabetes mellitus
GCs – glucocorticoids
Cort – corticosterone
HFD – high-fat diet
SD – standard diet
C:F – capillary-to-fibre ratio
SDH – succinate dehydrogenase
CSA – cross-sectional area
ORO – Oil Red O
FOXO1 – Forkhead box protein 01
AS160 – Akt substrate of 160 kDa
OGTT – oral glucose tolerance test
ITT – insulin tolerance test
AUC – area under the curve
FFAs – free fatty acids
NEFAs – non-esterified free fatty acids
11β-HSD1 – 1β-hydroxysteroid dehydrogenase type 1
GIP – Gastric inhibitory polypeptide
GLP-1 – Glucagon-like peptide-1
IRS-1 – Insulin receptor substrate-1
STZ – streptozotocin
GSIS – glucose-stimulated insulin-secretion
PGC1α – Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
AIR – acute insulin response
IMTG – intramuscular triglycerides
IMCL – intramuscular lipids
DAG – diacylglycerol
ISGU – insulin-stimulated glucose uptake

Chapter 1.

Literature Review and Introduction

Animal models of diabetes

1.1 Outline and introduction to diabetes.

Diabetes mellitus is a disease characterized by a relative or absolute lack of insulin, ultimately causing hyperglycemia. There are several types of diabetes, however the two main types are type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). Both T1DM and T2DM are multifaceted diseases in which a very complex genetic background interacts with environmental factors contributing to disease development (Ikegami, 2004). T1DM (commonly known as juvenile-onset diabetes) typically represents ~10% of all diabetes cases (Atkinson 2014; Cefalu, 2006) and is a chronic, autoimmune disease, targeting the insulin-producing pancreatic β-cells by effector lymphocytes (Mylona-Karayannia 2006). T2DM represents ~90% of all diabetes cases, and is a complex and diverse disease which occurs as the result of insulin resistance coupled with a failure of β-cell compensation (Jackerott 2006).

T1DM is characterized by a selective and specific involvement of the β-cells, without major immune filtration of other pancreatic cells, such as α-cells, which secrete glucagon, or δ-cells, which secrete somatostatin (Willcox 2009). In predisposed individuals, the initial 'activating' event is still unclear, however, it is suggested that early-life environmental triggers, such as infection, nutrition or chemical exposure, could initiate self-targeting immune cascades (Zaccardi 2016). In the initial stages of T1DM development, the progressive destruction of β-cells and serological positivity (presence of detectable autoantibodies) (Toivonen 2001) are not directly associated with alterations to blood glucose, as it seems the available β-cells are capable of maintaining euglycemia. Upon disease progression, there is further β-cell destruction followed

by insulin deficiency and hyperglycemia, with subsequent overt diabetes development. Tight glycaemic control after diagnosis is extremely important as it has been shown to reduce the risk of diabetic complications (Nathan 2005) in addition to the preservation of remaining β-cell mass/function (Picardi 2006).

T2DM, on the other hand, is more like a group of progressive diseases with different genetics and pathophysiologies but similar symptoms and outcomes; ultimately diminished pancreatic function over time creating chronic hyperglycemia. This progression to overt diabetes does not happen overnight, and typically is preceded by a deterioration of the insulin secretory capacity of the β-cells (Buchanan, 2003; Weyer 1999), which begins years prior to the clinical diagnosis of diabetes, characterized by the presence of insulin resistance (Kahn, 2000). The pancreatic dysfunction fails to compensate for the insulin resistance and leads to a state of relative insulin deficiency, progressing eventually to hyperglycemia. It is at this stage that impaired glucose tolerance and elevated fasting glucose (i.e. blood glucose ≥ 7.0 mmol/L) may be present. With worsening islet dysfunction and the inability for adequate compensation in relation to the degree of insulin resistance, clinically overt T2DM develops, defined by the Canadian Diabetes Association Clinical Practise Guidelines as a fasting blood glucose ≥ 7.0 mmol/L or a 2hr post oral glucose challenge of ≥ 11.1 mmol/L (Goldenberg 2013). Once hyperglycemia has manifest, multiple abnormalities are present, such as obesity, hyperlipidemia, hypercortisolemia, and increased levels of inflammatory cytokines (Defronzo, 2004). Ultimately, high levels of plasma glucose, free fatty acids (FFA), glucocorticoids (GCs) and cytokine concentrations can all worsen insulin resistance, making it quite difficult to identify the individual contributions of each of these metabolic defects in the pathogenesis of the disease.

Utilizing animal models that mimic diabetes pathophysiology provides a unique opportunity to study the onset, development and course of the disease in addition to the molecular mechanisms that lead to diabetes development. This thesis contains animal models of both T1DM and T2DM, as well as references to other animal models of diabetes.

1.2 Animal models of type 1 diabetes

As described above, T1DM is characterized by an autoimmune destruction of the β-cells, leading to impaired insulin production. Animal models of T1DM are insulin deficient through a number of different mechanisms ranging from chemical destruction of the β-cells to spontaneous development of autoimmune diabetes (Table 1).

1.2.1. Chemically-induced type 1 diabetes

The chemical induction of diabetes characteristically causes targeted necrosis to the pancreatic beta cells (Jorns, 1997; Peschke 2000), resulting in limited endogenous insulin production, hyperglycemia and weight loss (King, 2012). Diabetes is usually induced prior to the start of any experimental observation period to ensure stable hyperglycemia. Chemically-induced diabetes is not used for studies of the autoimmune processes in T1DM development, but may be used to examine the effects of insulin deficiency or hypoglycemia on various physiological processes. The two predominant compounds used to chemically-induce diabetes are streptozotocin (STZ) or alloxan, and while they induce cytotoxicity through distinct pathways, their β-cell selectivity is the same (Lenzen, 2008).

1.2.1.a. Streptozotocin-induced diabetes

STZ is a nitrosourea analogue, and its toxic action requires transport into the cell through the plasma membrane. STZ has also been used as a chemotherapeutic alkylating agent (Drímal 2006) and can be administered as a single large dose or as multiple smaller doses, with the

general goal of the induction of insulinopenic diabetes. Typically, multiple low doses of STZ (ranging from 20-40 mg/kg^{-1}/day, species dependant) have been used to study the immunological pathways causing insulitis and beta cell death (Mensah-Brown 2002; Yang 2003), while a single high dose can induce overt insulin-dependent diabetes in rodents. Once administered either subcutaneously or intraperitoneally, STZ, which is similar in structure to glucose, is selectively accumulated in pancreatic beta cells through the low-affinity GLUT2 glucose transporter situated in the plasma membrane (Karunanayake 1976). The toxicity of STZ is the result of its DNA alkylating action, which ultimately depletes cellular energy stores causing β-cell necrosis and insulin deficiency (Elsner 2000). It should be noted that STZ does not result in compete insulinopenia but does result in severe hyperglycemia.

Table 1.1. Rodent models of type 1 diabetes mellitus

Induction mechanism	Model	Main features	Elevated GCS
Chemical induction	High dose STZ Alloxan Multiple low dose STZ	Simple model of hyperglycemia Model of induced insulitis	✓ ✓(Selyatitskaya 2008) ✓(Zhang 2009a)
Spontaneous autoimmune	NOD mice BB rats	β-cell destruction due to an autoimmune process	✓(Fitzpatrick 1992) ✗ (Smith, 1991)
Genetically-induced	Akita mice	β-cell destruction due to ER stress	Not yet characterized
Virally-induced	Coxsackie B virus Encephalomyocarditis virus Kilham rat virus	β-cell destruction induced by viral infection of β-cells	Not yet characterized

1.2.1.b. Alloxan-induced diabetes

Alloxan is a pyrimidine derivative and toxic glucose analogue which selectively inhibits glucose-induced insulin secretion by inhibition of glucokinase (Lenzen, 2008). This agent also causes the selective destruction of β-cells by preferential accretion (GLUT2) within the β-cell and subsequent reactive oxygen species (ROS) generation (Lenzen, 2008). Due to its relative instability, alloxan has a narrow diabetogenic dose, as even nominal overdosing can cause general toxicity within the kidney (Szkudelski, 2001). The toxicity of alloxan occurs through a redox cycle that is initiated by its reduction to dialuric acid, and subsequent re-oxidation back to alloxan. This redox cycling generates the formation of superoxide radicals that undergo dismutation creating hydrogen peroxide and later reactive hydroxyl radicals that cause beta cell DNA fragmentation (Oberley, 1988; Szkudelski, 2001).

1.2.2. Spontaneous autoimmune models of type 1 diabetes

Autoimmunity is a multifaceted process with pathogenic mechanisms resulting from genetic predisposition and environmental modulation (Filippi 2005). As previously mentioned T1DM is a result of the destruction of the insulin-producing beta cells and is associated with infiltration of these cells by macrophages, dendritic cells and T and B cells (Giarratana, 2007). The spontaneously diabetic nonobese diabetic (NOD) mouse strain is the model of choice when studying the relationship between autoimmunity and T1DM, as its diabetes shares some of these characteristic autoimmune responses with human T1DM. The Biobreeding (BB) diabetic prone (BBDP) rat is the other commonly used autoimmune model of T1DM, which spontaneously develops hyperglycemia and ketoacidocis, characteristic of clinical T1DM (Mordes, 2004).

1.2.2.a. Nonobese diabetic mouse model

The NOD mouse was first developed in Osaka, Japan (Hanafusa 1994). Typically, these mice develop insulinitis and an associated infiltration of $CD4^+$ and $CD8^+$ lymphocytes, with the presence of B cells and natural killer (NK) cells, into the pancreatic β-cells around 3-6 weeks of age (Kim 2010). This insulitis is what causes β-cell destruction and severely limited insulin production around 10-14 weeks of age, resulting in weight loss and insulin therapy. Overt diabetes can develop up to 30 weeks of age and is more prevalent in females than males (Hanafusa, 1994; Pozzilli 1993). These mice also require fastidious housing to maintain the diabetic phenotype and must be kept in a specific pathogen-free (SPF) environment (King, 2012). While there are similarities to human T1DM, one factor that is inconsistent is the time point of intervention. A number of drugs that have been found to be effective in ameliorating hyperglycemia in the NOD mouse are ineffective when translated to humans (Von Herrath 2009), suggesting the autoimmune process in human disease is more complex than in this rodent model.

1.2.2.b. Biobreeding rat model

The BB rat was derived accidentally from outbred Wistar rats, and spontaneous diabetes was first identified in a Canadian colony. This lead to the creation of two founding colonies, from which all substrains are derived, one inbred (BBDP/Wor) and one outbred (BBDP) (Mordes, 2004). The development of diabetes occurs later in these animals, after puberty from 8-16 weeks of age, and is also the result of insulitis with infiltration of macrophages, T, B and NK cells. The animals are also lymphopenic with significant reduction in $CD4^+$ cells and an absence of $CD8^+$ cells, which is not characteristic of human T1DM (Mordes, 2004). Nonetheless, these animals have been deemed helpful in clarifying the genetics of the disease (Wallis 2009), and

have been used for numerous intervention studies (Hartoft-Nielsen 2009) and the study of diabetic complications such as neuropathy (Zhang 2007).

1.2.3. Other models of type 1 diabetes

One of the most basic ways of studying the effects of hyperglycemia in animals is to partially or completely remove the pancreas, termed a pancreatectomy. This surgery can be performed on both mice and rats, in addition to other animals such as pigs (Mellert 1998; Morel 1991), dogs (Fisher 2001) and primates (He 2011). While the reliability of hyperglycemia induction is quite high, it is a very invasive method which can increase the possibility of hypoglycemia and pancreatic exocrine insufficiency (He, 2011). Finally, viruses have been implicated in the pathogenesis of T1DM (Van Der Werf 2007) which has led to the utilization of viruses to induce β-cell destruction. Beta-cell death can be induced through direct infection or initiation of an autoimmune response against the β-cell (Jun 2003). The viruses used to induce diabetes in animal models are the coxsacki B virus (Jaidane 2009), Kilham rat virus (Ellerman 1996) and encephalomyocarditis virus (Shimada 2004). Using a virus model is complicated by the fact that the induction of diabetes is dependent on the replication of the virus in addition to the timing of the infection, and while a limited number of human T1DM cases have been linked to a single virus (Richardson 2009; Van Der Werf, 2007), the degree of the involvement of viruses in the pathogenesis of T1DM remains uncertain.

1.3 Animal models of type 2 diabetes

T2DM is typically characterized by peripheral insulin resistance, hyperinsulinemia and subsequent β-cell dysfunction (with or without a decrease in β-cell mass) and as such, insulin levels and glycemic control can be quite varied, ultimately depending on the phase of the disease and the compensation of hyperinsulinemia to improve high glucose levels. Another added

variable is that a large number of T2DM patients are not obese and can experience a reduced insulin secretion and less insulin resistance than the obese phenotype (Chatizigeorgiou 2009). Despite genetic predisposition, the risk of developing T2DM in humans increases with age, obesity, cardiovascular disease and a lack of physical activity (Cheng 2013; Meigs 2003). T2DM can manifest as multiple clinical and pathophysiological conditions, as there are a number of environmental factors and various genetic effects linked to its development (Chatizigeorgiou, 2009). To account for the individual differences in T2DM expression, animal models of T2DM must be capable of generating different degrees of both β-cell failure and insulin resistance, in addition to varying environmental and genetic triggers (Table 2).

Table 1.2. Rodent models of type 2 diabetes mellitus

Induction mechanism	Model	Main features	Elevated GCS
Obese models	$Lep^{ob/ob}$ mouse	Obesity-induced hyperglycemia	✓
	$Lept^{db/db}$ mouse		✓
	ZDF rat		✓
	KK mice		✓(Alberts 2005)
	NZO mice		Not yet characterized
	OLETF rat		× (Noguchi 2007)
Non-obese models	Cohen diabetic rat	Hyperglycemia induced by insufficient β-cell function/mass	Not yet characterized
	GK rat		Not yet characterized
Induced obesity	Israeli Sand rat	Obesity-induced hyperglycemia	✓(Amirat 1980)
	Spiny mouse		✓(Lamers W.H., 1986)
	C57/BL 6J mouse		✓(Alberts, 2005)
	Rapid onset diabetes (ROD) model		✓(Shpilberg 2012)
Genetically-induced models of β-cell dysfunction	Akita mice	B-cell destruction due to ER stress	Not yet characterized

1.3.1. Rodent models of type 2 diabetes

T2DM is likely a polygenic disease (Mccarthy, 2001) and the use of rodent models allows for a versatile approach in understanding the disease, through naturally occurring mutations, inbred and genetically engineered rodent models and more specifically in mice, global and tissue specific knockouts and transgenics. Like in humans, the phenotype of the rodent model also depends on the genetic background, sex and age of the rodent (Neubauer, 2006). While the affected genes in the rodent model might not perfectly match human disease, the increased propensity to develop diabetes based on a specific background is likely similar.

1.3.1.a. Obese rodent models of spontaneous type 2 diabetes

T2DM is well-known to be tightly connected to obesity, and there are a number of obese rodent models commonly used to further understand this association. Among the most widely studied models for T2DM are the ob/ob (obese) and db/db (diabetic) mice and the Zucker (fa/fa) rat. These models have mutations in leptin signaling (gene: ob/ob, and receptor: db/db, fa/fa), which is a hormone that induces satiety, and without functional signaling, these rodents develop hyperphagia and severe obesity (Chua 1996). With both mouse models, the expression of obesity predisposes these mice to diabetes, evidence which is important when studying the influence of obesity on the development of the disease. The db/db mouse typically becomes hyperinsulinemic early in life (within 2 weeks of age) followed by obesity by 3-4 weeks of age and hyperglycemia at 4-8 weeks of age succeeding β-cell failure, a similar progression as that seen in human T2DM (Bates 2005). The ob/ob mouse becomes hyperinsulinemic around 3-4 weeks of age, although the associated hyperglycemia is more mild than the db/db model, and is coupled with hyperphagia and insulin resistance (Folli 1993; Saad 1992). The Zucker fatty rat is similar in pathophysiology to the db/db mouse model, and is used to replicate human obesity coupled with

hyperlipidemia and hypertension (Ramarao, 2007). A mutation in this strain lead to the development of the inbred Zucker diabetic fatty (ZDF) rat, of which only the males develop severe diabetes around 8 weeks of age as the result of β-cell apoptosis. The reason for diabetes protection in the females is unclear.. Combined with the peripheral insulin resistance, the ZDF rat becomes hypoinsulinemic and hyperglycemic around 14 weeks of age (Pick 1998).

Genetic background, gender and age all play important roles on influencing the phenotype of both human and animal diseases, and selected inbreeding, which takes some of these variables into consideration, has yielded additional mouse models that correspond with the human condition. The KK mouse is moderately obese and hyperleptinaemic, and develops severe hyperinsulinemia and insulin resistance in both skeletal muscle and adipose tissue (Clee 2007; Reddi 1988). These animals also show signs of diabetic nephropathy (Ikeda, 1994). Additionally, there is a derivative of this strain, the KK-Aγ mouse, which was generated by the introduction of the yellow obese Aγ gene into the KK strain (Chakraborty 2009). These mice develop obesity later in life along with more severe hyperinsulinaemia. The New Zealand Obese (NZO) mouse is another polygenic model of obesity, also created through selected breeding. These animals are hyperphagic and obese as well as hyperleptinaemic by 9-12 weeks of age (Leiter 2004), as they have a defect in leptin transport across the blood-brain barrier (Halaas 1997). Generally, NZO mice develop hepatic insulin resistance from an early age which induces impairments in glucose tolerance, hyperglycemia and subsequent hyperinsulinemia, all of which progressively worsen with age (Haskell 2002).

In contrast with both the KK and NZO mice, the Otsuka Long Evans Tokushima Fatty (OLETF) rat, while also a model of obesity-induced T2DM, develops a more mild obese phenotype and late onset hyperglycemia (~18 weeks of age) (Ramarao, 2007). Diabetes develops

in males only, likely via a genetic link, with the general progression of diabetes development occurring with beta cell failure over three distinct stages. Cellular infiltration and degeneration of the β-cell occurs between 6-20 weeks of age, followed by hyperplasia between 20-40 weeks with the final stage causing the islets to become fibrotic and eventually replaced by connective tissue (Kawano 1994).

1.3.1.b. Nonobese rodent models of type 2 diabetes

As previously mentioned, not all humans with T2DM are obese, and it is very important to also have lean rodent models of the disease. These models usually center on β-cell failure, which is the primary impairment responsible for the subsequent development of overt diabetes in humans (Weir 2009). The Goto-Kakizaki (GK) rat is generated through the repetitive breeding of Wistar rats with the worst glucose tolerance, creating the development of a lean model of T2DM, characterized by glucose intolerance and impaired glucose-stimulated insulin secretion (Ohneda 1993). Interestingly, islet morphology and metabolism can be different between colonies, suggesting the hyperglycemia ensues from insulin secretory deficits that are genetically determined (Zong-Chao 1998). GK rats are used to study β-cell dysfunction in T2DM (Movassat 2007; Portha 2009) in addition to multiple diabetic complications associated with T2DM (Liepinsh 2009). The nonobese mutant (Akita) mouse is a genetically induced model of β-cell dysfunction. These mice contain a spontaneous mutation in the INS2 gene, which is the mouse homologue of human preproinsulin (Yoshioka 1997). Insulin dependent diabetes development is complemented by hyperglycemia, polydipsia and polyuria, which is usually more severe in males than females. The Akita mouse does not develop obesity or insulitis, and has been used mainly for β-cell transplantation studies and the study of diabetic retinopathy (Mathews 2002).

1.3.1.c. Other models of type 2 diabetes

T2DM is not only represented through spontaneous models but also through inducible paradigms of the disease, such as diet-induced diabetic models, chemically or surgically induced models or transgenic models that are associated with disease development (i.e. hyperglycemia and, in some cases, hyperinsulinemia).

Human studies have shown that increased fat intake is associated with body weight gain which can lead to obesity and other related diseases. High-fat feeding induced diabetes, which was established in 1988 (Surwit 1988) using C57BL/6J mice, has become a very popular and easily inducible model of T2DM, as rodents readily gain weight when fed high-fat diets (HFD). High-fat feeding results in obesity, hyperinsulinemia and impaired glucose homeostasis as the consequence of inadequate β-cell compensation (Winzell 2004). The standard rodent diet, on a caloric basis, is ~26% protein, 63% carbohydrate and 11% fat and general high-fat diets contain ~60% of energy derived from fat. Typically, rodents are fed the high-fat diet for several weeks to induce a more pronounced weight gain, which is associated with insulin resistance, impaired β-cell compensation and eventually glucose intolerance. Interestingly, not all rat models develop hyperglycemia on a HFD, likely because of insufficient β-cell compensation for the whole body insulin resistance (liver, muscle, adipose tissue). The Israeli sand rat is an established diet-induced diabetic animal model, which becomes obese and diabetic when given a standard laboratory chow diet, which is much higher in energy density than its natural diet (Ziv 1999). These rats develop hyperphagia, obesity, hyperinsulinemia, increased hepatic glucose production, glucose intolerance and peripheral insulin resistance (Cefalu, 2006; Kaiser 2005), in addition to a progressive loss of β-cell mass due to increased apoptotic mechanisms (Masiello, 2006). The sand rat is generally used to study the interaction between obesity and diabetes, the

effects of diet and exercise and also for pharmacological research (Kaiser, 2005). The C57BL/6J mouse and the spiny mouse are other diet-inducible rodent models (King, 2012), as background and strain ultimately work together to determine the susceptibility to diet-induced metabolic changes. Inbred C57BL/6J mice demonstrate a heterogeneic response to high-fat feeding, suggesting that the different outcomes are not solely genetic (Burcelin 2002). High fat feeding can also be used in transgenic or knock-out animals to generate the diabetic phenotype by adding further stress to the β-cells.

While both alloxan and STZ are potent diabetogenic agents, well known to induce T1DM, they have also been used in T2DM development. Alloxan or STZ injection, neonatally or immediately after birth in rats, generates adult onset T2DM (Portha 1974; Weir 1981). Of course each agent requires a specific dose and time of administration. As these chemicals are toxic to the β-cells, diabetes development is accompanied by decreased β-cell mass and subsequent impairments to insulin secretion. However, when combined with a genetically insulin resistant rodent strain or a high-fat diet, overt hyperglycemia and normal plasma insulin are produced, which is much more appropriate for the study of T2DM (Reaven 1991). There are also a number of therapeutic agents that can predispose to or precipitate diabetes development, especially in combination with pre-existing risk factors. These agents can be weakly diabetogenic, such as antihypertensive agents and statins, or more strongly diabetogenic, like steroids, antipsychotics and multiple immunosuppressive compounds.

The rapid onset diabetes (ROD) rodent model was very recently established in the Riddell laboratory (Shpilberg, 2012) to examine the collective effect of elevations in stress hormones, specifically cortisol, which is a potent diabetogenic stimulus, and high-fat feeding. With two weeks of exogenous corticosterone treatment (4x100mg pellets) plus a high fat diet,

male Sprague-Dawley rats become severely hyperglycemic, hyperinsulinemic, and insulin resistant (Shpilberg, 2012), through the development of hepatic steatosis (D'souza, 2011), impaired β-cell function (Beaudry 2013) and increased lipid deposition in both the liver and skeletal muscle. The ROD model mimics many of the symptoms observed in patients with T2DM, and provides evidence that elevations in GCs induce diabetes development, especially if susceptible individuals do not consume a balanced diet. The model is limited; however, in its translational application to humans undergoing exogenous GC therapy since the GC levels are supraphysiologic/pharmacologic in this model (Shpilberg, 2012).

As seen with T1DM animal models, partial removal of the pancreas is another possible mechanism for the induction of T2DM, however this method can cause the development of moderate hyperglycemia without overt changes in body weight or circulating insulin levels (Risbud 2002). In order to induce a more T2DM phenotype, partial pancreatectomies are typically combined with the administration of a diabetogenic agent, such as STZ, which is sometimes used for the study of transplantation or regeneration factors in T2DM (Risbud, 2002).

Finally, many transgenic models have been developed to study the complex and polygenic mechanisms involved in the pathogenesis of T2DM. As insulin and insulin growth factor-1 (IGF-1) both belong to the growth factor family and play important roles in metabolism and growth of almost every mammalian tissue, numerous mouse models of diabetes have focused on targeting proteins in the skeletal muscle insulin/IGF-1 signaling pathways. The role of genes implicated in muscle insulin resistance (IRS-1, IRS-2, GLUT-4) (Kido 2000; Stenbit 1997), lipid and glucose metabolism (Evans 2004b) and pancreatic insulin secretion (GLUT-2, glucokinase) (Cani 2007) are likely better understood through transgenic or knockout methods. However, global knockout models can be limited in their scope, as some genes can vary

considerably between tissues, necessitating a tissue-specific approach for some studies. Additionally, the organism has the capacity to compensate for the affected gene(s), possibly obscuring the precise phenotype, results that have been observed in both IRS-1 and GLUT-4 null mice (Stenbit 1996).

1.3.2. Nonrodent models of type 2 diabetes

Larger animals have also been used to understand the development of T2DM. In fact, the development of T2DM in the domestic cat is actually quite similar to the human condition (Henson 2006). Typically, diabetes onset occurs later in life and is influenced by similar environmental risk factors, such as inactivity and obesity (O'brien, 2002; Panciera 1990) and can be associated with diseases, pharmacological agents and hormones that impair peripheral tissue insulin sensitivity, such as treatment with corticosteroids or progestins (Rand 2004). Interestingly, humans and pigs seem to have comparable gastrointestinal structure and function, pancreas morphology and overall metabolic status (Larsen 2004), and several strains of pigs have a phenotype that resembles human T2DM (Bellinger 2006). obviously, primates are the most analogous animal model for humans, and T2DM in primates is common with obesity and with advanced age (Wagner 2001; Wagner 2006).

Effects of diabetes on skeletal muscle

This thesis focuses primarily on the effects of diabetes on skeletal muscle and as such an overview of the effects of the disease on this tissue is warranted here.

2.1. Overview of peripheral effects of diabetes

The ability to maintain normal blood glucose levels involves complex interaction between insulin secretion by pancreatic β-cells and insulin metabolic responsiveness in skeletal muscle, liver, adipose and other tissues. Insulin facilitates the uptake of glucose into the cell in addition to promoting storage through the stimulation of glycogen synthase (muscle and liver) and triglyceride production and suppression of lipolysis and proteolysis. Without insulin, the metabolic process is disturbed and a catabolic state is created. Skeletal muscle is particularly important in the maintenance of glucose homeostasis as it comprises ~40-50% of body mass and mediates over 75% of all insulin-mediated glucose disposal under normal physiologic conditions (Stump 2006).

2.2. Skeletal muscle insulin resistance

Insulin resistance, which is an impaired response of insulin-sensitive tissues to insulin, is a characteristic feature of T2DM and plays a key role in the pathogenesis of the disease (Defronzo, 2004; Defronzo 2009). While β-cell failure is the final outcome in the development of overt diabetes, skeletal muscle insulin resistance is typically observed prior to hyperglycemia and β-cell exhaustion, and is considered the primary defect in T2DM development (Lillioja 1988; Warram 1990). Systemic insulin sensitivity, under postprandial conditions, is mostly driven by skeletal muscle insulin sensitivity (Kelley 2001). As observed during physiological hyperinsulinemia (80-100uU/ml), there is an increase in leg muscle glucose uptake, which increases in a linear fashion, ultimately reaching a plateau after ~60 minutes (Defronzo 1981).

When examined in lean T2DM patients, the ability of insulin to maximally stimulate glucose uptake is attenuated (~50%) as the onset of insulin action is delayed. It has also been established that physiologic elevations in insulin concentrations over a period of 72 hours, in healthy subjects, can induce reductions to insulin-stimulated muscle glycogen synthase activity, total body glucose uptake and nonoxidative glucose disposal (which primarily reflects muscle glycogen synthesis) (Defronzo, 2009). Moreover, chronic hyperinsulinemia in conscious rats for 7 days decreases insulin-stimulated total body glucose uptake, glucose storage and glycolysis and fails to promote the normal suppression of hepatic glucose production (Koopmans 1999; Koopmans 1997). These findings suggest that, since the majority of glucose disposal occurs in skeletal muscle, even physiologic elevations in insulin concentrations can aggravate any underlying muscle insulin resistance. Or in other words, hyperinsulinemia may actually promote a further worsening of insulin sensitivity in diabetes, thereby promoting a vicious cycle that places an unrelenting demand on pancreatic β-cell function.

2.3. Skeletal muscle insulin signaling

The initial step in skeletal muscle glucose metabolism involves activation of the glucose transport system, which allows an influx of glucose into insulin target tissues. In both skeletal muscle and adipose tissue, insulin stimulates glucose transport into the cells through the activation of a complex signaling cascade. Integral to the mechanism of insulin signaling and action is the insulin receptor, which is expressed in insulin-responsive tissues, like the liver, adipose, β-cells and skeletal muscle, in addition to sites of diabetic complications. Mice with a global insulin receptor deletion show no obvious impairments at birth, however, develop early postnatal diabetes (Kitamura 2003). While tissue-specific deletion of the insulin receptor in pancreatic β-cells causes insulin secretion impairments comparable to what is observed in human

T2DM (Kulkarni 1999), deletion within skeletal muscle does not cause the development of hyperglycemia or overt diabetes (Brüning 1998). This result is likely due to both normal functioning of the insulin receptor in other insulin-sensitive tissues, and adaptive glucose uptake in peripheral organs mediated by glucose transporter (GLUT4) upregulation (Higaki 1999).

Once insulin binds to the insulin receptor, found in muscle, liver and adipose tissue, tyrosine phosphorylation of the insulin receptor is initiated, which causes movement of the insulin receptor substrate (IRS)-1 to the cell membrane. Tyrosine phosphorylation of IRS-1 causes an activation of the p85 regulatory subunit of phosphatidylinositol (PI)-3 kinase (PI3K) which activates the p110 catalytic subunit, increasing phosphatidylinositol-3,4,5 triphosphate. Abnormalities of IRS proteins or within second messengers, such as PI3K, can affect docking and subsequent downstream insulin signaling . The interaction of PI3K with the IRS proteins ultimately regulates cellular glucose uptake through a series of phosphorylation events (Gual 2005) and dysregulation of this interaction has been established as an important event in the development of the progressive defects in insulin secretion, insulin resistance and eventually overt T2DM development (Dominguez 2011). The resulting activation of protein kinase B (Akt) and phosphorylation of Akt substrate 160 (AS160), facilitates the translocation of GLUT4, an insulin-responsive glucose transporter, to the sarcolemma, which allows glucose to enter the cell (Taniguchi 2006). Changes in GLUT4 expression can occur in patients with vascular complications from diabetes mellitus, and decreased GLUT4 expression specifically, has been associated with impaired insulin receptor signaling (Friedman 1992). Furthermore, mice with a heterozygous deletion of the gene that encodes GLUT4 develop insulin resistance and T2DM (Stenbit, 1996), while skeletal muscle biopsies from T2DM patients show impaired intracellular GLUT4 translocation to the cell membrane (Kennedy 1999). Upon entry into the cell, glucose is

rapidly phosphorylated by hexokinase II to glucose-6-phosphate and shuttled to oxidative or non-oxidative pathways. As skeletal muscle is the predominant site of glycogen synthesis under physiologic conditions, almost two-thirds of all glucose-6-phosphate is converted to glycogen, while one-third enters glycolysis. Maintenance of the IRS-1/PI-3 kinase/Akt pathway is essential for normal, insulin-mediated glucose uptake within the skeletal muscle.

2.4. Mitochondrial oxidative phosphorylation

Mitochondria play a very important role in energy metabolism and ATP production in a number of tissues, including skeletal muscle, cardiac muscle, brain and liver. ATP generation occurs through tricarboxylic (TCA) cycle activity and the associated electron transport chain (ETC) of the inner mitochondrial membrane. Once the reducing equivalents (NADH and $FADH_2$) are produced, they are reoxidized via a process that involves the transfer of electrons through the ETC and associated translocation of protons across the inner membrane, ultimately creating the transmembrane electrochemical gradient. The electrochemical potential from this gradient generates ATP from ADP and Pi, through proton movement back through the ATP synthase complex. Under normal conditions, this proton gradient is also reduced by H^+ 'leak' to the matrix, which occurs via non-protein membrane pores, protein/lipid interfaces (H^+ leak) or though proton channels called uncoupling proteins (UCPs) (Newsholme 2012). Howwever, during normal oxidative phosphorylation, mitochondria can produce considerable reactive oxygen species (ROS) and reactive nitrogen species such as superoxide anions (O2-), peroxynitrite and other reactive species which are very powerful chemical oxidants (Turrens, 2003). To manage the potentially toxic levels of reactive species, cells require antioxidant systems to neutralize ROS. Enzymes such as manganese-superoxide dismutase (MnSOD), glutathione (GSH) and catalase are key antioxidants responsible for accepting unpaired electrons

to produce a stable product (Evans 2002; Evans 2004a). Although cells possess these different antioxidant systems, it is still possible for ROS to elude these defence mechanisms, resulting in a slow accumulation of chronic damage as the antioxidant network and repair systems become overwhelmed (Maiese 2007).

2.4.1. Fatty acid-induced mitochondrial dysfunction

Obesity is commonly associated with insulin resistance and T2DM development, and one of the most harmful effects of obesity is lipid deposition in non-adipose tissues such as skeletal muscle, cardiac muscle and liver. It has been suggested that the accumulation of lipids within skeletal muscle can directly interfere with insulin signaling, possibly contributing to the generation of insulin resistance. In agreement with this hypothesis is the strong association observed between lipid accumulation in the skeletal muscle (and liver) and insulin resistance in men (Mcgarry, 2002). There is also an established association between elevations in IMCL levels and muscular insulin resistance in T2DM patients (Goodpaster, 2004) and in high-risk non-diabetic patients with a family history of diabetes (Perseghin 2003). However, elevations in IMCL accumulation do not automatically cause insulin resistance, as high levels of IMCL can also be present in skeletal muscle from endurance-trained athletes, who are very insulin-sensitive (Goodpaster 2001b). The general concept is that elevations in lipid accumulation can become harmful when an increase in the availability or supply of lipids to the skeletal muscle is not compensated for through increased oxidative pathways, which results in the generation of toxic intermediates like ceramides and diacylglycerol (DAG), which then accumulate within the cell and subsequently interfere with the insulin signaling machinery (Shulman, 2000). Controversy remains as to how excess ROS production contributes to the induction of diabetes mellitus,

especially since there is data demonstrating that ROS signaling is actually required for normal insulin secretion (Macmillan-Crow 2001).

Excessive levels of ROS can damage cells by oxidizing DNA, protein and lipids as well as indirectly damaging cells through activation of a number of stress-sensitive intracellular signaling pathways such as NF-kB, p38 MAPK, JNK/SAPK and others. Stimulation of these pathways causes an increase in expression of various gene products that can cause cellular damage and have been established in the etiology of diabetic complications (Newsholme, 2012).

Mitochondria are the primary organelles that oxidize fatty acids, and there is evidence showing that fat oxidation is reduced in both T2DM patients and obese insulin-resistant nondiabetic individuals (Kelley, 2005), which suggests that skeletal muscle mitochondrial oxidative capacity is impaired. Evidence also shows that short-term increases in FFA, at fasting insulin levels, can induce insulin resistance in skeletal muscle, but does not impair mitochondrial activity (Chavez 2010), and highlights that physiological insulin resistance, in association with prolonged fasting, does not involve irregular mitochondrial activity. Additional data has demonstrated a reduction in the expression of key mitochondrial genes involved in the regulation of oxidative metabolism in the skeletal muscle of children of T2DM parents (Mootha 2003). Mitochondria isolated from the myotubes of T2DM patients demonstrate reduced ATP production when compared to lean controls (Minet 2010). Also, impaired TCA cycle flux has been shown in both insulin resistant offspring of T2DM patients (Befroy 2007) and exercising T2DM patients in vivo (Schrauwen and Hesselink 2008). Mitochondria, isolated from skeletal muscle biopsies of insulin-resistant individuals, was found to have reduced levels of mRNA for certain mitochondrial genes (Mootha, 2003) and lower protein expression of respiratory chain subunits (Heilbronn 2007).

2.5. Inflammatory signaling within the skeletal muscle

Expansion of adipose tissue as a consequence of a sedentary lifestyle and excess energy intake can also result in a local inflammatory response in the visceral adipose tissue, infiltration of immune cells and local and systemic increases in proinflammatory cytokines and adipokines (Eckardt 2014). Thus, obesity, which is considered the most common cause of insulin resistance and T2DM are associated with chronic low-grade, systemic tissue inflammation (Lumeng 2011; Olefsky 2010). In obesity, there is an established increase in M1-like-macrophages, adipose tissue macrophages that exert potent proinflammatory signals, and secrete a number of different cytokines including tumor necrosis factor a (TNFa), interleukin-1b (IL-1b) and others. These cytokines then act through both paracrine mechanisms, to directly inhibit insulin action in target cells, like skeletal muscle myocytes (Fève 2009), and endocrine mechanisms through activation of stress kinases including S6K, IKKb, JNK1 and PKCF, which subsequently phosphorylate IRS1 on the inhibitory serine residues, impairing downstream insulin action (Hotamisligil, 2006). Inhibition of insulin action by cytokines can also occur through transcriptional mechanisms, ultimately reducing the expression of certain insulin signaling proteins, in addition to enhanced ceramide formation, which directly inhibits Akt activation (Holland 2011).

There is clearly a close relationship between chronic inflammation, fatty acid metabolism and insulin resistance within the skeletal muscle in T2DM (De Luca, 2008). Biopsies from T2DM patients show evidence of infiltration of inflammatory cells into the skeletal muscle as the result of chronic inflammation in addition to elevations in inflammatory molecules/cytokines, including TNFa, IL-6, CRP and others (Marette, 2002). Additionally, skeletal muscle is capable of producing and secreting several inflammatory myokines, including a number of interleukins (IL-6, IL-8, IL-15) and TNFa, usually as the result of contraction (Bruunsgaard, 2005; Pedersen

2007). The effect of these contraction-mediated myokines on diabetic development is unclear, however, especially since regular exercise itself is protective against diabetes development (see section 2.6 below). However, skeletal muscles also possess a number of other immune system components, like cytokine receptors and TLRs (Lang 2003) which makes them a key immunomodulatory tissue.

2.6. Exercise and diabetes; skeletal muscle effects

Regular physical activity, both endurance and resistance exercise, has numerous health benefits for healthy individuals and patients living with diabetes. More effective than pharmacologic agents, regular physical activity can reduce T2DM development in humans at risk for the disease by as mush as 68% (Group, 2002). The health benefits of regular exercise include improved cardiovascular function, insulin sensitivity, muscle metabolism, body weight and lipid profile (Myers, 2003; Santos 2012) among numerous other physiological and psychological adaptations. As maintenance of daily euglycemia and lipid content are important areas of treatment in T2DM, exercise has proven to be a vital therapy as studies suggest that exercise could diminish the intake of high caloric foods, which would subsequently improve the metabolic profile (Benite-Ribeiro 2014) in addition to the fact that skeletal muscle contraction can improve glucose uptake independent of insulin stimulation. In contrast to the effects of insulin on stimulating skeletal muscle glucose disposal, exercise and contraction have no effect on tyrosine phosphorylation of IR and IRS-1, or PI3K activity (Goodyear 1995), and pharmacological inhibition of PI3K activity with wortmannin does not inhibit glucose transport in isolated rat muscles contracted in vitro (Hayashi 1998). Additionally, exercise is capable of increasing the activity of the insulin receptor and IRS proteins within the skeletal muscle, which further enhances the cellular uptake of glucose at rest (Christ-Roberts 2003). These results prove

that there are different signals leading to glucose transport by insulin and exercise in skeletal muscle which, either through individual or additive means, allow skeletal muscle contraction to ultimately override any established insulin resistance, causing increased glucose uptake and improved hyperglycemia.

Elevated insulin-independent glucose uptake during exercise is mostly reversed ~2–3 h postexercise, whereas enhanced skeletal muscle and whole body insulin sensitivity can persist for up to 24 – 48 h postexercise (Cartee 1990; Perseghin 1996). This substantially elevated skeletal muscle insulin-stimulated glucose uptake after acute exercise was first identified by Richter et al. (Richter 1982) using the perfused hindlimb model. This response was confirmed using in the euglycemic hyperinsulinemic clamp in humans (Mikines 1988; Sharoff 2010). Additional work established that prior exercise can directly regulate subsequent insulin-stimulated glucose transport in isolated muscles, and it is possible that prior exercise can also influence other processes that enhance in vivo glucose uptake, including elevated muscle blood flow and hexokinase activity (Fueger 2005; Richter 2013). More recent findings have examined the effects of an acute bout of exercise within insulin-resistant individuals and determined that a single bout of exercise can increase insulin-stimulated glucose uptake in insulin-resistant individuals (Perseghin 1996; Sharoff, 2010). However, when both insulin-resistant and healthy groups complete similar exercise protocols, exercise is consistently unable to equalize insulin-stimulated glucose uptake between groups (Castorena 2014; Pehmøller 2012).

Contractile activity also changes the fuel status of the skeletal muscle, causing varied decreases in both phosphocreatine and ATP levels, which can subsequently stimulate the AMP-activated protein kinase (AMPK) signaling pathway. AMPK is considered the master regulator of cellular energy homeostasis and can be activated in situations where cellular ATP

concentrations are depleted such as low cellular glucose availability, hypoxia and ischaemia (Winder 2000). In skeletal muscle, AMPK activation stimulates glucose uptake, fatty acid oxidation, GLUT4 translocation and mitochondrial biogenesis, while inhibiting protein and glycogen synthesis (Coughlan 2013), ultimately improving insulin sensitivity and glucose homeostasis, which makes it a possible target for therapeutic intervention in T2DM.

Regular exercise can also reduce inflammation as there is a strong inverse relationship between the level of physical activity and systemic low-grade inflammation (King 2003). As mentioned, regular physical activity leads to reductions in body weight and visceral adiposity, which is a key source of proinflammatory cytokines, and could be the reason behind reductions in C-reactive protein (Stewart 2007) and Toll-like receptor (TLR) expression (TLR2 and 4) in immune cells and skeletal muscle following long-term exercise. Additional benefits of exercise encompass the exercise-induced release of adrenaline (epinephrine), cortisol and other factors, like myokines, that have immunomodulatory effects ultimately acting on immune system function (Gleeson 2011).

The skeletal muscle is now identified as a secretory organ, able to synthesize and release proteins, termed myokines, capable of communicating systemically or locally within the muscle (Pedersen, 2011). Contraction stimulates the muscles' release of various myokines, including IL-6, IL-8 and IL-15 (Broholm, 2010), which have been suggested to mediate the acute and chronic adaptations induced with exercise. Muscle-derived IL-6 was the first identified myokine (Hiscock, 2004; Steensberg 2000), and is established as having the ability to affect other organs like the liver, brain and adipose tissue, as well as acting on muscle itself, as IL-6 induces glucose uptake (Saini 2014) and fatty acid β-oxidation (Pedersen, 2012) in muscle and can stimulate hepatic gluconeogenesis and lipolysis in adipose tissue. The plasma concentrations of the other

contraction induced myokines, IL-8 and IL-15 do not seem to increase proportionally to exercise (Broholm 2008; Nielsen 2007), suggesting that these factors may actually exert their actions locally. IL-15 is thought to be involved in muscle-adipose crosstalk (Pedersen 2008), while high concentrations of IL-8 might be involved in exercise-induced angiogenesis and capillarization (Pedersen, 2008). Enhanced glucose availability has been shown to attenuate IL-6 plasma concentrations after exercise, suggesting that fitness level can regulate IL-6 secretion (Pederson, 2005) similar to what is observed with cortisol after exercise training. Additionally, elevated levels of IL-6 have also been suggested to exert a protective effect on individuals with low levels of chronic inflammation, as myokines could mediate the suppression of proinflammatory signals at both the level of the skeletal muscle and peripheral tissues, like the liver (Bruunsgaard, 2005).

Glucocorticoids and diabetes; effects within skeletal muscle

3.1. Glucocorticoids, obesity and diabetes development

Glucocorticoids (GCs) are endogenous, steroid hormones responsible for mediating crucial and adaptive responses to stress. There is significant evidence that the glucocorticoid/glucocorticoid receptor (GC/GR) axis plays a critical role in maintaining overall glucose homeostasis, as maintenance of systemic carbohydrate metabolism requires complex regulation between various peripheral organs in addition to the central nervous system. It has been suggested that subtle alterations in both cortisol secretion and tissue-specific action is a possible link between insulin resistance, the metabolic syndrome and T2DM development (Björntorp 1999). Endogenous overproduction or exogenous elevations (Di Dalmazi 2012) in cortisol are associated with metabolic disturbances resulting in abdominal obesity, insulin resistance, β-cell dysfunction, hepatic steatosis, all of which can lead to T2DM development (Hwang 2014) (Figure 1). Typically, these effects are not the result of elevations in circulating GCs alone, as some studies have found cortisol concentrations to be near normal in obese patients (Hautanen 1997). There is data showing increased responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis, which is responsible for the secretion of GCs, to different stimuli, like food intake (Korbonits 1996), in patients with abdominal obesity, establishing a possible attenuation of negative feedback within the HPA axis (Pasquali 2002) subsequently affecting the diurnal secretion pattern of cortisol. A positive relationship in overweight/obese women between urinary free cortisol concentrations, waist circumference and caloric intake, when compared to normal women (Vicennati 2011) has been found. Additionally, increased adiposity itself has the capacity to alter cortisol concentrations as it contains the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD1), which converts inactive cortisone to active cortisol, and is

subsequently increased in conjunction with increases in abdominal obesity (Bujalska 1997). Elevations in 11β-HSD1 activity have also been found within the skeletal muscle (Zhang 2009b), suggesting the importance of understanding the regulation of GC action

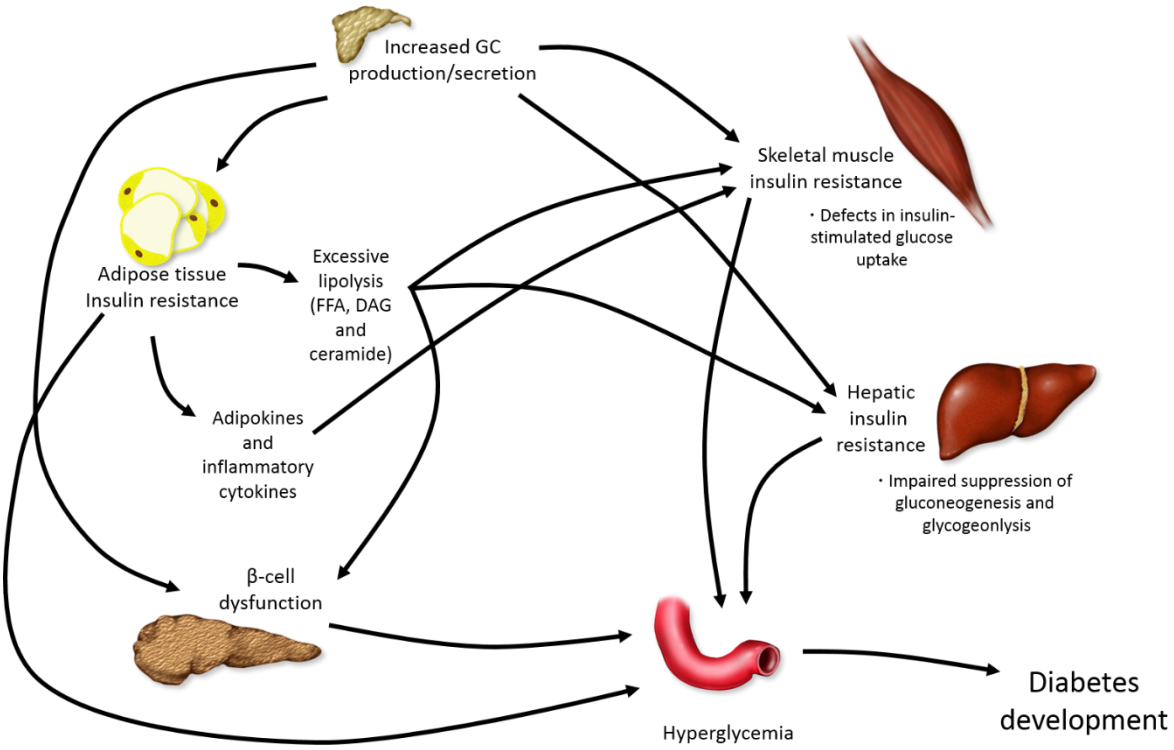

Figure 1.1. Pathophysiology of diabetes development.

The development of overt diabetes occurs through a number of mechanisms, all of which work together to affect elevations in blood glucose ultimately causing hyperglycemia. Glucocorticoids (GC), free fatty acids (FFA), diacylglycerol (DAG).

at the tissue level. As mentioned above, a new model of GC-induced diabetes has been developed (Shpilberg, 2012), but the effects of regular exercise in this mode are only now being investigated, as part of this thesis. As such, additional background on glucocorticoid metabolism is provided below in this thesis introduction.

3.2. Glucocorticoid synthesis and secretion

GCs are secreted by the adrenal cortex and their regulation is controlled by a neuroendocrine feedback system, the HPA axis (Stranahan 2008). Activation of the HPA axis occurs with the secretion of corticotrophin-releasing hormone (CRH) which stimulates the release of adrenocorticotropic hormone (ACTH) and initiates the synthesis and subsequent release of GCs from the adrenal cortex (Rose 2010). Cortisol is the predominant GC released in humans, while in rodents it is termed corticosterone, and its release is pulsatile and follows a circadian rhythm that is sensitive to light, sleep, stress and disease. Cortisol levels are highest during the early waking hours, and as the afternoon progresses, levels tend to decline; the normal range of cortisol in healthy humans is 60-230ng/ml (Chung 2011). Endogenous, hyper-secretion of GCs, the result of a benign pituitary or adrenal tumor, causes Cushing's disease and is characterized by visceral adiposity, hyperglycemia and peripheral muscle wasting (Juszczak 2013). In addition to endogenous cases, chronic pharmacological administration of synthetic GC analogs can also generate negative side effects which mirror the clinical pathology observed in patients with endogenous overproduction of GCs, termed Cushings syndrome. As steroid hormones, GCs are lipophilic and usually bound to carrier proteins (cortisol-binding globulin and albumin) (Chung, 2011). Up to 10% of total plasma cortisol circulates unbound and is considered the free fraction, which constitutes biologically active cortisol (Chung, 2011). Localized tissue metabolism contributes to the variation of physiological actions of GCs through the isoforms of

the enzyme 11β-HSD, which are responsible for regulating the active to inactive concentrations of cortisol. 11β-HSD type 1 (11β-HSD1) converts cortisone to its active form, cortisol, and is predominately expressed in liver and adipose tissue (Cooper 2009). Converting active cortisol to inactive cortisone occurs primarily in the kidney and is mediated by 11β-HSD type 2 (11β-HSD2). In a classic negative feedback loop, GCs act on the hypothalamus and pituitary to turn off neuroendocrine activity, restoring a steady state (Stratakis, 1995). In contrast to cortisol, synthetic GCs do not bind to CBG and are not classically metabolized by the IIβ-HSD2 isoform, which ultimately deactivates active cortisol to inactive cortisone (Oakley 2013).

3.3. Glucocorticoid signalling and the glucocorticoid receptor

The majority of the multi-systemic, physiological effects of GCs are mediated through cell-specific actions of the intracellular glucocorticoid receptor II (GR), a ligand-induced transcription factor belonging to the nuclear transcription superfamily (Rose, 2010) (Figure 2). Virtually all cells express the GR, and GR knockout mice do not survive (Rose, 2010) which further highlights the ubiquitous actions that GCs have throughout the body. The GR consists of two isoforms, GRα and GRβ, GRα is the subunit that binds active cortisol and translocates to the nucleus, while GRβ is expressed in lower levels and exerts a negative effect on GRα (Lee 2013). Active cortisol passively diffuses into the cell and binds to the cytosolic, intracellular GR complex, made up of the receptor, two molecules of hsp90 and some additional proteins (Oakley, 1996). Binding stimulates a conformational change, dissociating hsp90 and the associated proteins, and the new complex translocates into the nucleus where it can up- or down-regulate the expression of target genes (Kumar 2005). Once inside, the first mode of transcriptional regulation is through the binding of the liganded and dimerized GR to glucocorticoid response elements (GREs), found in the promoter regions of target genes (Oakley, 1996). Binding of the

GR to GREs increases chromatin remodelling and the rate of gene transcription through histone acetyltransferase activity from recruited coactivators, and is termed transactivation. Various transactivated genes have been associated with adipogenesis, muscle wasting and hyperglycemia, which are some side effects of GC treatment (Tomlinson 2006; Van Raalte 2009).

The second mode of transcriptional regulation is independent of GR dimerization and DNA binding, termed transrepression. Transrepression involves the interference of the GR monomer with other transcription factors. Transrepression of the proinflammatory transcription factors activator protein-1 (AP-1) and nuclear factor-B (NF-kB), for example, causes inhibition of the inflammatory signalling cascade and is considered a primary mechanism of the anti-inflammatory actions of GCs (Busillo 2013; Van Raalte, 2009).

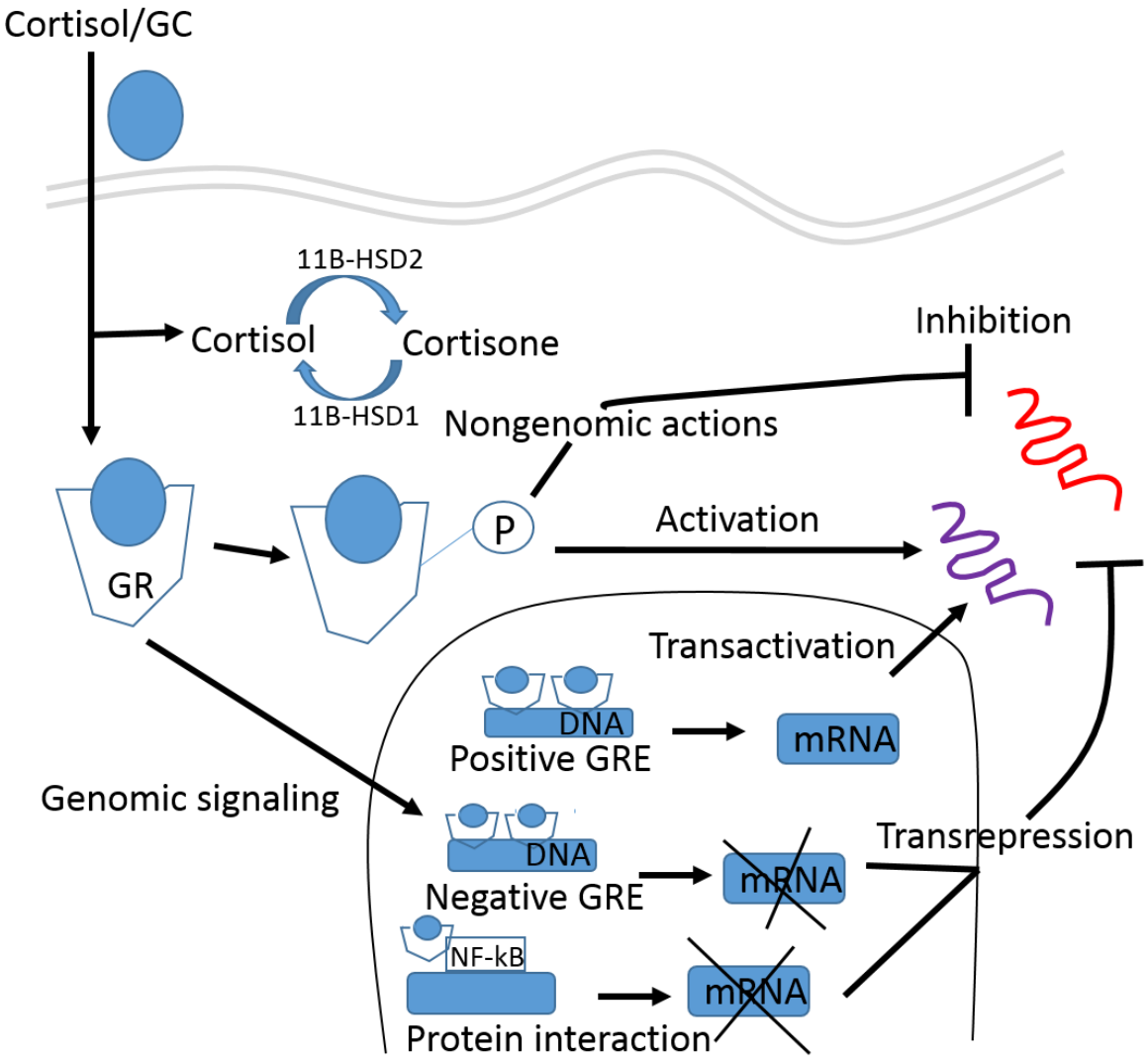
Cortisol/GC
11B-HSD2
Cortisol
Cortisone
11B-HSD1
Inhibition
Nongenomic actions
GR
P
Activation
Transactivation
DNA
Positive GRE
mRNA
Genomic signaling
DNA
Negative GRE
mRNA
Transrepression
NF-kB
mRNA
Protein interaction

Figure 1.2. Molecular mechanisms of glucocorticoid receptor action.

GCs regulate proteins through both genomic and non-genomic actions. Transactivation, or positive gene regulation, is mediated by binding of a ligand-activated glucocorticoid receptor (GR) to a GRE (GC response element). The ligand-activated GR can also bind to negative GREs resulting in repression of gene transcription. Inhibition of target genes can also occur through interaction of GCs with other transcription factors through protein-protein interactions, termed transrepression. (adapted from van Raalte et al. 2009).

3.4. Glucocorticoid effects on skeletal muscle

As previously mentioned, skeletal muscle is responsible for ~80% of postprandial glucose uptake from the circulation (Van Raalte, 2009), contains the body's largest glycogen stores, and is essential for optimal glucose metabolism. Skeletal muscle glucose uptake is reliant on insulin secretion, and is therefore termed insulin-stimulated glucose uptake (ISGU). Impairments in ISGU have been observed after GC exposure (Buren 2008; Dimitriadis, 1997) and highlight the significant effect that GCs can have on metabolism. GCs can affect ISGU directly, by causing insulin resistance via inhibition of insulin signalling proteins (Ruzzin 2005; Weinstein, 1998) or indirectly through effects on both fat (Elks, 1990) and protein metabolism (Krebs, 2002) (Figure 3).

3.4.1. Glucocorticoids and skeletal muscle insulin signaling

Elevated GCs have been shown to induce skeletal muscle insulin resistance, ultimately suppressing glucose uptake, primarily through the inhibition of GLUT4 translocation to the cell membrane (Weinstein, 1998). GC treatment can also impair glycogen synthesis through suppressed glycogen synthase activity (Buren, 2008; Dimitriadis, 1997; Weinstein, 1998), which suggests that abnormal activation of GC signaling within skeletal muscle may explain a part of dysfunctional glucose metabolism in the insulin resistant and/or diabetic state. This is especially true as GR mRNA levels in the skeletal muscle of diabetic patients correlate with the degree of insulin resistance, with a normalisation of GR expression following treatment to improve insulin sensitivity (Whorwood 2002).

Previous research has established that treatment of rats with dexamethasone (Dex), a GR agonist, causes a reduction in IR phosphorylation within skeletal muscle (Giorgino 1993) and subsequent inhibition of Akt phosphorylation/activity in both rat muscles after insulin

administration (Long 2003) and cultured C2C12 myotubes (Sandri 2004). Chronic elevations in GCs have also been found to induce both a decrease in IRS-1 protein content, responsible for transmitting signals from both insulin and insulin like growth factor-1 (IGF-1) through the activation of the PI3K and MAP kinase pathways, and decreased tyrosine phosphorylation of IRS-1 (Brown 2007; Girogino, 1993). GCs can also induce a reduction of PI3K activity (Saad, 1993), as the GR seems to compete with IRS-1 for association of PI3K subunits p110 and p85, which, when they are associated with the GR, causes a reduction in IRS-1 associated PI3K activity and subsequent Akt phosphorylation at both serine and threonine sites (Buren, 2008; Buren, 2002). The down-regulated Akt activity can affect GLUT4 translocation (Weinstein, 1998), glycogen synthesis and the muscle growth pathway, through decreased mammalian target of rapamycin (mTOR) activity ultimately decreasing protein synthesis and increasing protein degradation.

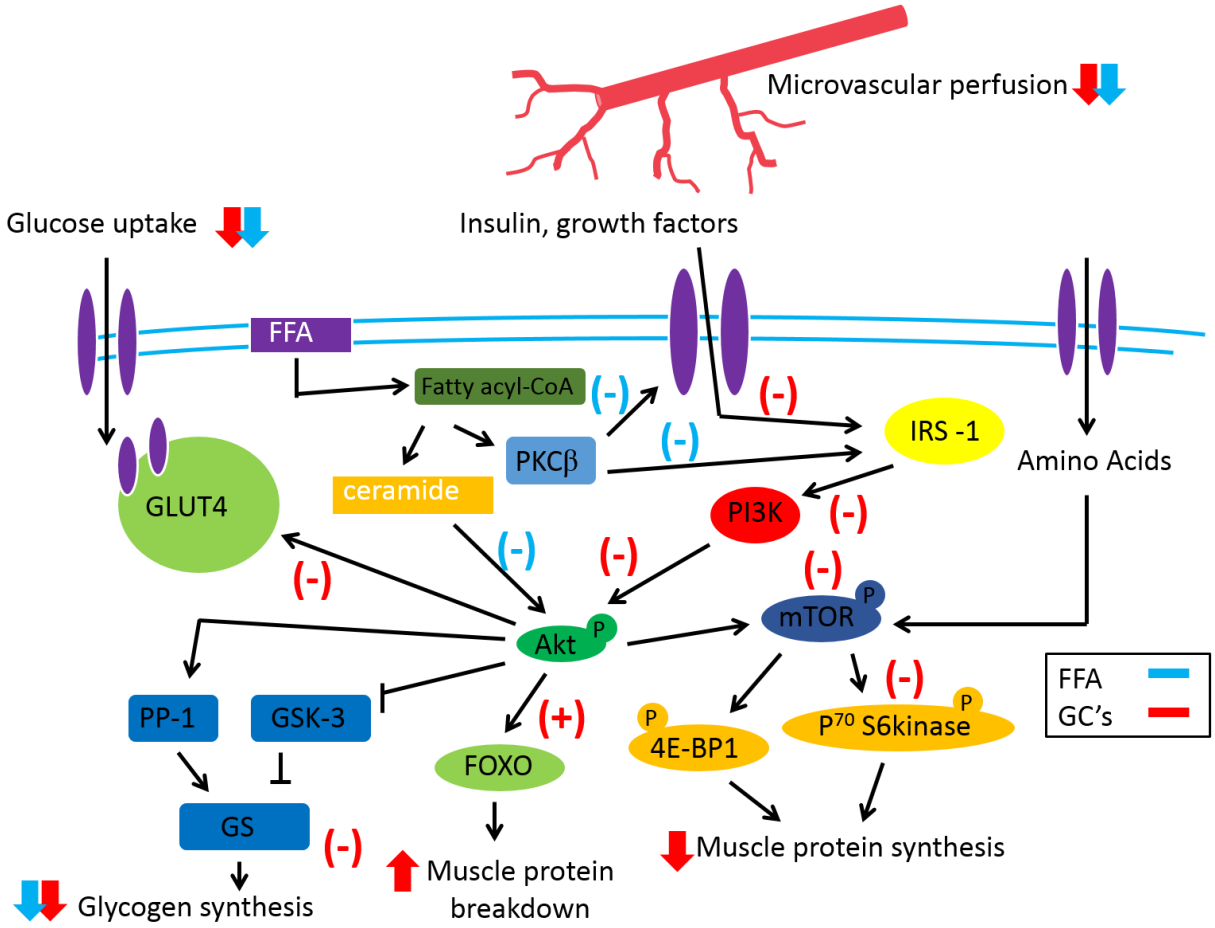

Figure 1.3. Glucocorticoid and intramuscular lipid-induced effects on skeletal muscle.

Insulin stimulates glucose uptake in skeletal muscle cells by mobilizing glucose transporter (GLUT4) receptors to the surface of the cell membrane. Once bound to the insulin receptor, insulin initiates GLUT4 translocation through activation of a cascade of cytosolic proteins: insulin receptor-1(IRS-1), PI3-K and Akt/protein kinase B although the entire pathway is not completely understood. Once glucose is transported across the membrane, it can be used for glycogen synthesis. Insulin inhibits glycogen synthase kinase (GSK)-3, an in activator of glycogen synthase. Elevated FFA's and GC's impair nutrient delivery in addition to several steps

of the insulin signaling pathway which decreases glucose uptake and glycogen synthesis, GCs also increase muscle protein catabolism and decrease protein synthesis.

(-) inhibition, (+) activation.

3.4.2. Glucocorticoid-induced skeletal muscle dyslipidemia

Cushing's syndrome patients are characterized by a redistribution of body fat from peripheral subcutaneous depots, to more central abdominal regions (Arnaldi 2010). This over activity of the HPA axis, which is also seen with obesity, could be causally related to insulin resistance and diabetes development through ectopic lipid deposition and the underlying effects of both whole body and tissue-specific lipid metabolism. GC exposure in wild-type mice causes inhibition of adiponectin receptor gene expression within skeletal muscle (De Oliveira 2011), possibly affecting positive downstream consequences of adiponectin signaling in skeletal muscle, like fatty acid oxidation and the prevention of IMCL accumulation. GCs increase whole body lipolysis which leads to elevated levels of nonesterified fatty acids (NEFA) and triglycerides (TG) (Wajchenberg, 2000). Elevations in NEFA concentrations increase the risk of accumulation of IMCL, like fatty acyl CoA, DAG, and ceramide which have emerged as important contributors to insulin resistance, through the inhibition of select proteins along the insulin signalling pathway (Bosma 2012; Singh 2003). IMCL may activate various serine kinases, like c-Jun amino-terminal kinase (JNK) and IkB kinase (IKK), which phosphorylate serine residues on IRS-1, causing a decrease in insulin signaling (Perseghin, 2003). Ceramide is thought to be a key mediator of many of the pathological events associated with GC-induced myopathy, like insulin resistance, atrophy and apoptosis (Dirks-Naylor 2009), and is capable of directly inhibiting Akt phosphorylation after high fat feeding (Chavez 2003).

3.4.3. Glucocorticoid-induced myopathy

GCs are one of the most common medications known to induce myopathy, especially when taken chronically at a high dose (Owczarek 2005). Individuals' prescribed long-term GC immunotherapy can have an incidence of muscle weakness as high as 50% (Bowyer 1985). GC-

induced myopathy is generally associated with muscle atrophy and weakness, insulin resistance, mitochondrial dysfunction and is proximal, symmetrical and can involve both upper and lower extremities (Dirks-Naylor, 2009). Steroid-induced atrophy has been shown to preferentially target fast, glycolytic muscle fibers (particularly type IIx and IIb) with almost no effect in type I fibers (Wang 2013). With acute atrophy there is an increase in the main degradation pathways, including the ubiquitin-proteasome system and the autophagy-lysosome pathway (Schiaffino 2013). The reduction of skeletal mass by GCs occurs through both decreased protein synthesis and increased protein breakdown (Goldberg Al, 1980; Schakman 2008a). Protein synthesis is inhibited through reduced amino acid transport and prevention of the anabolic effects of both insulin and IGF-1, particularly through decreased activation of eIF4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase 1 (S6K1). The resulting elevation in circulating amino acids can interfere with insulin signaling through the inhibition of insulin-stimulated IRS-1 tyrosine phosphorylation and activation of PI3K, and have also been shown to impair ISGU and glycogen synthesis in human muscle biopsies (Löfberg 2002). While increased proteolysis can occur through elevations in Muscle Ring Finger (MuRF)-1 and Atrogin-1 transcription (Gomes 2001), two important ubiquitin-proteasome system proteins (Bodine 2001), with subsequent up regulation of Forkhead box O (FoxO) 1 and 3. The combination of the atrophy-related decrease in total muscle area, and increased circulating amino acid levels, ultimately contribute to the attenuated ISGU observed with hypercortisolemia.

GCs have also been found to alter mitochondrial function (Du 2009), as defects in mitochondrial function are associated with GC-induced skeletal muscle wasting (Martens 1991). Although these mitochondrial alterations are usually the result of supraphysiological GC concentrations, as lower concentrations have actually been found to induce mitochondrial

biogenesis (Weber 2002). The general consensus is that, under physiological conditions, GCs work to enhance mitochondrial functions allowing the increased provision of energy to cells to adapt to acute stress challenges (Duclos 2004). However, chronic stress, which could lead to long-term elevations in GCs, has been shown to reduce mitochondrial function, through enlarged mitochondrial volume, decreased mitochondrial number and substrate utilization in addition to increased uncoupling of oxidative phosphorylation (Du, 2009; Martens, 1991).

3.5. Skeletal muscle-targeted therapeutic options

GCs are the number one prescribed immunosuppressive medication to combat rheumatoid arthritis (Andre 2011), COPD, asthma and lupus (Luijten 2013), in addition to being used in combination cancer treatment (Kfir-Erenfeld 2014) there are also patients who suffer from endogenous GC overproduction (Cushing's disease). Patients who are using exogenous GCs, like dexamethasone, do not want to limit GC action as they require the potent anti-inflammatory and immune suppressive properties that GCs possess which points to therapeutic strategies that can modulate the peripheral effects of GCs. Some options include physical activity or IIβ-HSD1 inhibition. While GRII antagonism, typically using mifepristone, is administered to Cushing's disease patients to combat the naturally high GC levels, as for these patients the goal is to block GC action. Both patient populations will experience the side effects associated with GC elevation, but each requires a different therapeutic focus.

3.5.1. Physical activity and GC metabolism

Exercise is widely used to control and/or treat several diseases because of its compensatory and regulatory actions on organisms as reviewed here (Pedersen 2015). Interestingly, exercise is itself a common form of metabolic stress which stimulates the HPA axis (Campbell 2009) causing increased circulating GCs to mobilize fuel sources and subdue the

immune system during exercise-induced muscle damage. While elevations in circulating GC levels induce insulin resistance, regular exercise enhances insulin sensitivity and causes reductions in both GR and IIβ-HSD1 expression within the skeletal muscle, adipose and liver (Coutinho 2006), ultimately reducing tissue-specific GC exposure. Regular exercise before and during two weeks of dexamethasone treatment improved glycogen loss (Barel 2010; Jung 2010), hyperinsulinemia and muscular atrophy (Barel, 2010) in some muscles, as well as increasing physical capacity. While others have found chronic exercise down regulates MuRF1 and atrogin-1 in atrophy induced through elevations in circulating GCs (Al-Nassan 2012; Hickson, 1981). Voluntary exercise is well known to improve skeletal muscle ISGU (Holten, 2004) and insulin resistance (Jung, 2010), induced by GC exposure, through attenuation of reductions in specific insulin signaling proteins, including Akt and IRS-1 activity and expression. Exercise also lowers ceramide content, while at the same time improving insulin sensitivity, possibly by directing lipids into the triglyceride pool (Amati 2011; Chavez 2012; Dube 2011). However, exercise training is not typically able to improve the extreme weight loss seen with dexamethasone or corticosterone treatment (Dionísio 2014) or some of the reductions observed in angiogenesis specifically decreased vascular endothelial growth factor (VEGF) expression (Barel, 2010).

3.5.2. Glucocorticoid receptor modulation and diabetes-related metabolism.

Mifepristone (RU486) is a non-selective GRII antagonist that competitively blocks the GRII and the progesterone receptor, but not the mineralocorticoid receptor (Johanssen 2007). RU486 has been extensively studied in the literature, from cell culture (Menconi 2008) to human trials, where it is primarily prescribed to Cushing's disease patients to aid in GC overproduction and hyperglycemia (Shibli-Rahhal 2006) as it has been shown to clinically improve glucose tolerance in these patients (Goldberg 1998). RU486 has also been studied in regards to GC-

induced skeletal muscle impairments and has been found to improve muscle atrophy (Bitar, 2001), in addition to attenuating the decreased Akt phosphorylation associated with GC-treatment.(Zhao 2009). However, RU486 administration requires constant patient observation as it can cause a number of side effects such as hypokalemia and aborted pregnancy due to the non-selectivity of its antagonism (Johanssen, 2007). The creation of more specific antagonists, selective for only the GRII, are in development and a few studies have been completed in rodent models (Beaudry 2014) analysing their therapeutic effectiveness. Overall, selective GII antangonists seem to be beneficial in improving whole-body steady state glucose metabolism and body mass gains in rodent models (Beaudry, 2014).

3.5.3. IIβ-HSD1 inhibitors and diabetes-related metabolism.

Elevations in GC activity within both the liver and adipose tissue have been implicated in the development of obesity and the metabolic syndrome (Staab 2010), and increased atrophy within the skeletal muscle (Schakman 2013), which sets the stage for inhibition of IIβ-HSD1 as a possible treatment strategy. Improving altered GC metabolism through the specific inhibition of IIβ-HSD1, especially within adipose tissue, has been proposed to result in favourable effects on body composition and the metabolic profile. Transgenic mice selectively overexpressing IIβ-HSD1 within white adipose tissue, developed visceral adiposity, dyslipidemia and insulin resistance in addition to elevations in local corticosterone levels (Masuzaki 2001), while IIβ-HSD1 null mice after being fed a HFD gained less weight and preferentially stored lipids in peripheral fat depots (Morton 2004). Pharmacological inhibition of IIβ-HSD1 activity in obese rodents improved glucose tolerance, insulin sensitivity and lipid profile (Kershaw 2005), while inhibition within C2C12 skeletal muscle cells abolished the effect of GCs on protein degradation and E3 ubiquitin ligases. There is additional evidence that IIβ-HSD1 inhibition restores IRS1

protein levels, decreased serine IRS-1 phosphorylation and enhanced Akt activation all of which contribute to augmented skeletal muscle insulin sensitivity (Morgan, 2009).

Role of the microvascular system within skeletal muscle

Alterations in skeletal muscle vasculature may help drive diabetes development, a phenomenon known to be influenced by both GCs and regular exercise. As such, this thesis introduction also highlights what is known about the effects of diabetes and GCs on the muscle vasculature.

4.1. Overview of the microvasculature in skeletal muscle

The microvasculature refers to the smallest systems of the body's blood vessels and can include, arterioles, which are small diameter blood vessels branching out from an artery, capillaries, which are the smallest blood vessels, metarterioles, which are vessels that link arterioles to capillaries and venules, which are blood vessels that allow deoxygenated blood to return from the capillaries to the larger veins (Krogh, 1919). Within the skeletal muscle, the microvasculature is the final interface from which circulating nutrients, hormones, gases and electrolytes must pass when moving to and from the systemic circulation (Wagner, 2000). These blood vessels have numerous structural and flexible adaptations to ultimately fulfill the optimization of muscle function and there is significant evidence that vascular function and skeletal muscle metabolic activity are closely linked, as alterations in blood flow and distribution can modulate nutrient delivery to the muscle and trans-endothelial transport can regulate the exchange of hormones and metabolites between plasma and the myocyte (Kolka 2012). Both regular exercise (Egginton, 2009; Hoier 2014; Karimian 2015) and circulating insulin levels (Akerstrom 2014; Egginton, 2011) have been well documented in the regulation of microvascular augmentation. A number of pathological conditions, including obesity (Bakker 2009; Gavin 2005), diabetes (Bakker, 2009; Roberts 2013), and hypercortisolemia (Halaby 2002; Shikatani 2012; Short 2004) negatively influence blood flow, muscle perfusion and new capillary formation.

4.1.1. Skeletal muscle structure

The architecture of the vascular network within the skeletal muscle is highly organized (Krogh, 1919) with arterioles branching off of primary arteries, down to terminal arterioles, which are perpendicular to the muscle fibres and work to supply them at regular intervals. Generally, the supply capacity of each terminal arteriole is ~15-20 capillaries which form a network around the muscle fibres. This is referred to as a microvascular unit and forms the smallest unit of control for capillary perfusion (Emerson 1997). The venules are found between two terminal arterioles and lymph vessels typically originate with the skeletal muscle and have the capacity to interact with both arterioles and venules. Within mammalian skeletal muscle, the organization of arterioles and venules is very similar, as the major differences in vascular structure are between aerobic and anaerobic skeletal muscles. Aerobic muscles require enhanced gas and nutrient exchange, in comparison to anaerobic muscles, which means they have generally more capillaries, larger capillary length and more capillary branches per fibre (Kubínová 2001) in addition to deeper microvessel situation within the sarcolemma and possibly greater perivascular mitochondria (Glancy 2015). Moreover, aerobic muscles typically have more mitochondria which rely on both nutrient and oxygen provision to generate energy for the cell. The proximity between muscle fibers and capillaries is a key factor in this process, as tissue delivery of oxygen, through passive diffusion, is actually limited to a distance of several micrometers (Hudlicka, 1992b), and recent findings have shown capillaries embedded in sarcolemmal grooves, which ultimately expands the contact area between the myocyte and the endothelial cells. Additional evidence shows mitochondria can be clustered within these grooves, which can reduce the distance that oxygen and fatty acids will require to diffuse into the myocyte (Glancy, 2015).

4.1.2. Exercise and muscle perfusion

The regulation of fuel fluxes into and out of skeletal muscle has been well studied in vivo in animals (Hellerstein, 2003; Weber 2004) and humans (Hawley 2011; Juel 1990) and subsequently it is understood that tissue perfusion delivers oxygen, FFA, glucose, triglycerides and amino acids to and removes metabolites (amino acids, lactate, carbon dioxide) from the skeletal muscle. During exercise there is an increase in bulk blood flow which is stimulated both by local factors including, potassium, adenosine, lactate, hydrogen ions, nitric oxide and prostaglandins (Clifford 2004; Mortensen 2009) which are released from the exercising muscle, and by neurovascular alterations that work to relax muscle vasculature. With very intense whole body exercise, the primary limiting factors are respiratory and cardiac function, while blood flow and oxygen delivery to the tissues are secondary factors (Mortensen 2005). When examined within isolated skeletal muscle, which removes the limitations to cardiorespiratory function, blood flow to the human quadriceps can actually increase >50-fold during short, intense exercise (Andersen 1985). Moreover, increasing the arterial oxygen content does not further enhance quadriceps oxygen extraction or peak work rate (Mourtzakis 2004). Both glucose and FFAs are important fuels during exercise, and during intense exercise, a >30-fold increase, from rest, in leg glucose uptake has been measured (Katz 1986) in addition to dramatic increases in glucose levels both within the muscle interstitium and the myocytes themselves. These results suggest that the delivery of glucose to the muscle microvasculature and trans-capillary transfer of glucose, are both fully capable of adapting to and subsequently meeting the glycemic metabolic demands during intense exercise.

When examining the effect of light to moderate exercise on the skeletal muscle microvasculature, there is less data, and the results that have been found typically provide a more

mechanistic insight into exercise-induced vascular regulation versus possible performance based effects. With light exercise, blood flow remains relatively unaffected; however there is an increase in capillary perfusion (Inyard 2007). Any additional increase in contractile activity, or an increase in exercise intensity, causes a corresponding increase in total blood flow which is mediated by a number of factors including venous pO2, which promotes nitric oxide (NO) production and release (Stamler 1997) and hemodynamic forces, such as shear stress-induced NO production. Both of these responses are enhanced by prostaglandins to further increase exercise-induced tissue perfusion (Mortensen 2007).

4.1.3. Insulin and muscle perfusion

Very early work examining the effect of insulin-stimulated glucose uptake in the forearm, saw an associated increase in forearm blood flow (Andres 1962). Additional work found that insulin-stimulated glucose uptake actually paralleled insulin's ability to increase total leg blood flow (Baron, 1994). Lower, more physiologic doses of insulin, in both healthy humans and animals, can increase microvascular perfusion, without an associated increase in total peripheral blood flow, while high physiological concentrations of insulin cause the relaxation of arterioles (vasodilation) and increased peripheral blood flow (Baron 1995). Further research has found that these effects are blunted in insulin resistant individuals with obesity (Laakso 1990), increased FFA levels (Steinberg 2000) and diabetes (Baron 1991). These pathological conditions impair insulin-stimulated peripheral blood flow and glucose uptake, and evidence suggests that impaired vasodilation in response to insulin could be a significant contributor to the metabolic complications associated with insulin resistance. Insulin is not the only hormone capable of stimulating the skeletal muscle microvasculature, as both GLP-1 (Subaran 2014) and adiponectin (Zhao 2013) increase microvascular volume through distinct biochemical pathways. Epinephrine

can increase skeletal muscle blood flow without altering microvascular perfusion volume, and corticosterone has been found to cause capillary rarefaction which ultimately reduces microvascular perfusion (Shikatani, 2012).

It is understood that there are differences in basal blood flow between different insulin-sensitive tissues. The liver and the myocardium each have a very substantial basal blood flow (~1 $ml/min^{-1} \cdot g\ tissue^{-1}$), while the islets of Langerhans have an even greater blood flow that is estimated to be about 5–12 mL/min/g of tissue (Iwase 2001). By contrast, skeletal muscle and adipose tissue basal blood flow averages only 0.03–0.04 $ml/min^{-1} \cdot g\ tissue^{-1}$ (Baron, 1994). As a result, in these tissues, blood flow (or for insulin and some other solutes, plasma flow) can become an important factor limiting nutrient delivery and solute exchange. As previously mentioned, there is an established link between the vascular action of insulin to enhance total limb blood flow and insulin's metabolic action, however, contrary findings have also been reported. In particular, those reports raised the concern that increasing blood flow did not result in augmentation of insulin's metabolic action in healthy or insulin-resistant individuals (Laine 1998; Natali 1998) and that the time course for onset of insulin-induced increases in limb blood flow (particularly at physiological insulin concentrations) lagged behind insulin-induced increases in skeletal muscle glucose uptake (Yki-Järvinen 1998).

The movement of insulin from the vascular to the interstitial compartment within skeletal muscle is rate limiting for insulin's metabolic action. Support for this argument is extensive and includes the early demonstration by Sherwin et al. (Sherwin 1974) that the disappearance kinetics of intravenously injected insulin indicated a rapid equilibration of injected insulin within plasma and visceral tissue compartments but a slow equilibration within skeletal muscle. Additional data notes that when the insulin concentration was increased muscle insulin clearance

declined, and suggests a saturable process for insulin transport. All of this evidence highlights the interesting relationship between insulin and microvascular recruitment.

The vasodilatory action of insulin occurs through its binding to the insulin receptor expressed on endothelial cells, which stimulates the PI-3K/Akt pathway into activating endothelial nitric oxide synthase (eNOS) (Jiang 1999) and induces NO-mediated vasodilation. Insulin can also activate the MAPK pathway in addition to enhancing the production of the vasoconstrictor endothelin-1 (Mather 2013). In pathological conditions like obesity and T2DM, there is an established endothelial dysfunction which is associated with increased inflammation, FFAs, oxidative stress and a significant reduction in perivascular adiponectin release. All of these factors have the capacity to inhibit the activation of the PI3K pathway within the endothelial cell, resulting in a reduced vasodilatory response, or sometimes even vasoconstriction in the face of hyperinsulinemia (Clerk 2006). There is additional evidence to support the idea that insulin exerts a physiological action at all levels of the arterial vascular tree, and that insulin resistance is accompanied by dysfunction at each level. At the level of conduit arteries, an extensive body of data indicates that metabolic insulin resistance, as seen in obesity, metabolic syndrome, and type 1 and type 2 diabetes, is accompanied by dysfunction that is manifested by increased vascular stiffness and/or impaired NO-mediated vasodilation (Stehouwer 2008; Webb 2010). Interestingly, in insulin-resistant individuals, pharmacologically increasing blood flow, through the use of prazosin, an α_1-adrenergic antagonist that induces hyperaemia through enhanced shear stress (Dawson, 1989b), does not always improve insulin-stimulated glucose uptake. It has been suggested that although peripheral tissue perfusion is augmented, insulin resistance within the myocyte remains and the acute correction of perfusion

is not enough to overcome that metabolic defect, however further understanding of this phenomenon is required.

4.2. Skeletal muscle angiogenesis

In addition to blood flow and perfusion, the formation of new blood vessels from pre-existing vessels, termed angiogenesis, is another level of skeletal muscle microvascular regulation. Angiogenesis is typically responsible for most, if not all, new blood vessel growth during adult development and disease. This process is in contrast to vasculogenesis, which is the de novo formation of blood vessels essential to the initial development of embryonic microcirculation (Olfert 2016). The growth of new blood vessels, or capillaries, occurs though endothelial sprouting, from the abluminal surface of endothelial cells, or non-sprouting (intussusceptive) microvascular growth (IMG) from the luminal surface of endothelial cells (Ribatti 2012), and is involved in tissue injury and wound healing (Arnold 1991; Billingham 1955), the endometrial cycle (Gambino 2002) and skeletal muscle adaptation to stress and exercise (Egginton, 2009), among other physiological events. There are a number of important signals that induce skeletal muscle angiogenesis, including tissue hypoxia in both adult and embryonic capillary growth, increased contractile activity, as seen with endurance exercise (Bloor, 2005), and physiological or pathological reductions in contractile activity, such as hind limb unloading (Li 2004; Mariotti 2008) or in obesity (Gavin, 2005; Goodwill 2012) or diabetes (Bakker, 2009; Vessieres 2012).

4.2.1. Capillary growth in adult skeletal muscles

The basic mechanisms of blood vessel growth and development have been established over the past couple decades (Folkman, 1997) and as such, angiogenesis can be divided into a number of specific phases, ultimately based upon the responses of the endothelial cells to certain

angiogenic stimuli, including 1) protease production, 2) migration or chemotaxis, 3) proliferation, 4) capillary morphogenesis, and 5) vascular maturation or stabilization.

In response to an angiogeneic stimulus (injury, hypoxia, mechanical stretch) endothelial cells become activated and attract and bind leukocytes and blood platelets that release a number of pro- and anti-angiogenic factors. Proteolysis is then required for the endothelial cells to pass through the basement membrane, which surrounds the capillary (Haas, 2002), and the secretion of these factors cause the stimulation of local endothelial cells which begin to produce proteases, including urokinase-type plasminogen activator and collagenase, to subsequently degrade the basement membrane which, once degraded, will allow the movement of the endothelial cells (Kalebic 1983). The basement membranes are composed of a scaffold of collagens, fibronectins and laminins, while the interstitial matrix contains fibrillar collagens. All of these matrix proteins are substrates for the matrix metalloproteinase (MMP) family of enzymes which influence cell adhesion through enhanced cell proliferation or migration through the release and/or activation of sequestered growth factors (Pepper, 2001). The endothelial cells then migrate out of the original vessel towards the source of the angiogenic stimulus, which produces nascent vascular sprouts. After which, the endothelial cells undergo mitosis, or proliferation, to align and form a capillary lumen in a process termed capillary morphogenesis (Kalebic, 1983). The cells then go through vascular maturation, or stabilization, which causes the formation of cell-cell junctions, the recruitment of supporting peri-endothelial cells (pericytes and smooth muscle cells) and the development of organ-specific endothelial characteristics which renders the endothelial cells quiescent.

4.2.1.a. Angiogenic factors

Physiological angiogenesis is controlled through equilibrium between both pro- and anti-angiogenic factors. Presently, there are more than 40 stimulators and inhibitors of angiogenesis (Olfert 2011), and there are numerous examples within the literature demonstrating that an imbalance between the two contrasting factors causes either uncontrolled angiogenic growth or capillary rarefaction within various pathologies (Esemuede 2004).

There are a number of different factors that stimulate endothelial cell proliferation in vitro and mediate angiogenesis in vivo, however the major putative angiogenic factor, vascular endothelial growth factor (VEGF) and it's receptors, seems to increase to a greater extent, and more consistently than other measured angiogenic factors, including the fibroblast growth factor family (FGF), platelet derived growth factor (PDGF), the angiopoietins and TIE-receptors, the integrins and a number of others (Pepper, 1997; Yancopoulos 1998). These factors typically mediate their angiogenic actions through binding to specific cell-surface receptor tyrosine kinases, of which, the receptors for VEGF are expressed almost exclusively on endothelial cells. Receptor tyrosine phosphorylation stimulates signaling cascades that translate into specific endothelial cellular responses necessary for angiogenesis, like chemotaxis and proliferation.

While significantly less studied, endogenous inhibitors of angiogenesis are integral to the regulation of capillary growth. These angiostatic factors can be derived from within the extracellular matrix, such as thrombospondin, and they can also be from outside of the matrix, whether from other cells or within the blood such as angiostatin. There is still a significant amount of research required to fully ascertain the relationship between both angiogenic and angiostatic factors, however there is evidence implicating these anti-angiogenic factors in various pathologies, including ischemia-induced skeletal muscle injury (Milkiewicz 2011b; Silvestre

2002), diabetes (Krisp 2013; Simo 2006) and chronic obstructive pulmonary disease (Gouzi 2012; Suzuki 2012). They have also been proposed as the 'brakes' of exercise-induced skeletal muscle angiogenesis, possibly in order to maintain precise angio-adaptive mechanisms (Olfert, 2011).

4.2.2. Exercising muscle

The growth of capillaries in response to exercising muscle is a complex, highly regulated process which is stimulated by increased blood flow and shear stress, mechanical stretch of the tissue in addition to the signals generated through increased metabolic demand or reduced oxygen delivery (Brown 2003). These signals modulate the activity of the pro-angiogenic and angiostatic factors previously described and many others, which act together to accurately regulate angiogenesis. Typically, capillarization within the skeletal muscle can be transformed in relation to the extent that the muscle is used, as aerobic (Andersen 1977), anaerobic (Egginton 2000) and resistance training (Gavin 2007) can induce angiogenesis. Capillarization is associated with fibre size and can be measured using the capillary-to-fibre (C:F) ratio. Generally, aerobic training can increase C:F either with or without a parallel change in fibre area, resistance training typically causes increased fibre area and matched alterations in C:F while inactivity causes a reduction in both fibre area and C:F (Musacchia 1990).

4.2.2.a. Shear stress, mechanical stretch and metabolically related factors

When there is an increase in blood flow there is a corresponding increase in shear stress, which is a frictional force acting on the luminal side of the endothelial cells (Egginton 2014). The endothelial cells respond to this force through activation of signaling pathways which stimulate vascular growth factors and vasoactive compounds that alter blood flow. Vasodilatation, using chronic administration of the α_1-adrenergic receptor antagonist prazosin, is

a widely used laboratory model of elevated shear stress, which has been shown to induce capillary growth in rat skeletal muscle (Dawson, 1989b), usually without modification of the metabolic activity of the skeletal muscle fibres. The use of this compound in rodents causes long term hyperaemia which stimulates the increased expression of angiogenic factors and increased endothelial cell proliferation resulting in angiogenesis, with the largest increases in capillarity occurring where the hyperaemic response was greatest (Egginton, 2001). Shear stress as a solitary, initial angiogenic stimulus is different from what is typically observed with contracting muscle, and subsequently causes a different pattern of capillary growth. Increased shear stress induces growth by longitudinal splitting, or intussusceptive microvascular growth (IMG) (Egginton, 2001). The process of intussusception is when the capillary network increases its vascular surface through invagination of the capillary wall allowing opposing endothelial cells to make contact across the lumen and form intercellular junctions. The new capillaries are subsequently formed by perforation and invasion of the region of contact by interstitial tissue (Burri 1990). This method of angiogenesis appears to be faster, less complex and possibly less energetic than sprouting angiogenesis, as it requires minimal degradation of the basement membrane and extracellular matrix, less endothelial cell proliferation, and capillary function seems to be uncompromised during growth (Prior 2004). However, the level of shear stress induced through pharmacological vasodilatation is probably much higher than what would be observed with functional hyperaemia induced by electrical stimulation in rodents.

Endothelial cells are tethered to myocytes through the extracellular matrix and connective tissue and contraction-induced alterations of the sarcomere, such as stretch or overloading (contraction), can stimulate the endothelial cells and induce capillary growth, primarily through sprouting angiogenesis (Egginton, 2011). Typically, alterations to blood flow are minimal, and in

contrast to endothelial detection of shear stress, muscle stretch or overload actually affects all cells within the skeletal muscle (myocytes, satellite cells, interstitial fibroblasts, vascular smooth muscle cells, pericytes and endothelial cells) (Haas, 2002). During both shear stress and muscle stretch, tensile forces are generated that stimulate mechanotransduction signaling pathways by activating integrin receptors. Protein and mRNA analysis of angiogenic factors within contracting skeletal muscle, demonstrate that factors associated with matrix remodelling, which primarily occur in sprouting growth, are upregulated in response to muscle stretch but not shear stress (Milkiewicz 2005). The growth factor VEGF is important for both luminal splitting and sprouting angiogenesis (Williams 2006b).

It is not only elevations in blood flow, shear stress and mechanical contraction that are important for the initiation of angiogenesis, alterations in metabolism and oxygen utilization and supply are other very important physiological stimuli. In an exercising model, it is difficult to elucidate the exact role of metabolism in angiogenesis, as changes in metabolic flux generally induce corresponding alterations to blood flow. It is well known that pro-angiogenic compounds are released from the exercising muscle during contraction, like the vasodilators adenosine and prostacyclin (Egginton, 2001), although the exact signal for this release, whether it is coupled with enhanced metabolism, alterations in oxygen levels or the mechanical aspects of contraction are not fully understood.

4.2.3. Angiogenesis during diabetes mellitus

Alterations in skeletal muscle capillarity can have a significant impact on metabolism, the endocrine system and muscle function. Typically, elevations in capillary density (CD) or C:F, suggest capillary growth or angiogensis and are associated with functional improvements, while decreased CD or C:F (capillary rarefaction) are associated with many chronic diseases including

diabetes (Kivela 2006), peripheral artery disease (Kikuchi 2014), cachexia and chronic obstructive pulmonary disorder (Bašić, 2014).

Diabetes is associated with both micro- and macrovascular disorders which can result in adult-onset blindness, limb amputations, stroke and peripheral artery disease (Kilpatrick 2006; Olfert, 2016), among a host of others. There is evidence that capillarity in skeletal muscle is altered in both T1DM (Hansen 2002; Raskin 1983) and T2DM (Rasio 1992), typically in the form of endothelial dysfunction resulting in capillary rarefaction. Endothelial dysfunction encompasses a number of different functional changes in the vascular endothelium, including impaired vasodilation, angiogenesis, inflammatory activation and elevations in plasma levels of endothelial products (Bakker, 2009). Endothelial dysfunction is an early and significant event in the pathogenesis of micro- and macrovascular pathologies in both forms of diabetes, and is primarily due to chronic hyperglycemia, although insulin resistance, pro-inflammatory signaling and oxidative stress have also been implicated. Impaired endothelial function tilts the physiological balance towards vasoconstrictive and angiostatic effects which accelerate the development of atherosclerosis, a major complication of diabetes (Landmesser 2004).

4.2.4. Effect of exercise on skeletal muscle capillary rarefaction in diabetes mellitus

Exercise training is a viable therapeutic option to improve the capillary rarefaction associated with insulin resistance (Hansen 2010), as it has been shown to stimulate local microvascular adaptations within the skeletal muscle in addition to systemic adaptations due to improved glycemia. In both humans and rodent models of insulin resistance and T2DM, exercise mediates local and systemic improvements in endothelial (Milkiewicz 2011a) and smooth muscle function which is typically evident through enhanced vasodilator signaling. Additionally, regular exercise promotes increases in NO synthesis and bioavailability while simultaneously

lowering levels of ROS which otherwise would counteract NO activity (Fuchsjäger-Mayrl 2002), other possible mechanisms which could ameliorate the endothelial dysfunction associated with diabetes. To date, there are limited studies examining the effect of exercise within the skeletal muscle in T1DM, although there is evidence that chronic T1DM causes capillary rarefaction and decreased skeletal muscle perfusion (Kindig 1998b; Laakso 1992; Tibiriçá 2007). Kivela et al. (Kivela, 2006) is one of the few published works examining the effect of T1DM on skeletal muscle angio-adaptive molecules, capillarization, and the potentially therapeutic role of exercise training. The authors did find that 5 weeks of regular exercise caused a partial restoration of the diabetes-induced reduction in VEGF mRNA, however, further characterization of exercise-induced alterations within the skeletal muscle microvasculature are required.

Summary of Intent

Overall Purpose

The overall purpose of the studies presented in this thesis was to investigate the relationship between elevations in glucocorticoids and diabetes development on skeletal muscle insulin resistance and angiogenesis, using two different rodent models of diabetes; T2DM and exogenous elevations in GCs combined with a HFD (ROD model) and T1DM using streptozotocin to induce β-cell destruction.

Specific Purpose

The specific purposes of the experiments conducted were to:

1) Examine the effect of elevations in exogenous GCs combined with a HFD on insulin-stimulated glucose disposal and other markers of skeletal muscle insulin resistance (chapter 2).
2) Determine whether voluntary exercise is a viable therapeutic strategy to combat the significant diabetogenic and atrophic side effects associated with elevations in GCs and a HFD (chapter 3).
3) Investigate whether prazosin administration, a potent pharmacologic stimulant of muscle angiogenesis, improves glucose intolerance, insulin sensitivity and other skeletal muscle specific defects associated with hypercortisolemia (chapter 4).
4) Explore the induction of, and possibly enhancement in, angiogenesis from the combination of prazosin and voluntary exercise on the capillary rarefaction associated with untreated, chemically-induced, insulin-dependent diabetes.

Overall Hypothesis

The overall hypothesis for these studies was that elevations in glucocorticoids would cause skeletal muscle insulin resistance, atrophy and capillary rarefaction and correlate with diabetes development. A secondary hypothesis is that both exercise and prazsoin would help ameliorate the diabetogenic effects of hypercortisolemia.

Specific hypotheses

The specific hypotheses for the studies listed above were that:

1) Exogenous glucocorticoids and high-fat feeding synergistically cause skeletal muscle insulin resistance through decreased insulin stimulated glucose transport, impaired insulin signaling and glycolytic atrophy.
2) Voluntary exercise will improve whole body glucose metabolism, normalize glycolytic fibre atrophy, and skeletal muscle insulin signaling without altering circulating glucocorticoid concentrations.
3) Improved skeletal muscle capillary rarefaction, using prazosin administration, will enhance insulin sensitivity through increased skeletal muscle perfusion.
4) The combination of three weeks of voluntary exercise and prazosin administration will lead to a magnified improvement in hyperglycemia and skeletal muscle capillary rarefaction, associated with untreated streptozotocin-induced diabetes.

Chapter 2

Severe skeletal muscle metabolic impairments occur in response to concurrent high-fat feeding and glucocorticoid treatment in male Sprague Dawley rats

Author Contributions: I designed the study and wrote the paper along with input from Dr. David Dyck, Dr. Ian Ritchie and my supervisor Dr. Michael Riddell. I performed all animal handling, including pellet surgeries, oral glucose tolerance tests and blood draws. Dr. Ian Ritchie and I completed the ex vivo glucose transport analysis, with input from Dr. David Dyck. I completed all plasma analysis (insulin ELISA, corticosterone RIA) and histochemistry (CSA, SDH, ORO). Western blotting of insulin signaling proteins was completed by myself and Deanna Porras; while GR and 11β-HSD1 was completed by myself. Carbonylated protein assessment was perfomed by Sofhia Ramos, with input from Dr. Christopher Perry. Dr. Michael Riddell oversaw the entire study and assisted in manuscript preparation.

Rationale: It was well established that combining elevations in GC concentrations (pathophysiological) with a diet high in fat (ROD model) caused significant metabolic and tissue-specific side effects. The liver, pancreas and adipose tissue of this rodent model had been extensively investigated, although the exact mechanism of the synergistic initiation of diabetes development was not fully elucidated. This study was undertaken to analyze the skeletal muscle, which is a major organ responsible for glucose metabolism, through the measurement of *ex vivo* insulin stimulated glucose transport, in addition to examination of the glycolytic fibre-specific atrophy, indicative of GC action. In executing this study, we were able to establish that the glycolytic (white) skeletal muscle of these ROD animals (Cort-HFD) demonstrated severely impaired insulin stimulated glucose uptake and atrophy, possibly due to greater 11β-HSD1 protein content, which could cause increased tissue-specific exposure to GCs.

Severe skeletal muscle metabolic impairments occur in response to concurrent high-fat feeding and glucocorticoid treatment in male Sprague Dawley rats.

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Author Contributions

Conceived and designed the experiments: E.C.D., I.R.R., D.J.D., and M.C.R. Performed the experiments: E.C.D., I.J.R, S.V.R., and D.P.P. Analyzed data: E.C.D., I.R.R., S.V.R., D.P.P., and M.C.R. Wrote the paper: E.C.D. and M.C.R. Edited the manuscript: I.R.R., S.V.R., D.P.P., D.J.D., C.G.R.P., and M.C.R

Preface

Glucocorticoids (GCs) are naturally occurring stress hormones that are frequently prescribed to treat immune and inflammatory conditions. The administration of GCs can promote skeletal muscle insulin resistance, hepatic steatosis, ectopic fat accumulation as well as β-cell dysfunction. We have previously shown that a high-fat diet (HFD) in combination with GC treatment amplifies these detrimental metabolic effects, but the effects of this co-treatment have not been examined within the skeletal muscle *per se*. Young, male Sprague-Dawley rats were subcutaneously implanted with corticosterone (Cort), the main GC in rodents, (400mg/rat) or wax pellets and provided with either a rodent standard diet (SD) or a HFD (n=5/group). As expected, two weeks of HFD/Cort treatment resulted in profound hyperinsulinemia/hyperglycemia. Muscle insulin resistance was assessed *in situ* through direct measurement of insulin-stimulated glucose uptake (ISGU) in both the extensor digitorum longus (EDL) and soleus muscles. Cort-HFD-treatment resulted in ~50% less ISGU compared to the control-HFD within the EDL, with surprisingly no impairment observed within the soleus. Cort-SD treatment caused ~4-fold decrease in IRS-1 protein within the EDL, while Cort-HFD animals had ~2-fold increase in AS160 protein, an adaptation that was unable to compensate for the decreased ISGU. Concurrent Cort-HFD treatment also resulted in greater type IIb/x fibre atrophy within the tibialis anterior and elevations in 11β-HSD1 protein within the EDL. These findings emphasize the significant adverse effects that combining Cort-treatment with a HFD can cause within the skeletal muscle, particularly in white muscle, which likely contributes to the development of insulin resistance.

Introduction

Synthetic glucocorticoids (GC) are commonly prescribed as anti-inflammatory and immunosuppressant medications for auto-immune diseases such as rheumatoid arthritis and lupus, (Rhen, 2005) and are also used in combination with cancer treatments (Walsh, 1992). Chronic or high dose GC administration is associated with numerous side effects such as: steroid myopathy (Owczarek, 2005), peripheral insulin resistance (Stojanovska 1990), visceral adiposity (Asensio 2004) and diabetes development (Pivonello 2010). These metabolic disturbances occur because GCs are inherently catabolic and can liberate fuel substrates and promote ectopic lipid accumulation (Peckett 2011). Increased exposure to GCs in rodents (Dallman, 2010; Dallman 2005) or chronic stress in humans (Tomiyama 2011) have been shown to stimulate the desire for energy dense 'comfort' foods (high fat, simple sugars) and are often associated with excessive weight gain (Zakrzewska, 1999). Independently, chronically elevated GCs and high-fat feeding cause dysregulated lipid metabolism in the skeletal muscle of both rodents and humans (Koyama, 1997; Pan, 1997), specifically through accumulation of intramyocellular triglycerides (IMTGs), diacylglycerides (DAGs) and ceramides. Accumulation of these fatty acid intermediates in skeletal muscle can induce a reduction in insulin signaling through direct inhibition of insulin signaling proteins (IRS-1, Akt/PKB) (Ruzzin, 2005) and can alter insulin stimulated glucose uptake through inhibition of GLUT4 receptor translocation (Buren, 2008; Dimitriadis, 1997).

Previous work from our lab has shown that combining exogenous GCs (corticosterone in rodents; Cort) with a high-fat diet (HFD) drastically augments these metabolic impairments, rapidly inducing severe, whole body glucose intolerance, hepatic steatosis and a type 2 diabetes mellitus (T2DM) phenotype within days of treatment in young male Sprague-Dawley rats

(Beaudry, 2013; D'souza, 2011; Shpilberg, 2012). With exogenous Cort treatment only, or with just a HFD, the animals do not develop a diabetic phenotype in this same time-frame, suggesting a synergistic effect occurs with the combination of the two metabolic insults. In this paper, we examine the impact of these two metabolic stresses (diet, exogenous GCs) separately and in combination on skeletal muscle, which is a key determinant of whole body insulin sensitivity and T2DM development. We further determine if there are any muscle-specific differences between predominantly glycolytic and oxidative muscle groups. Our findings clearly demonstrate that fast-twitch, glycolytic muscle fibres are a key target for the induction of atrophy and insulin resistance in response to the combination of high fat feeding and exogenous GC treatment.

Methods

This study was carried out in accordance with the recommendations of the Canadian Council for Animal Care guidelines and was approved by the York University Animal Care Committee (2013-5).

Experimental Design and Animals: Male Sprague-Dawley rats (Charles River Laboratories, initial mass of 225-250 g, six weeks post-weaned, n=15) were individually housed (lights on 12 hour: lights off 12 hour cycle) after one week of acclimatization to room temperature (22-23°C) and humidity (50-60%) controlled facilities. An overview of the experimental design is depicted in Figure 2.1. Briefly, on day 0, basal (AM) Cort was measured and then animals were randomly divided into one of three groups: corticosterone-treated with a rodent standard diet (Cort-SD), corticosterone-treated with a high-fat diet (Cort-HFD), or placebo-treated with a high-fat diet (Control-HFD). Each rat received a subcutaneous implantation of either corticosterone (the major GC found in rodents) (Cort) pellets (4 x 100 mg; Sigma-Aldrich, Canada, Cat# C2505) or wax pellets (Control), as previously described

(Shpilberg, 2012). Following recovery from surgery, rats were placed into standard sterile cages and were maintained on either a rodent standard diet (14% fat, 54% carbohydrate, 32% fat; 3.0 calories/g) or a HFD (60% total calories from fat; 5.1 calories/g). All animals were fed *ad libitum* during the treatment period, which lasted for 15 days. Body mass and food intake were measured daily for each rodent using an electronic scale (Mettler Toledo, Canada) and any changes in the rodents' health were noted and monitored.

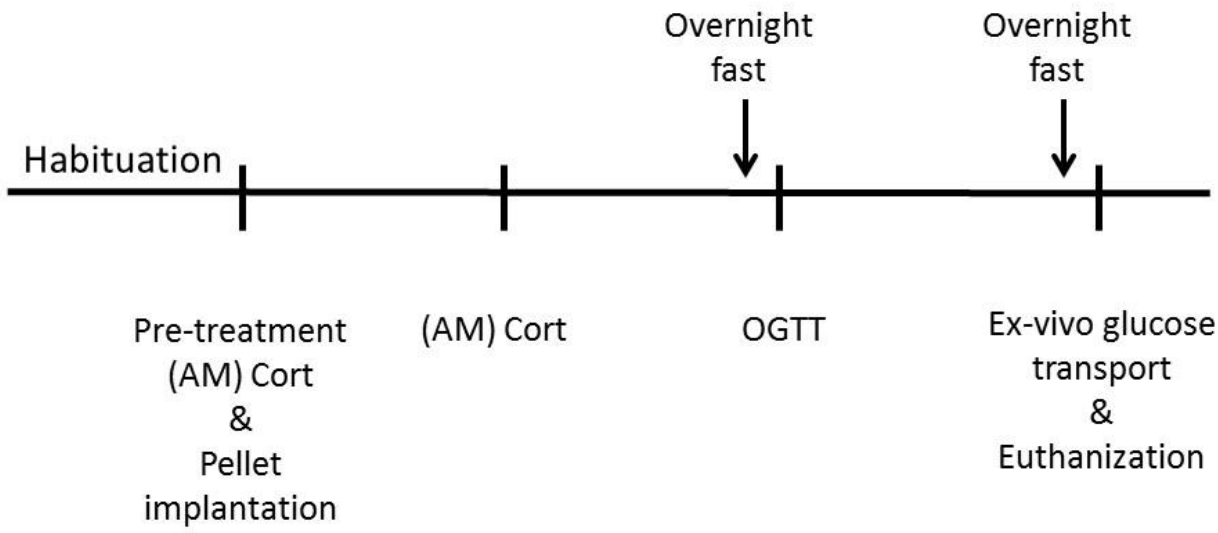

Figure 2.1. Schematic of experimental design.

Saphenous vein measurement of pre-treatment corticosterone (Cort, AM) occurred on day 0, immediately prior to pellet implantation (wax/Cort). A second saphenous vein Cort measurement was taken on day 7. An oral glucose tolerance test (OGTT) was administered on day 12, after an overnight fast. Animals were fasted overnight and then euthanized on day 14 or 15, following ex-vivo analyses of insulin-stimulated glucose transport.

Cort sampling: On day 0 and day 7, at ~0800 hour (AM), blood samples were collected from each animal via saphenous vein for Cort concentration measurements. A small area of hair on the lateral upper hind limb was shaven with an electric razor and wiped with a thin layer of petroleum jelly. Once the saphenous vein was located, a sterile 25-gauge needle was used to puncture the vein and whole blood was collected in lithium-heparin coated microvette capillary tubes (Sarstedt, Canada, Cat# 16.443.100) and centrifuged at ~ 9, 500 x g (Eppendorf Mini-Spin Plus, Brinkman Instruments Inc., USA) for 5 minutes, transferred into polyethylene tubes and stored at -80°C until further analysis of Cort concentrations using a radioimmunoassay kit (MP Biomedical, USA).

Oral glucose tolerance test: Animals were fasted overnight (~12-14 hours) on day 11 and were then administered an oral glucose tolerance test on day 12 (OGTT, 1.5 g/kg body mass), as previously described (Shpilberg, 2012). Each animal's fasting whole blood glucose concentration was measured (time 0) via saphenous vein bleed (~10 µl) using a sterile needle (25G) and glucometer (Bayer One Touch). Plasma was also isolated (~50 µl) from saphenous bleed collection at this same time point for subsequent analysis of insulin concentrations via an ELISA 96-well kit (Crystal Chem, USA, Cat# 90060). Whole blood glucose was also measured and recorded from saphenous vein bleed at T=5, 15, 30, 60, 90 and 120 minutes post oral gavage. Glucose area under the curve (AUC) was calculated for each animal and these data were expressed for each group, relative to the fasting glucose levels of the control animals.

Basal and insulin-stimulated glucose transport: After an overnight fast, following 14 or 15 days of treatment (as not all animals could be studied on the same day for practical purposes), basal and insulin-stimulated glucose transport and protein activation were assessed in paired, isolated soleus and EDL strips (~25 to 30 mg each). As previously published (Ritchie, 2011), a

27-gauge needle was used to carefully strip the soleus and EDL longitudinally, from tendon to tendon, into three sections, which were incubated under their respective condition. Pre-gassed (95% O2-5% CO2) Medium 199 containing 0.1% BSA and heated to 30ºC was used as a base for all glucose uptake buffers. Insulin (10 mU/ml) (Humulin R; Eli Lilly, Toronto, Ontario, Canada) was added to all buffers for the insulin-stimulated condition. Immediately following excision, the soleus or EDL strips were placed in 20-ml glass vials containing pre-incubation buffer for 30 min in the presence or absence of insulin (10mU/ml; concentration maintained in following buffers). The pre-incubation buffer was a base buffer containing 8 mM glucose and 32 mM mannitol. Both the soleus and EDL strips were washed in two glucose-free buffers, containing 4 mM pyruvate and 36 mM mannitol, for 10 min each, then the muscles were incubated in base buffer with 4 mM pyruvate, 8 mM 3-O-[^{3}H]methyl-$_{D}$-glucose (0.5 µCi/ml; American Radiolabeled Chemicals, St. Louis, MO), and 28 mM [^{14}C] mannitol (0.2 µCi/mmol; American Radiolabeled Chemicals, St. Louis, MO) for 20 min (insulin-stimulated) or 40 min (basal) conditions. Soleus and EDL muscles were then removed, thoroughly blotted to remove extra buffer, and had their tendons removed. Muscles were then weighed and subsequently digested for 10 min in 1 ml of 1 M NaOH at 95ºC. Muscle digest from each sample (200 µl) was sampled in duplicate and analyzed by liquid scintillation counting, from which glucose transport was calculated.

Histology: Skeletal muscle (tibialis anterior, TA) tissues from animals euthanized after ~ 2 weeks of treatment were embedded in tissue freezing medium, frozen in liquid nitrogen and cryosectioned (10 µm thick). The TA was stained for fiber type and SDH activity as previously described (Beaudry 2015).

Fibre Type, SDH and Oil Red O Analysis: To identify skeletal muscle fibre type a metachromatic myosin ATPase stain was performed on cross-sections of the TA muscle using a modified protocol (Ogilvie 1990). Sections were pre-incubated in an acidic buffer (pH = 4.25) to differentially inhibit myosin ATPases within the different fibre types. In this protocol, type I fibres appear dark blue, type IIa appear very light blue and type IIb and IIx are not apparent from each other and are thus classified as IIb/x. These fibres appear bluish-purple. A representative image of the TA for each group was acquired for analysis. The distribution of the CSA in a fiber population was assessed and depicted as a histogram.

Succinate dehydrogenase (SDH) activity was then assessed using histochemical analysis (Shortreed, 2009) and expressed as total optical density. The same muscle regions of the TA that were used for fibre typing were used for SDH activity determination. Serial sections were used to directly compare levels of SDH in each fibre with each fibre type. SDH activity was assessed with Adobe Photoshop CS6, converted to grayscale, and reported as the average optical density (sixty fibers were counted per muscle section). The grayscale is evaluated on a range of completely black (set a zero) to white (set at 225).

TA sections were fixed with 3.7% formaldehyde for 1 hour at room temperature while an ORO solution composed of 0.5 g ORO powder (Sigma-Aldrich, Canada) and 100 ml of 60% triethyl phosphate (Sigma-Aldrich, Canada) was mixed and filtered. Following fixation in 3.7% formaldehyde, slides were immersed in filtered ORO solution for 30 minutes at room temperature. Slides immediately underwent five washes with ddH_2O, were allowed to dry for 10 minutes and were sealed with Permount (Sigma-Aldrich, Canada). All images were acquired at 10 x magnifications, using a Nikon Eclipse 90i microscope (Nikon, Canada) and Q-imaging MicroPublisher 3.3 RTV camera with Q-capture Software.

Western Blot Analysis: All procedures for derivatization of protein carbonyls with DNPH (2,4-dinitrophenylhydrazine) were conducted using the Oxy blot kit (Millipore, S7150). Briefly, 10 μg and 15 μg of protein was loaded from EDL and soleus muscles respectively. Proteins were denatured with 12% SDS followed by derivatization for 20 min with gentle agitation at room temperature. Neutralization solution was then added to each sample to stop the derivatization reaction and samples were reduced with 2-mercaptoethanol (5%v/v). Proteins were separated on a 12% polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Canada). Membranes were blocked in odyssey blocking buffer for 1 hour prior to overnight incubation with primary antibody provided with the kit (1:300, Rabbit Anti-DNP). On the following day membranes were washed with Tris-buffered saline with Tween 20 (TBST) and incubated with goat anti-rabbit IgG 680 (Licor, 926-68071) for 1 hour and imaged (Odyssey, Licor Bioscience). EDL and soleus muscles were homogenized and separated on a 10% (GR, 11β-HSD1, GLUT4, total Akt, pSer^{473} and pTh^{308}) or 6% (total PGC1-α, IRS-1, and AS160) SDS-page gel according to previously published work (Beaudry, 2015). Proteins were transferred to a PVDF membrane (Bio-Rad, Canada) and were blocked in 5% powdered milk and TBST at room temperature for 1 hour. Membranes were then incubated overnight at 4°C with their respective primary antibodies at a concentration of 1:1000 (GR, Cat#sc-12763, Santa Cruz Biotechnology, USA; 11β-HDS1, Cat#10004303, Cayman Chemical, USA; GLUT4, Cat#ab65267, Abcam, Toronto, ON; Akt, Cat#ab8805, Abcam, Toronto, ON; pSer^{473}, Cat#ab28821, Abcam, Toronto, ON; pTh^{308}, Cat#ab5626, Abcam, Toronto, ON; PGC1-α, Cat#4359, Cell Signaling, USA; total IRS-1, Cat#2382, Cell Signaling, USA; AS160, Cat #C69A7, Cell Signaling, USA). The following morning, the membranes were washed with TBST and incubated with anti-mouse (1:10000, Cat#ab6789, Abcam, Toronto, ON) or anti-

rabbit (1:10000, Cat#ab6721, Abcam, Toronto, ON) secondary antibodies for 1 hour at room temperature. Membranes were then washed and imaged. Images were detected on a Kodak In vivo FX Pro imager and molecular imaging software (Carestream Image MI SE, version S.0.2.3.0, Rochester, New York) was used to quantify protein content. GAPDH (1:10000, Cat#ab9484, Abcam, Toronto, ON) and α-tubulin (1:10000, Cat#ab7291, Abcam, Toronto, ON) were used as loading controls.

Statistical Analysis: All data are represented as means ± SE, with a criterion of $P \leq 0.05$ or $P \leq 0.01$ and were assessed as stated using one-way ANOVAs as a means of statistical significance. Individual differences were calculated using Tukey post-hoc test (Prism4; Graphpad software Inc; La Jolla, CA, USA).

Results

Body mass and food intake: Day 11 was used as a marker of body mass and food intake as the evening of that day the animals were fasted in preparation for the OGTT on day 12, which would alter their body composition. Cort-treatment resulted in a significant loss of total body mass (Table 2.1, $P \leq 0.01$), regardless of diet, as measured on day 11 of the protocol. Absolute food intake (g/day) was higher in Cort-SD animals compared to the other two groups ($P \leq 0.01$), while relative food intake (kcal/kg body mass/day) was higher in the two Cort-treated groups compared with the controls (both $P \leq 0.01$). Cort-HFD had significantly lower relative EDL mass compared to the other two groups ($P \leq 0.01$), while Cort-treatment in both groups (chow and HFD) caused an increase in relative soleus mass (Table 2.1, $P \leq 0.05$), as has previously been shown by us (Beaudry, 2014). Interestingly, the addition of HFD to Cort treatment appeared to attenuate the rise in soleus mass compared to Cort treatment alone (Table 2.1, $P \leq 0.01$).

Plasma cort, and fasted glucose and insulin concentrations: As expected, Cort levels measured at 0800, 7 days after pellet implantation, were elevated in both Cort groups (Table 2.1, $P \leq 0.05$), similar to what we have previously reported in this model (Shpilberg, 2012). There was no significant effect of the HFD on Cort concentrations within either the Control or Cort-treated groups (Table 2.1). Fasting blood glucose values were significantly elevated prior to the oral glucose challenge within the Cort-HFD group only (Fig. 2.2A, $P \leq 0.01$). Fasting insulin values were 3-4 fold higher in the Cort-treated animals compared to controls, with the Cort-HFD group having the highest values, albeit not statistically different from the Cort-SD group (Fig. 2,2B, $P \leq 0.05$).

Oral Glucose Tolerance Test: Cort-treatment resulted in elevated blood glucose values during the oral glucose challenge compared to the Control-HFD group (Fig. 2.2C), however, only Cort-HFD animals had significantly higher glucose AUC compared to controls when the AUC was adjusted for baseline values within each group (Fig. 2.2C`, $P \leq 0.05$).

Skeletal muscle glucose transport (ex-vivo): Basal glucose transport in the EDL was ~40-50% lower in the Cort-treated groups compared to control-HFD animals, although these differences failed to reach statistical significance. Basal glucose uptake was similar in all three groups within the soleus muscle. Insulin stimulation significantly increased glucose transport within both the EDL and soleus in all treatment groups (Fig 2.3A, B; $P \leq 0.05$). This effect was blunted within the EDL muscle only in the Cort-HFD animals (Fig. 2.3A`, ($P \leq 0.01$). Intriguingly, insulin-stimulated glucose transport was unaffected by either diet or Cort-treatment in the soleus muscle, albeit the delta change from baseline was the lowest in the Cort-HFD group (Fig. 2.3B`).

Table 2.1. Corticosterone concentrations, body composition and food intake.

	Control-HFD	Cort-SD	Cort-HFD
Fasted body mass (g)	394.3 ± 6.0	301.6 ± 12.0*	298.3 ± 15.5*
Absolute food intake (g/day)	22.4 ± 0.7	34.6 ± 3.3†	24.3 ± 2.5
Relative food intake (kcal/g body mass/day)	0.29 ± 0.01	0.4 ± 0.03*	0.42 ± 0.04*
EDL mass (g/kg/BM)	0.46 ± 0.01	0.49 ± 0.04	0.38 ± 0.01#
Soleus mass (g/kg/BM)	0.35 ± 0.03	0.48 ± 0.02†	0.41 ± 0.03#
(AM) Plasma corticosterone (ng/ml)	29.3 ± 18.0	426.1 ± 77.5*	485.7 ± 80.5*

Notes: Body weight and food intake were measured every day, the data here is from day 11, one day prior to the OGTT. Plasma corticosterone values were measured 7 days after pellet implantation and high-fat diet administration. Body mass on day 11 with one-way ANOVA. *Significantly different from control-HFD $p<0.001$ with Tukey's post Hoc, † significantly different from control and Cort-HFD $p<0.001$ with Tukey post Hoc, #significantly different from control-HFD and Cort-SD $p<0.001$ with Tukey post Hoc.

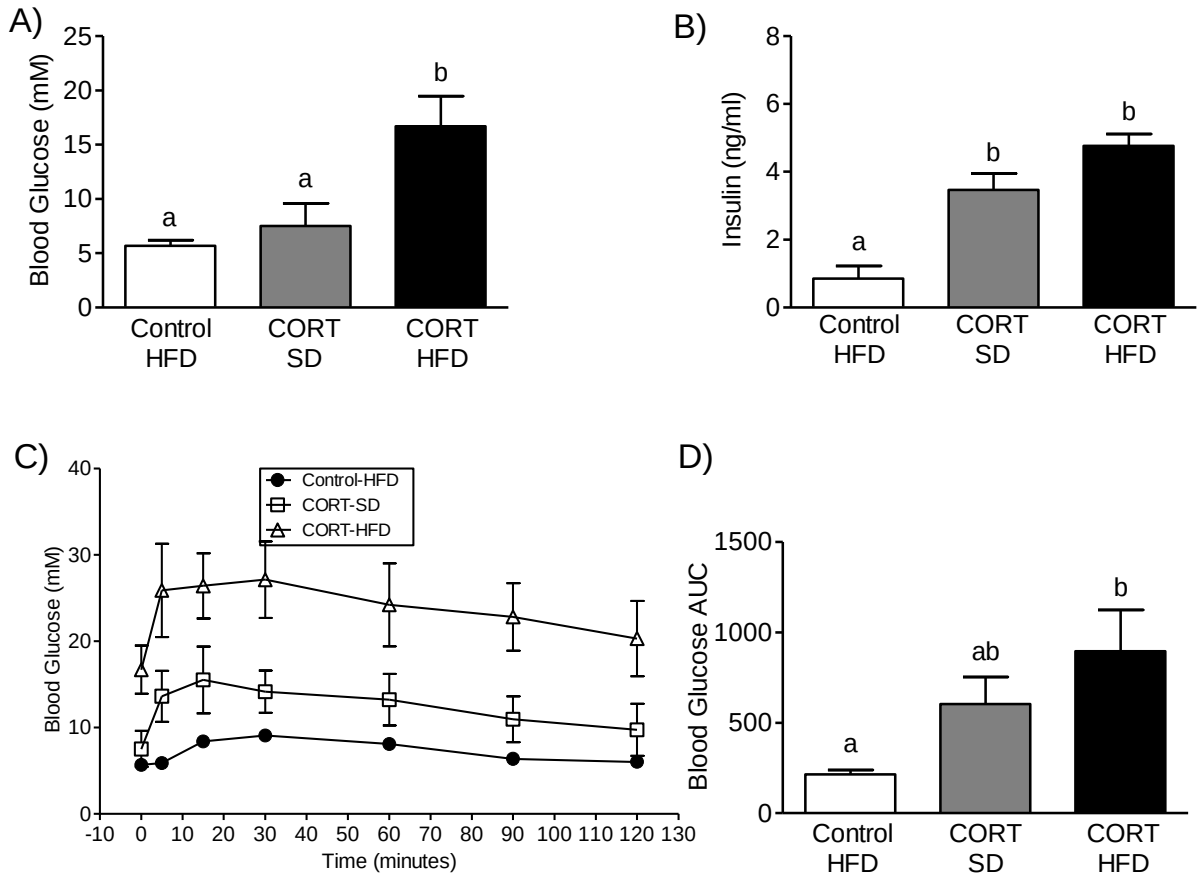

Figure 2.2. Corticosterone and a high-fat diet cause severe hyperglycemia and glucose tolerance.

Fasted glucose (A) and insulin (B) concentrations were measured at the onset of an oral glucose tolerance test (OGTT) (C, D); n=5. Bars that do not share similar letters denote statistical significance ($P<0.05$) using a one-way ANOVA with Tukey's (A and D), ($P<0.01$) (B). All values are means ± SE.

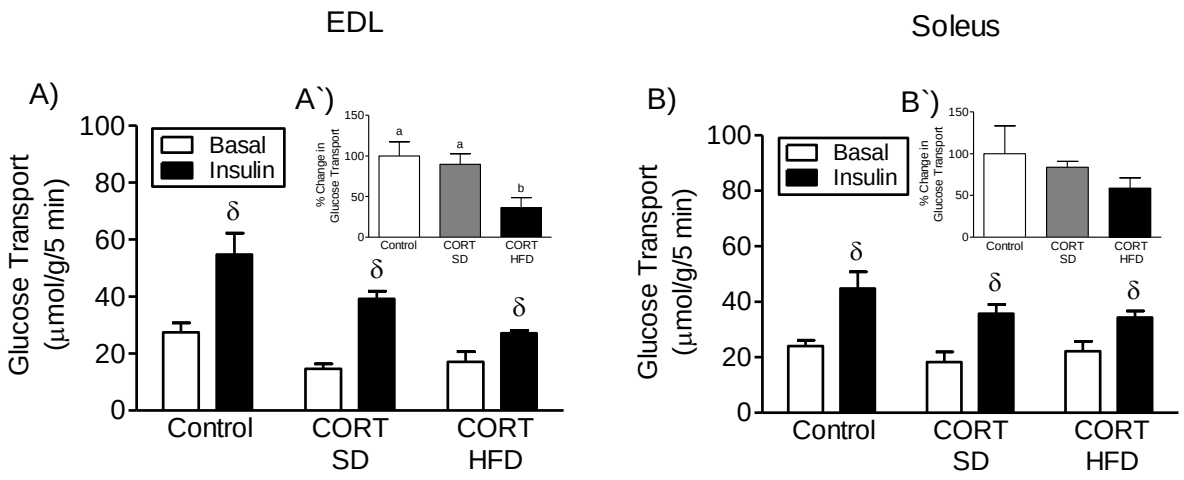

Figure 2.3. Corticosterone and a high-fat diet impair glucose transport in the extensor digitorum longus but not in the soleus.

Glucose transport was assessed in the EDL (A, A`) and the soleus (B, B`) at basal and insulin stimulated conditions; n=4. δ Significantly different from respective basal state ($P \leq 0.05$) using an unpaired, two-tailed, T-test. Bars that do not share similar letters denote statistical significance ($P \leq 0.05$) using a one-way ANOVA with Tukey post Hoc (A'). All values are means ± SE.

Insulin signaling protein content: Within the EDL muscle, IRS-1 total protein was markedly lower in both Cort-treatment compared to control-HFD animals (Fig. 2.4A, $P \leq 0.01$). In contrast, AS160 total protein was significantly increased in the Cort-SD group in comparison to the Control-HFD group, and even more elevated within the Cort-HFD group (Fig. 2.4B, $P \leq 0.01$). There were no significant differences in GLUT4 total protein concentration across all three treatment groups with the EDL muscle (Fig. 2.4C). Within the soleus, there was no effect of Cort-treatment on total IRS-1 or AS160 protein content (Fig. 2.4D-E). However, total GLUT4 content was higher in the Cort-SD group compared to Control-HFD group, and paradoxically lower in the Cort-HFD group compared to the control-HFD group (Fig. 2.4F, $P \leq 0.01$).

To determine whether there was an effect of insulin stimulation on components of the insulin signaling pathway, total and phosphorylated Akt was assessed both with and without insulin stimulation *ex vivo*, in both the EDL and soleus muscles. Within the EDL, there was an effect of insulin to increase $pSer^{473}$ in all three treatment groups, with Cort-treatment blunting this effect (Fig. 2.5A, $P \leq 0.01$). When the percent change in phosphorylation status from basal to insulin stimulated was measured relative to control-HFD, there was a greater impairment with Cort-HFD treatment compared to the other two groups (Fig. 2.5A`, p=0.0591). Interestingly, this result was not found within the soleus (Fig. 2.5C, C`), as there was no difference in insulin stimulated $pSer^{473}$ with either Cort-SD or Cort-HFD. While there was no effect of Cort-treatment to decrease pTh^{308} after insulin stimulation in the EDL (Fig. 2.5B), when the percent change in phosphorylation status was measured relative to control-HFD, there was a negative effect of insulin to stimulate pTh^{308} in the Cort-HFD group (Fig. 2.5B`, p=0.073), suggesting that pTh^{308} is activated even before insulin administration. When measured in the soleus, there was no effect

of insulin stimulation to increase pTh^{308} phosphorylation within any treatment group (Fig. 2.5D, D`).

Muscle cross-sectional area, fibre frequency, succinate dehydrogenase and ORO content: To examine the effect of Cort-HFD-treatment within the skeletal muscle, the TA muscle of representative animals from each treatment group was stained for fibre type to measure CSA, SDH, and ORO content. CSA, indicating individual muscle fibre size, was lowest in type IIb/x fibres of Cort-HFD-treated animals (Fig. 2.6A, B, $P \leq 0.05$). While the CSA of the type IIb/x fibres of the Cort-SD animals was also decreased, it did not reach statistical significance. A shift to smaller muscle fibres with Cort treatment, particularly in the Cort-HFD group, is displayed in the percent frequency histogram in the various fibre size categories shown (Fig. 2.6C). SDH staining, which correlates to oxidative capacity, was not significantly different among the three treatment groups (Fig. 2.6D, E). ORO staining of TA muscle sections was used to visually examine lipid accumulation in all treatment groups (Fig 2.6F). Lipid staining appeared to be highest in Cort-HFD-treated animals, which correlates with previous data examining the effect of Cort-HFD-treatment (Beaudry, 2015; Shpilberg, 2012).

Carbonylated protein and PGC1-α content: Total protein carbonylation was examined in both the EDL and soleus to determine whether there were any elevations in oxidative stress due to either a HFD or Cort-treatment. There were no significant differences in total protein carbonylation within either the EDL or soleus (Fig. 2.7A, C). Additionally, total PGC1-α content, a marker of mitochondrial biogenesis, was analyzed yielding no significant differences between groups within either the EDL or the soleus (Fig. 2.7B, D).

Glucocorticoid receptor and 11β-HSD1 protein content: GR protein content, within the EDL and soleus muscles, was unaffected by two weeks of Cort-treatment, regardless of diet (Fig.

2.8A). Overall, however, soleus muscle expressed ~2-fold higher GR concentration than EDL muscle, which was somewhat surprising since much of the selective effects on white muscle may have been explained by a higher GC receptor density. 11β-HSD1, the enzyme responsible for converting inactive 11-dehydrocortisone to active cortisol, protein content was highest in the EDL of the Cort-HFD group (Fig. 2.8B, $P \leq 0.05$), but was not statistically different from that found within the EDL of the Cort-SD group. In contrast to the receptor expression, 11β-HSD1 protein expression was higher in EDL than in the soleus in all three treatment groups.

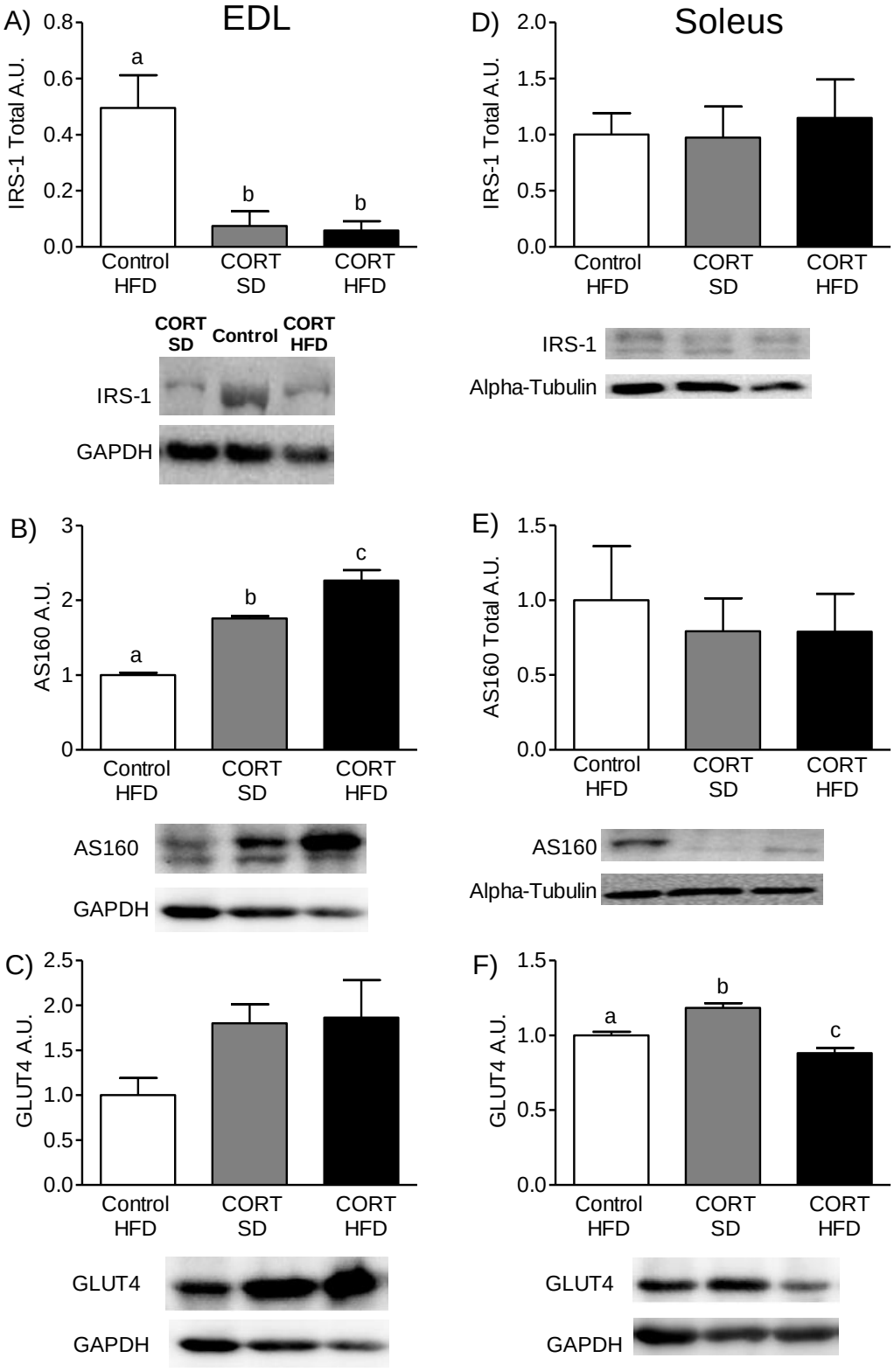
A)
EDL
IRS-1 Total A.U.
0.8
0.6
0.4
0.2
0.0
a
b
b
Control HFD
CORT SD
CORT HFD
CORT SD
Control
CORT HFD
IRS-1
GAPDH
D)
Soleus
IRS-1 Total A.U.
2.0
1.5
1.0
0.5
0.0
Control HFD
CORT SD
CORT HFD
IRS-1
Alpha-Tubulin
B)
AS160 A.U.
3
2
1
0
a
b
c
Control HFD
CORT SD
CORT HFD
AS160
GAPDH
E)
AS160 Total A.U.
1.5
1.0
0.5
0.0
Control HFD
CORT SD
CORT HFD
AS160
Alpha-Tubulin
C)
GLUT4 A.U.
2.5
2.0
1.5
1.0
0.5
0.0
Control HFD
CORT SD
CORT HFD
GLUT4
GAPDH
F)
GLUT4 A.U.
1.5
1.0
0.5
0.0
a
b
c
Control HFD
CORT SD
CORT HFD
GLUT4
GAPDH

Figure 2.4. Insulin signaling proteins within the extensor digitorum longus are affected by corticosterone-treatment.

Total IRS-1, AS160, and GLUT4 were measured in the EDL (A-C) and the soleus (D-F); n=5. Bars that do not share similar letters denote statistical significance ($P \leq 0.01$) using a one-way ANOVA with Tukey post Hoc. All values are means ± SE.

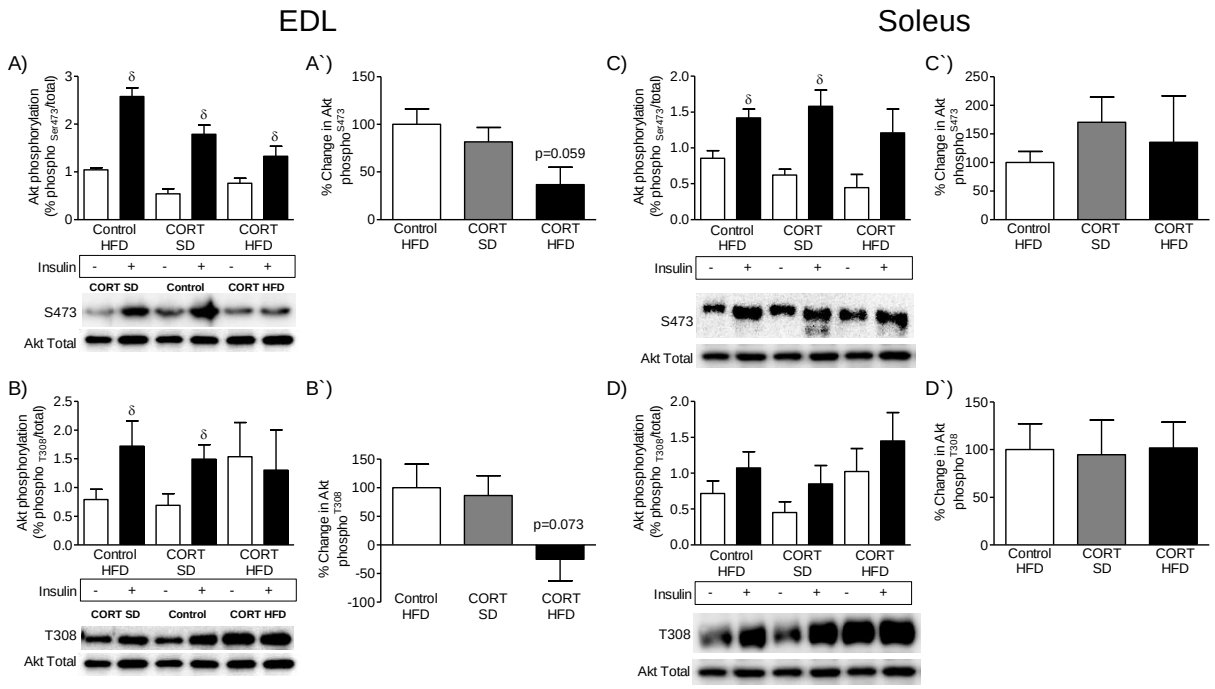

Figure 2.5. Effect of corticosterone and a high-fat diet on Akt phosphorylation.

Akt pSer^{473} was analyzed in the EDL (A, A') and the soleus (C, C`) at basal and insulin-stimulated conditions. Akt pTh$^{308}$ was analyzed in the EDL (B, B`) and the soleus (D, D`) at basal and insulin-stimulated conditions; n=5. δ Significantly different from respective basal state ($P \leq 0.05$) using an unpaired, two-tailed, T-test. Bars that do not share similar letters denote statistical significance ($P<0.05$) using a one-way ANOVA with Tukey post Hoc. All values are means ± SE.

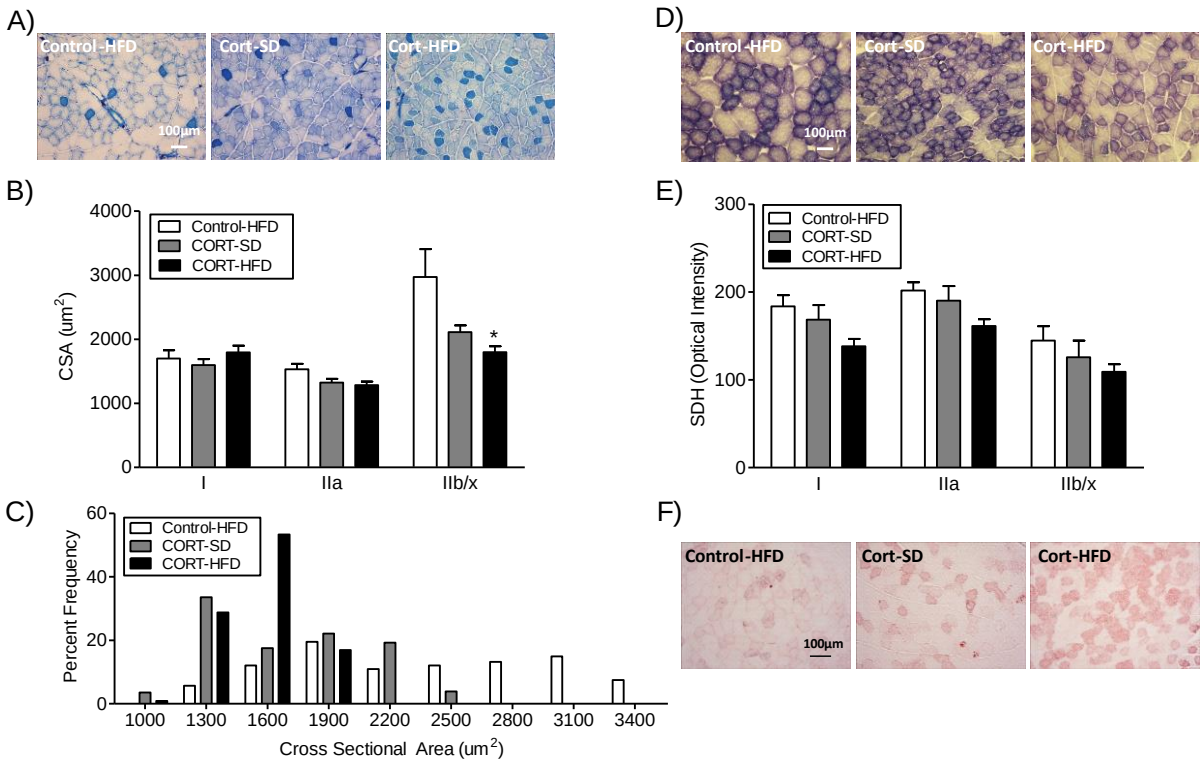

Figure 2.6. Corticosterone and a high-fat diet cause glycolytic fibre atrophy.

Representative images of the tibialis anterior (TA) depicting fiber type staining (A), measurement of individual muscle fiber cross-sectional area (CSA) (B) and assessment of individual fiber frequency (C) within each treatment group. Representative images of the TA depicting succinate dehydrogenase (SDH) staining (D), quantification of the intensity of SDH staining within all three treatment groups (E), and Oil Red O (ORO) neutral lipid staining (F); n=5. *Significantly different from control-HFD ($P \leq 0.05$) using a one-way ANOVA with Tukey post Hoc. All values are means ± SE.

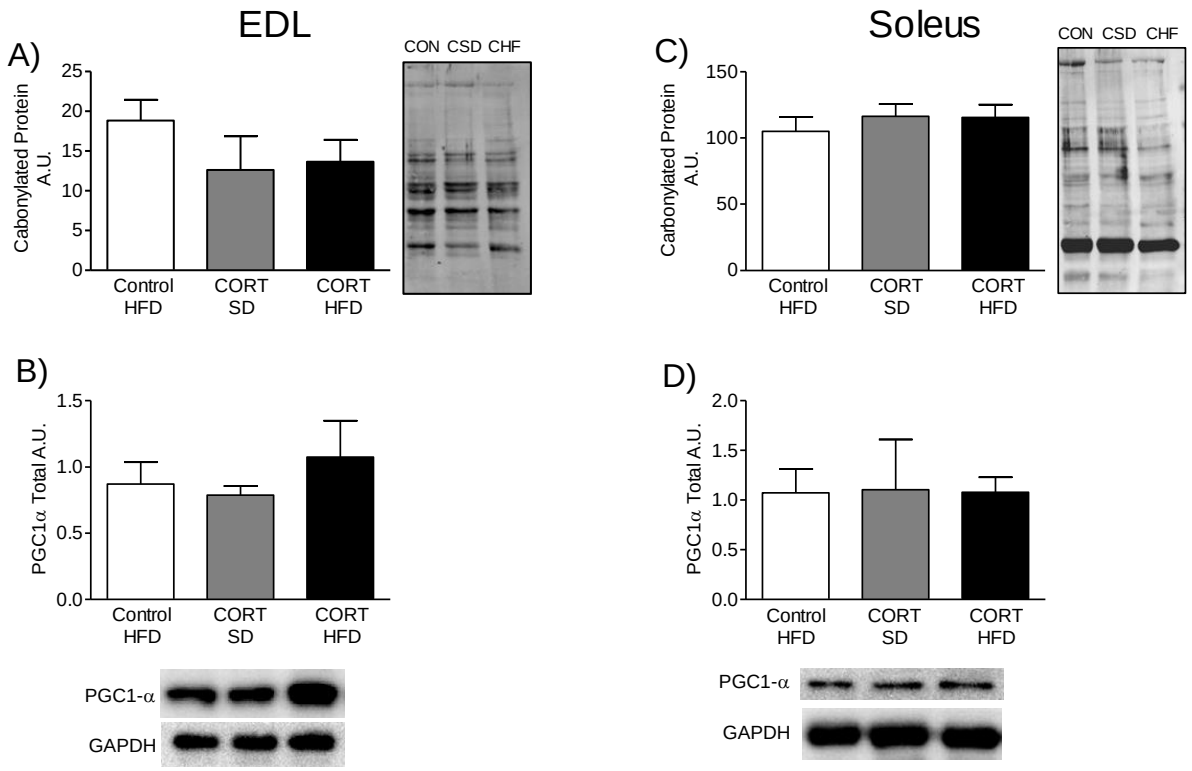

Figure 2.7. Corticosterone and a high-fat diet do not alter protein carbonyl or PGC1-α content.

Protein carbonylation in EDL (A) and soleus (C). Relative total PGC1-α in EDL (B) and soleus (D); n=4-5. Analyzed by one-way ANOVA with Tukey post Hoc. All values are means ± SE.

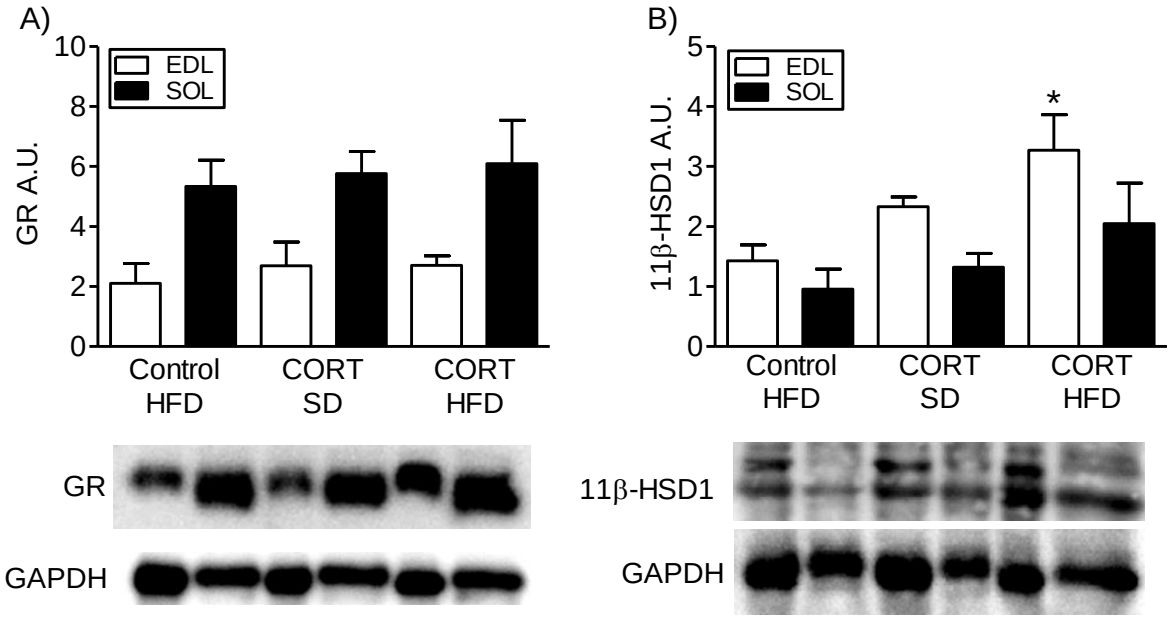

Figure 2.8. Glucocorticoid receptor and 11β-HSD1 content are elevated in the soleus and extensor digitorum longus after corticosterone and high-fat diet treatment.

Glucocorticoid receptor expression was assessed in both the EDL and soleus of all three treatment groups (A) at basal conditions. 11β-HSD1 total protein expression was measured in both the EDL and soleus of all three treatment groups (B) at basal conditions; n=5. *Significantly different from control-HFD ($P \leq 0.05$) using a one-way ANOVA with Tukey post Hoc. All values are means ± SE.

Discussion

This study clearly demonstrates the deleterious effects of combining exogenous Cort-treatment with a HFD can have on skeletal muscle, particularly in those muscle groups that expressed a high degree of glycolytic fibres. Moreover, we show here that the combination of Cort and a HFD causes severe whole body glucose intolerance, muscle fibre type specific impairments in insulin signaling and glucose transport and considerable glycolytic muscle atrophy. The striking impairments observed, primarily in glycolytic (white) muscle, are not explained by a higher glucocorticoid receptor expression in that tissue compared with oxidative (red) muscle, but may be explained by a greater regeneration of intracellular GC via the increased expression of the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) levels in the former muscle type.

The metabolic impairments caused by Cort-HFD-treatment have been previously examined by our lab in a number of other publications focusing on whole body glucose metabolism, liver and pancreatic tissues (Beaudry, 2013; Beaudry, 2015; Beaudry, 2014; D'souza, 2011; Shpilberg, 2012). The focus of this study was to determine whether skeletal muscle is a primary organ affected by this co-treatment, which would help to further explain the animals' severe hyperinsulinemic/hyperglycemic phenotype. To help determine if skeletal muscle was a primary organ of metabolic defect with GC and/or HFD treatment, we used an ex-vivo glucose transport analyses that allows for more direct measures of muscle specific glucose transport under a standardized insulin exposure. This technique was advantageous since measuring insulin signaling and transport in vivo would have suffered from the inability to control for the markedly differing circulating insulin (and glucose levels) in these three animal

groups (Fig 2.2). Using this technique also allowed us to determine if different muscle groups (i.e. white vs red) differ in their responses to prolonged GC-HFD treatment.

The combination of exogenous Cort and a HFD caused a novel and significant reduction in insulin-stimulated glucose uptake (ISGU) within the EDL but not within the soleus. The effect of dexamethasone, a synthetic GC, to impair skeletal muscle ISGU has previously been assessed in the epitrochlearis (glycolytic) and soleus muscles of male rats (11, 41). The authors found that both muscle types were significantly impacted after ~11 days of dexamethasone treatment, resulting in impaired ISGU. Here, we examined the addition of a HFD, as well as a lower ex-vivo insulin concentration, on ISGU, also within both a glycolytic (EDL) and an oxidative (soleus) muscle. The insulin concentration used in this study was chosen to represent a lower, more physiological value, with the objective of elucidating the mechanism behind the impairments in glucose transport.

GCs are well-known stimulators of whole body lipolysis (Peckett, 2011), causing increased non-esterified free fatty acid (NEFA) and triglyceride levels. We have previously shown that Cort-HFD-treatment is capable of causing significant elevations in circulating triglycerides, with minimal effects on NEFA concentrations, in addition to intramuscular lipid accumulation (Shpilberg, 2012) which was improved with 2 weeks of Mifepristone administration (Beaudry, 2014). The reduction in insulin sensitivity and subsequent impairment to ISGU within the skeletal muscle induced by GC is thought to occur through direct perturbation of insulin signaling in combination with additional effects on protein and lipid metabolism. This suggests that the added variable of a HFD could compound the established impairments associated with GC treatment, especially since, in accordance with previously

published work (Beaudry, 2015; Shpilberg, 2012), we observed grater neutral lipid accumulation through ORO staining within the TA muscle (Fig. 2.6F).

GCs can affect muscle insuin signaling at several levels along the insulin signaling cascade, notably decreasing total IRS-1 content (Saad, 1993) while increasing Ser^{307} phosphorylation (Morgan, 2009). Increased serine phosphorylation can subsequently decrease affinity of IRS-1 for the insulin receptor, increasing IRS-1 degradation (Aguirre, 2000; Aguirre 2002), which could be the reason why both Cort-treated groups had deceased IRS-1 content within the EDL. Under basal conditions, AS160 is located within the vesicles that contain GLUT4 and controls the availability of guanosine triphosphate (GTP), which is required for vesicle translocation to the cell membrane, ultimately initiating glucose entry. Upon phosphorylation, initiated by Akt/PKB, AS160 dissociates from the vesicle, which allows the binding of GTP and subsequent vesicle translocation (Sano 2003). Previous research has established that GCs increase AS160 protein and GLUT4 mRNA content (Ewart, 1998; Morgan, 2009) as a compensatory mechanism to preserve insulin sensitivity to compensate for inhibition of insulin signaling through decreased IRS-1. Additionally, the phosphorylation of AS160 by Akt regulates GLUT4 translocation which is the main mechanism that permits glucose entry into insulin-target tissues. Intriguingly, we found that with Cort-HFD treatment there was an upregulation of AS160 total protein, yet there were still impairments in glucose transport within the EDL. We also observed a trend for increased GLUT4 protein due to Cort-treatment, within the EDL. Neither of these results were found within the soleus, however, possibly suggesting a protective effect of oxidative muscle on Cort-HFD treatment. Interestingly, the blockade of 11β-HSD1, the major enzyme responsible for activation of GCs, in both human and rodent skeletal muscle (selective and nonselective) is able to restore IRS-1 levels similar to controls, improve

Akt/PKB activation and caused an insulin-sensitizing effect (Morgan, 2009). Moreover, selective inhibition of 11β-HSD1 caused a decrease in lipogenic gene expression (ACC1, DGAT, FAS) and increased free fatty acid (FFA) utilization (Berthiaume 2007) with the net effect being a decrease in intramuscular lipid accumulation, as well as a decrease in local FFA and DAG generation. These conclusions, added to the fact that we did not see any major differences between key insulin signaling proteins in either Cort-treated group, suggest that the added fat content from the diet did not have a substantial effect on insulin signaling machinery in this 2-week treatment period.

Pronounced skeletal muscle atrophy is a common side effect of prolonged GC treatment with corticosteroids or with Cushing's disease (Khaleeli, 1983). There are a number of possible mechanisms explaining skeletal muscle atrophy with elevated GCs. These steroids are known to be both catabolic, through stimulation of muscle proteolysis and anti-anabolic, through inhibition of insulin and IGF1-related muscle protein synthesis (Lofberg, 2002). The increase in circulating amino acids, caused by the inhibition of amino acid transport into the skeletal muscle by GC, in addition to the atrophy-related decrease in cross-sectional area (CSA), likely all contribute to the reduced ISGU associated with elevations in plasma GC. GCs are typically associated with the preferential targeting of glycolytic skeletal muscle for atrophy (Dekhuijzen, 1995; Fournier 2003). While we did not measure protein synthesis or degradation, we did analyze the CSA and found that the Cort-HFD group had the lowest type IIb/x fibre CSA, which manifested as a possible fibre type shift when analyzed as a histogram. These results suggest that the skeletal muscle of the Cort-HFD group was more affected by the atrophic stimulus typically conveyed through elevations in GCs. This could be a contributing factor to the obvious glucose intolerance as there was less muscle available to take up the added plasma glucose.

In addition to muscle size, alterations in muscle quality may be an important component in explaining the synergism associated with Cort-HFD-treatment. Excessive lipid accumulation within the skeletal muscle has been associated with sarcopenia (Tardif, 2014), in addition to chronic diseases such as diabetes (Goodpaster, 2004), and muscular dystrophy (Lott 2014) while also considered an important component of insulin resistance (Hegarty, 2003). Elevations in intramuscular lipid infiltration have been acknowledged as a contributor to declining strength and mobility, independent of muscle size (Goodpaster, 2001a), and in situations of reduced insulin action, it has been identified that irregular mitochondrial fatty acid metabolism is linked to intramuscular lipid accumulation (Kelley, 2002; Vladimir B. Ritov, 2005). Mice fed a HFD for ~2 months or less were found to have no impairments in basal skeletal muscle protein metabolism (Anderson 2008) but expressed whole body insulin resistance and impaired mitochondrial function (Boudina 2012). GCs have also been implicated in mitochondrial dysfunction, as long-term corticosteroid treatment causes mitochondrial damage (Mitsui 2002) and overproduction of ROS in human myogenic cells (Oshima 2004), while acute administration of Cort in broiler chickens was shown to augment lipid peroxidation, ultimately inducing oxidative stress (Lin 2009). To determine whether the mitochondria, or elevations in protein oxidation, could be contributing factors to the synergism we see with the Cort-HFD combination, we measured basic markers of mitochondrial biogenesis and reactive oxygen species (ROS) production. We did not find any statistically significant effects of Cort-HFD-treatment on SDH staining, a marker of oxidative capacity, although the Cort-HFD group did appear to have lighter staining intensity. Additionally, PGC1-α protein expression, a marker of mitochondrial content, was unaffected by Cort-HFD-treatment, as was total protein carbonylation. Protein carbonylation was tested as a measure of protein modification (Antunes 2014), and is not considered specific to

ROS produced within either the mitochondria or cytosol. As such, it is possible that while we were unable to detect any differences in total protein carbonylation, future measurement of protein nitrosylation or lipid membrane peroxidation, or other cellular ROS accumulation measurements, could link ROS elevations within the skeletal muscle to the major metabolic effects induced by the combination of Cort-treatment with a HFD.

There is much interest in the management of local GC action as a therapeutic strategy, as while it is known that excessive GC levels can play a role in the pathogenesis of obesity and T2DM development (Tomlinson 2007; Walker 2006), circulating plasma GC concentrations are not always elevated in obese individuals. It is possible that an increase in local reactivation of GC in a tissue-specific manner could be the driving force behind the associated metabolic dysregulation. The enzyme responsible for modulating the tissue-specific effects of circulating GC is 11β-HSD1, which activates inactive GCs (i.e. cortisone in humans and 11-dehydrocortisone in rodents) in muscle, liver and adipose tissue so that they can then bind and activate the glucocorticoid receptor (GR) (Tomlinson 2004). Other studies have examined the effect of both deleting and over-expressing 11β-HSD1 in metabolism, showing that the high expression of this enzyme in adipose tissues drives the development of the metabolic syndrome (Drake 2005; Livingstone 2003; Wamil, 2011). In our study, both Cort-treated groups had elevations in 11β-HSD1 expression, particularly in EDL muscle of the Cort-HFD group (Fig. 8B). In humans, increased muscle 11β-HSD1 has been linked to reduced insulin sensitivity (Whorwood, 2002) and cortisone itself has been shown to induce insulin resistance via upregulation of 11β-HSD1 in C2C12 cells (Park 2014). We have previously found there to be increased 11β-HSD1 mRNA and protein expression in the subcutaneous and visceral fat of these

animals (Beaudry, 2014; Shpilberg, 2012), indicating that this treatment causes a widespread up regulation of this enzyme, which likely worsens the metabolic phenotype in a number of tissues.

In summary, we show that Cort-HFD-treatment causes a more severe reduction in ISGU within glycolytic muscle, a consequence that appears to be linked to a greater glycolytic atrophy than Cort treatment alone. Furthermore, Cort-treatment decreases total IRS-1 content and $pSer^{473}$ phosphorylation within glycolytic muscle, a result that was not paralleled within the soleus nor affected by HFD. Interestingly, 2 weeks of Cort-HFD-treatment seemed to have the highest increase in 11β-HSD1 total protein, again within glycolytic muscle, but surprisingly showed no effects to induce elevations in protein oxidation. This study underscores the significant adverse effects that combining Cort-treatment with a HFD can have within the skeletal muscle, particularly in white muscle, which likely contributes to the diabetic phenotype. These findings also underscore the importance of a low fat diet when individuals are given a course of GC treatment.

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Chapter 3

Voluntary exercise improves metabolic profile in high-fat fed glucocorticoid treated rats

This chapter is an original published article. It is presented in its published format.

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**Co First Author*

Author Contributions: Dr. Jacqueline Beaudry and I designed the study and wrote the paper along with input from our supervisor Dr. Michael Riddell. Dr. Jacqueline Beaudry and I performed all animal handling, including pellet surgeries, oral glucose tolerance and insulin tolerance tests, and plasma analysis (insulin ELISA, corticosterone RIA, NEFAs, GIP, GLP-1). Dr. Erwan Leclair assisted with measuring GIP and GLP-1. Dr. Jacqueline Beaudry completed all islet analyses. I sectioned and stained all skeletal muscle samples for measurement of CSA and SDH, Ashley Peckett assisted with SDH analysis, while Erin Mandel completed the capillary to fibre analysis, with input from Dr. Tara Haas. I performed western blotting of all skeletal muscle proteins with assistance from Dr. Erwan Leclair. Dr. Michael Riddell oversaw the entire study and assisted in manuscript preparation.

Rationale: Regular exercise is well known to induce considerable physiological improvements in a number of pathological situations. Chapter 3 focussed on the application of voluntary wheel running, as a more practical exercise stimulus, to the ROD model (elevated GC and HFD). The completion of this study demonstrated the viability of aerobic exercise as a possible therapeutic strategy for individuals who suffer from endogenous GC overproduction or are taking exogenous GC therapy and indulge in poor diet choices.

Voluntary exercise improves metabolic profile in high-fat fed glucocorticoid treated rats

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Author Contributions

Conceived and designed the experiments: J.L.B., E.C.D. and M.C.R. Performed the experiments: J.L.B., E.C.D., E.L., E.R.M., and A.J.P. Analyzed data: J.L.B., E.C.D., E.L., E.R.M., A.J.P. and M.C.R. Wrote the paper: J.L.B. and E.C.D. Edited the manuscript: E.C.D., E.L., E.R.M., T.L.H. and M.C.R.

Preface

Diabetes is rapidly induced in young male Sprague-Dawley rats following treatment with exogenous corticosterone (CORT) and a high-fat diet (HFD). Regular exercise alleviates insulin insensitivity and improves pancreatic β-cell function in insulin resistant/diabetic rodents, but its effect in an animal model of elevated glucocorticoids is unknown. We examined the effect of voluntary exercise (EX) on diabetes development in CORT-HFD treated male Sprague-Dawley rats (~ 6 weeks old). Animals were acclimatized to running wheels for 2 weeks, then given a HFD, either wax (placebo) or CORT pellets and split into 4 groups: placebo-SED or -EX and CORT-SED or -EX. After 2 weeks of running combined with treatment, CORT-EX animals had reduced visceral adiposity, increased skeletal muscle type IIb/x fibre area, oxidative capacity, capillary to fibre ratio and insulin sensitivity compared to CORT-SED animals (all $p<0.05$). Although CORT-EX animals still had fasting hyperglycemia, these values were significantly improved compared to CORT-SED animals (14.3±1.6 vs. 18.8±0.9 mM). In addition, acute *in vivo* insulin response to an oral glucose challenge was enhanced ~ 2-fold in CORT-EX vs. CORT-SED ($p<0.05$) which was further demonstrated *ex vivo* in isolated islets. We conclude that voluntary wheel running in rats improves, but does not fully normalize, the metabolic profile and skeletal muscle composition of animals administered CORT and HFD.

Introduction

Sustained elevations in circulating glucocorticoids (GCs) cause major physiological consequences to the body including severe insulin resistance (Shibli-Rahhal, 2006) central obesity (Peckett, 2011) non-alcoholic fatty liver disease (D'souza, 2011) and hyperglycemia (Lansang 2011). Both rodent (Campbell 2010; Király 2008) and human (Cameron 1984; Chiodini 2006; Lansang, 2011) studies of type 2 diabetes mellitus (T2DM) show a strong correlation between hypercortisolemia and the development of T2DM. Examination of possible therapeutic options to ameliorate GC-induced metabolic conditions is of utmost importance as exogenous GCs are among the most commonly prescribed anti-inflammatory and immunosuppressive medications (Rosen 2005).

Our laboratory has developed an animal model of rapid-onset diabetes induced through treatment with exogenous corticosterone, (CORT, the primary active GC in rodents) and a high-fat diet (HFD, 60% calories from fat) (Beaudry, 2013; Beaudry, 2014; Shpilberg, 2012). We found that modest elevations in CORT, comparable to peak diurnal levels observed in healthy rodents, when combined with a HFD, result in severe hyperglycemia and hyperglucagonemia, despite hyperinsulinemia and increased pancreatic β-cell mass. These results are largely due to extreme whole-body insulin insensitivity and impaired insulin responsiveness to oral glucose challenge (Beaudry, 2013). Recently, we have also demonstrated that this diabetic phenotype can be corrected by the administration of a nonspecific GC receptor II antagonist, RU486, without modification to the animal's diet (Beaudry, 2014). However, RU486 is also highly selective for the progesterone receptor (Johanssen, 2007) and may present complications with pregnancy and other undesirable side effects. To date, other more selective GR antagonists have failed to completely reverse the diabetic phenotype in GC-treated animals (Beaudry, 2014). Thus, alternative therapies for reducing the metabolic effects of hypercortisolemia are needed.

Regular exercise in diabetic prone rats can improve glucose homeostasis by increasing whole body insulin sensitivity (Király 2010), β-cell function (Király, 2008) and by lowering circulating basal (non-stimulated) GC levels (Campbell, 2010; Király 2007). Interestingly, in Zucker Diabetic Fatty (ZDF) rats, the prevention of hyperglycemia with regular exercise coincides with the prevention of basal hyperglucocorticoidemia (Campbell, 2010). This may indicate that some of the metabolic advantages of exercise in diabetes prevention and treatment occur through a reduction in chronic GC elevation thereby limiting the GC action at the tissue level. Little is known about the effect of voluntary exercise on a model of exogenously administrated GCs, as acute exercise would not be expected to lower GC concentrations (Fediuc 2006). Interestingly, dexamethasone-treated Wistar rats that underwent treadmill training for 8 weeks, showed restoration of muscle glycogen levels and improved muscular atrophy, despite sustained insulin resistance (Barel, 2010), thereby suggesting exercise training may offer some protection against hyperglucocorticoidemia.

Voluntary exercise normalizes glucose tolerance and islet glucose sensitivity (Beaudry 2012) through up-regulated mechanisms of skeletal muscle glucose uptake, i.e. GLUT4 translocation (Burr 2012; Van Raalte, 2009), oxidative capacity (Nagatomo 2012) and pancreatic islet function (Delghingaro-Augusto 2012). In this rodent model of elevated GCs and high fat feeding, we hypothesized that voluntary exercise would alleviate hyperglycemia, impaired muscle insulin signalling and skeletal muscle wasting while helping to preserve β-cell function. As elevated GCs are caused by exogenous treatment in our model, voluntary exercise would not be expected to lower GC concentrations sufficiently to promote full metabolic recovery. To examine the metabolic effects of exercise in this rodent model, we compared the effects of ~2.5 weeks of voluntary wheel running with sedentary behaviour on various markers of energy

metabolism including: 1) whole-body glucose homeostasis, 2) body composition and skeletal muscle tissue histology, 3) in vivo and ex vivo islet responsiveness to glucose challenge and 4) skeletal muscle atrophic/insulin signalling proteins.

Methods

This study was carried out in accordance with the recommendations of the Canadian Council for Animal Care guidelines and was approved by the York University Animal Care Committee (2013-6).

Animals: First cohort of animals consisted of forty (40) male Sprague-Dawley rats (Charles River Laboratories, initial mass of 225-250 g, six weeks post-weaned) were individually housed (lights on 12 hour: lights off 12 hour cycle) after one week of acclimatization to room temperature (22-23°C) and humidity (50-60%) controlled facilities. Animals were initially divided equally into either sedentary (SED) or wheel running exercise (EX) groups (day -14) for a two week habituation phase, and then further subdivided into either CORT or placebo treated groups (i.e. 4 groups in total with 10 in each subgroup). Thus, the four experimental groups consisted of two sets of EX animals; one group received wax pellets (placebo-EX) and the other group received CORT pellets (CORT-EX) and two sets of SED animals; one group received wax pellets (placebo-SED) and the other group received CORT pellets (CORT-SED). A second cohort of animals (n=20) were utilized to measure incretin levels, muscle histology and western blot data. All animals were placed on an *ad libitum* HFD (see below) following the two-week habituation period and were killed between days 17-22 to accommodate for islet isolation experiments.

Experimental Design: A timeline of the experimental protocol is shown in Fig 3.1. Each EX animal was placed into specialized rodent cages with 24-hour access to a running wheel

(Harvard Apparatus), while SED animals were housed in standard cages. Each wheel was equipped with a magnet and sensor that was wired to an electronic counter. Wheel revolutions were counted each time the magnet passed the sensor and the numbers on the counters were recorded and reset to zero daily. The wheel revolutions were collected daily and were multiplied by the wheel circumference (106 cm) to estimate the running distances of each animal (previously reported in (Campbell, 2009). During the two-week cage habituation period, rodents were given standard rodent chow (Purina chow #5012) and water *ad libitum*.

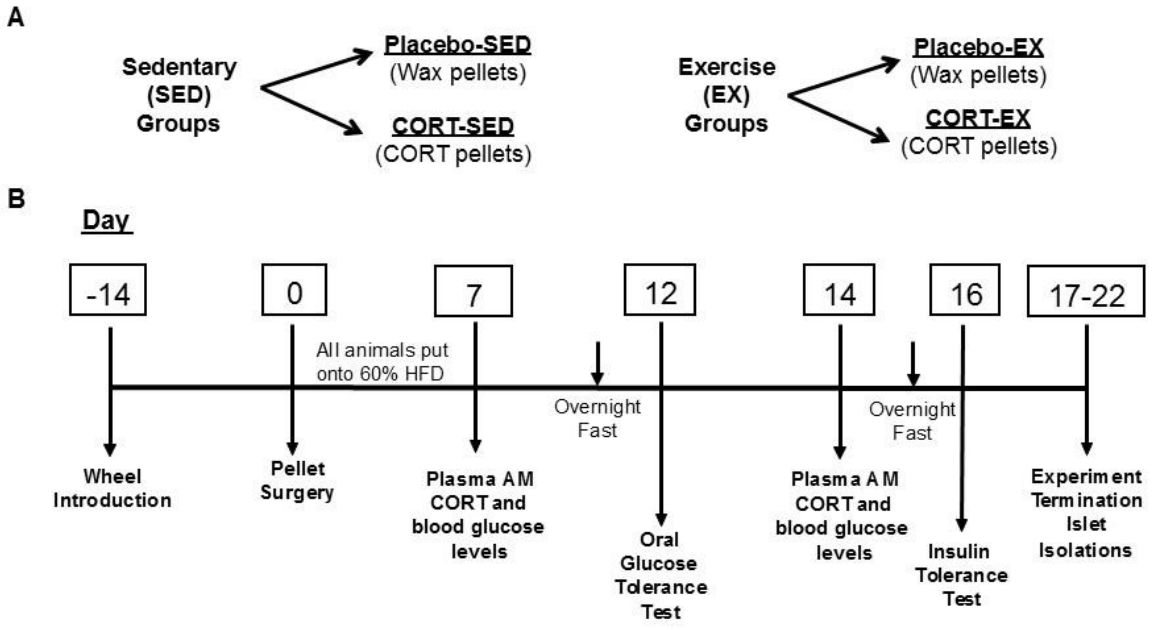

Figure 3.1. Schematic of experimental groups and design.

Experimental treatment groups (A) and schematic of experimental design (B). On day -14, all animals were individually housed and divided into one of four groups; placebo-EX, CORT-EX, placebo-SED and CORT-SED (EX, exercise and SED, sedentary). At this time, all EX animals were introduced to running wheels in their cages and allowed 2 weeks to acclimate to the experimental conditions. On day 0, either wax or CORT pellets were subcutaneously implanted into the animals according to the assigned experimental group and all animals were given a HFD (60%) *ad libitum*. Seven days post pellet implantations (~ 0800 hour), blood samples were taken to measure plasma CORT and blood glucose levels. On day 11 animals were fasted overnight (~ 16 hour) and the following day (12) were administered an oral glucose tolerance test. On day 14 (~ 0800 hour), blood samples were taken again to measure plasma CORT and blood glucose levels. On the evening of day 15, animals were fasted and on day 16 animals were administered an insulin tolerance test. All animals were euthanized between days 17-22 of the experimental protocol.

Pellet Surgeries: On day 0, each rat received a subcutaneous implantation of either corticosterone (CORT) pellets (4 x 100 mg; Sigma-Aldrich, Canada, Cat# C2505) or wax pellets (placebo), as previously described (Shpilberg, 2012). Immediately following surgery, rats were placed into standard sterile cages with chow and water for 24 hour to recover and then placed back in their home cages and were given *ad libitum* access to a high-fat diet (HFD) and water as previously described (Shpilberg, 2012).This diet consisted of 60% of the total calories from fat and 5.1 kcal per gram (D12492, Research diets); this diet was maintained until the end of the experimental protocol.

CORT sampling: As in previously published protocols (Beaudry, 2013; D'souza, 2011; Shpilberg, 2012). On day 7, at ~0800 hour, blood samples were collected from each animal via saphenous vein for CORT concentration measurements. A small area of hair on the lateral upper hind limb was shaven with an electric razor and wiped with a thin layer of petroleum jelly. Once the saphenous vein was located, a sterile 25-gauge needle was used to puncture the vein and whole blood was collected in lithium-heparin coated microvette capillary tubes (Sarstedt, Canada, Cat# 16.443.100) and centrifuged at ~ 9, 500 x g (Eppendorf Mini-Spin Plus, Brinkman Instruments Inc., USA) for 5 minutes, transferred into polyethylene tubes and stored at -80°C until further analysis of CORT concentrations using a radioimmunoassay kit (MP Biomedical, USA). Fed blood glucose concentrations (mM) were measured on day 7 using a single drop of blood (2 µl) with a handheld glucometer (Bayer Contour, USA), after blood was collected for CORT measurements. This sampling was repeated on day 14 so that CORT concentrations could be assessed two weeks after pellets were implanted.

Oral glucose and intraperitoneal insulin tolerance test: Animals were fasted overnight (16 hour) on day 12 and were administered an oral glucose tolerance test (OGTT, 1.5 g/kg body

mass) as previously described in (Shpilberg, 2012). Each animal's fasting blood glucose (time 0) was measured via saphenous vein bleed (~ 50 μl) using a sterile needle (25G) and glucometer (Bayer One Touch) and plasma was also collected for later analysis of insulin concentrations via an ELISA 96-well kit (Crystal Chem, USA, Cat# 90060). Blood glucose was also measured and recorded for time 5, 15, 30, 60, 90 and 120 minutes post oral gavage. Blood was collected to measure plasma insulin for time 15, 30, 60 and 120 minutes post oral gavage. In separate experiments, total GIP and GLP1 levels were measured at time 0 and 10 min post glucose gavage and analyzed using ELISA kits (Millipore, USA, Cat #EZRMGIP-55K) and (Meso Scale Discovery, MD, USA Cat #K150JVC-1, version 2), respectively. An insulin tolerance test (ITT) was performed on day 16 after an overnight fast by intraperitoneal insulin injection (0.75 units/kg body mass) as previously reported (Shpilberg, 2012). For these tests, ~ 50 μl of blood was collected to measure glucose concentrations were measured with a glucometer at time 0, 5, 10, 20 and 30 minutes post insulin injection. Non-esterified fatty acid (NEFAs) concentrations were measured from overnight fasted plasma collections, collected before OGTT (NEFA kit, HR Series NEFA-HR, Wako Chemicals, USA). Glucose and insulin area under the curve (AUC) was measured relative to the lowest fasting glucose and insulin levels of a placebo-SED animal. The acute insulin response (AIR) was determined between the difference in basal plasma insulin (fasting insulin levels) and 15 minutes following the oral glucose gavage (previously reported in (Beaudry, 2013; Holness 2005)). This measurement represents the secretion of insulin in response to an exogenous glucose load (Rafacho 2011) Homeostatic Model Assessment for β-cells (HOMA-β) as previously reported in (Beaudry, 2013; Shpilberg, 2012) was calculated based on the following equation: 20 x Insulin (μ units·L)/Glucose (mM)-3.5 (Wallace 2004).

Glucose stimulated insulin secretion (GSIS) experiments: All animals were killed by decapitation between days 17 to 22 of the experimental protocol, in a counterbalanced fashion, and islet isolations were carried out as previously reported (Shpilberg, 2012). Collagenase pancreas digestion was followed by Histopaque-1077 (H8889, Sigma-Aldrich, Canada) pellet suspension followed by re-suspension in KREBs buffer (125 mM NaCl, 4.7 mM KCl, 1.2 mM, 5 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 2.4 mM $MgSO_4$, 10 mM Hepes, 0.5% BSA, pH-7.4). Islets were hand selected and cultured in filtered RPMI buffer (Wisent) overnight (24 h) at 37°C, 5% CO_2. Islets were separated into a 12-well culture plate (6-10 islets/well in 3 batches) and given a 30-minute pre-incubation period as previously described (Shpilberg, 2012). Islets were given fresh KREB's buffer with 2.8 mM glucose + 0.1% BSA for 1 hour at 37°C, 5% CO_2. Media was changed to KREBs buffer with 16.7 mM glucose + 0.1% BSA for 1 hour at 37°C, 5% CO_2. Immediately following each incubation period, media was collected, centrifuged and stored at -20°C for further analysis. At the termination of experiments, islets were placed in 1 ml cold lysis buffer (acid-ethanol solution), sonicated (15 s), and centrifuged at ~ 13,500 x g at 4°C for 10 minutes. Supernatant was collected and stored at -20°C until further analysis of insulin content. All insulin secretion data was normalized to total insulin content. Insulin was measured using radioimmunoassay kit (Millipore, USA).

Histology: Liver and skeletal muscle tissue (tibialis anterior, TA) from euthanized animals were embedded in tissue freezing medium, frozen in liquid nitrogen, cryosectioned (10 µm thick) and stained with Oil Red O (ORO) for neutral lipid content as previously described (Koopman 2001). Staining for fibre type, SDH activity and capillary to fibre ratio (Ogilvie, 1990) was also completed.

ORO staining: Liver and TA sections were fixed with 3.7% formaldehyde for 1 hour at room temperature while an ORO solution composed of 0.5 g ORO powder (Sigma-Aldrich, Canada) and 100 ml of 60% triethyl phosphate (Sigma-Aldrich, Canada) was mixed and filtered. Following fixation in 3.7% formaldehyde, slides were immersed in filtered ORO solution for 30 minutes at room temperature. Slides immediately underwent five washes with ddH_2O, were allowed to dry for 10 minutes and were sealed with Permount (Sigma-Aldrich, Canada). Liver and TA images were acquired at 20 x and 10 x magnifications respectively, using a Nikon Eclipse 90i microscope (Nikon, Canada) and Q-imaging MicroPublisher 3.3 RTV camera with Q-capture Software.

Fibre Type and SDH Analysis: To identify skeletal muscle fibre type a metachromatic myosin ATPase stain was performed on cross-sections of the TA muscle using a modified protocol (Ogilvie, 1990). Sections were pre-incubated in an acidic buffer (pH = 4.25) to differentially inhibit myosin ATPases within the different fibre types. In this protocol, type I fibres appear dark blue, type IIa appear very light blue and type IIb and IIx are not apparent from each other and are thus classified as IIb/x. These fibres appear bluish-purple. A representative image of the TA for each group was acquired for analysis. Succinate dehydrogenase (SDH) activity was then assessed using histochemical analysis and expressed in relative optical density to placebo-SED (Shortreed 2009). The same muscle regions of the TA that were used for fibre typing were used for SDH activity determination. Serial sections were used to directly compare levels of SDH in each fibre, to each fibre type. SDH activity was assessed with Adobe Photoshop CS6, converted to greyscale and reported as the average optical density (sixty fibres were counted per muscle section). The greyscale is evaluated on a range of completely black (set as

zero units) to white (set at 255 units). All images were acquired with a Nikon Eclipse 90i microscope and Q-Imaging MicroPublisher with Q-Capture software at 10x magnification.

Capillary-to Fibre Analysis: To determine capillary-to-fibre ratio, TA sections (10 μm thick) were fixed with 3.7% para-formaldehyde prior to being stained with fluorescein isothiocyanate-conjugated Griffonia simplicifolia isolectin B4 (1:100; Vector Laboratories, Canada). Sections were viewed using a Zeiss M200 inverted microscope with a 20x objective and images were captured using a cooled CCD camera using Metamorph imaging software. Capillary-to-fibre counts were averaged from 5-7 independent fields of view per animal by a blinded observer.

Western Blot Analysis: We quantified protein expression for key determinants of skeletal muscle insulin signalling including total IRS-1, total AKT, pAKT (T308), GLUT4, FOXO1 and PGC1α to assess the atrophic and mitochondrial biogenesis pathways. Western blot analysis was carried out according to previously published work (Shpilberg, 2012). In brief, 50 μg of protein lysate from the TA was run on a 10% (total AKT, pAKT (T308), GLUT4, FOXO1) or 6% (total IRS-1 and PGC1α) SDS-page gel and proteins were transferred to a PVDF membrane (Bio-Rad, Canada). Membranes were blocked in 5% powdered milk and Tris-buffered saline with Tween 20 at room temperature for 1 hour. Membranes were then incubated overnight at 4°C with their respective primary antibodies (total AKT, 1:1000, ab47610, Abcam, Toronto, ON; T308, 1:1000, ab5626, Abcam, Toronto, ON; GLUT4, 1:1000, ab65267Abcam, Toronto, ON; FOXO1, 1:1000, Cat #2880, Cell Signaling, USA; PGC1α, 1:1000, Cat #4259, Cell Signaling, USA; total IRS-1, 1:1000, Cat #2382, Cell Signaling, USA). The following morning, the membranes were washed with TBST and incubated with anti-mouse (1:10000, Cat#ab6789, Abcam) or anti-rabbit (1:10000, Cat#ab6721, Abcam) secondary antibodies for 1 hour at room temperature.

Membranes were then washed and imaged. Images were detected on a Kodak In vivo FX Pro imager and molecular imaging software (Carestream Image MI SE, version S.0.2.3.0, Rochester, New York) was used to quantify protein content. GAPDH (1:10000, ab9484, Abcam, Toronto, ON) was used as a loading control for all proteins.

Statistical Analysis: All data are represented as means ± SE, with a criterion of $p<0.05$ and $p<0.001$ and were assessed as stated using two-way ANOVAs as a means of statistical significance. Individual differences were calculated using Bonferroni's post-hoc test unless stated as using a Duncan's post-hoc test (Statistica 6.0 software).

Results

Body mass. Relative body mass was reported between 0-10 days as metabolic profiling (OGTT, ITT) of these animals in a fasted state was performed on days 11-17. All animals had similar body mass prior to pellet surgery (day 0, ~ 350 g). Both placebo-treated groups gained mass after pellet surgery from day 0 to 10 (Fig. 3.2A). However, on experiment termination day (days 17-22) placebo-SED animals tended to weigh more than placebo-EX animals (472.7±14.4 vs. 438.7±31.5 g). Similar to previously reported findings (Beaudry, 2013; Beaudry, 2014; D'souza, 2011; Shpilberg, 2012), both CORT-treated groups lost body mass after pellet surgery compared to placebo animals ($p<0.01$, Fig. 2A). No differences were found in relative body mass between CORT-EX and -SED animals on day 10; however, CORT-EX animals had heavier absolute body mass than CORT-SED animals by the termination of treatment (333.5±16.2 vs. 265.9±13.0 g, $p<0.05$).

Running Distances. On average, CORT-treated runners ran longer distances than placebo runners (6541.7±467 vs. 3308.4±260 m, $p<0.05$, Fig. 3.2B). However, this difference was also noted prior to pellet implant surgery; thereby suggesting that these two groups of rats differed

slightly in their preference for wheel running prior to the experimental treatment (CORT vs. wax implantation). When running distances were expressed relative to day -2 (i.e. before surgery), CORT treatment, per se, did not appear to influence running distances significantly (Fig. 3.2C).

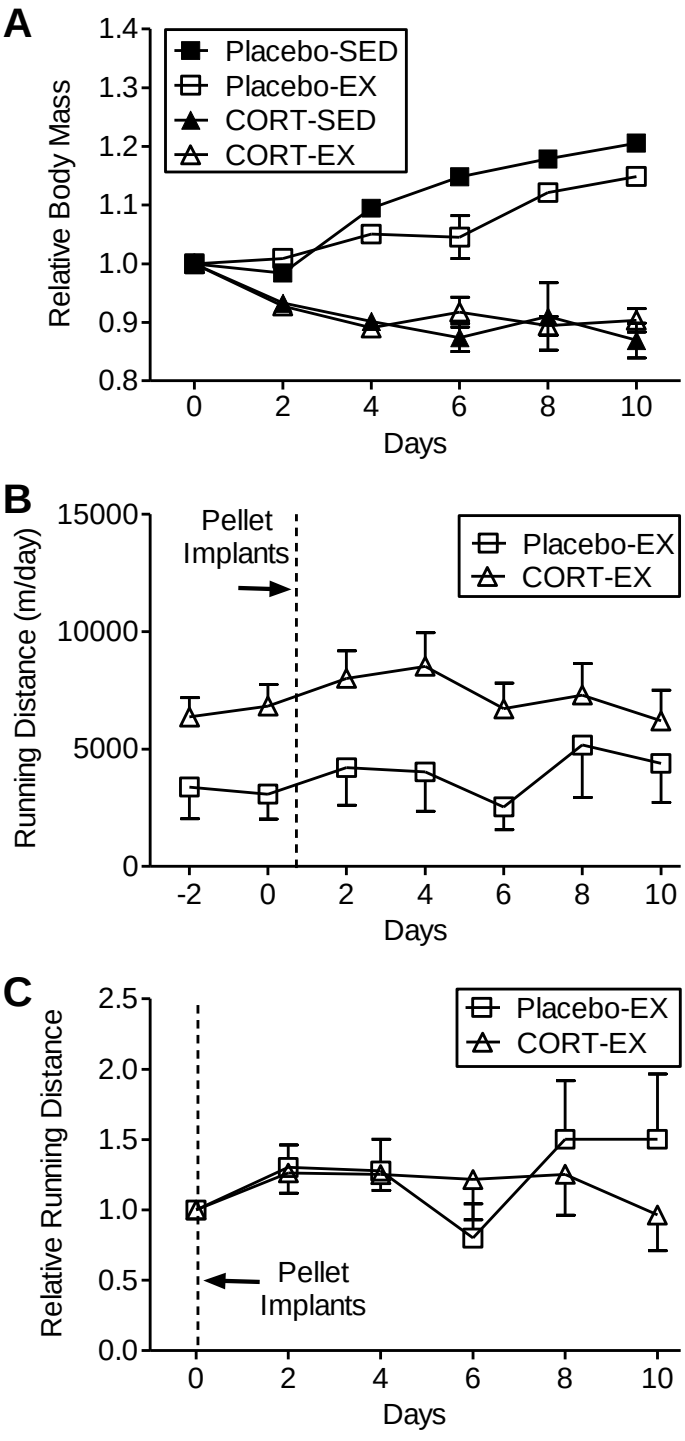
A
Placebo-SED
Placebo-EX
CORT-SED
CORT-EX
Relative Body Mass
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0
2
4
6
8
10
Days
B
Pellet
Implants
Placebo-EX
CORT-EX
Running Distance (m/day)
15000
10000
5000
0
-2
0
2
4
6
8
10
Days
C
Placebo-EX
CORT-EX
Relative Running Distance
2.5
2.0
1.5
1.0
0.5
0.0
Pellet
Implants
0
2
4
6
8
10
Days

Figure 3.2. Corticosterone-treatment caused reductions in body mass with no effect on running distances.

Body mass relative to pre-surgery mass during pellet treatment (A), absolute daily running distances during pellet treatment (B) and daily running distances relative to pre-surgery running distances (day 2) (C). CORT-treated animals experienced ~ 10-15% reduction in relative body mass throughout the pellet treatment period while placebo-treated animals continuously gained mass from day 2 onward ($p<0.05$). CORT-EX animals ran further distances per day than placebo-EX animals ($p<0.05$), however, CORT-EX animals ran more prior to surgery. Pellet implants had no impact on relative running distances. n=7-12. All values are means ± SE.

Plasma CORT, fasting NEFAs, glucose and insulin concentrations. As expected, CORT levels measured at ~ 0800 hour on day 7 were higher in all CORT treated groups as compared to placebo treated animals ($p<0.05$, Table 3.1) and similar to our previously published results (64). Placebo-SED animals had the lowest CORT levels compared to all other animal groups ($p<0.05$). Levels measured at ~ 0800 h on day 14 were also higher in CORT-treated groups compared to placebo-SED and not significantly impacted by exercise in the CORT treated groups. On day 14, CORT levels in placebo-EX animals were higher than in placebo-SED animals, and similar to what was observed in the CORT-SED group, but still lower than the CORT-EX animals ($p<0.05$, Table 3.1). Fasted NEFA levels were higher in CORT-treated animals compared to placebo animals ($p<0.05$, Table 3.1), while CORT-EX animals tended to have lower fasting NEFAs than CORT-SED animals. Placebo-SED animals tended to have higher fasting NEFAs than placebo-EX animals, although this difference failed to reach statistical significance.

Fasting blood glucose levels prior to oral glucose challenge were ~ 4-fold higher in CORT-SED animals than in placebo-SED ($p<0.05$, Table 3.1). CORT-EX animals had lower fasting blood glucose levels compared to CORT-SED animals, although concentrations were still significantly higher when compared to placebo-treated groups ($p<0.05$, Table 3.1). CORT-SED animals had ~ 5.4-fold increase in fasting plasma insulin levels compared to placebo-SED and CORT-EX animals had ~ 8-fold increase in fasting plasma insulin levels compared to placebo-EX animals ($p<0.05$, Table 3.1). CORT-EX animals also tended to have higher fasting plasma insulin levels than CORT-SED animals, although these values did not achieve statistical significance.

Table 3.1. Corticosterone, fasted non-esterified free fatty acidss, glucose and insulin concentrations.

	Placebo		CORT	
	SED	**EX**	**SED**	**EX**
Day 7 CORT	66.0±17.1	271.3±42.9*	566.2±65.6*,#	548.2±45.0*,#
Day 14 CORT	70.6±12.3	375.0±44.7*	468.6±38.3*	504.9±38.7*,#
Fasted NEFAs	0.45±0.1	0.39±0.9	1.0±0.1*	0.85±0.1*
Fasted Glucose	4.9±0.3	5.1±0.2	18.8±0.9*	14.3±1.6*,**,#
Fasted Insulin	1.24±0.3	1.1±0.5	6.8±0.6*	8.9±1.2*

Note: All values are means ± SE in ng/ml for CORT and insulin. Values are in mM for glucose and NEFAs. The * indicates the mean was statistically significant from placebo-SED, the ** indicates significance from CORT-SED and the # indicates significance from placebo-EX. All analysis was carried out by a two-way ANOVA with a Tukey's post-hoc, where $p<0.05$. n=7-12.

Oral glucose tolerance test. CORT-SED animals had markedly elevated blood glucose levels before and during the oral glucose tolerance challenge compared to all other treatment groups ($p<0.05$, Fig. 3.3A). CORT-EX animals had improved glucose tolerance compared to CORT-SED animals ($p<0.05$); however, they still were glucose intolerant when compared to the two placebo groups ($p<0.001$, Fig. 3.3A'). Interestingly, placebo-EX rats had similar fasting glucose and glucose tolerance as placebo-SED rats, suggesting that exercise failed to improve glucose homeostasis in these high-fat fed animals. Plasma insulin concentrations were highest in CORT-EX animals before and during the oral glucose tolerance challenge compared to all treatment groups ($p<0.001$, Fig. 3.3B). CORT-SED animals also had increased levels of plasma insulin at baseline but levels failed to respond to oral glucose challenge ($p<0.01$, Fig. 3.3B'). Acute insulin response (AIR) to oral glucose gavage was measured to determine the islet sensitivity to exogenous glucose challenge (Fig. 3.3C). CORT-SED animals had a negative AIR to glucose challenge, indicating impaired islet responsiveness ($p<0.05$), as has been recently demonstrated in this model (Shpilberg, 2012). Voluntary wheel running in both placebo- and CORT-treated groups increased AIR by ~ 2-fold above placebo-SED animals ($p<0.05$). To determine the influence of basal blood glucose levels on plasma insulin concentrations in each treatment group, these parameters were plotted against each other (Fig. 3.3D). The r^2 with all groups included was 0.47 and there was a significant correlation relationship ($p<0.0001$) suggesting that higher fasted blood glucose concentrations were driving higher insulin levels in the CORT-treated animals. It should be noted that fasting blood glucose and fasting plasma insulin values in the placebo animals did not deviate markedly, clustering at around 3.5-5.5 mM of glucose and less than 4 ng/ml of insulin, while the CORT treated animals had much higher (and wider) ranges in both fasting glucose and insulin levels (Fig. 3.3D). Total GIP and GLP1

plasma levels were measured at 0 and 10 min during a separate OGTT to determine differences in insulin secretion amongst treatment groups (Fig. 3.3E and F). Both incretins tended to be elevated in CORT-treated animals, compared to placebo animals. However, there were no differences found in GIP or GLP1 levels between CORT-EX and SED groups at 0 or at 10 min post gavage.

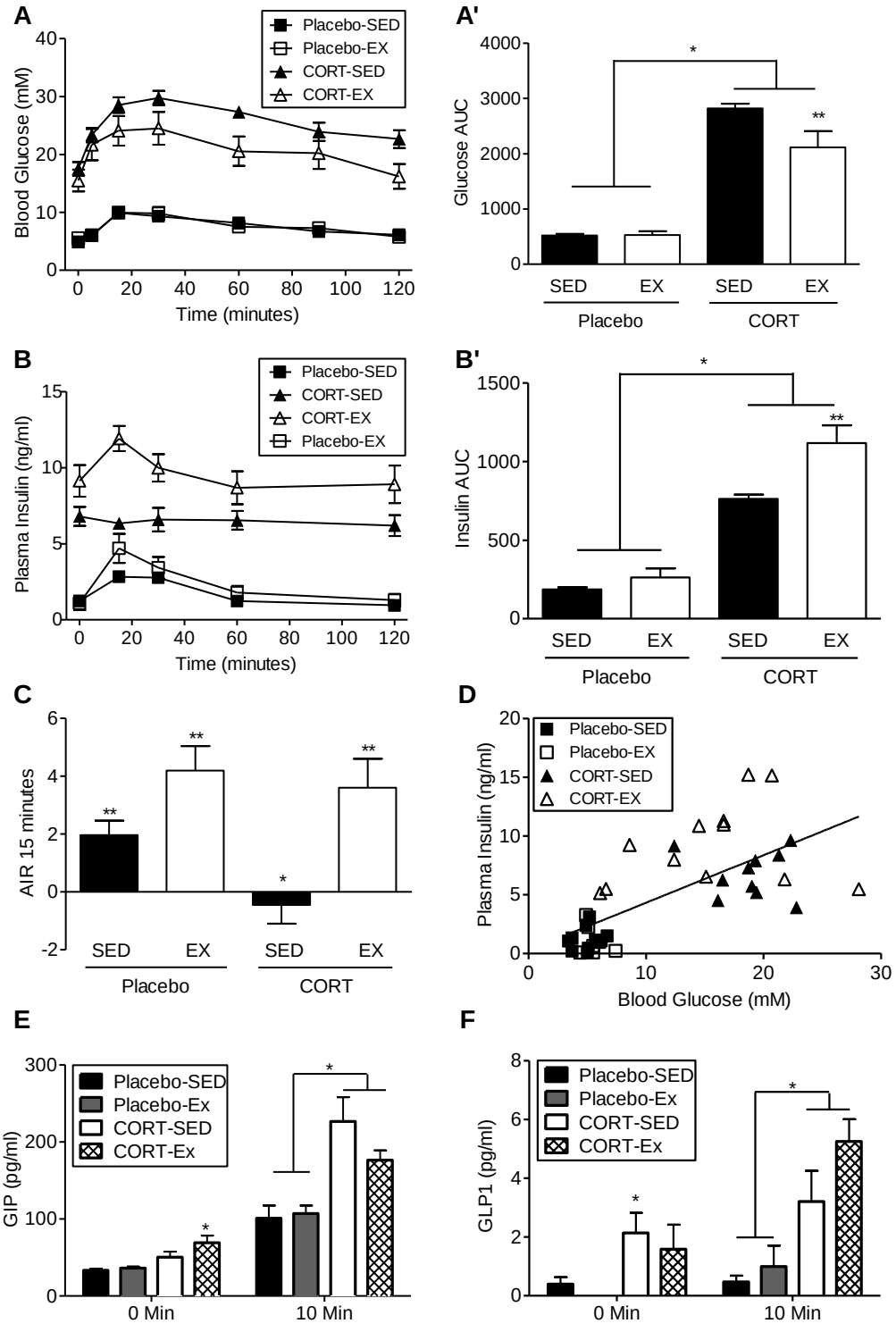
A
Placebo-SED
Placebo-EX
CORT-SED
CORT-EX
Blood Glucose (mM)
Time (minutes)
A'
Glucose AUC
SED
EX
SED
EX
Placebo
CORT
B
Placebo-SED
CORT-SED
CORT-EX
Placebo-EX
Plasma Insulin (ng/ml)
Time (minutes)
B'
Insulin AUC
SED
EX
SED
EX
Placebo
CORT
C
AIR 15 minutes
SED
EX
SED
EX
Placebo
CORT
D
Placebo-SED
Placebo-EX
CORT-SED
CORT-EX
Plasma Insulin (ng/ml)
Blood Glucose (mM)
E
Placebo-SED
Placebo-Ex
CORT-SED
CORT-Ex
GIP (pg/ml)
0 Min
10 Min
F
Placebo-SED
Placebo-Ex
CORT-SED
CORT-Ex
GLP1 (pg/ml)
0 Min
10 Min

Figure 3.3. Effect of voluntary wheel running on glucose tolerance, insulin secretion and plasma gastric inhibitory polypeptide and glucagon-like peptide-1 levels.

Glucose tolerance (A and A'), insulin secretion (B and B'), acute insulin responses (AIR, C and D), plasma GIP and GLP1 levels during 0 and 10 min of OGTT (E and F). Blood glucose levels were elevated with CORT-SED treatment ($p<0.001$) and voluntary wheel running decreased glucose intolerance in CORT-treated animals. Plasma insulin levels post oral glucose challenge were elevated with CORT treatment compared to placebo-treated animals and exacerbated with the addition of wheel running ($p<0.01$). n=7-12 for all above graphs. Acute insulin response (AIR) between basal and 15 minutes post exogenous glucose challenge was elevated with EX and reduced in SED-treated animals (n=6-12). Higher fasting plasma insulin concentrations were correlated with higher fasting blood glucose concentrations in both CORT-EX and SED groups ($r^2=0.47$, $p<0.0001$). n=6-12. CORT animals had higher fasting and stimulated GIP and GLP1 levels than placebo groups. n=4-5. The * indicates values are statistically significant from placebo groups and ** indicates significance from CORT-SED ($p<0.05$) using a two-way ANOVA with Bonferroni's (A', B', E and F) and Duncan's (C) post hoc tests. All values are means ± SE except in D, where values are individual.

In vivo assessment of β-cell function. HOMA-β was used to calculate basal β-cell function where higher HOMA-β scores indicate greater β-cell function (Fig. 3.4A). No significant differences in HOMA-β levels were found between CORT-SED and placebo-SED groups, although values tended to be higher in the CORT-SED group compared to the placebo-SED group ($p=0.1481$). Voluntary wheel running improved β-cell function in CORT animals by ~ 2.5-fold compared to CORT-SED groups ($p<0.05$, Fig. 3.4A). It also increased β-cell function in placebo-EX animals compared to placebo-SED animals by a similar extent ($p<0.05$).

Islets were isolated from each animal upon euthanization (day 17-22) and challenged with low (2.8 mM) and high (16.7 mM) glucose containing media. We found that in both low and high glucose media, CORT-SED islets had higher relative glucose-stimulated insulin secretion (GSIS) than all the other groups ($p<0.05$, Fig. 3.4B). CORT-EX tended to have lower relative GSIS levels in low glucose media when compared to the CORT-SED group and had significantly lower GSIS in the high glucose media compared to the CORT-SED group.

An insulin tolerance test was conducted *in vivo* to measure insulin sensitivity primarily in the skeletal muscle (Fig. 3.4C). CORT-SED animals had the highest glucose AUC levels compared to all treatment groups ($p<0.05$, Fig. 3.4C'). Voluntary wheel running reduced glucose AUC in CORT-EX animals indicating improved peripheral insulin sensitivity ($p<0.05$).

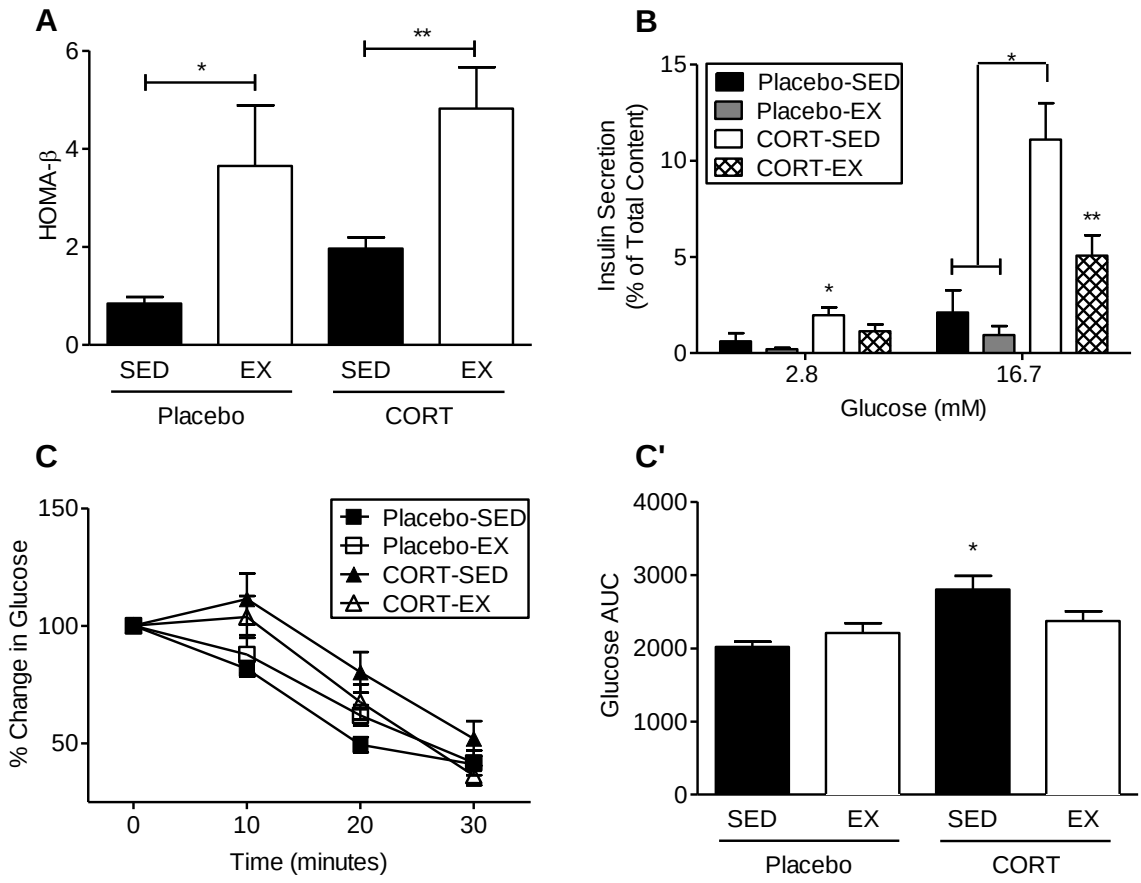

Figure 3.4. Effect of voluntary exercise on β-cell function.

β-cell function (A) as measured by HOMA-β, glucose stimulated insulin secretion (GSIS) (B) and insulin sensitivity (C and C'). HOMA-β, an indicator of β-cell function was increased with voluntary wheel running in placebo and CORT-treated animal groups. CORT-SED group had higher insulin secretion in low (2.8 mM) and high (16.7 mM) glucose media compared to placebo-treated groups. Voluntary exercise normalized GSIS in CORT-treated animals. n=3-7 per group. Insulin sensitivity measured by the response to an insulin bolus was improved with voluntary wheel running in CORT-treated animals ($p<0.05$). n=7-12. The * indicates statistical significance from placebo-SED and ** indicates statistical significance from CORT-SED ($p<0.05$) using two-way ANOVA with Duncan's (B) and Bonferroni's (C') post hoc tests. All values are means ± SE.

Food intake and body composition. As expected, relative food intake tended to be slightly higher in placebo-EX animals than placebo-SED animals, although it was not significant ($p>0.05$, Table 3.2). There was no significant difference in relative food intake between CORT-SED and -EX animals; however, there was a main effect of pellet treatment across groups with the CORT-treated animals having a higher relative food intake than placebo-treated animals ($p<0.05$). Visceral adiposity, as measured by relative epididymal fat pad mass, greatly increased in CORT-treated animals but was lower in CORT-EX compared to CORT-SED group ($p<0.05$, Table 3.2). There were no differences in visceral adiposity between placebo-SED and placebo-EX groups. Relative liver mass was increased with CORT treatment compared to placebo-treated animals ($p<0.05$) but no differences were found with the addition of exercise in placebo or CORT-treated animals (Table 3.2). Relative gastrocnemius mass was lower with CORT-SED treatment compared to placebo-treated animals ($p<0.05$) and tended to be higher with CORT-EX treatment. No differences were found with relative TA mass between any treatment groups. Relative soleus mass, a marker of oxidative muscle mass, was higher in CORT-SED animals compared to CORT-EX animals and placebo-SED animals ($p<0.05$, Table 3.2). In contrast, relative epitrochlearis mass, a marker of glycolytic muscle mass, was significantly lower in CORT-SED animals compared to placebo-SED animals. CORT-EX animals had higher relative epitrochlearis mass in comparison to CORT-SED animals, with values similar to those observed in the placebo-treated groups ($p<0.01$, Table 3.2).

Table 3.2. Food intake and body composition.

	Placebo		CORT	
	SED	**EX**	**SED**	**EX**
Food intake	0.25±0.02	0.30±0.02	0.45±0.01*	0.44±0.02*
Epididymal Fat	17.0±1.0	17.0±1.6**	26.9±1.1*	21.8±1.4*, **
Liver	37.4±1.6	37.7±1.6	69.7±2.4*	69.3±6.8*
Gastrocnemius	4.7±0.2	5.4±0.5	3.7±0.1*	4.2±0.2#
Tibialis Anterior	1.5±0.04	1.6±0.20	1.35±0.05	1.50±0.1
Soleus	0.40±0.01	0.45±0.1	0.52±0.03*	0.43±0.02
Epitrochlearis	0.14±0.01	0.17±0.02	0.11±0.01*	0.20±0.03*,**

Note: Values are means ± SE in kcal/g of body mass per day for food intake and g tissue/kg of body mass. The * indicates the mean was statistically significant from placebo-SED, the ** indicates significance from CORT-SED and the # indicates significance from placebo-EX. All analysis was carried out by a two-way ANOVA with a Tukey's post-hoc, where $p<0.05$. n=7-12.

Muscle and liver lipid staining. ORO staining of TA muscle sections was used to visually examine lipid accumulation in all treatment groups (Fig 3.5 A). Lipid staining appeared to be higher in placebo-EX animals compared to placebo-SED animals, as expected since endurance exercise generally increase muscle lipid storage in healthy rats (Goodpaster, 2001b). CORT-treated animals had increased lipid staining compared to placebo-treated animals, indicating more lipid accumulation in the muscle and no obvious differences were observed between CORT-SED and CORT-EX groups. CORT-treated animals increased liver ORO staining compared to placebo-treated animals, indicating more hepatic lipid accumulation (Fig. 3.5 B). There were no differences in ORO staining between CORT-SED and -EX groups as well between placebo-SED and -EX groups.

Muscle oxidative capacity, PGC1α protein, capillary to fibre ratio, cross-sectional area and FOXO1 protein levels. To determine the effects of exercise training, the TA muscle of representative animals from each treatment group was stained for SDH activity (Fig. 3.6 A and B). SDH is an oxidative complex bound to the inner mitochondrial membrane of skeletal muscle cells and is responsible for oxidizing succinate to fumarate in the citric acid cycle (Hederstedt 1981). Strong SDH staining correlates with higher muscle fibre oxidative potential. The TA from both placebo-EX and CORT-EX treatment groups showed darker SDH staining compared to placebo-EX and CORT-SED muscles ($p<0.001$, Fig. 6A and B). In type I and IIa muscle sections, CORT-SED animals had lighter SDH staining than all other treatment groups ($p<0.05$, Fig. 3.6 A and B). PGC1α is a known protein marker for mitochondrial biogenesis (Wing 2011) and was measured to help explain the observations of impairments in SDH staining in CORT-SED animals. Although data failed to reach statistical significance, PGC1α tended to be elevated in both EX groups compared to their respective SED groups (Fig. 3.6C).

CORT-treatment alone causes significant reduction in capillary to fibre ratio within skeletal muscle (Shikatani, 2012). Capillary to fibre ratio was reduced in CORT-SED animals but normalized in CORT-EX animals compared to placebo-treated animals (Fig. 3.6 D and E). Cross-sectional area, indicating individual muscle fibre size, was lowered in type IIa and IIb/x muscle fibres from the TA in CORT-SED animals (Fig. 3.6 F and G) and tended to increase with CORT-EX treatment. CORT treatment is known to induce atrophic signalling pathway leading to muscle wasting (Lecker 1999). Therefore, FOXO1 protein levels were measured as an indicator of muscle degradation. CORT treatment alone increased FOXO1 protein content (Fig. 3.6 H, $p<0.05$); however, EX failed to normalize FOXO1 levels.

Insulin signalling and glucose metabolic protein levels. Regular exercise is known to stimulate skeletal muscle insulin signalling (Goodyear 1998b) therefore protein markers of these pathways were analyzed. Voluntary wheel running increased total IRS-1 protein content in placebo animals by ~2-fold and tended to increase total IRS-1 in CORT-EX animals compared to CORT-SED animals (Fig. 3.7A). Total AKT protein content was increased in CORT-EX animals compared to placebo-SED animals ($p<0.05$, Fig. 3.7B). However, no differences were found in phosphorylated AKT (T308) or GLUT4 protein expression between any of the treatment groups (Fig. 3.7C and D).

Running distance vs. blood glucose, plasma insulin and CORT. To determine if fasting blood glucose, plasma insulin and CORT are associated with individual running distances in the exercising rats, each value from each exercising animal in both treatment groups was plotted and a linear regression was calculated (data not shown). No significant correlations were found between running distances and fasting blood glucose levels ($r^2=0.083$, $p>0.05$), fasting plasma insulin levels ($r^2=0.023$, $p>0.05$), nor with plasma basal CORT levels ($r^2=0.003$, $p>0.05$).

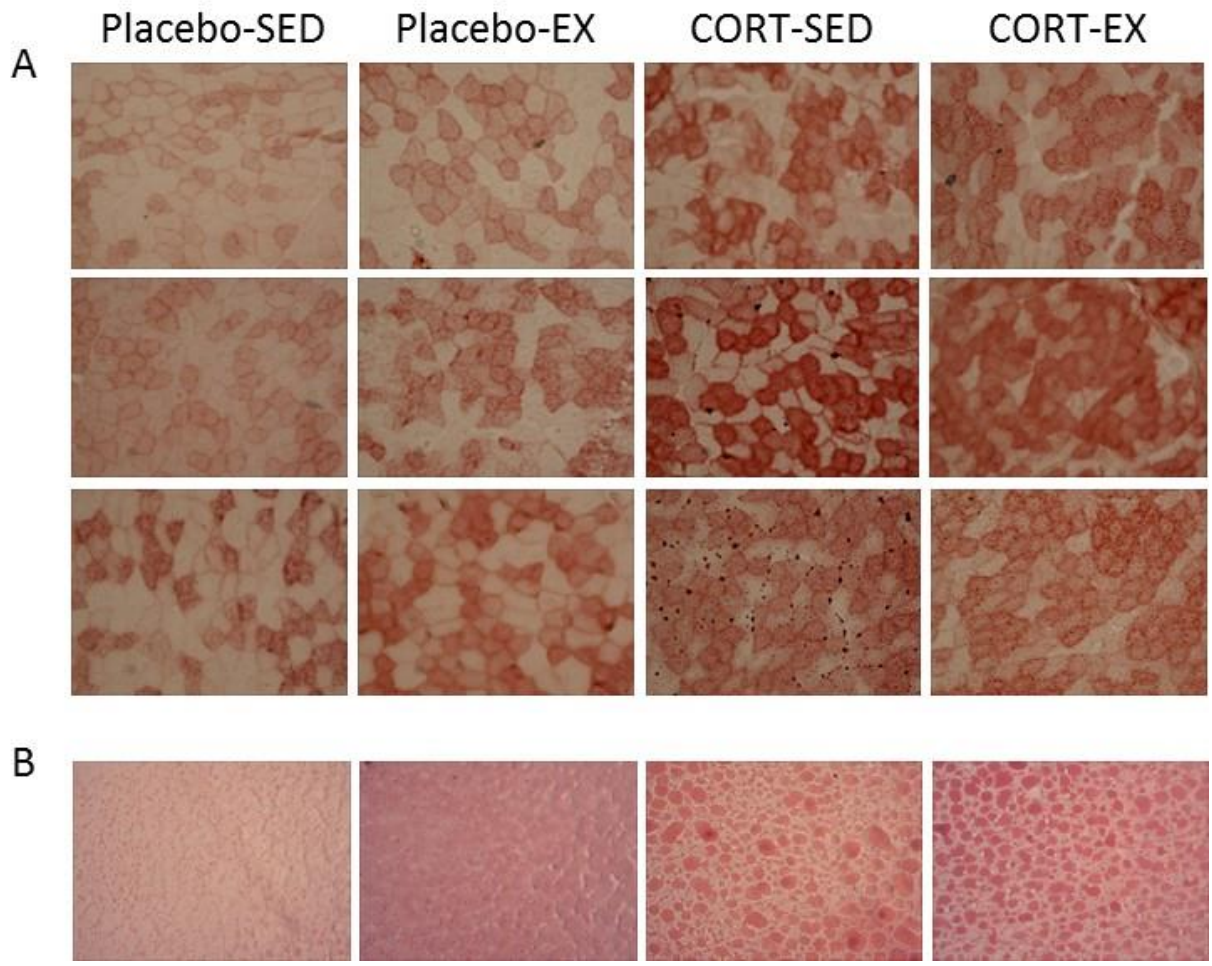

Figure 3.5. Voluntary exercise does not improve lipid deposition within either the skeletal muscle or liver.

Muscle (A) and liver (B) lipid accumulation was completed through ORO staining. CORT treatment increased lipid deposition in both muscle (tibialis anterior) and liver. There was no effect of exercise to lower lipid accumulation in either tibialis anterior or the liver in CORT-treated animals. Representative images are shown from an n=6.

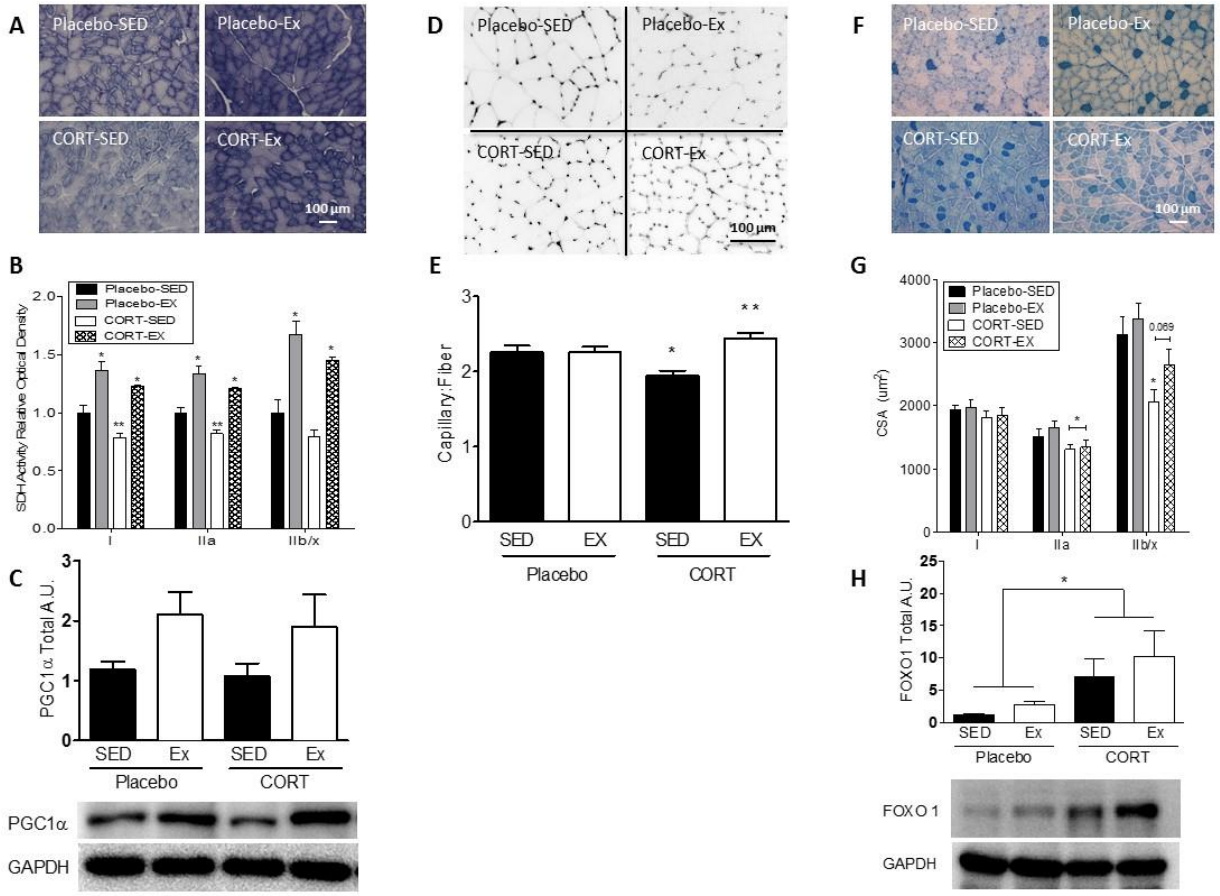
A
Placebo-SED
Placebo-Ex
CORT-SED
CORT-Ex
100 µm
B
Placebo-SED
Placebo-EX
CORT-SED
CORT-EX
SDH Activity Relative Optical Density
I
IIa
IIb/x
C
PGC1α Total A.U.
SED
Ex
SED
Ex
Placebo
CORT
PGC1α
GAPDH
D
Placebo-SED
Placebo-Ex
CORT-SED
CORT-Ex
100 µm
E
Capillary:Fiber
SED
EX
SED
EX
Placebo
CORT
F
Placebo-SED
Placebo-Ex
CORT-SED
CORT-Ex
100 µm
G
Placebo-SED
Placebo-EX
CORT-SED
CORT-EX
CSA (um²)
0.069
I
IIa
IIb/x
H
FOXO1 Total A.U.
SED
Ex
SED
Ex
Placebo
CORT
FOXO1
GAPDH

Figure 3.6. Effect of voluntary exercise on skeletal muscle oxidative capacity, capillary content and glucocorticoid-induced atrophy.

Muscle oxidative capacity as measured through succinate dehydrogenase (SDH) histology (A and B), PGC1-alpha protein content (C), capillary to fibre ratio (D and E), cross-sectional area (CSA) in I, IIa, and IIb/x muscle fibre types (F and G) and FOXO1 protein content (H). Muscle oxidative capacity was increased in all placebo- and CORT-EX muscle types. n=6. In type I and IIa muscle sections, CORT-SED had lower oxidative capacity. PGC1-alpha tended to be higher in EX treated animal groups compared to SED groups. n=5. CORT-SED treatment resulted in a significant reduction in capillary to fibre ratio compared to all treatment groups and CORT-EX animals exhibited greater capillary-to-fibre ratio compared to CORT-SED animals ($p<0.05$, n=6-9), demonstrating that CORT-induced capillary rarefaction is prevented by volitional exercise. This assay stained for capillaries via Griffonia Simplicifolia isolectin-fluorescein and image color was inverted to enhance muscle fiber visualization. CSA tended to normalize with exercise in CORT treated animals in IIb/x muscle fibres. FOXO1 protein content increased with CORT-SED and EX treatment. n=4-5. The * indicates statistical significance from placebo-SED and ** indicates statistical significance from CORT-SED ($p<0.05$) using two-way ANOVA with Bonferroni's post hoc test. All values are means ± SE.

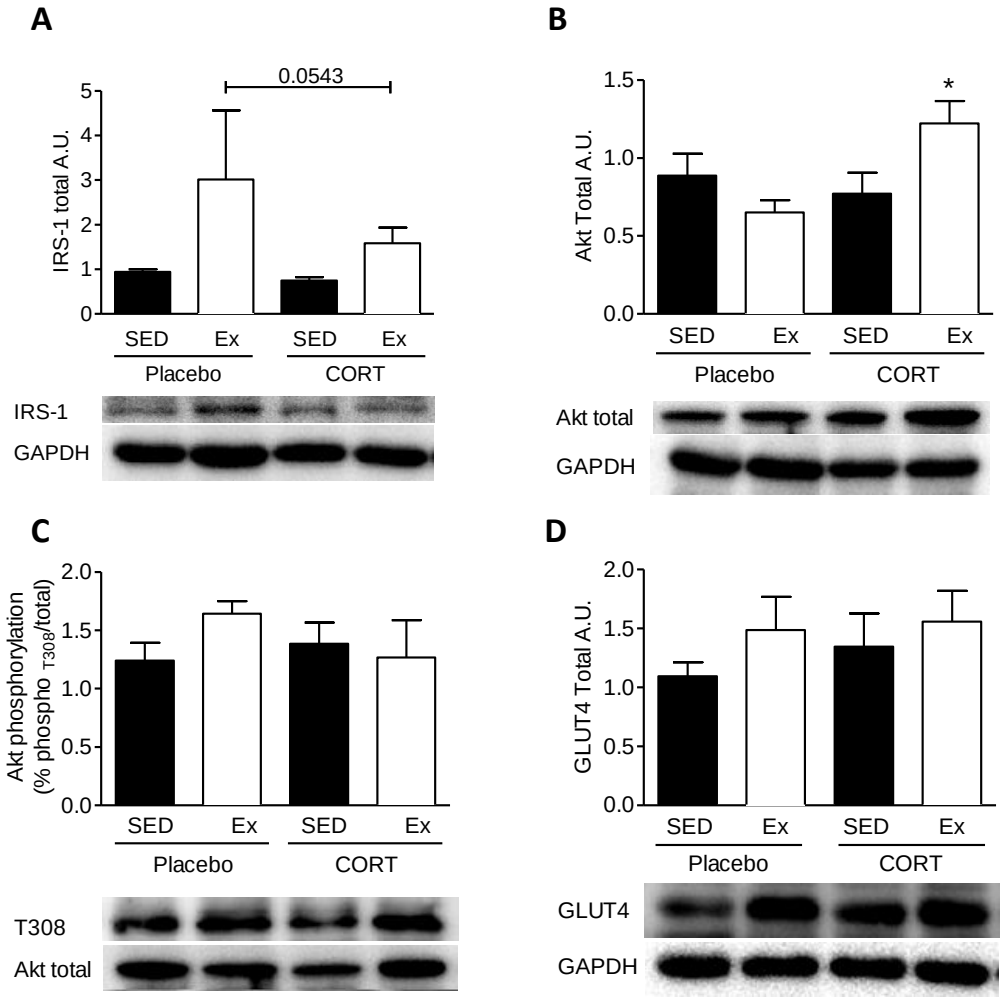

Figure 3.7. Effect of voluntary exercise on insulin signaling protein content.

IRS-1 (A), AKT Total (B), AKT phosphorylated T308 (C) and GLUT4 protein content (D) in tibialis anterior (TA) muscle. Exercise tended to increase IRS-1 protein content in placebo and CORT animals. CORT-EX animals had higher AKT Total protein content compared to CORT-SED animals but no differences in AKT phosphorylated T308 protein content were found amongst groups. No differences were found in GLUT4 protein content between the treatment groups. n=4-5. The * indicates statistical significance from CORT-SED ($p<0.05$) using two-way ANOVA with Bonferroni's post hoc test. All values are means ± SE.

Discussion

The effect of voluntary wheel running was assessed to determine if exercise has the ability to reduce, or offset, the detrimental metabolic outcomes typically observed in rodent models of exogenous GC treatment. In this study, we demonstrate that voluntary exercise (for ~ 2.5 weeks) reduced visceral adiposity, glucose intolerance, insulin insensitivity and impaired glucose responsiveness. Furthermore, many skeletal muscle specific abnormalities such as oxidative capacity, atrophy and capillary rarefaction associated with sustained GC treatment were improved with voluntary exercise. These novel findings suggest that regular exercise is able to promote healthier metabolic function, even in situations of hypercortisolemia, i.e. Cushing's syndrome or patients treated with exogenous GCs.

Generally, GCs decrease absolute body mass but increase relative fat mass, an outcome that appears to be dependent upon animal model/type, dosing and duration of treatment (Coderre 2007; Kaasik 2007; Karatsoreos 2010; Menezes 2007; Rafacho 2009; Yau 2012). Much of the loss in total mass is speculated to be due to reductions in skeletal muscle mass, although body length (or height) is also reduced if GCs are elevated in young growing animals (Shpilberg, 2012) or in human adolescence (Abad 2001). In this study, and in our previously published studies (Beaudry, 2013; Beaudry, 2014; Shikatani, 2012; Shpilberg, 2012), we found that CORT treatment induces a dramatic (~ 50%) reduction in total body mass when compared to placebo-treated animals and this effect was not mitigated by a high fat diet. Normally, voluntary wheel running in healthy young animals lowers body mass gain (Campbell, 2009; Fediuc, 2006; Yau, 2012) and reduces adiposity in obesity prone animals (Bi 2005; Patterson 2008). However, in our study, voluntary exercise did not cause a further reduction in body mass nor did it prevent the body mass loss that is typically observed in CORT-treated animals. Indeed, both CORT-SED

and -EX-treated groups had similar body masses throughout the 10 days of treatment (Fig. 3.2A). Although, it was obvious that the exercising animals had much less adipose mass and greater skeletal muscle mass (i.e. altered body composition) compared to the CORT-SED animals (Table 3.2). Some evidence indicates that the lower body mass observed in GC-induced models is the result of reduced food intake (Rafacho 2008). Others suggest that GCs increase the preference for foods with high-fat content, overriding the anorexigenic effects of insulin (Dallman 2007) and leading to larger fat accumulation. We found that CORT-treated animals, regardless of being SED or EX, consumed more calories relative to body mass than the placebo-treated animals (~ 1.5-fold change, Table 3.2). Although exercise improved body composition (decreased adiposity and increased skeletal muscle mass) in the CORT treated animals, possibly due to greater running distances than placebo animals (Fig. 3.2), the growth impairments and changes in eating behaviour accompanying CORT treatment were seemingly unaffected by exercise.

Exercise training and adaptations to restraint stress reduce hyperglucocorticoidemia and prevent diabetes development in ZDF rats (Bates 2008; Király, 2008) and in other insulin resistant rodent models (Linden 2014). In this study, and in our previously published work (Beaudry, 2013; Shpilberg, 2012), we show that elevated GCs induce severe glucose intolerance, hyperinsulinemia, impaired β-cell function and severe insulin resistance. Here, we show that daily voluntary wheel running, for ~2.5 weeks, improves glucose intolerance and insulin responsiveness to oral glucose challenge in GC-treated high-fat fed rats (Fig. 3.3). However, increased fasting and stimulated insulin levels were observed. In comparison to other published work on dexamethasone treatment and treadmill exercise (Barel, 2010), we found that CORT-EX animals had elevated fasted and stimulated insulin levels during the glucose challenge compared to all other treatment groups (Fig. 3.3B).

In an attempt to explain the higher levels of circulating insulin in CORT-treated animals, we measured plasma incretin hormone (GIP and GLP1) levels, which are known to stimulate insulin secretion upon glucose ingestion (Campbell 2013) and are also known to be elevated with CORT treatment (Jensen 2012). In our study, CORT treatment increased GIP and GLP1 levels in both the basal and glucose stimulated states but there were no definitive distinctions found between SED and EX treatments. As such, we conclude that the improvements in insulin responsiveness to oral glucose feeding in the CORT-exercising animals cannot be attributed to differences in incretin secretion. However, similar to our observation of increased GIP and GLP-1 with CORT treatment in general, others have shown increased fasting insulin levels after 11 weeks of swim training in ZDF rats compared to SED and lean controls, results which were paralleled by increases in β-cell mass, through increased β-cell proliferation (hypertrophy and hyperplasia) mechanisms (Király, 2008). Although β-cell mass was not measured directly in this study, we do demonstrate greater AIR and HOMA-β levels with CORT exercise treatment (Fig.4.), suggesting that our exercise model was sufficient to improve β-cell function possibly via proliferation or β-cell specific pathways.

We have recently shown that CORT-SED treatment impairs β-cell responsiveness to glucose *in vivo* but not *ex vivo* (Beaudry, 2013). Regardless of exercise protocol, endurance training (swimming, voluntary wheel running, treadmill running), results in reduced insulin release in isolated healthy rodent islets (Almeida 2012; Galbo 1981; Tsuchiya 2013; Zawalich 1982; Zoppi 2011) and more specifically in the β-cell itself (Farrell 1992). Our findings are in line with these previous observations, since placebo-EX animals tended to have lower GSIS compared to placebo-SED islets (Fig. 3.4). In addition, voluntary wheel running (6 weeks) improves GSIS in ZDF rat islets as well as prevents complete depletion of insulin stores within

the islet (Delghingaro-Augusto, 2012). Our study is the first to show that voluntary exercise lowers relative GSIS in CORT-treated animals; however, it does not normalize to that of placebo-treated SED animals. Our results differ somewhat from those found in active ZDF rats, as GSIS was elevated in high glucose and palmitate media compared to inactive ZDF rat islets (Delghingaro-Augusto, 2012). This may be because ZDF rats have larger compensatory β-cell mass in response to exercise causing higher insulin levels.

GCs help to regulate adipose tissue by promoting mechanisms of differentiation and ectopic fat distribution (Peckett, 2011). In this model of elevated GCs and HFD, there is an excessive accumulation of visceral fat that is ~ 1.5-fold higher in CORT-SED animals when compared to placebo treatment (Table 3.2), which parallels previous work (Björntorp, 2001; Chandola 2006; Rebuffe-Scrive 1992; Shpilberg, 2012). Exercise is known to help reduce fat mass gain (Danielsen 2013; Gollisch 2009; Stiegler 2006) even in instances of high-fat feeding (Gauthier 2004; Gollisch, 2009). In the present study, we demonstrate that voluntary wheel running is sufficient to attenuate visceral fat accumulation in CORT/HFD-treated animals, but not muscle nor hepatic neutral lipid accumulation (Fig. 3.5A and B, respectively). Perhaps this may explain why we demonstrate some relief from GC-induced abnormalities with exercise but not complete reversal of the various features of insulin resistance and glucose intolerance.

GCs are well known to induce muscle atrophy (Menezes, 2007), likely through negative protein balance due to increased protein degradation and decreased protein synthesis (Hasselgren, 1999). Both GCs and diabetes advance muscle atrophy by targeting fast-twitch (type II), glycolytic muscle (Schakman, 2013) and reducing muscle mitochondrial content (Lewis 1992). In this model of CORT/HFD, we found lower skeletal muscle oxidative capacity, as measured by SDH staining, in type I and type II fibres of the CORT-SED animals (Fig. 3.6 A

and B). This impaired oxidative capacity, along with the obvious muscle atrophy and capillary rarefaction, likely contributes to the poor glycemic control and skeletal muscle lipid accumulation in these animals. Similar to previous findings (Nagatomo, 2012), we found that regular exercise improved muscle oxidative capacity in all fibre types of the TA muscle. We suspected that these exercise-mediated improvements could be caused by up-regulation of PGC1α, a transcription factor that turns on oxidative metabolic proteins that contribute to greater mitochondrial biosynthesis (Puigserver 2003) and slow-twitch (i.e. type I) muscle fibre formation (Lin 2002). We found that EX treatment tends to increase PGC1α protein levels in the TA muscle indicating that greater levels of oxidative capacity may be due to elevated mitochondrial biosynthesis and possibly more slow-twitch fibre recruitment, helping to utilize higher levels of plasma glucose. In addition, it is a continued observation in our model that relative soleus muscle mass increases with CORT and HFD treatment (Table 3.2). The soleus muscle is composed of slow-twitch fibres that are less sensitive than fast-twitch fibres under conditions of atrophy (reviewed in (Schiaffino 2011) and increased compensation by the soleus muscle may result. Interestingly, CORT-EX treatment normalized soleus muscle mass, indicating decreased compensation, possibly elicited by the activation of oxidative muscle fibre types with voluntary exercise.

One of the most novel aspects of this study was the finding that CORT-EX treatment was able to induce angiogenesis by increasing the number of capillaries in a given area of muscle thereby evading CORT-induced capillary rarefaction, which is a hallmark of GC treatment (Shikatani, 2012). The increased capillarization suggest that exercise improves nutrient and insulin delivery to skeletal muscle in GC treated animals, and that this adaptation may contribute to the augmentation in glucose tolerance and muscle insulin sensitivity detected in these animals.

In line with this, we also observed a tendency for improved cross sectional area in the type IIb/x fibres of the TA muscle with regular exercise and CORT treatment. While it is still not known why GCs target type IIb/x fibres, higher GC receptor expression in these fibres may be a likely mechanism (Shimizu 2011). Indeed, skeletal muscle specific GC receptor knockout animals are resistant to GC-induced muscle atrophy (Watson 2012). However, these knockout studies are not entirely conclusive, as they do not allow determination of exact mechanisms of GC-induced atrophy. Therefore, it remains that either genomic (atrophic genes, i.e. FOXO1) or non-genomic (inhibition of insulin signalling) pathways are likely contributors to muscle wasting with GC treatment. In this study, we confirm increases in FOXO1 protein levels in CORT-treated animals (Fig. 6H); however, unlike previous literature (Yuan 2011) regular exercise was unable to normalize FOXO1 levels. It is possible that although these FOXO1 levels were not normalized in the CORT-EX animals, voluntary wheel running was sufficient to stimulate increase protein synthesis mechanisms (i.e. IGF-1 or mTOR signalling) independent of elevated FOXO1 levels (reviewed in (Atherton 2012). Although IGF-1 was not measured in this study, we have previously measured and found lower plasma IGF-1 levels in CORT-SED animals (unpublished observations) indicating less activation of the growth pathways. We suggest that regular exercise in CORT treated animals may promote higher levels of IGF-1 overriding protein degradation pathways, such as FOXO1. Moreover, we recognize that we used whole muscle homogenates to measure FOXO1 protein levels and these methods may not have allowed us to specifically capture true nuclear FOXO1 translocation. The up-regulation of non-genomic signalling pathways, such as IRS-1 and AKT, may help to reverse GC-induced muscle atrophy mechanisms and improve glucose intolerance (Schakman, 2013). We demonstrate increased protein levels of total AKT, as well as the tendency to increase total IRS-1 protein levels, in CORT-EX animals as

compared to CORT-SED animals. GCs are known to inhibit P13K/AKT signalling, which down-regulate protein synthesis pathways (Schakman 2008b) and increase muscle catabolism, in addition to propagating insulin resistance through impaired GLUT4 translocation (Goodyear, 1998b). Together, we conclude that voluntary exercise is an adequate stimulus to improve skeletal muscle oxidative capacity, capillary to fibre ratio and shows tendencies to reverse muscle atrophy in type IIb/x fibres in situations of elevated GCs and HFD. These improvements in skeletal muscle composition are most likely mediated via the atrophic mechanisms beyond FOXO1 protein expression, as insulin-signalling proteins (i.e. GLUT4 and IRS-1) remained relatively unaffected.

In summary, we have shown that elevated CORT exposure, in conjunction with a HFD, induces severe hyperglycemia, hyperinsulinemia and muscle wasting along with impaired insulin response and islet glucose sensitivity; features that can all be attenuated with volitional activity. Our study shows that voluntary exercise attenuates symptoms of T2DM induced by elevated CORT and HFD and provides evidence that exercise can independently influence tissues even when the exercise-associated reductions in basal GC levels are prevented. Exercise intervention may be an ideal form of rehabilitation in individuals with hypercortisolemia or Cushing's syndrome as these individuals suffer from hyperglycemia, insulin insensitivity, muscular atrophy and increased central obesity. Therefore, these individuals may benefit from the positive effects of regular exercise that may help to control or attenuate symptoms of diabetes development.

Chapter 4

The metabolic effects of prazosin on insulin resistance in glucocorticoid-treated rats

Author Contributions: I designed the study and wrote the paper along with input from Erin Mandel, Dr. Tara Haas and my supervisor Dr. Michael Riddell. Erin Mandel and I completed all animal anthropometry, including pellet surgeries, oral glucose tolerance tests, and some plasma analyses (insulin ELISA, corticoserone RIA). I performed the NEFA measurements, skeletal muscle IRS-1 and liver G-6-pase western blots and sectioned all skeletal muscle samples and stained them for measurement of CSA and SDH. Erin Mandel completed the capillary to fibre assessment and FOXO1 western blots. Sepideh Mohajeri performed liver sectioning and H and E assessment under my supervision, while I completed the ORO on the liver sections. Dr. Michael Riddell, with input from Dr. Tara Haas, oversaw the entire study and assisted in manuscript preparation.

Rationale: Glucocorticoids have potent anti-angiogenic action, ultimately causing capillary rarefaction, especially within the skeletal muscle. There is additional information suggesting that alterations to the microvasculature can significantly impact insulin sensitivity. In Chapter 3, voluntary exercise enhanced insulin resistant skeletal muscle angiogenesis, with an associated improvement in glucose disposal and increased insulin secretion. The completion of this study (Chapter 4) allowed independent assessment of GC-induced capillary rarefaction (without HFD), and provides evidence that pharmacologically enhanced hyperemia, in insulin resistant skeletal muscle, can stimulate capillary growth which corresponds with improved insulin sensitivity. However, alterations in angiogenic capacity are unable to overcome the GC-induced pathologies caused within the myocyte itself.

The metabolic effects of prazosin on insulin resistance in glucocorticoid-treated rats.

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Author Contributions

Conceived and designed the experiments: E.C.D., E.R.M., T.L.H. and M.C.R. Performed the experiments: E.C.D., E.R.M., and S.M. Analyzed data: E.C.D., E.R.M., S.M. and M.C.R. Wrote the paper: E.C.D. Edited the manuscript: E.R.M., S.M., T.L.H. and M.C.R

Preface

High dose usage of glucocorticoids (GC) induces skeletal muscle atrophy, insulin resistance and reduced muscle capillarisation. Identification of new treatments to prevent or even reverse the capillary rarefaction and metabolic deterioration that occurs in the presence of prolonged elevations in GCs would be of major therapeutic benefit. Chronic administration of prazosin, an α_1-adrenergic antagonist, increases skeletal muscle capillarization, in both healthy rodents and recently, in a rodent model of elevated GCs and hyperglycemia. The purpose of this study was to determine whether prazosin administration would improve glucose tolerance and insulin sensitivity, as a result of prazosin mediated sparing of capillary rarefaction, in this rodent model of increased GC exposure. Prazosin was provided in drinking water (50mg/L) to GC-treated or control rats (400 mg implants of either corticosterone or a wax pellet) for 7 or 14 days (n=5-9/group). Whole body measures of glucose metabolism were correlated with skeletal muscle capillarisation (C:F) at 7 and 14 days in the 4 groups of rats. Individual C:F was found to be predictive of insulin sensitivity (r^2=0.4781), but not of glucose tolerance (r^2=0.1601) and when compared with water only, prazosin treatment significantly decreased insulin values during oral glucose challenge by~1/3rd in the Cort-treated animals. Cort-treatment, regardless of duration, induced significant glycolytic skeletal muscle atrophy (P<0.05), decreased IRS-1 protein content (P<0.05), and caused elevations in FOXO1 protein expression (P<0.05), which were unaffected with prazosin administration. In summary, it appears that α_1-adrenergic antagonism improves Cort-induced skeletal muscle vascular impairments and reduces insulin secretion during an OGTT, but is unable to improve the negative alterations directly affecting the myocyte itself, including muscle size and muscle signaling protein expression.

Introduction

Glucocorticoids (GCs) are naturally occurring stress hormones, which are frequently prescribed for a number of immune and inflammatory conditions (Rhen, 2005) and in combination with cancer treatments (Walsh, 1992). Acute high dose or chronic usage of GCs has been shown to induce skeletal muscle atrophy (Menconi, 2008), insulin resistance (Buren, 2008; Schakman, 2013), capillary rarefaction (Shikatani, 2012), nonalcoholic fatty liver disease (D'souza, 2011) and the development of type 2 diabetes mellitus (T2DM) (Katsuyama 2015). GCs also have various effects on vascular function, inhibiting inflammation and cell proliferation (Shikatani, 2012), preventing endothelium dependent vasodilatation (Vogt 2001) and altering vasoconstrictor responses (Ullian, 1999).

The profound metabolic deterioration caused by high GCs may be related, at least in part, to changes in the vascular network. Indeed, a key characteristic of both insulin resistance and T2DM is an inability for the peripheral vascular networks to sufficiently perfuse skeletal muscle during periods of elevated metabolic demand (Lind, 1993), ultimately leading to poor muscle oxygenation (Greene, 1992; Stainsby, 1988) and likely decreased hormone and nutrient provision (Kusters 2015b; Lillioja 1987). The relationship between insulin resistance within the skeletal muscle and microvascular perfusion continues to suggest an important role for the microvasculature as a target for insulin action, and that when impaired, insulin's vasodilatory action within the microvasculature can significantly affect glucose metabolism.

New capillary growth within the skeletal muscle occurs via existing capillaries through sprouting or nonsprouting angiogenesis (Egginton, 2011). Exercise training and mechanical loading triggers sprouting angiogenesis, while vasodilation through α-adrenergic antagonism promotes nonsprouting angiogenesis. The α_1-adrenergic antagonist prazosin has been used in rodent studies (Dawson, 1989b; Ziada, 1989b), to selectively increase skeletal muscle capillary-

to-fiber ratio, and very recent work from our lab has established that 14 days of prazosin treatment is enough to prevent capillary rarefaction associated with elevated GCs through maintenance of multiple aspects of shear stress signaling (manuscript under review). There is further evidence that α_1-adrenergic blockade can enhance hepatic function (Dubuisson 2002; Oben 2003), insulin sensitivity and the lipid profile in hypertensive and non-insulin dependent diabetes mellitus patients (Hirano, 2001; Maruyama 1991a), in addition to decreasing blood pressure. Furthermore, subjects with T2DM, when compared to healthy lean and obese controls, have elevated resting sympathetic activation (Huggett 2005) and demonstrate an augmented plasma glucose response after a noradrenaline load, primarily due to elevated hepatic glucose output, suggesting these individuals could be more sensitive to sympathetic stimulation (Bruce, 1992).

Recent work has established that the administration of prazosin to healthy rats results in enhanced insulin sensitivity (Akerstrom, 2014), while in aging adults at risk for T2DM development, augmented skeletal muscle capillarization due to aerobic exercise training caused improved insulin sensitivity which persisted even after 2 weeks of detraining (Prior 2015). To examine the relationship between improved microvasculature and insulin sensitivity in a model of skeletal muscle insulin resistance, we used the α_1-adrenergic antagonist prazosin to increase skeletal muscle capillarization and measured markers of glucose metabolism and skeletal muscle atrophy. We hypothesized that the increased angiogenesis due to α_1-adrenergic antagonism would increase peripheral tissue perfusion, enhancing insulin sensitivity and skeletal muscle glucose uptake.

Methods

This study was carried out in accordance with the recommendations of the Canadian Council for Animal Care guidelines and was approved by the York University Animal Care Committee (2013-5).

Animals: Male Sprague-Dawley rats (Charles River Laboratories, initial mass of 225-250 g, six weeks post-weaned, n=50) were individually housed (lights on 12 hour: lights off 12 hour cycle) after one week of acclimatization to room temperature (22-23°C) and humidity (50-60%) controlled facilities. After 7 days of acclimation, animals were randomly divided into one of four groups: corticosterone-treated or wax-treated (controls), with either regular drinking water (Cort-water and Control-water), or drinking water containing prazosin hydrochloride (50 mg/L; P7791, Sigma Aldrich Canada) (Cort-prazosin and Control-prazosin). Animals were then randomly allocated to complete either 9 or 16 days of the protocol (7 or 14 days of water/prazosin treatment). All animals were fed a standard rodent chow diet (14% fat, 54% carbohydrate, 32% fat; 3.0 calories/g) ad libitum.

Pellet Surgeries: On day 0, each rat received a subcutaneous implantation of either corticosterone (the major GC found in rodents) (Cort) pellets (4 x 100 mg; Sigma-Aldrich, Canada, Cat# C2505) or wax pellets (control), as previously described (Shpilberg, 2012). Immediately following surgery, rats were placed into standard sterile cages with chow and water for 24 hours to with *ad libitum* access to chow and water. Two days after Cort or wax pellet implantation, prazosin was administered to those groups allocated to receive it, a timeline of the experimental protocol is shown in Fig. 4.1.

Blood sampling: On day 7 or 14, at ~0800 hour, blood samples were collected from each animal via saphenous vein for the determination of basal cort concentrations. For this, a small

area of hair on the lateral upper hind limb was shaven with an electric razor and wiped with a thin layer of petroleum jelly. Once the saphenous vein was located, a sterile 25-gauge needle was used to puncture the vein and whole blood was collected in lithium-heparin coated microvette capillary tubes (Sarstedt, Canada, Cat# 16.443.100) and centrifuged at ~ 9, 500 x g (Eppendorf Mini-Spin Plus, Brinkman Instruments Inc., USA) for 5 minutes, transferred into polyethylene tubes and stored at -80°C until further analysis of cort concentrations using a radioimmunoassay kit (Cat #07120102, MP Biomedicals, Solon Ohio).

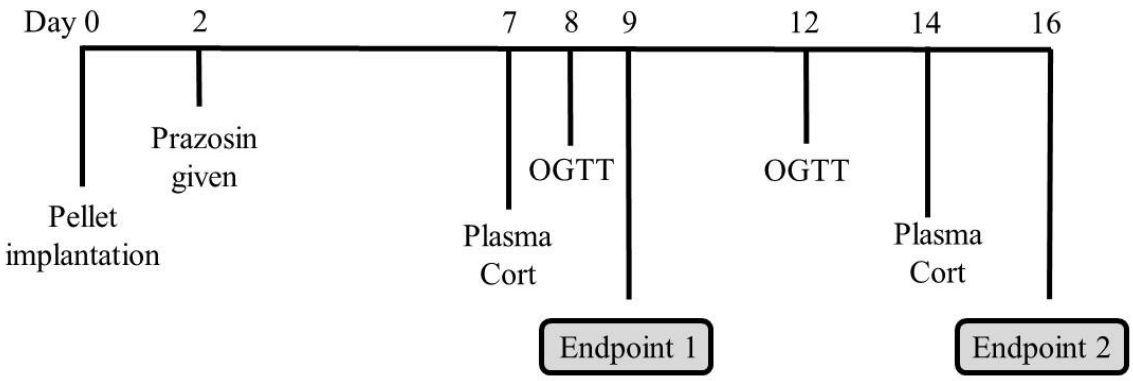


Figure 4.1. Schematic of experimental design.

Pellet implantation of either wax or cort pellets occurred on *day 0*, followed by prazosin administration on *day 2*. Saphenous vein measurement of plasma cort took place on *day 7*. An oral glucose tolerance test was administered on *day 8* after on overnight fast to examine the effects of one week of concurrent cort and prazosin treatment. The first round of animals were euthanized on *day 9* (endpoint 1). The second round of animals were administered their oral glucose tolerance test on *day 12*, followed by plasma cort measurement on *day 14*. The second round of animals were euthanized on *day 16* (endpoint 2).

Oral glucose tolerance test: Animals were fasted overnight (16 hour) on days 7 and 11 and were administered an oral glucose tolerance test on the following day (OGTT, 1.5 g/kg body mass), as previously described (Shpilberg, 2012). Each animal's fasting blood glucose (time 0) was measured via saphenous vein bleed (~ 50 µl) using a sterile needle (25G) and glucometer (Bayer One Touch meter, Contour New York). Plasma was also collected from saphenous vein bleed (see above for this procedure) for subsequent analysis of insulin concentrations via enzyme-linked immunosorbent assay (ELISA kit, Cat# 90060, Crystal Chem, Downer's Grove, Illinois). Blood glucose was also measured from saphenous vein at 5-, 15-, 30-, 60-, 90- and 120-minutes post oral glucose gavage. In the four groups completing 16 days of the protocol, blood was collected to measure plasma insulin at 15-, 30-, 60- and 120- minutes post oral gavage. Insulin concentrations were not measured in the OGTT in the 9 day treated group for technical reasons. NEFA concentrations were measured from overnight fasted plasma collections, collected before OGTT (NEFA kit, Cat #999-34691, Wako Diagnostics, Richmond, Virginia). Glucose area under the curve (AUC) was measured relative to the lowest fasting glucose of a control-water animal, while insulin AUC was measured relative to each animals individual fasting insulin level. Homeostatic Model Assessment for β-cells (HOMA-β) and insulin resistance (HOMA-IR) as previously reported in (Beaudry, 2013; Shpilberg, 2012; Wallace, 2004) was calculated based on the following equations: HOMA-β = 20 x Insulin (µ units·L)/Glucose (mM)-3.5, HOMA-IR = [Insulin (µUnits·ml) x glucose (mmol·L)]/22.5.

Histology: Skeletal muscle (tibialis anterior, TA) and liver tissue from euthanized animals were embedded in tissue freezing medium, frozen in liquid nitrogen and cryosectioned (10 µm thick). The TA was stained for fibre type, SDH activity and capillary-to-fibre ratio while

the liver was stained with Oil Red O (ORO) for neutral lipid content as previously described (Beaudry, 2015) and hematoxylin and eosin for structural assessments.

Fibre Type and SDH Analysis: To identify skeletal muscle fibre type a metachromatic myosin ATPase stain was performed on cross-sections of the TA muscle using a modified protocol (Ogilvie, 1990). Sections were pre-incubated in an acidic buffer (pH = 4.25) to differentially inhibit myosin ATPases within the different fibre types. In this protocol, type I fibres appear dark blue, type IIa appear very light blue and type IIb and IIx are not apparent from each other and are thus classified as IIb/x. These fibres appear bluish-purple. A representative image of the TA for each group was acquired for analysis. Succinate dehydrogenase (SDH) activity was then assessed using histochemical analysis and expressed as total optical density. SDH activity was assessed with Adobe Photoshop CS6, converted to greyscale and reported as the average optical density (two 10x images were averaged per animal). The greyscale is evaluated on a range of completely black (set as zero units) to white (set at 255 units). All images were acquired with a Nikon Eclipse 90i microscope and Q-Imaging MicroPublisher with Q-Capture software at 10x magnification.

Capillary-to Fibre Analysis: TA capillary-to-fibre measurements, as reported by Mandel et al (2016), were utilized in the current study to correlate with factors related to insulin sensitivity and glucose uptake.

ORO staining: Liver sections were fixed with 3.7% formaldehyde for 1 hour at room temperature while an ORO solution composed of 0.5 g ORO powder (Sigma-Aldrich, Canada) and 100 ml of 60% triethyl phosphate (Sigma-Aldrich, Canada) was mixed and filtered. Following fixation in 3.7% formaldehyde, slides were immersed in filtered ORO solution for 30 minutes at room temperature. Slides immediately underwent five washes with ddH_2O, were

allowed to dry for 10 minutes and were sealed with Permount (Sigma-Aldrich, Canada). Liver images were acquired at 20x magnification using a Nikon Eclipse 90i microscope (Nikon, Canada) and Q-imaging MicroPublisher 3.3 RTV camera with Q-capture Software.

H&E staining: Liver sections were hydrated with ethanol, washed with running tap water, then stained with hematoxylin (Sigma-Aldrich, Canada), differentiated (0.1% HCl, 0.1% NH_4OH) and stained with eosin. After staining was complete, the sections were dehydrated, allowed to dry, and then sealed with Permount (Sigma-Aldrich, Canada). Liver images were acquired at 20x and 10x magnifications respectively, using a Nikon Eclipse 90i microscope (Nikon, Canada) and Q-imaging MicroPublisher 3.3 RTV camera with Q-capture Software.

Western Blot Analysis: We quantified protein expression for IRS-1 and FOXO1 to assess the insulin signaling and atrophic pathways in the skeletal muscle, and G6Pase to assess the gluconeogenic pathway in the liver. Western blot analysis was carried out according to previously published work (Beaudry, 2015). In brief, 20-40 μg of protein lysate from the TA or the liver was run on a 10% (FOXO1 and G6Pase) or 6% (total IRS-1) SDS-page gel and proteins were transferred to a PVDF membrane (Bio-Rad, Canada). Membranes were blocked in 5% powdered milk and Tris-buffered saline with Tween 20 at room temperature for 1 hour. Membranes were then incubated overnight at 4°C with their respective primary antibodies (FOXO1, 1:1000, Cat#2880, Cell Signaling, USA; G6Pase 1:1000, Cat#sc-25840, Santa Cruz Biotechnology, USA; total IRS-1, 1:1000, Cat#2382, Cell Signaling, USA). The following morning, the membranes were washed with TBST and incubated with anti-mouse (1:10000, Cat#ab6789, Abcam) or anti-rabbit (1:10000, Cat#ab6721, Abcam) secondary antibodies for 1 hour at room temperature. Membranes were then washed and imaged. Images were detected on a Kodak In vivo FX Pro imager and molecular imaging software (Carestream Image MI SE,

version S.0.2.3.0, Rochester, New York) was used to quantify protein content. GAPDH (1:10000, ab9484, Abcam, Toronto, ON) was used as a loading control for all proteins.

Statistical Analysis: All data are represented as means ± SE, with a criterion of $P<0.05$ and $P<0.001$ and were assessed as stated using two-way ANOVAs as a means of statistical significance. Individual differences were calculated using Bonferroni's post-hoc test (Prism4; Graphpad software Inc; La Jolla, CA, USA).

Results

Body composition and muscle mass: A significant reduction in body weight and in the relative muscle weight of hind limb muscles (TA, plantaris, and gastrocnemius) was observed after exogenous Cort-treatment at both time points (P<0.05, Table 4.1), consistent with the known influence of Cort on body mass and muscle atrophy (Schakman, 2013; Shpilberg, 2012). Prazosin did not modify body weight or relative muscle weights at either time point (P<0.05, Table 4.1).

Plasma Cort, fasting NEFAs, glucose and insulin concentrations: Cort levels measured at 0800 on days 7 and 14 were elevated in both cort groups ($P<0.05$, Table 4.2), as expected and previously observed by our lab (Beaudry, 2014). There was no effect of prazosin on Cort concentrations within either the control or cort-treated groups. Fasted NEFA levels were similar in all groups at both day 7 ($P<0.05$, Table 2) and day 14 ($P<0.05$, Table 4.2).

Fasting blood glucose values were significantly elevated prior to the oral glucose challenge after both 8 ($P<0.05$, Table 4.2) and 12 days ($P<0.05$, Table 4.2) of cort treatment. No effect of prazosin to alter fasting blood glucose concentration was observed in either groups (Cort or controls). Fasting insulin values were ~ 4-fold higher after cort-treatment compared to placebo-treated groups regardless of treatment duration ($P<0.05$, Table 4.2). Prazosin treatment

of only 7 days caused a slight reduction in fasted insulin concentrations in the Cort-prazosin animals compared to the Cort-water animals (3.7±0.4 vs 5.3±0.6 ng/ml, $P<0.05$, Table 4.2), however this reduction was not maintained after 14 days of prazosin treatment (3.8±0.6 vs 3.7±0.2, Table 4.2).

Table 4.1. Animal characteristics

	1 Week			
	Control-Water	**Control-Prazosin**	**Cort-Water**	**Cort-Prazosin**
Body weight (g)	361.7±10	357.2±7.7	260.7±5.5#	251.3±2.3#
TA relative weight (g/kg BW)	1.77±0.06	1.85±0.04	1.71±0.05	1.71±0.08
EDL relative weight (g/kg BW)	0.5±0.02	0.52±0.01	0.52±0.02	0.51±0.01
Plantaris relative weight (g/kg BW)	0.87±0.75	0.93±0.04	0.84±0.04	0.87±0.03
Soleus relative weight (g/kg BW)	0.41±0.01	0.38±0.02	0.49±0.01#	0.50±0.02#
Gastroc relative weight (g/kg BW)	4.5±0.3	4.9±0.1	4.3±0.1	4.4±0.2
	2 Weeks			
	Control-Water	**Control-Prazosin**	**Cort-Water**	**Cort-Prazosin**
Body weight (g)	373.4 ± 4.9	369.4±10	245.0±7.7#	254.9±8.5#
TA relative weight (g/kg BW)	1.88 ± 0.04	1.88±0.03	1.57±0.04#	1.58±0.05#
EDL relative weight (g/kg BW)	0.49 ± 0.01	0.52±0.01	0.48±0.02	0.47±0.02
Plantaris relative weight (g/kg BW)	0.91±0.04	0.92±0.02	0.82±0.03	0.81±0.03
Soleus relative weight (g/kg BW)	0.45±0.009	0.40±0.02	0.54±0.02#	0.51±0.02#
Gastroc relative weight (g/kg BW)	5.0±0.2	5.2±0.1	4.2±0.2#	4.3±0.1#

Notes: All values are means ± SE. #Significantly different compared to respective control group. All analysis was completed by two-way ANOVA with Bonferroni's post hoc, where $P<0.05$; n=5 for 1 week, n=7-9 for 2 weeks.

Table 4.2. Plasma and metabolic effects of corticosterone- and prazosin co-treatment.

	1 Week			
	Control-Water	**Control-Prazosin**	**Cort-Water**	**Cort-Prazosin**
Basal plasma corticosterone (ng/ml)	44.3 ± 17.6	61 ± 46.1	28 ± 17.5	37.6 ± 14.7
Plasma corticosterone (ng/ml)	17.5 ± 1.8	18.9 ± 7.1	583.1 ± 51.3*	485.5 ± 93.3*
Capillary-to-fiber ratio	1.9 ± 0.1	2.0 ± 0.1	1.7 ± 0.2*	1.7 ± 0.1*
Fasted glucose (mmol/L)	4.8 ± 0.1	5.2 ± 0.2	6.56 ± 0.26*	6.6 ± 0.8*
Fasted insulin (ng/ml)	0.39 ± 0.2	0.29 ± 0.2	5.3 ±0.6*	3.7 ± 0.4*#
HOMA-IR	1.6 ± 1.3	1.9 ± 1.2	44.7 ±5.5*	36.3 ± 6.9*
HOMA-β	222.6 ± 131.4	112 ± 74	1009.9 ± 94.9*	1136.8 ± 396*
	2 Weeks			
	Control-Water	**Control-Prazosin**	**Cort-Water**	**Cort-Prazosin**
Basal plasma corticosterone (ng/ml)	39.8 ± 25.1	47.3 ± 25.2	64.5 ± 20.6	51.6 ± 19.5
Plasma corticosterone (ng/ml)	16.2 ± 6.5	9.6 ± 0.6	296.3 ± 34.5*	284.7 ± 34.2*
Capillary-to-fiber ratio	2.1 ± 0.1	2.3 ± 0.03†	1.6 ± 0.1*	2.0 ± 0.1*†
NEFAs (mmol/L)	0.67 ± 0.1	0.66 ± 0.1	0.79 ± 0.1	0.65 ± 0.1
Fasted glucose (mmol/L)	5.5 ± 0.2	5.8 ± 0.2	6.7 ± 0.3*	8.1 ± 0.5*
Fasted insulin (ng/ml)	0.9 ± 0.2	1.1 ± 0.2	3.8 ± 0.6*	3.7 ± 0.2*
HOMA-IR	7.0 ± 1.8	8.6 ± 1.7	35 ± 4.7*	42.3 ± 4.2*
HOMA-β	266.8 ± 55.6	294 ± 60	739.7 ± 83.4**	435.4 ± 81.0

Notes: All values are means ± SE. †Main effect of prazosin consumption. #Mean was statistically significant from control-prazosin. *Main effect of cort-treatment. **Significantly different from all groups. All analysis was completed by two-way ANOVA with Bonferroni's post hoc, where $P<0.05$, $P<0.001$ for HOMA- β; n=5 for 1 week, n=7-9 for 2 weeks.

Oral Glucose Tolerance Test: Cort, regardless of the duration of treatment, resulted in elevated fasting blood glucose values and deterioration in glucose tolerance when compared to the control groups ($P<0.05$, Fig. 4.2A, 2B). Seven and 14 days of prazosin treatment did not improve glucose tolerance significantly, although AUC appeared to be slightly reduced with prazosin exposure and values at the 120-minute time point were normalized with prazosin treatment ($P<0.05$, Fig. 4.2A', Fig. 2B'). Plasma insulin concentrations were highest in the cort water-treated animals before and throughout the oral challenge ($P<0.05$, Fig. 4.2C). Interestingly, prazosin administration lowered the insulin AUC during oral glucose gavage at the 2-week time point, primarily by restoring the insulin levels back to baseline by the 30-minute time point ($P<0.05$, Fig. 4.2D'). HOMA-IR was used to determine the level of fasting insulin resistance, with both 9 and 16 days of cort-treatment causing significant insulin resistance when compared to both control groups, with prazosin not having any significant effect in either group ($P<0.001$, Table 4.2). HOMA-β was used to calculate basal β-cell function where higher HOMA-β scores indicate greater β-cell function. Cort-treatment, after 9 days, caused ~4-5-fold increase in HOMA- β compared to controls, with prazosin having no significant effect ($P<0.001$, Table 4.2). Two weeks of cort water treatment was also associated with higher HOMA-β score, while the cort-prazosin animals had an attenuated rise in HOMA-β ($P<0.05$, Table 4.2), suggesting that prazosin administration was able to release the demand on β-cell function.

Capillary-to-fibre ratio vs. glucose tolerance and insulin sensitivity: The C:F analysis was originally reported in Mandel et al (2016); a series of representative images (Fig. 4.3A) and reference C:F values (Table 2) were reported here to allow for regression analysis. To determine if glucose tolerance and insulin sensitivity are associated with C:F in the 14 day treatment groups, individual glucose AUC. Fasted insulin and insulin AUC values from each animal were

plotted and a linear regression was calculated for each variable (Fig. 4.3B, 3C, 3D). C:F was found to be predictive of insulin sensitivity (r^2= 0.5783, P<0.05, Fig. 3C; r^2=0.4781, *P*<0.05, Fig. 3D) but not of glucose tolerance (r^2=1.601, *P*<0.05, Fig. 4.3B).

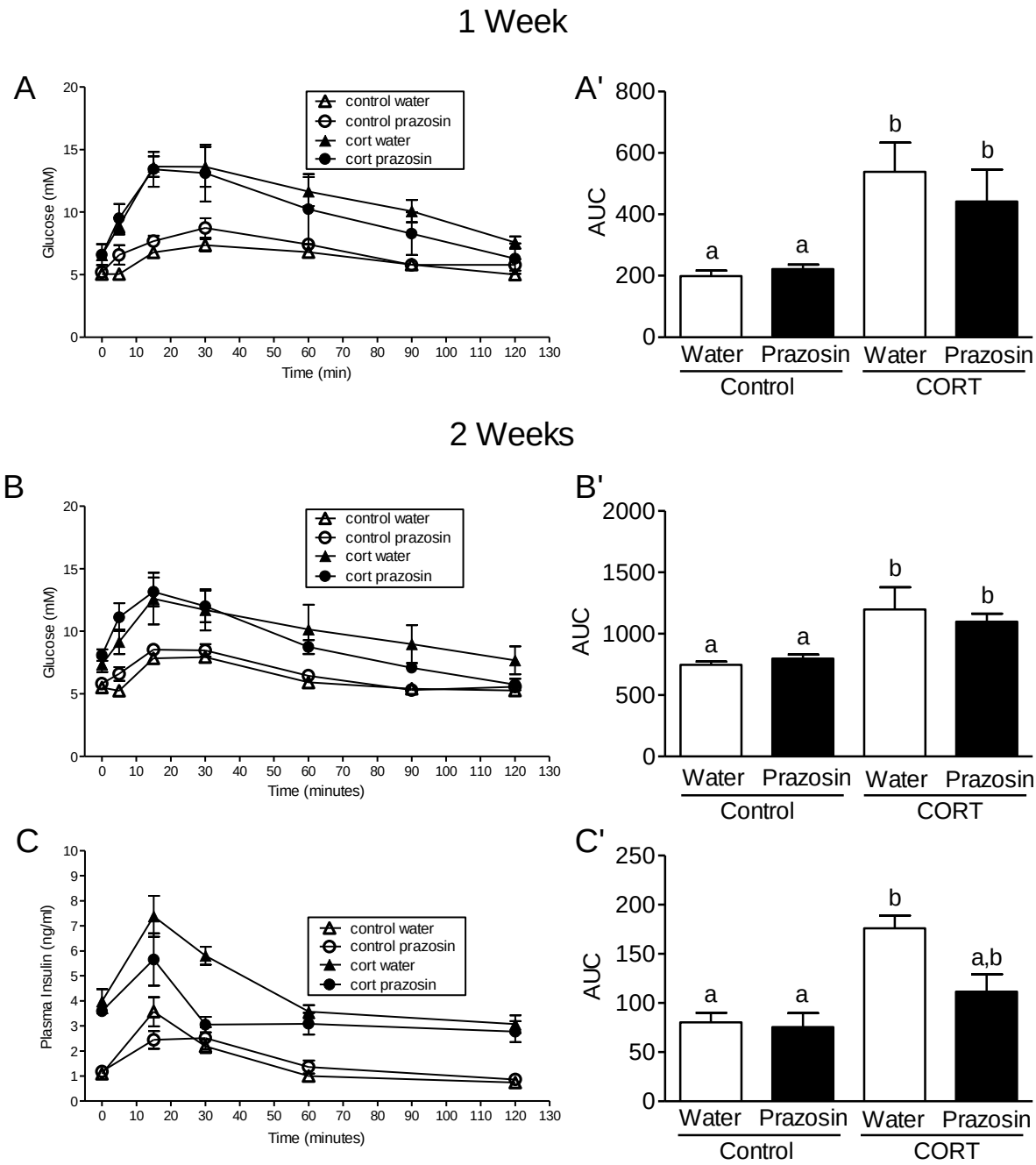


Figure 4.2. Glucose tolerance and insulin secretion after corticosterone and prazosin co-treatment.

Glucose tolerance and insulin secretion after 8 (A and A') and 12 days (B, B', C and C') of corticosterone and prazosin treatment. An oral glucose tolerance test (OGTT) was administered after 8 and 12 days of cort-treatment. The cort-water animals were glucose intolerant after only 8 days of treatment, (P<0.05); n=5. Both cort-treated groups were glucose intolerant after 12 days of cort-treatment, regardless of prazosin consumption (P<0.05); n=7-9. Insulin secretion throughout the OGTT was significantly elevated in the cort-water group, and was greatly improved with prazosin consumption (P<0.05); n=7-9. Bars that do not share similar letters, denote statistical significance (P<0.05) using a two-way ANOVA with Bonferroni's (A', B' and C'). All values are means ± SE.

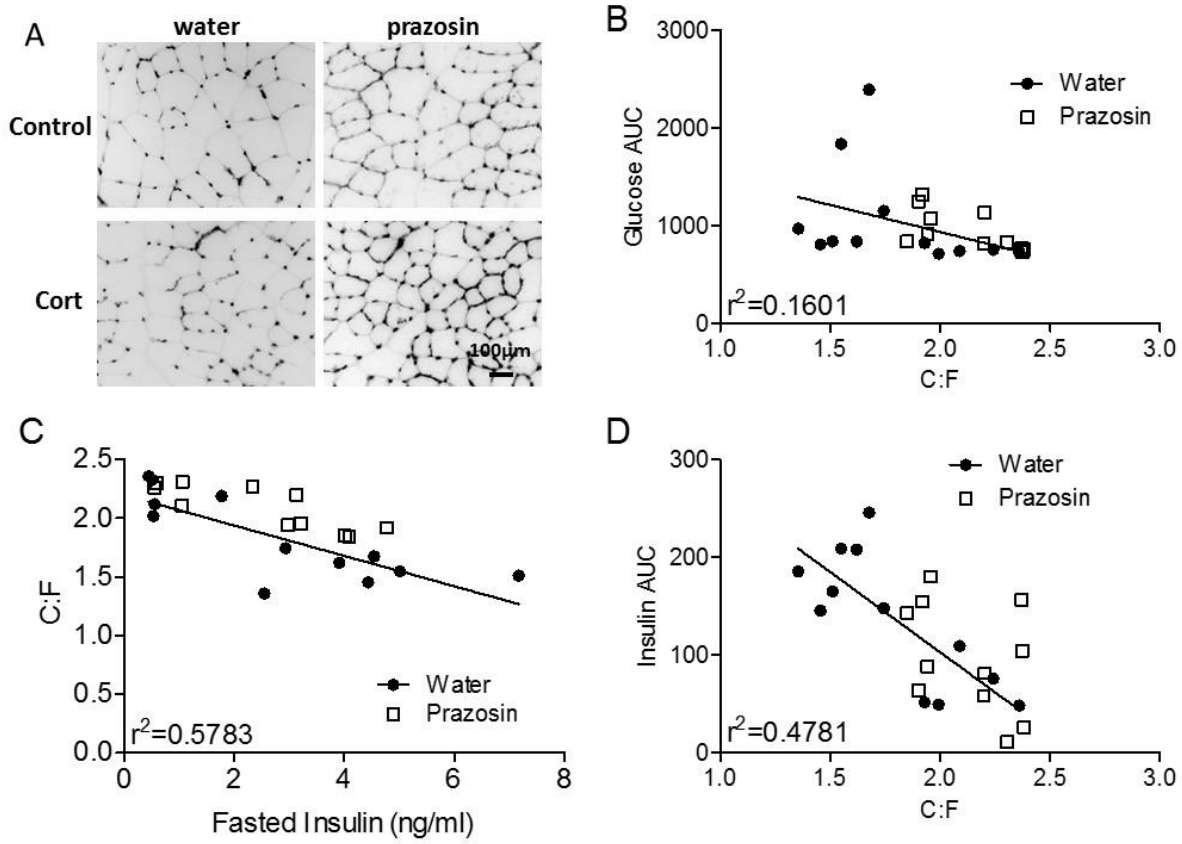


Figure 4.3. Capillary-to-fiber ratio (C:F) is predictive of insulin sensitivity but not glucose tolerance.

Representative images depicting C:F within the TA muscle (A) after 2 weeks of co-treatmnt. Individual C:F, glucose area under the curve (AUC) (B), fasted insulin (C) and insulin AUC (D) values after two weeks of concurrent cort and prazosin treatment were plotted and a linear regression was calculated. C:F was found to have no relationship with glucose tolerance (r^2=1.601, P<0.05), but was predictive of insulin sensitivity (r^2= 0.5783, P<0.05; r^2=0.4781, P<0.05); n=7-9. All values are individual.

Muscle cross-sectional area and succinate dehydrogenase content: The TA muscle of representative animals from each treatment group was stained for CSA and SDH. CSA, indicating individual muscle fiber size, was lowest in type IIb/x fibers of cort-treated animals after both 9 and 16 days (Fig. 4.4A-D, $P<0.01$). The control-prazosin animals had the largest muscle fibers across all three fiber types after 9 days ($P<0.05$), but this difference was unsustained by 16 days of treatment. SDH staining, which correlates with muscle oxidative capacity (Seick 1986), was not different among all treatment groups after both 9 and 16 days (Fig. 4.6 A-D).

FOXO1 and IRS-1 protein content: Cort-treatment is known to stimulate muscle atrophy and impair insulin signaling, therefore FOXO1 and IRS-1 protein concentrations were analyzed since these are considered critical signaling molecules in these two pathways. After 9 days of cort-treatment, there was a significant increase in FOXO1 protein content and ~2-fold decrease in total IRS-1 (Fig. 4.5A, 5B, $P<0.05$). These changes were sustained after 16 days, regardless of prazosin treatment (Fig. 4.5C, 5D, $P<0.05$).

Liver histology and enzymatic content: H&E staining of liver sections was used to visually examine possible structural damage in all treatment groups after both 9 (Fig. 4.7A) and 16 (Fig. 4.7B) days. Nuclei are visualized as purple (dark) and the cytosol is pink (light). Cort-treatment appeared to cause damage to the liver, seen as vacuoles or holes in the cytosol, at both 9 and 16 day end points, and 14 days of prazosin consumption seemed to have a small visual beneficial effect in reducing the number of vacuoles. Liver sections after 16 days of treatment were also stained for ORO, to examine lipid accumulation (Suppl. Fig. 4.1A). Lipid staining was highest in the Cort-water treated group compared with both control groups and the Cort-prazosin group. Cort-prazosin animals had visibly less lipid accumulation in comparison to Cort-water

animals, but when compared to both control groups the lipid droplets were still obvious. Glucose-6-phosphatase (G6Pase) protein content was measured to assess the gluconeogenic pathway in the 16 day treatment groups (Fig. 4.7C). There was an effect of Cort-treatment to significantly increase G6Pase content (P<0.05) in comparison to both control groups, with no obvious effect of prazosin treatment in either controls or Cort-treated groups.

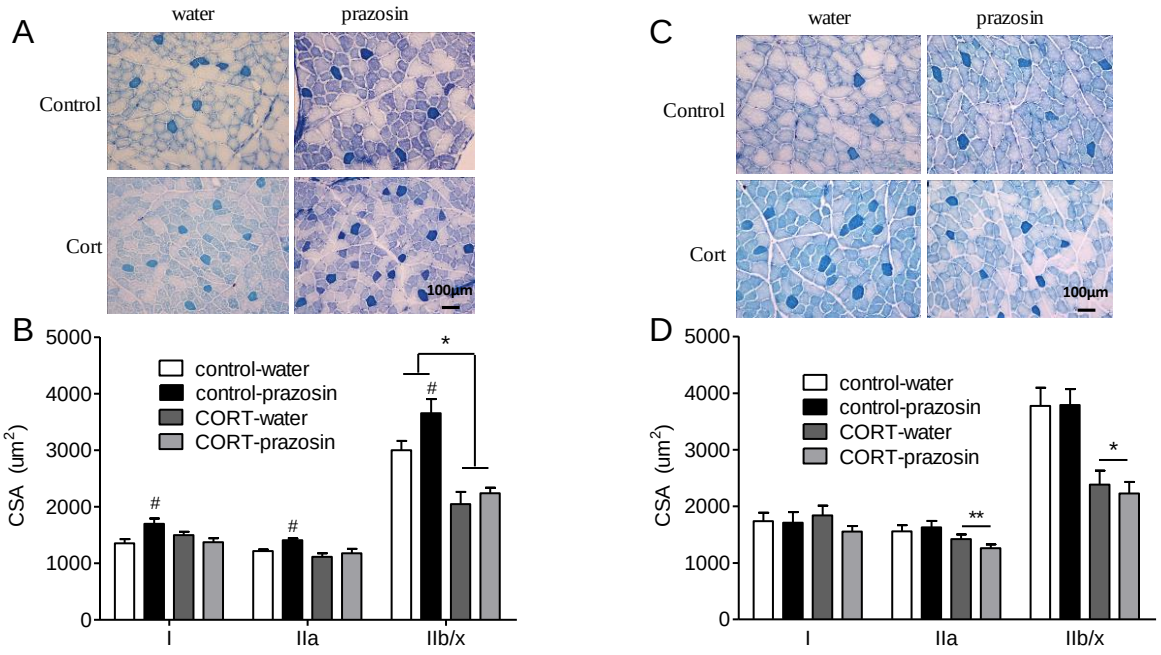


Figure 4.4. Corticosterone treatment causes glycolytic fibre atrophy.

Cross-sectional area (CSA) in I, IIa, and IIb/x fibre types of the tibialis anterior (TA) after 9 (A, B) and 16 days (C, D) of concurrent prazosin and corticosterone treatment. Cort-treatment, regardless of duration, resulted in significant atrophy of the IIb/x fibers (P<0.01). The control-prazosin animals had the largest muscle fibers across all three fiber types after 9 days (P<0.05), which was not sustained after 16 days of co-treatment.; n=4-5. # Significantly different from all other groups (P<0.05), * significantly different from control groups (P<0.01), ** significantly

different from control-prazosin ($P<0.05$) using a two-way ANOVA with Bonferroni's. All values are means ± SE.

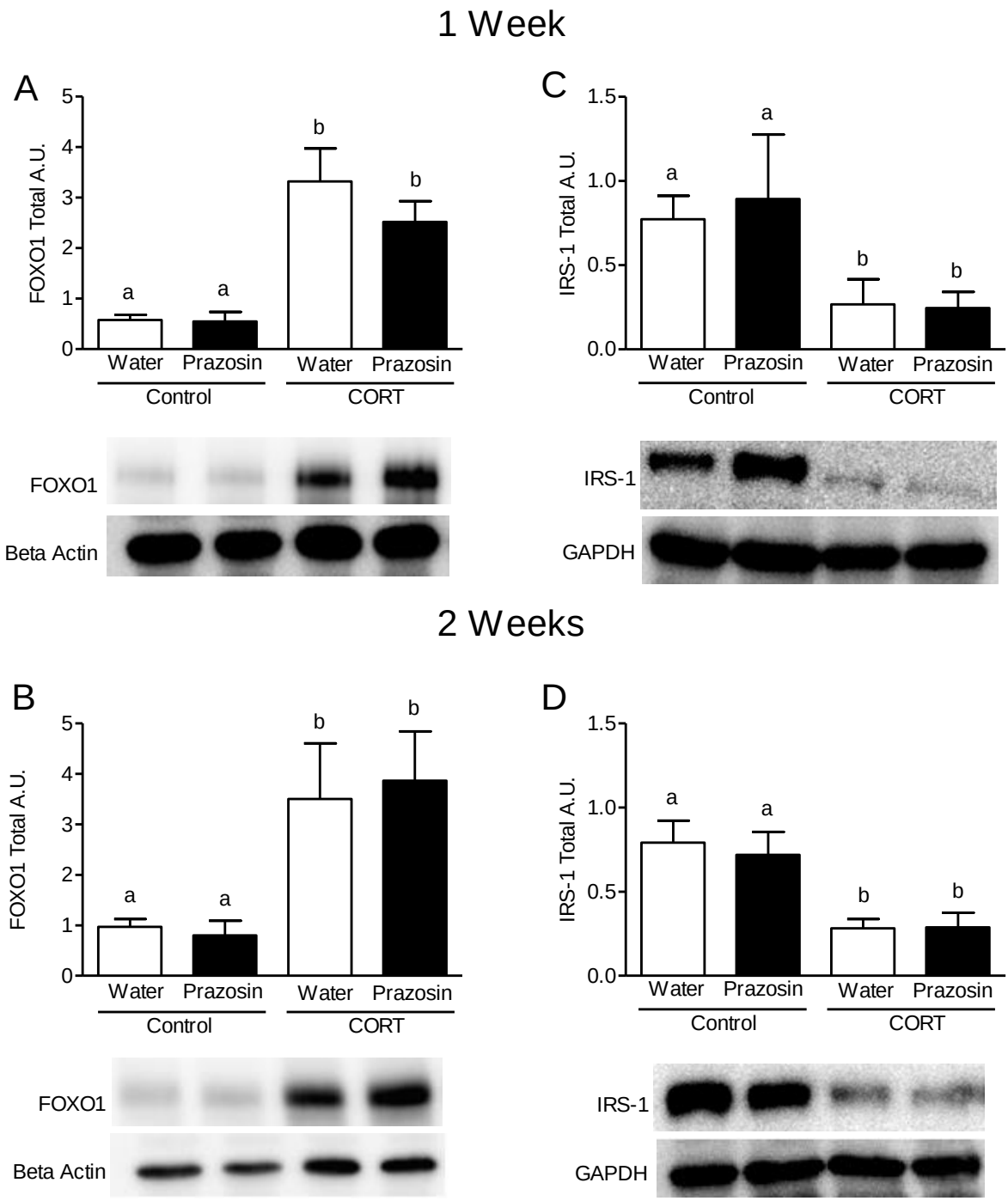

1 Week
A
FOXO1 Total A.U.
0
1
2
3
4
5
a
a
b
b
Water
Prazosin
Water
Prazosin
Control
CORT
FOXO1
Beta Actin
C
IRS-1 Total A.U.
0.0
0.5
1.0
1.5
a
a
b
b
Water
Prazosin
Water
Prazosin
Control
CORT
IRS-1
GAPDH
2 Weeks
B
FOXO1 Total A.U.
0
1
2
3
4
5
a
a
b
b
Water
Prazosin
Water
Prazosin
Control
CORT
FOXO1
Beta Actin
D
IRS-1 Total A.U.
0.0
0.5
1.0
1.5
a
a
b
b
Water
Prazosin
Water
Prazosin
Control
CORT
IRS-1
GAPDH

Figure 4.5. The effect of corticosterone and prazosin on FOXO1 protein content.

Effect of prazosin and corticosterone on FOXO1 (A,C) and IRS-1 (B,D) total protein content in the tibialis anterior (TA) after 9 (A,B) and 16 (C,D) days of co-treatment. Regardless of protocol duration, cort-treatment caused increased FOXO1 content and decreased total IRS-1 content, impairments which were unaffected by prazosin administration; n=5. Bars that do not share similar letters, denote statistical significance ($P<0.05$) using a two-way ANOVA with Bonferroni's. All values are means ± SE.

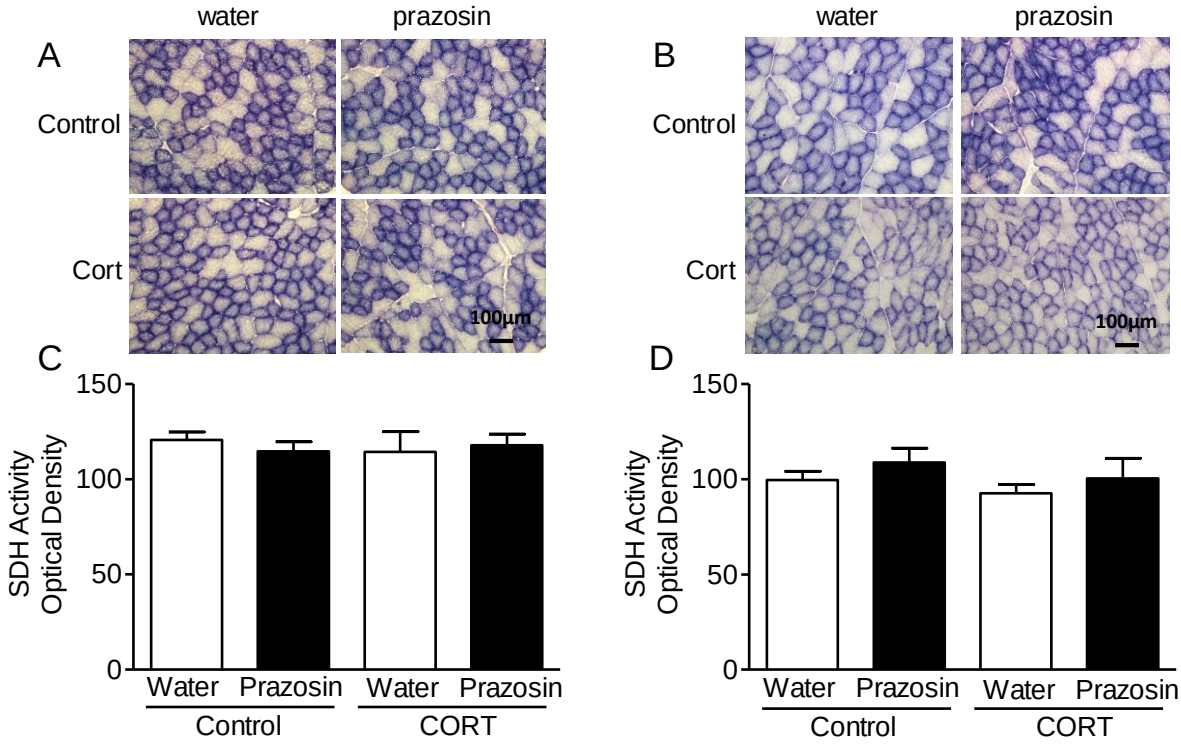


Figure 4.6. The effect of corticosterone and prazosin co-treatment on succinate dehydrogenase content.

Representative cross-sections of the tibialis anterior (TA) depicting succinate dehydrogenase (SDH) content after 9 (A, B) and 16 days (C, D) of concurrent prazosin and corticosterone treatment. Muscle oxidative capacity was unaffected by either cort- or prazosin- treatment at either time point; n=4-6. A two-way ANOVA was used. All values are means ± SE.

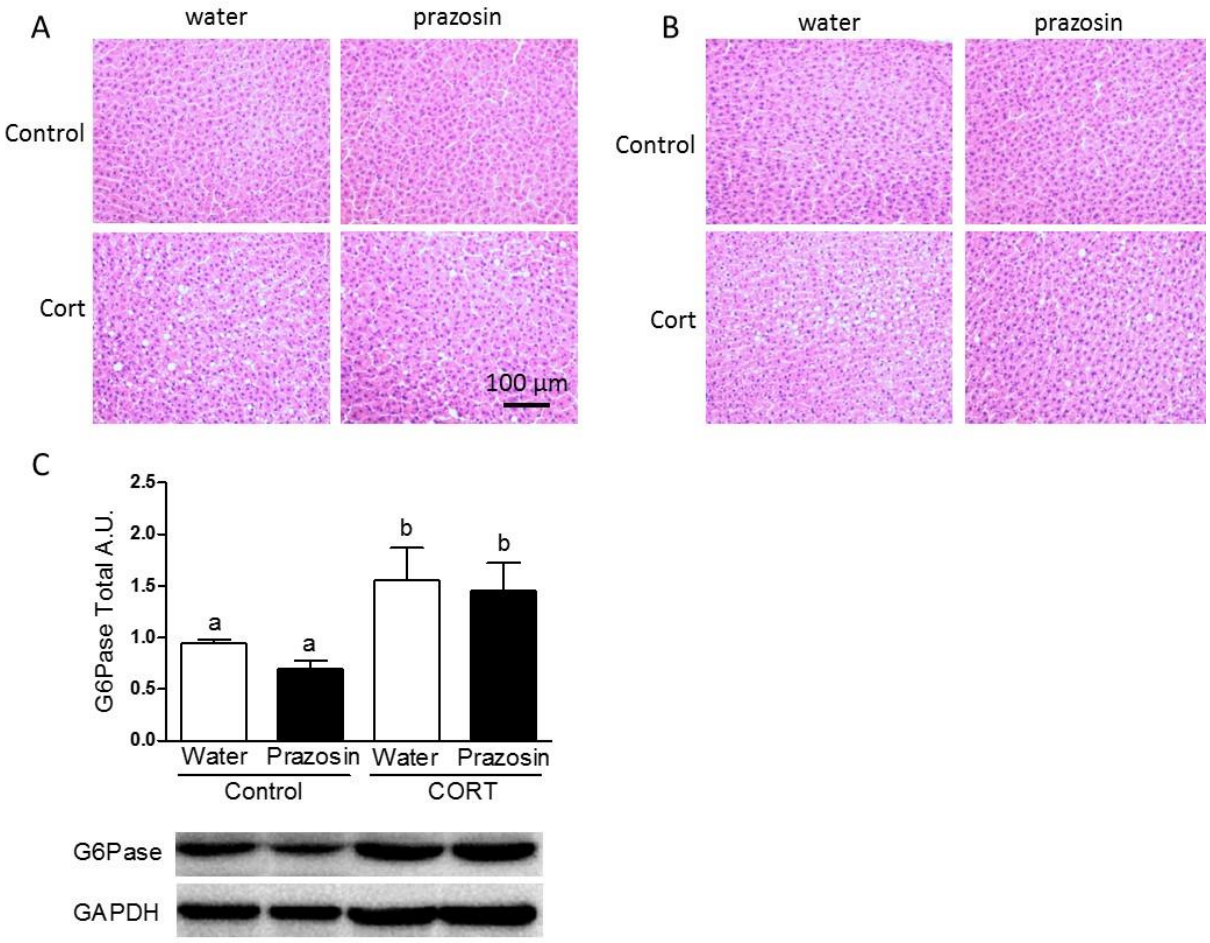
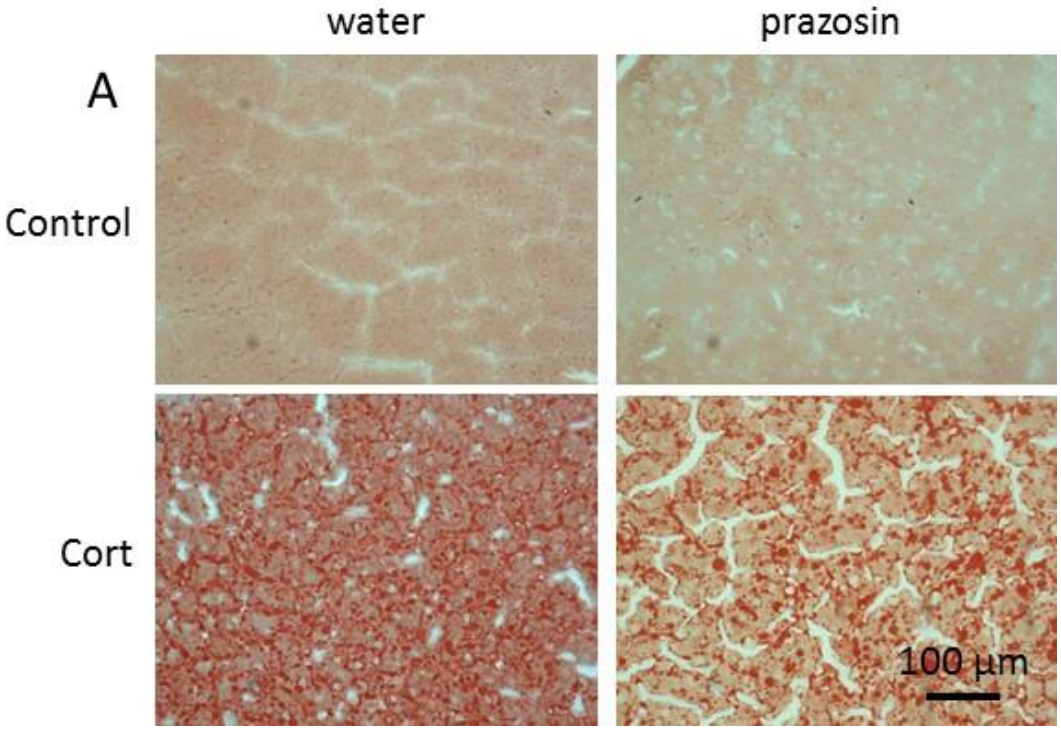


Figure 4.7. Liver structure and G-6-Pase content is unaffected by prazosin treatment.

Representative images of the liver depicting hematoxylin and eosin staining after 9 (A) and 16 (B) days and protein content of the gluconeogenic enzyme glucose-6-phosphatase (G-6-Pase) (C) after 16 days of co-treatment. Cort-treatment of both 9 and 16 days caused vacuole deposition in the liver, which appeared to be slightly improved with two weeks of prazosin consumption. Two weeks of cort-treatment caused a significant elevation in liver G-6-Pase protein content, which was unaffected by prazosin consumption; n=5. Bars that do not share similar letters, denote statistical significance ($P<0.05$) using a two-way ANOVA with Bonferroni's. All values are means ± SE.



Supplementary Figure 4.1. Liver lipid content is unaffected by prazosin treatment.

Representative images showing Oil Red O staining of the liver (A) after 16 days of co-treatment. Prazosin seemed to improve the fat accumulation observed in the liver after 16 days of cort-treatment; n=5-9.

Discussion

This study demonstrates, for the first time, that the improvement in skeletal muscle capillary number with prazosin treatment, observed in GC-treated rats, corresponded with a distinct improvement in whole body insulin sensitivity. However, in spite of the marked improvements in muscle capillarization and insulin sensitivity with prazosin treatment, GC-induced muscle atrophy and severe glucose intolerance was still observed in this animal model. These findings suggest that simply promoting muscle capillarization might not translate to significant metabolic improvements in the Cushing's like phenotype in this particular animal model.

Elevations in GCs for a two-week period in male rats negatively impacts ectopic fat deposition, glucose tolerance, insulin sensitivity and skeletal muscle growth and capillary number (Beaudry, 2014; D'souza, 2011; Shikatani, 2012; Shpilberg, 2012). Recently, we have established that regular exercise improves the metabolic profile and skeletal muscle phenotype in male Sprague-Dawley rats exposed to prolonged corticosterone treatment (Beaudry, 2015). Prazosin is an established method to increase skeletal muscle angiogenesis, through interference with peripheral sympathetic function, selectively blocking postsynaptic α-adrenoceptors (Graham, 1977), without any of the other metabolic and morphological adaptations that can occur with exercise training. Blockade of the α-adrenergic pathway with prazosin leads to angiogenesis and enhanced capillary flow in skeletal muscle in healthy rats, a phenomenon that is linked to improvement in whole body insulin sensitivity without apparent changes in skeletal muscle insulin signaling *per se* (Akerstrom, 2014). We have also established that prazosin administration is able to prevent the capillary rarefaction associated with pathophysiological elevations in GCs, through maintenance of multiple aspects in shear stress signaling (manuscript

in review). While muscle capillarization was improved by prazosin treatment, as was whole body insulin sensitivity, glucose tolerance and skeletal muscle size were unaffected. These findings suggest that an increase in skeletal muscle capillarization alone may not improve glucose tolerance or significantly alter the metabolic profile in organisms suffering from elevated GCs.

We have previously shown that two weeks of Cort-treatment causes glucose intolerance and here we show that this response can also occur as early as 9 days of Cort-treatment. Early work examining the effect of prazosin in human hypertensive subjects found that 12 weeks of prazosin consumption was associated with altered carbohydrate metabolism, specifically improving both glucose uptake and insulin sensitivity (Pollare 1988). A number of studies have since been completed further verifying the beneficial effect that prazosin can have on glucose tolerance and insulin sensitivity in human subjects (Maruyama, 1991a; Swislocki, 1989). The relationship between insulin sensitivity and capillarisation was recently explored, also using prazosin to alter skeletal muscle angiogenesis, in sedentary, non-diabetic, male rats (Akerstrom, 2014). The authors found that after 21 days of prazosin treatment there was an increase in capillarisation, accompanied by an improvement in insulin sensitivity and glucose uptake, but no effect on major insulin signaling proteins within the skeletal muscle. The authors attribute the improvement in insulin sensitivity to improved glucose diffusion due to enhanced surface area for exchange. In line with these observations, we also saw significant improvements in both insulin sensitivity and capillarisation after only 14 days of prazosin administration, but found only modest alterations in glucose tolerance, perhaps because hepatic glucose production was not improved or because the muscle atrophy was still apparent in this animal model. To further examine the link between capillarisation and insulin sensitivity, we analyzed individual capillary-to-fiber and insulin and glucose AUC values and found that the individual capillary-to-

fiber ratio was predictive of insulin sensitivity but not of glucose tolerance. The lack of significant improvement to glucose tolerance could be due to the fact that Cort-treatment caused such pronounced skeletal muscle atrophy and hepatic insulin resistance that the modest improvements in skeletal muscle vasculature were unable to overcome these impairments.

The alterations in glucose tolerance observed in the Cort-treated animals appear to be mediated by activation of the atrophic pathway identified by significantly decreased skeletal muscle cross-sectional area, specifically within the glycolytic (IIb/x) fibers and elevations in total FOXO1 protein content, an atrophic protein with a glucocorticoid response element (GRE). We also observed an obvious deficit in IRS-1 total protein content which was evident after just 9 days of treatment. These results parallel the general findings of the effects of elevated Cort on the skeletal muscle machinery (Buren, 2002; Saad, 1993; Schakman, 2013). While we know prazosin was able to improve the capillary rarefaction observed after Cort-treatment and increase insulin sensitivity, the Cort-treated animals were still glucose intolerant, insulin resistant and were physically smaller than their placebo counterparts. In general, GCs decrease absolute body mass but increase relative fat mass, a result which is especially pronounced in young growing animals (Shpilberg, 2012) or in human adolescence (Abad, 2001). The loss in total mass is usually attributed to reduced skeletal muscle mass, but can also represent reductions in body length or height. While we did not expect α_1-adrenoceptor antagonism to completely reverse these symptoms of GC excess, it was hypothesized that increasing the skeletal muscle capillary supply could in fact enhance nutrient and hormone provision, possibly attenuating some growth impairments within the muscle itself.

We hypothesized that pharmacologically increasing the capillarisation of insulin resistant rodent skeletal muscle could subsequently impact its oxidative capacity. After basic

quantification of SDH staining within the TA, neither 7 nor 14 days of prazosin administration caused augmentation to SDH staining intensity within either the control or Cort-treated groups. These results suggest that the capillary supply to skeletal muscle can be enhanced independently of the metabolic profile, oxidative capacity and fiber size, an interesting finding also observed in the skeletal muscle of young men (Bosutti 2015). As such, it is likely that regular exercise, which can increase both muscle capillarisation and oxidative capacity as well as improve skeletal muscle insulin sensitivity, might be a preferred means to improve metabolic function in this particular animal model of sustained hyperglucocorticoidemia.

The promoter of the glucose-6-phosphatase (G6Pase) gene also has a GRE which suggests initiation of gene transcription by activated glucocorticoid receptors, stimulating an increased rate of gluconeogenesis (Yoshiuchi, 1998). Our results show increased G6Pase protein content in the liver of Cort-treated rats, suggesting elevated gluconeogenesis, which was unaffected by prazosin administration. Very early research determined that the glycogenolytic and gluconeogenic actions of epinephrine and norepinephrine in the rat liver were primarily mediated through an α-adrenergic, cAMP-independent mechanism (Chan, 1979), and that after adrenalectomy, there was a reduction in α-adrenergic action in rat livers. Additionally, hepatic triglyceride secretion rate was reduced in prazosin-treated rats (Chait, 1979), which correlated with reduced plasma triglyceride concentrations and points to a direct effect of α-adrenergic blockade on liver triglyceride secretion rate. Additionally, antagonism of the α-adrenergic pathway leads to increased availability of lipoprotein lipase, which also can decrease plasma triglycerides and triglyceride-rich lipoproteins (Nash, 1990). These are possible mechanisms for the improved lipid profile observed in multiple chronic α-adrenergic blockade studies , and also a reason why we measured NEFAs and lipids in the liver. While there was no effect of Cort to

increase NEFA concentrations, there was also no effect of prazosin to lower NEFA concentrations in either the control or Cort groups. Histochemical analysis of the liver shows slight improvements in both structural integrity and neutral lipid deposition after 14 days of prazosin administration in the Cort-treated rats. There was a fair amount of variability involved in the ORO analysis, as some Cort-treated rats given prazosin did not improve to the extent as others, a result that was additionally observed with both glucose tolerance and insulin sensitivity assessments. This is not uncommon with our model of elevated GCs, as some rats tend to respond to the Cort pellets much more aggressively than others, and points to a possible enhanced therapeutic action of prazosin if administered for a longer period of time, as here we only examined either 7 or 14 days of treatment. Interestingly, 9 days of Cort produced minimal visible defects within the liver. Furthermore, 16 days of Cort-treatment caused elevations in fasted glucose values, regardless of prazosin administration, which also points to the conclusion that two weeks of α_1-adrenoceptor antagonism was unable to provide major functional improvements to the liver of Cort-treated rodents.

The Cort concentration administered to the rats was a pathophysiological dose and could have been too high to allow for prazosin to reverse the key metabolic impairments, a possible reason why there were no major improvements observed after the significant increase in capillary-to-fiber ratio. The fact that prazosin was able to influence insulin sensitivity and increase capillary number indicates that it did have an effect, but suggests that the systemic consequences of GC's (skeletal muscle atrophy, increased liver gluconeogenesis, glucose intolerance and insulin resistance) were too aggressive for prazosin to have a substantial beneficial effect. This study also shows how the short-term correction in skeletal muscle capillarisation, specifically through α_1-adrenergic blockade, in a model of Cort-induced insulin

resistance is insufficient to overcome the other metabolic and physiologic impairments associated with this condition.

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Chapter 5

The effects of voluntary exercise and prazosin on capillary rarefaction and metabolism in streptozotocin-induced diabetic male rats

Author Contributions: Dr. Erwan Leclair and I designed the study and wrote the paper along with input from Erin Mandel, Dr. Tara Haas, Dr. Olivier Birot and my supervisor Dr. Michael Riddell. Dr. Erwan Leclair and I completed all animal handling, including STZ injections, blood glucose and exercise monitoring, and plasma analyses (NEFAs, corticosterone RIA). I performed all skeletal muscle sectioning, Erin Mandel completed the capillary to fibre analyses, Julian Aiken completed the western blot analyses. Dr. Michael Riddell, with input from Dr. Tara Haas, oversaw the entire study and assisted in manuscript preparation.

Rationale: Diabetes is associated with both micro- and macrovascular complications, generally due to chronic hyperglycemia. Skeletal muscle capillary rarefaction, a microvascular complication, was shown to be attenuated in GC-induced insulin resistant rats through voluntary exercise in Chapter 3, and prazosin-induced hyperemia in Chapter 4. There is currently very limited research examining the effect of exercise on the microvasculature of diabetic skeletal muscle. The completion of this study highlights the importance of exercise on vascular complications associated with diabetes, through the use of a chemically-induced (STZ) rodent model of diabetes. Additionally, the apparent synergism of exercise and prazosin co-administration on further angiogenic and glycemic enhancements, underscores the importance of combination therapies designed to target the vasculature.

The effects of voluntary exercise and prazosin on capillary rarefaction and metabolism in streptozotocin-induced diabetic male rats

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* E.C. Dunford and E. Leclair contributed equally to this work

Author Contributions

Conceived and designed the experiments: E.C.D., E.L., and M.C.R. Performed the experiments: E.C.D., E.L., E.R.M, and J.A. Analyzed data: E.C.D., E.L., E.R.M., J.A, O.B. and M.C.R. Wrote the paper: E.C.D. and E.L. Edited the manuscript: E.C.D., E.L., E.R.M., T.L.H., J.A., O.B., M.C.R.

Preface

Type-1 diabetes mellitus causes impairments within the skeletal muscle microvasculature. Both regular exercise and prazosin have been shown to improve skeletal muscle capillarization and metabolism in healthy rats, through distinct angiogenic mechanisms. The aim of this study was to evaluate the independent and additive effects of voluntary exercise and prazosin treatment on capillary-to-fiber ratio (C:F) in streptozotocin (STZ)-treated diabetic rats. STZ (65mg/kg) was intraperitoneally administered to male Sprague-Dawley rats (n=36) to induce diabetes, with healthy, non-diabetic, sedentary rats (n=10) used as controls. The STZ-treated rats were then divided into sedentary (SED) or exercising (EX, 24h access to running wheels) groups and then further subdivided into prazosin (Praz) or water (H_2O) treatment groups: non-diabetic-SED-H_2O, STZ-SED-H_2O, STZ-EX-H_2O, STZ-SED-Praz, and STZ-EX-Praz. After 3 weeks, untreated diabetes significantly reduced the C:F within both the tibialis anterior (TA) and soleus muscles in the STZ-SED-H_2O animals (P<0.05). Both voluntary exercise and prazosin treatment independently resulted in a normalization of C:F within the TA (1.86±0.12 & 2.04±0.03 vs 1.71±0.09, P<0.05) and the soleus (2.36±0.07 & 2.68±0.14 vs 2.13±0.12, P<0.05). The combined STZ-EX-Praz group resulted in the highest C:F within the TA (2.26±0.07, P<0.05). Voluntary exercise volume was correlated with fed blood glucose levels (r^2=0.7015, P<0.01), and when combined with prazosin, caused further enhanced glycaemic control (P<0.01). Additionally, exercise and prazosin reduced circulating NEFAs more than either stimulus alone (P<0.05). These results suggest that the distinct stimulation of angiogenesis, with both regular exercise and prazosin treatment, causes a cooperative improvement in the microvascular complications associated with type-1 diabetes.

Introduction

Type-1 diabetes mellitus is a chronic autoimmune disease targeting the pancreatic beta-cells causing little to no insulin production, resulting in hyperglycaemia (Cryer, 2012). Despite exogenous insulin therapy, individuals with diabetes have an increased risk for long-term microvascular and macrovascular complications which can significantly impact their morbidity and mortality (Coleman 2015; Dawson 2008). Type-1 diabetes is associated with impaired angiogenesis in skeletal muscle (Abaci 1999; Rivard 1999). It is generally held that hyperglycaemia itself, or some metabolic byproduct of hyperglycaemia, induces remodeling of capillaries within the skeletal muscle, resulting in lower capillary-to-fiber ratio (C:F), and ultimately affecting regional hemodynamic regulation (Kindig 1998a; Sexton 1994). In addition to insulin therapy, regular exercise is an established management strategy for type-1 diabetes, improving glucose uptake and insulin sensitivity within both the skeletal muscle and adipose tissue (Galassetti 2013). Additional health benefits include increased cardiorespiratory fitness, improved endothelial function, increased vascular health and quality of life (Chimen 2012; Galassetti, 2013). Regular endurance exercise has been shown to increase skeletal muscle capillarization in healthy individuals (Hudlicka 1992a). However, the effects of endurance training on changes in capillarization in diabetic animals and patients are contradictory (Henriksson, 1992a; Kivela, 2006; Leinonen 1982; Wallberg-Henriksson 1984). Vascular function has been shown to improve with physical activity in both animal models of diabetes (Kivela, 2006) and in patients with type-1 diabetes (Chimen, 2012), but these alterations were unable to fully restore the diabetes-induced defects (Fuchsjager-Mayrl 2002; Mason 2006).

New capillary growth within the skeletal muscle occurs via existing capillaries through two morphologically different, and separately inducible, forms of physiological angiogenesis termed sprouting or non-sprouting angiogenesis (Egginton, 2011). Exercise training and

mechanical loading trigger sprouting angiogenesis, while vasodilation, such as through α-adrenergic antagonism, promotes non-sprouting angiogenesis (Egginton 2001; Milkiewicz 2001). Prazosin, an α_1-adrenergic antagonist, has been used in many rodent studies (Akerstrom, 2014; Dawson 1989a; Ziada 1989a), to selectively increase skeletal muscle C:F, but it has yet to be used as an agent to stimulate angiogenesis in an animal model of type-1 diabetes. However, chronic treatment with prazosin in streptozotocin (STZ)-induced diabetic rats reduces elevations in blood pressure and cholesterol levels and improves cardiac function (Goyal 1996). There is further evidence that α_1-adrenergic blockade, using prazosin, enhances hepatic function (Dubuisson, 2002; Oben, 2003), insulin sensitivity and the lipid profile in hypertensive and type-2 diabetic patients (Hirano 2001; Maruyama 1991b), in addition to decreasing blood pressure.

The aim of this study was to evaluate the independent and combined effects of exercise and prazosin administration on skeletal muscle capillary content in the STZ-induced rodent model of type-1 diabetes.

Methods

This study was carried out in accordance with the recommendations of the Canadian Council for Animal Care guidelines and was approved by the York University Animal Care Committee (2013-5). The Guide for the care and use of laboratory animals, Eighth edition (2011) was followed.

Animals: Adult, male Sprague-Dawley rats (Charles River Laboratories, initial mass of 225-250g, n=46) were individually housed (lights on 12 hour: lights off 12 hour cycle) after one week of acclimatization to room temperature (22-23°C) and humidity (50-60%) controlled facilities.

Experimental design: A timeline of the experimental protocol is shown in Fig. 5.1. All animals had access to voluntary running wheels for one week. Each exercising animal was placed into rodent cages with 24-hour access to a running wheel (Harvard Apparatus), while sedentary animals were housed in standard cages. Running distance was measured and reset daily. Seven days after wheel assignment, a single injection of streptozotocin (STZ) (65 mg/kg) was administered to induce diabetes. Animals were also provided with sugar water (20% sucrose) to assist with diabetes development, and any exercising animals had their wheels removed overnight to further promote diabetes induction. Two days after diabetes inducement animals were divided into one of five groups: Non-diabetic-SED-H_2O (Control), STZ-SED-H_2O, STZ-EX-H_2O, all given regular water, STZ-SED-Praz and STZ-EX-Praz, all given drinking water containing prazosin hydrochloride (5 mg/kg; P7791, Sigma Aldrich Canada). Five days after diabetes induction, all animals had their food reduced to 30% of their total body weight, to improve glycaemia in the diabetic animals. Body mass, food intake and fluid consumption were measured daily for each rodent and any changes in the rodents' health were noted and monitored.

Blood glucose, NEFA and corticosterone sampling: Blood glucose values were measured with a glucometer (AlphaTRAK, Abbott Laboratories, IL, USA) and 5ul of blood from the tip of the tail. Blood samples were collected from each animal via saphenous vein, for NEFA (NEFA kit, HR Series NEFA-HR, Wako Chemicals) and corticosterone (MP Biomedical, USA) concentration measurements on Days 1 (basal) and 32. Glucose area under the curve (AUC) was measured relative to each animal’s individual Day 11 glucose value and the net area was used to account for the lowering in blood glucose observed in both exercising groups.

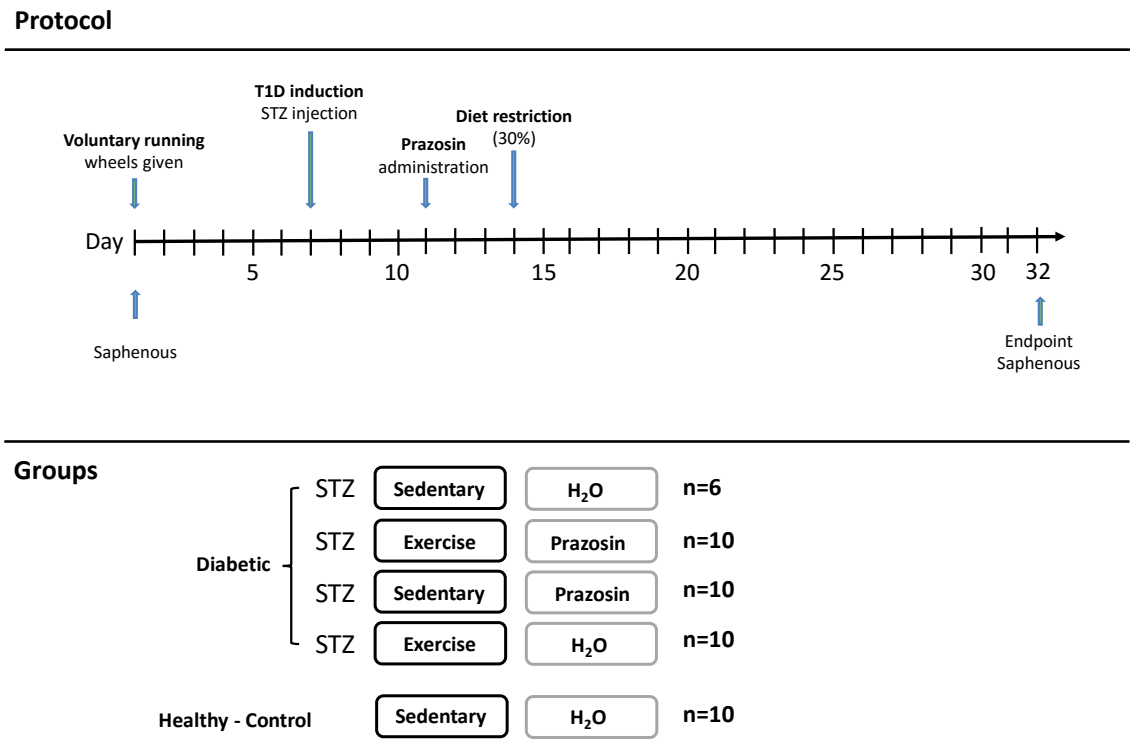


Figure 5.1. Schematic of the experimental design.

Basal saphenous vein measurement for corticosterone and non-esterified fatty acid (NEFA) concentrations occurred on *day 1*, prior to the provision of voluntary running wheels. Basal blood pressure was measured on *day 5*, while streptozotocin (STZ) was administered on *day 7* followed by another saphenous blood draw and blood pressure measurement on *day 9*. Prazosin was given on *day 11* and on *day 14* animals had their food reduced to 30% of their total body weight, to improve glycemia in the diabetic animals. *Days 16* and *23* were two more saphenous draws, while *day 19* was the final blood pressure measurement. Animals were euthanized on *days 29-32* (endpoint).

Capillary-to Fiber Analysis: Skeletal muscle, tibialis anterior (TA) and soleus, from euthanized animals, was embedded in tissue freezing medium, frozen in liquid nitrogen and cryosectioned (10 μm thick). TA and soleus sections (10 μm thick) were fixed with 3.7% para-formaldehyde prior to being stained with fluorescein isothiocyanate-conjugated Griffonia simplicifolia isolectin B4 (1:100; Vector Laboratories, Canada). Sections were viewed using a Zeiss M200 inverted microscope with a 20x objective and images were captured using Metamorph imaging software. Capillary-to-fiber counts were averaged from 5-7 independent fields of view per animal by a blinded observer.

Western Blotting: Immunoblotting was carried out on protein extracts from rat soleus or TA muscles as previously described (Aiken 2016). Frozen muscle (20 to 40 mg) was mixed at 4°C with RIPA buffer. For each sample, protein extracts were prepared using two stainless carbide beads (Retsch, Fisher Scientific, Montreal, Canada) in the Retsch MM400 tissue lyser (30 pulses/sec, Retsch GmbH, Haan, Germany). Denatured samples (30 μg/well) were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. After blocking with 5% fat-free milk at room temperature for 45 min, the blots were probed overnight at 4°C with primary antibodies against the following proteins: β-actin (sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Mdm2 (non-commercial clone 2A10; (Aiken, 2016; Roudier 2013; Roudier 2012), VEGF (clone VG-1; 05-1117; Millipore, Etobicoke, ON, Canada), or TSP-1 (clone A6.1; MS-421-P0; Invitrogen, Burlington, ON, Canada). After incubation with secondary antibody [cat. no. P0260; Dako, Carpinteria, CA, USA], proteins were visualized with chemiluminescence (Millipore) on Imaging Station 4000MM Pro (Carestream Health, Rochester, NY, USA) or on X-ray film (CL-XPosure Film; prod. no. 34090; Thermo Scientific, Rockford, IL, USA). Blots were analyzed with Carestream software.

Statistical Analysis: All data are represented as means ± SE, with a criterion of $P<0.05$ and $P<0.01$ and were assessed as stated using two-way ANOVAs as a means of statistical

significance. Individual differences were calculated using Bonferroni's post-hoc test (Statistica, StatSoft, Poland). A ttest was also used to compare values for non-diabetic and STZ-treated rats.

Results

Capillary-to-fiber content: The effect of STZ-induced diabetes on the skeletal muscle microvasculature was evaluated using the capillary-to-fiber ratio (C:F) expressed relative to muscle mass (Fig. 5.2). There was obvious capillary rarefaction in the TA of the STZ-SED-H_2O group when compared to the non-diabetic, control group (Fig. 5.2A and Fig 5.2A', $P<0.001$), which was paralleled within the soleus of the STZ-SED-H_2O animals (Fig. 5.3A and Fig. 5.3A', $P<0.001$).

C:F for both the STZ-SED-Praz and the STZ-EX-H_2O groups were significantly higher from the STZ-SED-H_2O group (Fig. 5.2B and 5.2B', $P<0.001$), although they were not significantly different from each other, suggesting that individually, both prazosin and exercise were capable of improving the diabetes-induced loss of capillaries toward that observed in non-diabetic control rats. The STZ-EX-Praz animals had significantly higher C:F in comparison to the STZ-EX-H_2O and STZ-SED-Praz animals ($P<0.001$), signifying that co-treatment exerted an additive effect on angiogenesis. We also observed an increase in C:F within the soleus of the STZ-SED-Praz animals ($P<0.01$) and the STZ-EX-Praz animals (Fig. 5.3B and Fig. 5.3B', $P<0.05$), however exercise was unable to improve the diabetes-induced rarefaction independently.

Circulating glucose concentrations: Hyperglycaemia was evident in all STZ-treated animals (Fig. 5.4A). When analyzed as an AUC from Day 11 onward (the date of prazosin initiation), there was a significant main effect of both exercise and prazosin to improve daily fed

glucose concentrations, with the most favourable response occurring in the STZ-EX-Praz group (Fig. 5.4A', $P<0.01$), suggesting that the co-treatment produced the most beneficial result.

Running distance: Daily running distance was graphed in relation to prazosin administration (Fig. 5.5A) and no difference was found between the average running distance in the two exercising groups (Fig. 5.5B). A significant correlation was observed between individual mean blood glucose concentrations and cumulative running distances (Fig. 5.5C, $P<0.01$).

Corticosterone, NEFAs: STZ-induced diabetes affected the concentrations of circulating corticosterone and NEFAs. Prior to prazosin administration, corticosterone and NEFA concentrations were not different across the STZ-treatment group, so these data were pooled (Initial basal, Fig. 5.6A and 5.6B). All STZ-treated animals developed significantly elevated corticosterone concentrations (Fig. 5.6A, $P<0.001$) at the end of the treatment period, and only the prazosin group showed significantly decreased values (Fig. 5.6A', $P<0.05$). There was a significant effect of voluntary exercise, prazosin and their combination, to improve NEFA values (Fig. 5.6B', $P<0.05$).

Mdm2, VEGF-A, and TSP-1: VEGF-A and TSP-1, key pro- and anti-angiogenic molecules in skeletal muscle, are influenced by Mdm2, and these three markers have been used to understand the changes observed in skeletal muscle C:F. In both the TA and soleus muscles, STZ-treated rats had significantly decreased Mdm2 protein content vs. non-diabetic rats (Fig. 5.7A, A', 5.7B, B', $P<0.05$). Within the TA, Mdm2 protein content was lowest in the STZ-SED-H_2O group, but was only significantly lower than the STZ-SED-Praz group (Fig. 5.7A", $P>0.05$). Soleus Mdm2 protein content was not significantly modified in the four different treatment groups (Fig. 5.7B"). STZ-treatment tended to decrease VEGF-A protein content within both muscles vs. the non-diabetic group (Fig. 5.8A, A', 5.8B, B', $P>0.05$). While prazosin treatment

(STZ-SED-Praz) induced the highest VEGF-A protein content within the TA, no significant difference was obtained when compared to the other groups (Fig. 5.8A"). VEGF-A protein content within the soleus was not altered with either exercise or prazosin treatment (Fig. 5.8B"). TSP-1 protein content was found to be increased in STZ-treated rats in both muscles, but not significantly (Fig. 5.9A, A', 5.9B, B', $P>0.05$). Although no significant effect of exercise and prazosin was found within either the TA or soleus, TSP-1 tended to be higher in the TA from the STZ-EX-Praz group and in the soleus for both STZ-EX-H_2O and STZ-EX-Praz groups (Fig. 5.9A", B", $P>0.05$).

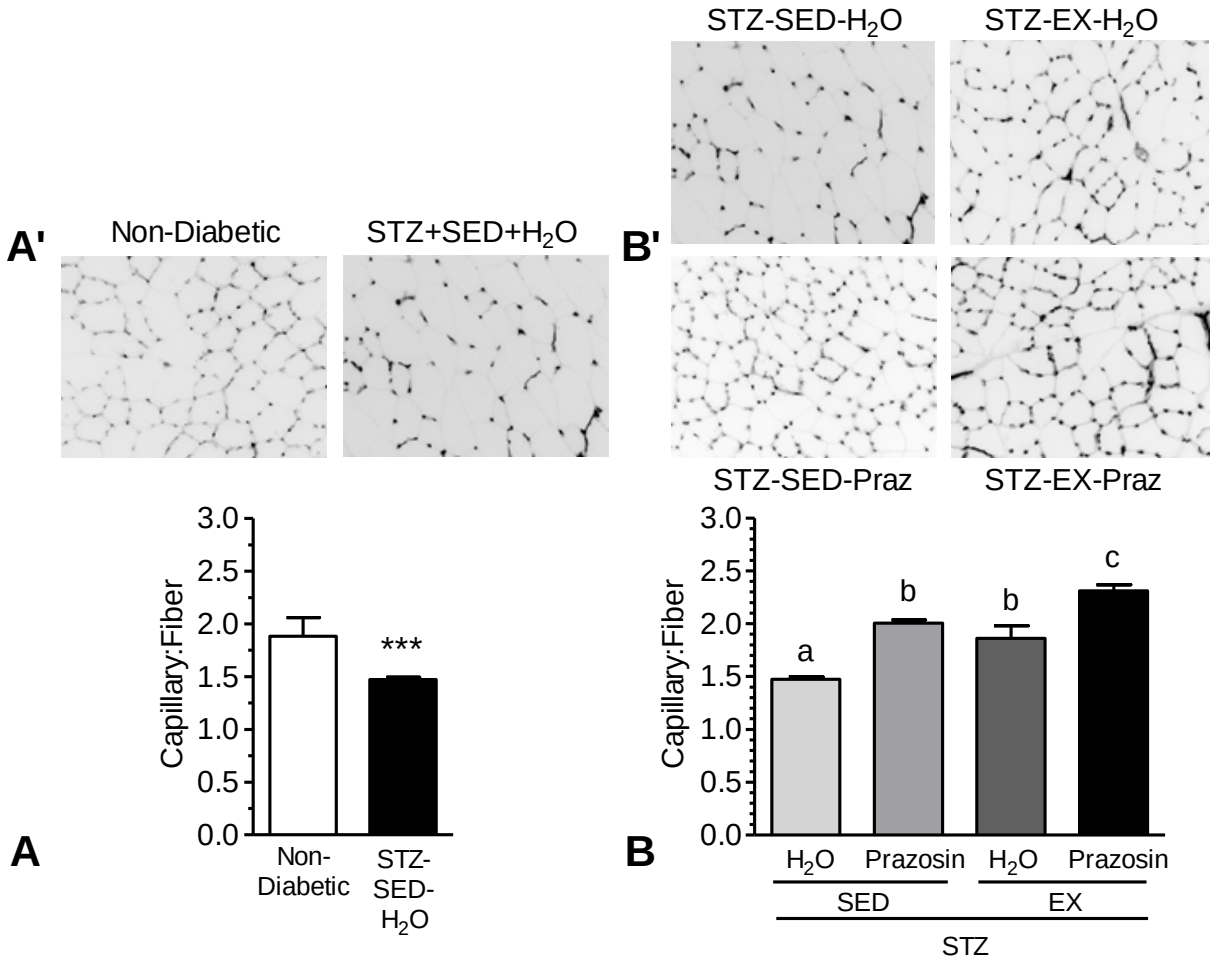


Figure 5.2. Capillary-to-fibre ratio within the tibialis anterior is improved after voluntary exercise and prazosin co-treatment.

Capillary-to-fiber ratio (C:F) in the tibialis anterior (TA) after 19 days of voluntary exercise and prazosin co-treatment (A, B); n=5. C:F was analyzed using a two-way ANOVA with Bonferroni's post hoc. A planned comparison was completed within the exercising groups (two-tailed T-test) to examine the effect of the co-treatment on C:F. * Significantly different from all groups (P<0.05), ** significantly different from STZ-EX-H_2O (P=0.02). All values are means ± SE.

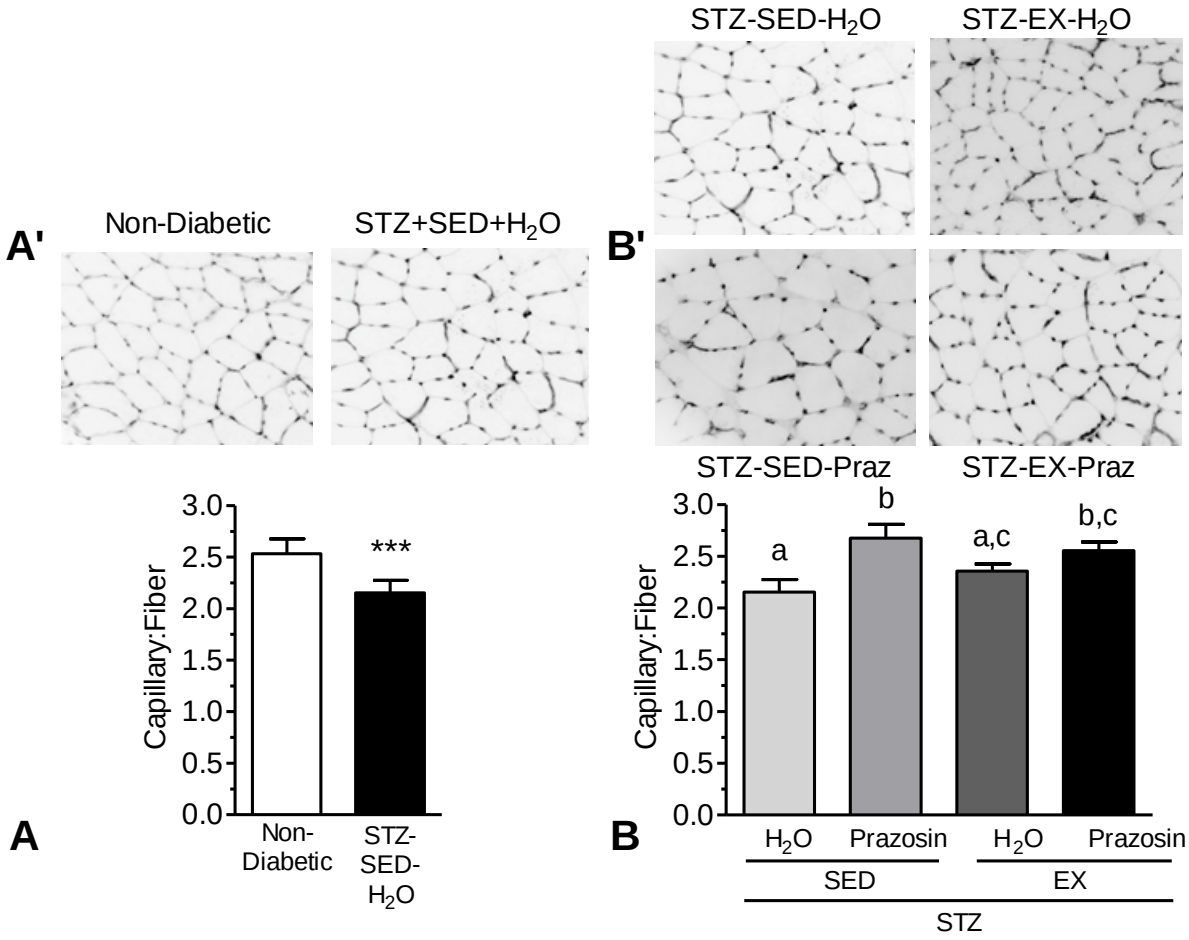


Figure 5.3. Capillary-to-fibre ratio within the soleus is improved with voluntary exercise and prazosin treatment.

Capillary-to-fiber ratio (C:F) in the soleus after 19 days of voluntary exercise and prazosin co-treatment (A, B); n=5. C:F was analyzed using a two-way ANOVA with Bonferroni's post hoc. *Significantly different from all groups (P<0.05). All values are means ± SE.

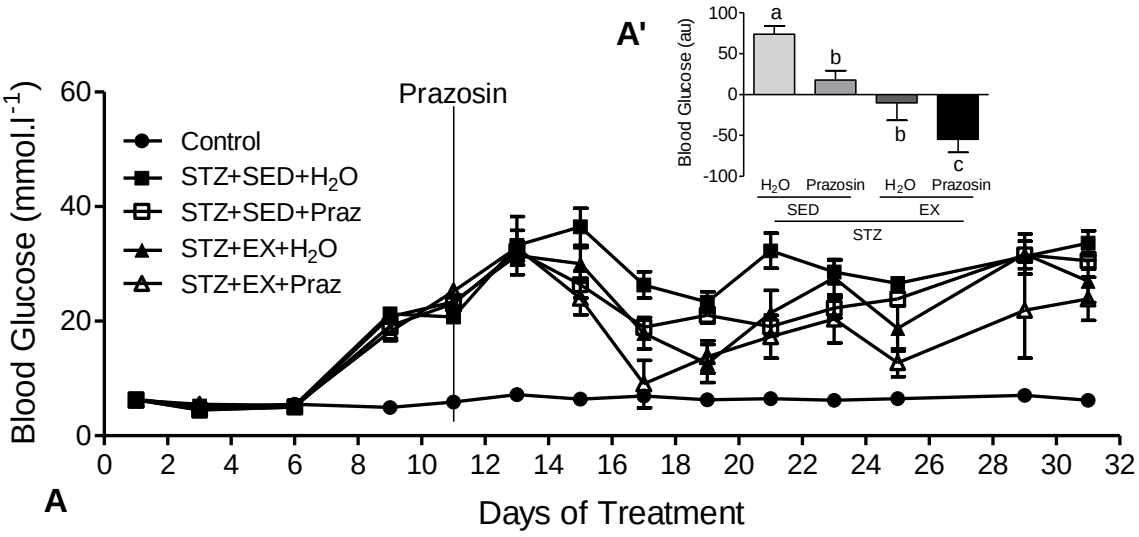


Figure 5.4. Voluntary exercise and prazosin co-treatment improves blood glucose concentrations.

Blood glucose values across the experimental timeline (A) and expressed as area under the curve (AUC; A`). AUC values are calculated from *day 11* (prazosin administration) until *day 32* (endpoint). A main effect of both prazosin (P<0.01) and exercise (P<0.01) to improve daily glucose concentrations was found, with the co-treatment (STZ-EX-Praz) producing the most significant improvement; n=6-10. The dotted line represents control AUC. AUC was analyzed using a two-way ANOVA with Bonferroni's post hoc. All values are means ± SE.

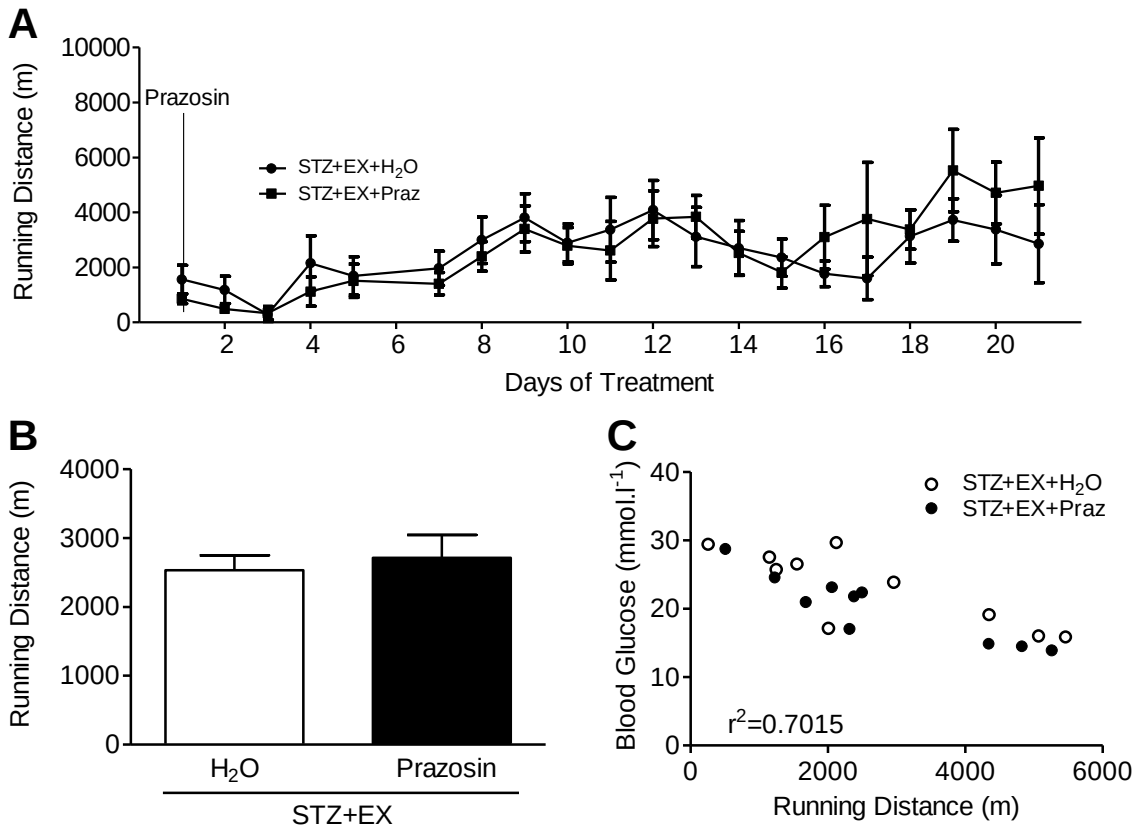


Figure 5.5. Voluntary running distance is correlated to improved blood glucose concentrations.

Voluntary running distances over time (A) and the total mean values (B) were graphed from the initiation of the co-treatment (prazosin administration). The mean values were correlated to mean blood glucose values (C) (r^2=0.7015, P<0.01); n=6-10. All values are means ± SE (A, B).

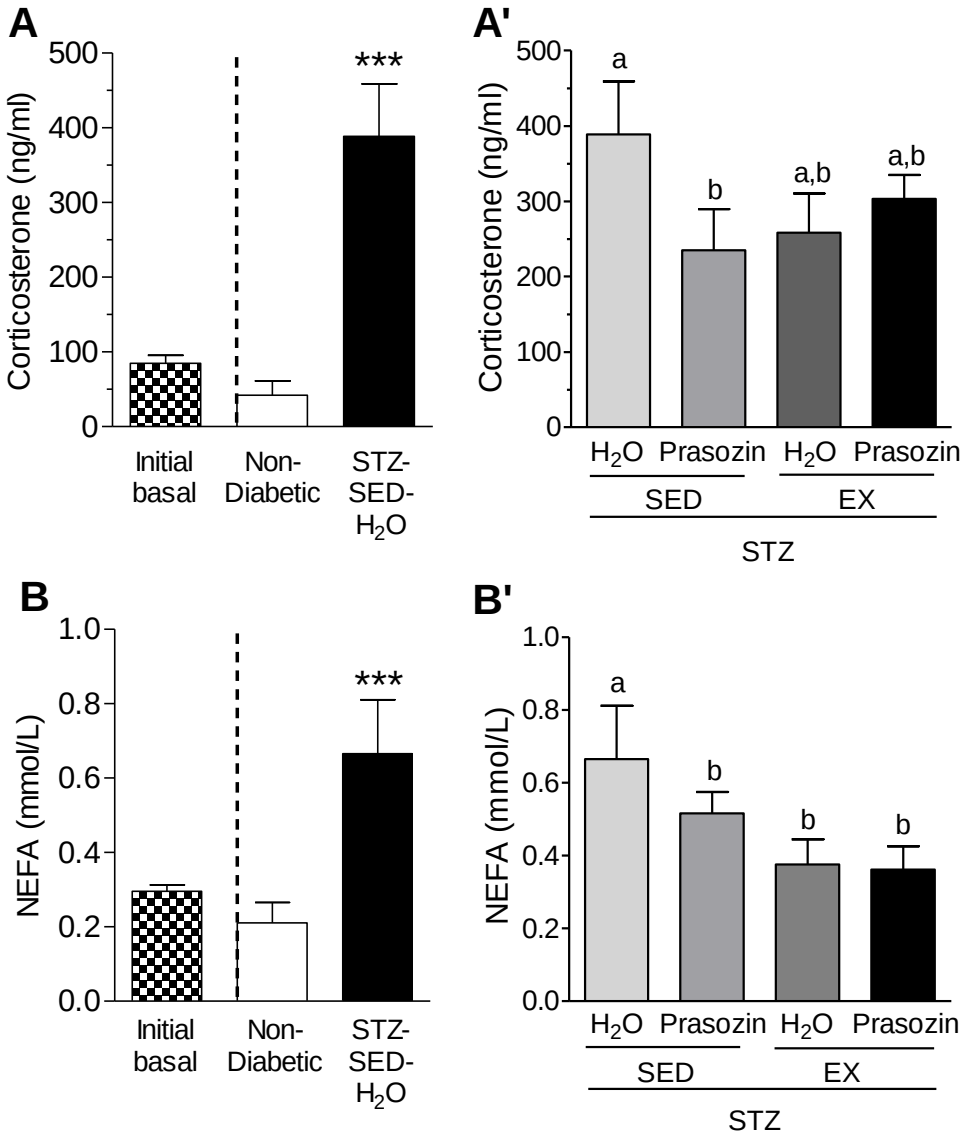


Figure 5.6. Corticosterone and non-esterified free fatty acid concentrations.

Corticosterone (A) and non-esterified fatty acid (NEFA) (B) concentrations were measured throughout the experimental protocol. The number of days relates to STZ injection. Basal is the cumulative data from 2 weeks prior to STZ injection. Prazosin was initiated on *day 11*. Bars that do not share similar letters, denote statistical significance ($P<0.05$) using a two-way ANOVA with Bonferroni's post hoc; n=6-10. All values are means ± SE.

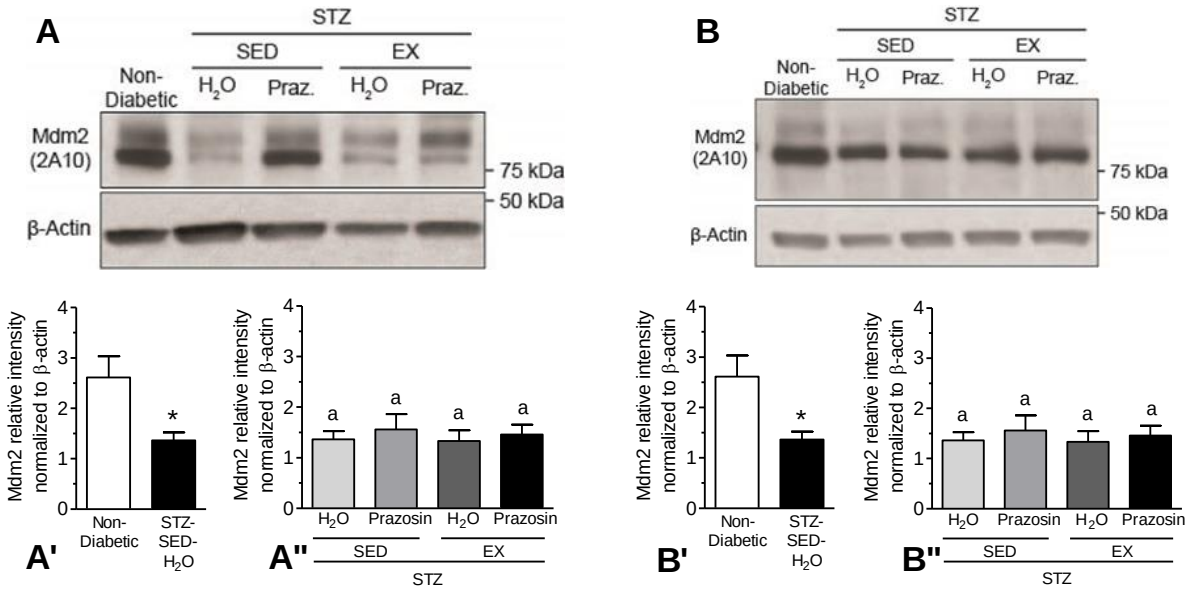


Figure 5.7. Skeletal muscle Murine Double Minute-2 protein levels are decreased in streptozotocin-induced type-1 diabetes.

A) Murine Double Minute-2 (Mdm2) protein in control (non-diabetic) and STZ-treated rat tibialis anterior (TA) muscles. A') Mdm2 protein in non-diabetic and STZ-treated rat TA muscle. $^{**}P \leq 0.01$ following unpaired Student's t-test analysis. A") Mdm2 protein in the TA muscles of sedentary, prazosin-treated sedentary, exercised, or exercised and prazosin-treated STZ-treated rats. Different letters indicate significant difference, $P \leq 0.05$ following 2-way ANOVA. B) Mdm2 protein in non-diabetic and STZ-treated rat soleus muscles. B') Mdm2 protein in non-diabetic and STZ-treated rat soleus muscle. $^{*}P \leq 0.05$ following unpaired Student's t-test analysis. B") Mdm2 protein in the soleus muscles of sedentary, prazosin-treated sedentary, exercised, or exercised and prazosin-treated STZ-treated rats. Same letters indicate no significant differences were found between groups following 2-way ANOVA. All data are means ± SEM (n=6 per group).

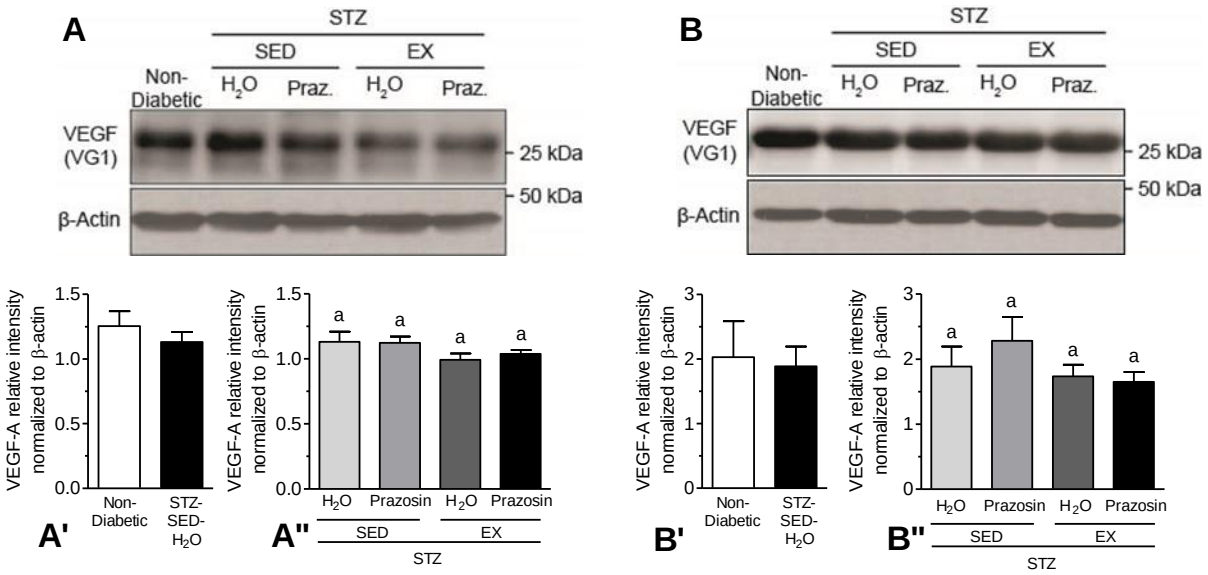


Figure 5.8.Streptozotocin-induced diabetes does not alter skeletal muscle vascular endothelial growth factor protein levels.

A) Vascular Endothelial Growth Factor (VEGF) protein in control (non-diabetic) and STZ-treated rat tibialis anterior (TA) muscles. A') VEGF protein in non-diabetic and STZ-treated rat TA muscle. No significant difference following unpaired Student's t-test analysis. A") VEGF protein in the TA muscles of sedentary, prazosin-treated sedentary, exercised, or exercised and prazosin-treated STZ-treated rats. B) VEGF protein in non-diabetic and STZ-treated rat soleus muscles. B') VEGF protein in non-diabetic and STZ-treated rat soleus muscle. No significant difference following unpaired Student's t-test analysis. B") VEGF protein in the soleus muscles of sedentary, prazosin-treated sedentary, exercised, or exercised and prazosin-treated STZ-treated rats. Same letters indicate no significant differences between groups following 2-way ANOVA. All data are means ± SEM (n=6 per group).

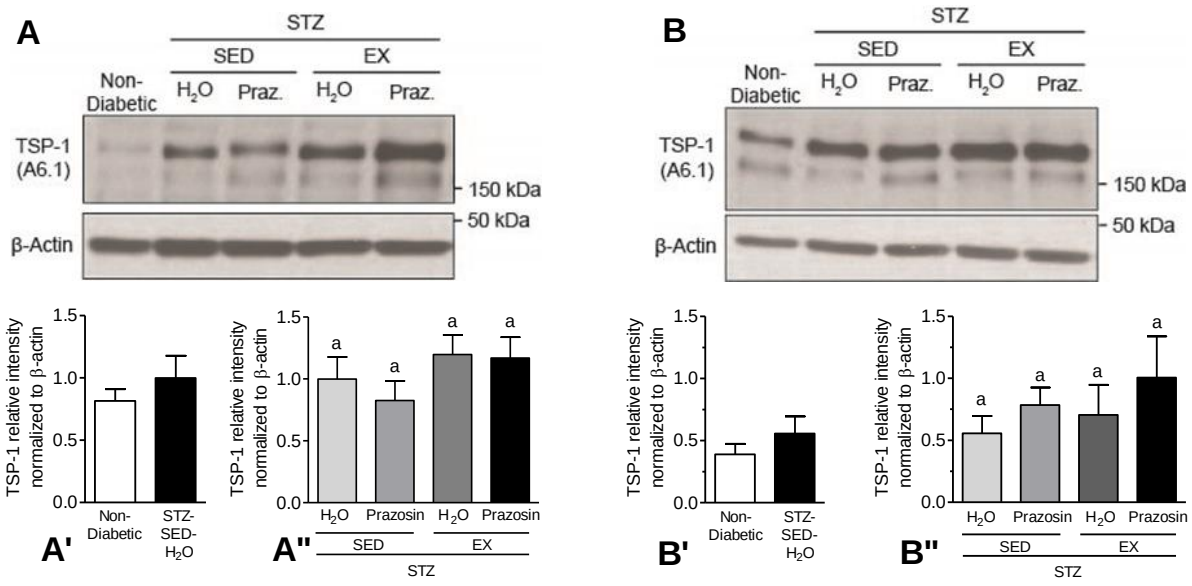
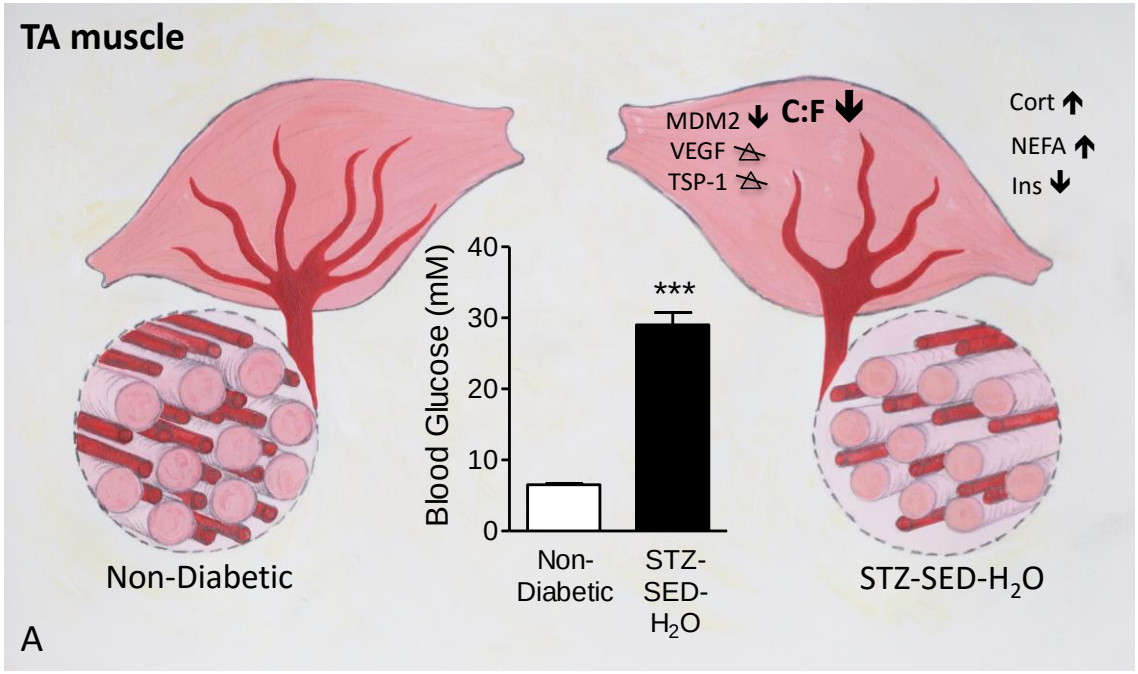


Figure 5.9. Streptozotocin-induced diabetes does not alter skeletal muscle thrombospondin-1 protein levels.

A) Thrombospondin-1 (TSP-1) protein in control (non-diabetic) and STZ-treated tibialis anterior (TA) muscles. A') TSP-1 protein in non-diabetic and STZ-treated rat TA muscle. No significant difference following unpaired Student's t-test analysis. A") TSP-1 protein in the TA muscles of sedentary, prazosin-treated sedentary, exercised, or exercised and prazosin-treated STZ-treated rats. B) TSP-1 protein in non-diabetic and STZ-treated rat soleus muscles. B') TSP-1 protein in non-diabetic and STZ-treated rat soleus muscle. No significant difference following unpaired Student's t-test analysis. B") TSP-1 protein in the soleus muscles of sedentary, prazosin-treated sedentary, exercised, or exercised and prazosin-treated STZ-treated rats. Same letters indicate no significant differences between groups following 2-way ANOVA. All data are means ± SEM (n=6 per group).


TA muscle
MDM2
C:F
VEGF
TSP-1
Cort
NEFA
Ins
Blood Glucose (mM)
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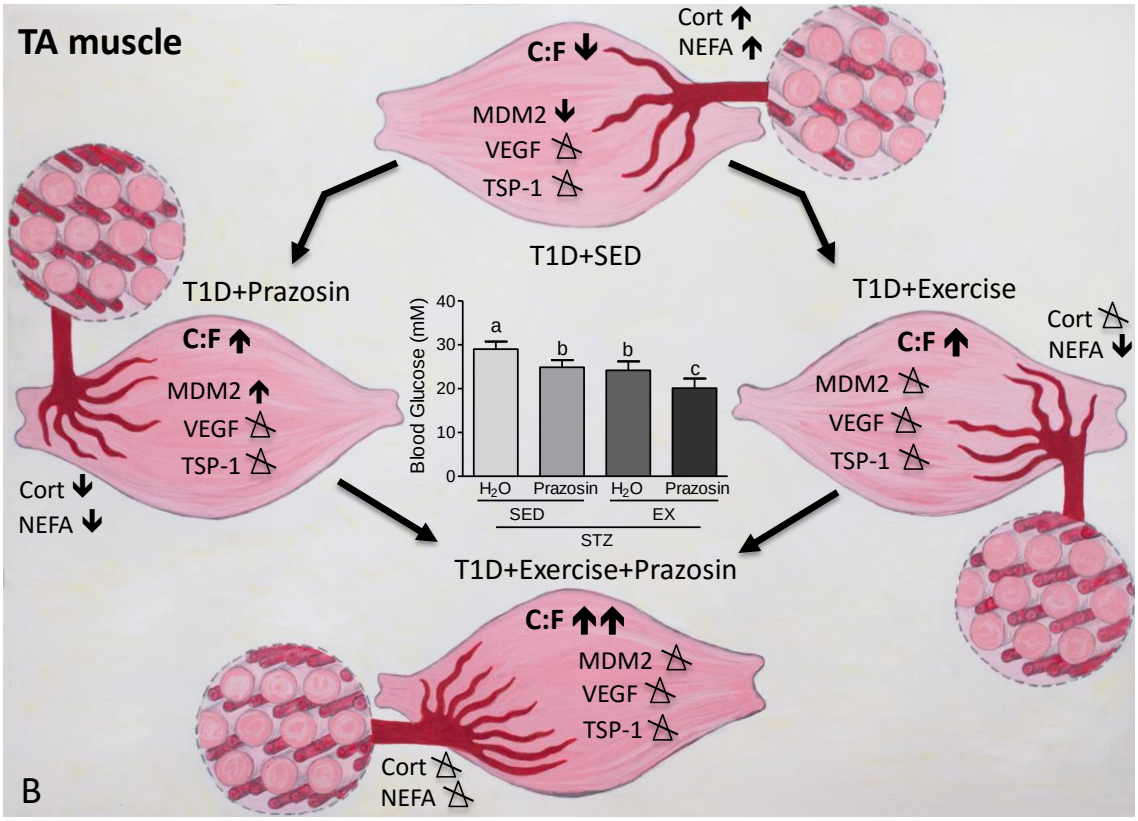
TA muscle
Cort
NEFA
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MDM2
VEGF
TSP-1
T1D+SED
T1D+Prazosin
T1D+Exercise
Cort
NEFA
C:F
MDM2
VEGF
TSP-1
Blood Glucose (mM)
40
30
20
10
0
a
b
b
c
H2O
Prazosin
H2O
Prazosin
SED
EX
STZ
Cort
NEFA
C:F
MDM2
VEGF
TSP-1
T1D+Exercise+Prazosin
C:F
MDM2
VEGF
TSP-1
Cort
NEFA
B

Figure 5.10. Summary of the effects of prazosin and exercise within the tibialis anterior (TA) skeletal muscle of streptozotocin-treated rats.

A) STZ-treatment causes increased fed blood glucose levels, elevations in corticosterone (Cort), NEFA and decreased insulin (Ins) content. Within the TA, STZ-treatment decreased capillary-fiber ratio (C:F) and Murine Double Minute-2 (Mdm2) protein content while both Vascular Endothelial Growth Factor (VEGF) and Thrombospondin-1 (TSP-1) were unaffected. (B). STZ-treated rats were exposed to either prazosin treatment, voluntary exercise, or a combination of both. Individually, prazosin and voluntary exercise increased C:F and decreased both NEFA and fed blood glucose concentrations. Prazosin treatment increased Mdm2 protein content while VEGF and TSP-1 were unaffected. Voluntary exercise caused no alterations to angiogenic proteins. The combination of prazosin with voluntary exercise resulted in the most improved fed blood glucose concentrations and C:F. Both Cort and NEFA concentrations were unaffected and there were no alterations to angiogenic proteins.

Discussion

Uncontrolled type-1 diabetes has been shown to cause significant impairments within the skeletal muscle vasculature, primarily due to hyperglycaemia and endothelial dysfunction (Costantino 2015). Using a combined therapeutic approach, we have shown that exposure to 19 days of combined voluntary exercise and prazosin treatment improved the C:F within the TA muscle and normalized the C:F within the soleus muscle of STZ-treated rats. The co-treatment caused additional improvements in glycaemic control and circulating lipid levels, thereby suggesting that the two treatments can act in an additive fashion to improve muscle capillarization and metabolism in this animal model of type-1 diabetes. To our knowledge, this is the first time that these two therapeutic modalities have been combined in an animal model (health or disease) and suggests that there is likely an additive effect of both prazosin and exercise on angiogenesis and metabolic rescue in diabetes.

The growth of new capillaries in response to exercising muscle is a highly regulated process (Gavin, 2009), which is stimulated by increased functional hyperaemia and shear stress (Hellsten 2014), mechanical stretch of the tissue in addition to increases in metabolic demand or reduced oxygen delivery (Egginton, 2009). These signals modulate the expression and activity of pro-angiogenic and angiostatic factors which act together to regulate sprouting angiogenesis (Egginton, 2011). Prazosin, an α_1-adrenergic antagonist, increases skeletal muscle capillarization via a VEGF- and endothelial nitric oxide synthase-dependent pathway (eNOS) (Baum 2004; Williams 2006a), ultimately causing longitudinal splitting of existing capillaries (Zhou 1998), which is distinct from the angiogenic mechanism that is dominantly observed in exercising muscle.

It is well established that endurance exercise promotes new capillary growth (Hudlicka, 1992a) in both animals and healthy humans (Olfert, 2016), however, literature regarding the

effect of exercise on the pathological effects of type-1 diabetes within the skeletal muscle vasculature is inconclusive (Henriksson, 1992a; Wallberg-Henriksson, 1984). In healthy rats, exercise training causes increased VEGF mRNA and protein content (Gavin, 2009), which stimulates further enhancements in capillarization. When examined in STZ-induced diabetic mice, treadmill endurance training was unable to improve quadriceps femoris cross-sectional fiber area or C:F after five weeks of exercise training (Kivela, 2006). In our study, uncontrolled STZ-induced diabetes caused significant capillary rarefaction in both the TA (Fig. 5.2A, A') and soleus muscles (Fig. 5.3A, A'), which is consistent with previous studies (Krause 2009; Sexton, 1994). When given access to voluntary exercise wheels, STZ-induced diabetic rats showed a significant increase in C:F in the TA (Fig. 5.2B, B') and a slight, but not significant, tendency to increase C:F in the soleus (Fig. 5.3B, B') when compared to sedentary STZ-treated rats. The exercise modality in our study was voluntary wheel running and this might have elicited different muscle recruitment between the TA vs. soleus muscles (Kano 1997), although there was no difference in volume of exercise between treatment groups (Fig. 5.5A, B). Additionally, angiogenic potential appears to be inversely proportional to the original capillarity, i.e. it is easier to induce in fast muscle than slow or cardiac muscle (Egginton, 2009), another possible explanation for the muscle type-specific differences observed in capillarity.

The enhancement of skeletal muscle capillarization, through non-sprouting angiogenesis with prazosin treatment, results in an increase in capillarity in glycolytic and oxidative skeletal muscles in healthy rats (Akerstrom, 2014; Dawson, 1989a; Ziada, 1989a). However, to our knowledge, this study is the first to illustrate the beneficial effects of prazosin on skeletal muscle capillary rarefaction in STZ-induced diabetic rats. We observed a normalization of C:F in both TA and soleus muscles of sedentary, STZ-treated rats as their C:F values were equivalent to

those measured in the control group. When coupled with wheel running, there was an additive effect on the angiogenic response, specifically in the TA. It is worth noting that the skeletal muscle angiogenesis observed within both the TA and soleus (the normalization) occurred amidst significantly elevated circulating corticosterone levels (Fig. 5.6A, A'), a hormone which has been linked to capillary rarefaction (Shikatani, 2012). In addition to the fact that both treatment modalities result in distinct forms of angiogenesis, there is data suggesting that the combination of high-volume, low-intensity exercise, like voluntary wheel running, actually elicits a more rapid angioadaptive response than what is observed after treadmill exercise training (Olfert, 2016).

Exercise is a widely recognized strategy for improving glycaemia in diabetes (Chimen, 2012), as regular exercise leads to the improvement of whole-body glucose and lipid metabolism and enhanced skeletal muscle glucose disposal (Goodyear 1998a) and insulin sensitivity (James 1983; James 1985). We observed a significant effect of voluntary exercise to improve glycaemic control in the STZ-treated rats, and this result was amplified when the animals were co-administered prazosin (Fig. 5.4A, A'). These improvements in blood glucose, regardless of prazosin administration, were also correlated to running distance (Fig. 5.5C), suggesting that higher running volumes caused better glycaemic control. In our study, sedentary STZ-induced diabetic rats treated with prazosin also showed improved plasma glucose values throughout the experimental protocol, an unexpected observation as the literature regarding the action of prazosin on glycaemia is inconsistent (Barbieri 1980; Goyal, 1996; Martin 1989). It seems that alterations to glycaemic status are generally accompanied by skeletal muscle angiogenesis, an effect that was recently observed in healthy rats (Akerstrom, 2014) thereby suggesting that increased capillarization likely enhances insulin sensitivity.

The angiogenic response is controlled by a dynamic balance between anti- and pro-angiogenic factors (Olfert, 2011). VEGF-A and TSP-1 are key pro- and anti-angiogenic molecules (Malek 2009; Olfert 2009). Interestingly, we have recently brought evidence that the E3 ubiquitin ligase Mdm2 could be a central regulator of skeletal muscle angiogenesis, partly by regulating VEGF-A and TSP-1 expression (Aiken, 2016; Roudier, 2012).

Our current results show, for the first time, that STZ-treated rats had a significant decrease in Mdm2 protein levels in both the TA and soleus muscles, as summarized in Fig. 5.10A. This reduction in Mdm2 could explain the lowered skeletal muscle capillarization in both muscles in sedentary diabetic rats, as Mdm2 protein levels have been shown to be closely related to endothelial content within the skeletal muscle (Roudier, 2012). It was previously observed that both capillarization and Mdm2 protein levels were significantly lower in a model of type-2 diabetes and in skeletal muscles from Zucker diabetic fatty (ZDF) rats (Roudier, 2012). Further results demonstrated that voluntary running efficiently restored both the impaired skeletal muscle capillarization and Mdm2 protein levels within the ZDF rats. In contrast to these findings, the voluntary exercise stimulus applied within our study did not rescue the observed decrease in Mdm2 protein in the diabetic animals (Fig. 5.10B), and suggests that the physiological response of Mdm2 to exercise could be lost in uncontrolled, type-1 diabetic muscle.

No significant alterations in TSP-1 or VEGF-A protein levels in response to diabetes induction, prazosin treatment or voluntary exercise were observed in either the TA or soleus muscles. In contrast, Kivela and colleagues (Kivela, 2006) have shown that STZ-induced diabetic mice had lower VEGF-A protein levels at 3 and 5 weeks post-diabetes induction and a concomitant increase in TSP-1 expression. In that study, while exercise served to delay the reduction in VEGF-A levels, it was not sufficient to attenuate the elevation in TSP-1 mRNA.

The C:F is increased as early as 14 days of treadmill running in rodent skeletal muscle (Slopack 2014), therefore we hypothesize that alterations in VEGF-A and TSP-1 protein levels could have occurred at an earlier time point to stimulate the growth of capillaries. While VEGF-A protein could have been elevated prior to the measured time point in order to induce the angiogenic process, the trend for an increase in TSP-1 protein levels could indicate the stopping or slowing down of capillary growth, as the balance between oxygen and nutrient supply and demand had been achieved in the skeletal muscle.

In summary, the significant improvement to glycemic control observed after co-treatment with voluntary exercise and prazosin administration could be the result of enhancements in insulin sensitivity and lipid metabolism, increased skeletal muscle glucose disposal and possibly improved diffusion conditions for glucose in the muscle as the result of heightened skeletal muscle angiogenesis. These results suggest that the combination of both voluntary exercise and prazosin administration could lead to a cooperative improvement in peripheral vascular complications linked to type-1 diabetes and may perhaps prevent future complications through the augmentation of skeletal muscle capillarization and glycaemic control.

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Chapter 6

Summary of findings and general discussion

The effect of diabetes mellitus within the skeletal muscle is profound and involves a number of key alterations that typically occur over a prolonged period of time. Both T1DM and T2DM are associated with elevations in GC concentrations, which are well-known to cause a variety of negative systemic side effects. The focus of this dissertation was to understand how elevations in GC concentrations can be associated with skeletal muscle insulin resistance and diabetes development and whether augmentation to the muscle microvasculature is beneficial or even possible during conditions that generate significant exposure to elevations in GCs. To examine these situations, two rodent models of diabetes were used; exogenous elevations in GCs either combined with a HFD (ROD model) or provided a standard chow diet, to reproduce T2DM, and STZ-treated rats to induce β-cell destruction, simulating T1DM.

GCs are associated with increased preference for energy dense foods (Dallman, 2010; Dallman, 2005) (high fat, simple sugars) and are often associated with excessive weight gain (Zakrzewska, 1999). Dexamethasone, a synthetic analogue of cortisol, administered to diet-induced obese mice, caused whole body glucose intolerance, insulin resistance and significant ectopic fat redistribution, especially within the TA muscle (Gounarides 2008). To further understand the mechanisms of altered metabolism in conditions of exogenous GC therapy, a rodent model of elevated GCs and high fat feeding was developed (Shpilberg, 2012). With two weeks of exogenous corticosterone treatment (4x100mg pellets) plus a HFD, male SD rats become severely hyperglycemic, hyperinsulinemic (Beaudry, 2013), and insulin resistant, they develop hepatic steatosis (D'souza, 2011), visceral adiposity with increased nonesterified free fatty acids (NEFAs) and ectopic fat deposition. What is interesting about this model is that with

corticosterone or a HFD alone, the animals do not develop the same impairments, but when the two treatment conditions are combined, there is a synergistic effect that amplifies the detrimental actions. However there is incomplete data examining this relationship within skeletal muscle.

Manuscript #1, entitled, 'Severe skeletal muscle metabolic impairments occur in response to concurrent high-fat feeding and glucocorticoid treatment in male Sprague Dawley rats' investigated the effect of exogenous corticosterone and a HFD, separately and in combination, on skeletal muscle, as it is a major determinant of whole body insulin sensitivity and T2DM development. Our findings confirm that high concentrations of GCs cause pronounced and targeted glycolytic skeletal muscle atrophy and insulin resistance, alterations which are augmented in response to the added lipid content associated with high-fat feeding. More specifically, the results expressed within this manuscript, suggest that while elevated GCs in isolation can cause significant skeletal muscle modifications, including impaired ISGU, reduced CSA and insulin signaling protein expression, the added HFD which caused much more IMCL accumulation, intensified all of these already detrimental effects. This information is critical for patients who are receiving exogenous GC therapy, as it shows how damaging poor nutrition or diet choices can be on skeletal muscle metabolism.

There is significant data proving that regular exercise is capable of improving glucose tolerance, insulin sensitivity, skeletal muscle insulin signaling and atrophy, in addition to ectopic lipid deposition both during and post exogenous GC administration (Barel, 2010; Beaudry, 2012; Czerwinski 1987; Lapier, 1997; Thomasson 2011). Much less is known in regards to the effect of regular exercise in rats fed a HFD while undergoing exogenous GC treatment. To further investigate possible therapeutic options for those individuals taking exogenous GCs and possibly consuming a diet high in fat, manuscript #2 entitled 'Voluntary exercise improves metabolic

profile in high-fat fed glucocorticoid treated rats' was focussed on assessing voluntary exercise as a potential mechanism to improve the metabolic side effects, including whole body glucose intolerance and insulin resistance, caused by this co-treatment. There was an additional focus on the skeletal muscle, as the significant atrophy and impaired glucose uptake now associated with this rodent model could be attenuated with regular exercise.

After 4 weeks of voluntary wheel running, the Cort-HFD animals showed improved glucose tolerance, insulin secretion, hyperglycemia, hyperinsulinemia and HOMA-IR values. There were also specific skeletal muscle improvements, with increased glycolytic muscle mass, TA CSA, oxidative capacity and capillarisation. An interesting aspect to this study was that these results were exercise volume specific, as less running correlated with a reduced amount of metabolic improvements, which provides practical human application. The results found within this manuscript offer further proof of the concept 'exercise is medicine' as a number of the improvements observed have also been seen with RU486 treatment, which is a pharmacological option for the treatment of hypercortisolemia. Improvements including glucose tolerance and insulin sensitivity (Bitar, 2001; Taylor 2009), lipid deposition, both within the viscera and skeletal muscle (Langley 1990; Okada 1992), and enhancements seen in skeletal muscle atrophy (Tischler, 1994), such as increased CSA (Wu 2010).

Both diabetes and chronic exposure to elevations in GCs are capable of causing capillary rarefaction through impaired skeletal muscle angiogenesis (Kivela, 2006; Shikatani, 2012). There is also a recognized relationship between circulating insulin levels, muscle microvascular perfusion and angiogenesis, which suggests that physiologic insulin concentrations can enhance muscle microvascular perfusion, without affecting total blood flow, subsequently enhancing muscle angiogenesis (Akerstrom, 2014; Kusters 2015a; Lambadiari 2015). There is additional

information that has found certain metabolic conditions, including obesity and diabetes, to blunt this response (Kivela, 2006; Li 2007) while pharmacologically enhanced hyperaemia can augment muscle microvascular recruitment (Cristiano Barbieri 1980; Dawson, 1989b). Manuscript # 3 entitled 'The metabolic effects of prazosin on insulin resistance in glucocorticoid-treated rats' investigated whether it was possible to stimulate skeletal muscle angiogenesis, using a model of chronic hyperaemia through α_1-adrenergic antagonism, in a rodent model of elevated GCs and insulin resistance, and whether the pharmacologically-induced vasodilation influenced glucose tolerance and insulin sensitivity. After 14 days of prazosin administration, concurrent with 16 days of exogenous Cort-treatment, there was a significant effect of prazosin to enhance the Cort-induced capillary rarefaction. The individual capillary-to-fibre values were correlated to improved insulin secretion throughout an OGTT, however there were no corresponding improvements in glucose uptake. These results are the first to examine the effect of prazosin administration in a model of GC-induced insulin resistance, and provide further evidence that there is a link between increased capillarization and insulin sensitivity. This study raised additional questions regarding the decreased insulin secreted during the OGTT. While technically these results imply an improvement in insulin sensitivity, when examined in concert with the glucose data, there seems to be a decrease in insulin secretion, even though there is still moderate hyperglycemia initiated by the OGTT. This suggests a possible impairment at the β-cell level, which was outside of the scope of this manuscript. Interestingly, after further examination of the skeletal musculature, it was evident that the pathophysiological concentrations of circulating corticosterone caused significant damage, through increased atrophic signaling, decreased CSA and IRS-1 protein expression, which could not be treated with amelioration to the microvasculature.

Impaired skeletal muscle vascularisation is not only the result of elevations in GCs, as both T1DM and T2DM result in micro- and macrovascular consequences thought to be induced through hyperglycemia and endothelial dysfunction. There are a number of studies that have examined the effect of exercise on the vascular system in healthy and T2DM subjects, and found exercise beneficial (increased VEGF expression, improved endothelial dysfunction and enhanced angiogenesis), while the research examining exercise within T1DM skeletal muscle is more limited and contradictory (Henriksson, 1992b; Leinonen. H., 1982; Wallberg-Henriksson, 1984). Regular exercise has been shown to improve vascular function in both animals (Kivela, 2006) and patients (Chimen, 2012) with T1DM, but these alterations were unable to fully restore the diabetes-induced defects (Fuchsjager-Maryl 2002).

There is recent evidence highlighting the concept that intusseusception/longitudinal splitting, which can be induced through prazosin administration or 'passive' training, requires less energy and therefore results in more rapid capillary adaptation when compared to exercise-induced sprouting angiogenesis (Prior, 2004). This has led to the investigation into the utilization of multiple modes of capillary growth to provide further magnification of the angiogenic stimulus. Currently, the potential different stimuli and associated molecular responses in relation to the combination of both pharmacologically-induced and exercise-induced angiogenesis are still poorly understood and future investigation will help to improve models of exercise to maximize the benefits of rehabilitation therapies in the clinic.

The objective of manuscript #4, entitled 'The effects of voluntary exercise and prazosin on capillary rarefaction and metabolism in streptozotocin-induced diabetic male rats' was to determine whether it was possible to enhance skeletal muscle angiogenesis in a model of chemically-induced diabetes (STZ-treated rats) through the co-administration of prazosin and

voluntary exercise, which cause new capillary growth through distinct mechanisms. The results found within this manuscript are the first to demonstrate both a normalisation and significant improvement in the skeletal muscle capillary rarefaction caused by untreated chemically-induced T1DM, with prazosin administration and voluntary wheel running. These important angiogenic effects also occurred during chronic exposure to elevations in GC levels, an unfortunate consequence of untreated diabetes. There was an additional benefit of improved glycaemia, which was correlated to exercise volume, and was the most improved in the prazosin-exercise group. The exercising animals also saw considerable reductions in NEFA levels over the treatment period, which could further enhance glycaemic control. The results observed in this manuscript underscore the therapeutic potential of inducing capillary growth through multiple pathways which could possibly prevent the peripheral vascular complications that are associated with T1DM.

Overall this thesis reveals that there is a significant relationship between elevations in GC concentrations, skeletal muscle insulin resistance and diabetes development; an association which becomes magnified with the addition of a HFD. This thesis provides considerable evidence proving that manipulation of the skeletal muscle microvasculature, either through voluntary exercise or pharmacologically enhanced hyperemia, can attenuate the hypercortisolemia-induced skeletal muscle capillary rarefaction and improve glucose metabolism through enhanced glucose uptake and insulin sensitivity (Figure 6.1).

Limitations

Although the research completed within this thesis reached its objectives, there are a number of important limitations associated with the major findings established within this dissertation. The study design in Chapter 2 is lacking a placebo standard diet (SD) control group. The primary measurement within this manuscript was the insulin-stimulated glucose transport data, and unfortunately there were some technical difficulties associated with the collection of the placebo-SD group, culminating in an n of 2. Overall, the lack of a placebo-SD control group makes it difficult to definitively determine the effects of Cort-treatment and a HFD, however, previously published work demonstrates that there is minimal to no difference between the control-HFD group and the control-SD group (Beaudry, 2013; Shpilberg, 2012) in regards to glucose tolerance and insulin sensitivity. This suggests that the control-HFD group would make an adequate primary control group. Further, within this same manuscript, the use of 3 O-H methyl glucose instead of 2 deoxyglucose, as a radioactive tracer to measure insulin-stimulated glucose transport, means it is possible that the effects of GCs on hexokinase action within the skeletal could have been missed. Overall, it is possible that the observed results under-represent the already significant skeletal muscle impairments induced with Cort-treatment, as it would be hypothesized that the Cort-treated rats would have impaired hexokinase activity. Finally, the study design of Chapter 3, through the utilization of voluntary running wheels, did not allow for the analysis of either exercise intensity or any possible acute effects of voluntary exercise. While the use of voluntary running wheels provided a more realistic exercise stimulus, as the rats were not forced to exercise train and could more accurately represent human activity, there was not a consistent exercise intensity for which to compare te obsereved adaptations. Also, as the animals were sacrificed throughout the day, and not immediately following wheel running, any possible

acute adaptations associated with exercise were not measured. However, the voluntary exercise stimulus used showed an obvious effect to improve a number of metabolic and muscular detriments caused by Cort-HFD treatment.

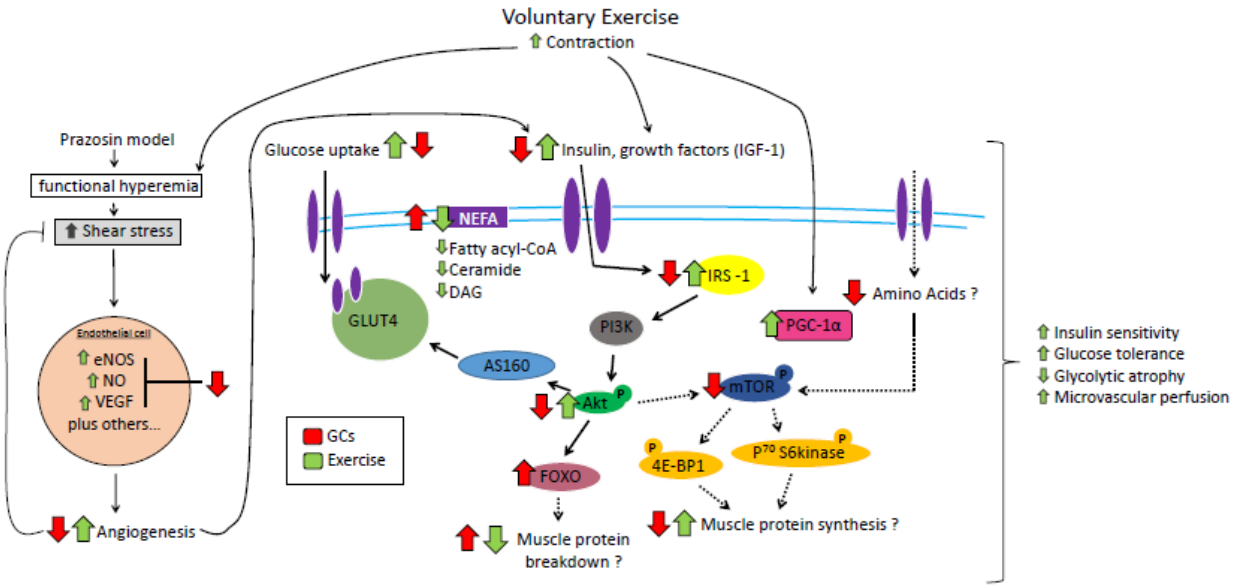

Figure 6.1. Proposed effects of voluntary exercise and prazosin on insulin resistant skeletal muscle.

Glucocorticoids (GCs) exert specific actions on select proteins within the skeletal muscle cell regulating insulin signaling and the endothelial cell regulating angiogenesis (highlighted with). The targeted response being decreased insulin sensitivity leading to impaired insulin-stimulated glucose uptake, significant glycolytic atrophy and capillary rarefaction. The introduction of exercise (voluntary wheel running) and increased shear stress (prazosin) (highlighted with), caused the attenuation and in some cases, reversal, of the specific impairments associated with elevations in GCs. Voluntary exercise resulted in the largest improvements, causing enhanced insulin sensitivity, glucose tolerance and angiogenesis, in addition to attenuated glycolytic atrophy, however further examination of the mechanism behind this improvement is warranted.

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