Diet-Induced Obesity Myopathy

IMPACT OF DIET-INDUCED OBESITY ON MOUSE SKELETAL MUSCLE

HEALTH: METABOLISM, GROWTH AND REGENERATION

By

KARIN ELIZABETH TRAJCEVSKI, B.Sc, M.Sc

A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the

Requirements for the Degree Doctor of Philosophy

McMaster University DOCTOR OF PHILOSOPHY (2013) Hamilton, Ontario (Medical Sciences)

TITLE: Impact of diet induced obesity on mouse skeletal muscle health: metabolism, growth and regeneration.

AUTHOR: Karin E. Trajcevski, B.Sc. (York University), M.Sc. (York University)

SUPERVISOR: Professor Thomas J. Hawke

NUMBER OF PAGES: xiii, 165

ABSTRACT

Prediabetes can lead to Type II Diabetes Mellitus, yet Prediabetes is a disease in its own right with its own physiological complications. Despite the pervasiveness of Prediabetes in our society and the negative impact on current and future health the extent of myopathy, short of muscle insulin resistance, and the mechanisms behind development of muscle insulin resistance remains unclear. Animal models of diet-induced obesity (DIO) have been employed to assess development of muscle insulin resistance and changes to muscle health. However there is a lack of clarity as to the molecular mechanisms leading to muscle insulin resistance. The goal of the studies presented here was to elucidate changes to muscle health and potential mechanisms contributing to muscle insulin resistance in response to DIO. Since the ability to perform exercise is to date one of the best therapies for Prediabetes and exercise contributes to a healthy muscle mass, the ability of muscle to undergo proper regeneration was also assessed following DIO. The results presented in this work demonstrate that skeletal muscle tissue adapts to increased dietary lipid by an early increase in functional lipid oxidation, mitigating IMCL deposition, despite glucose intolerance. Unfortunately this adaptive response is reversed with prolonged dietary fat intake and the development of muscle insulin resistance. Of note was the stronger link between IMCLs and muscle insulin resistance, compared to inflammation. DIO also led to decrements in satellite cell functionality which, along with physiological changes to HGF content and signaling, likely resulted in the observed impairment in regenerative ability. The results reported here improve our understanding of changes to muscle health and the mechanisms behind development of muscle insulin

resistance with DIO. These findings have implications for therapies and treatments for Prediabetes.

ACKNOWLEDGEMENTS

my husband, words cannot express the importance of your support and encouragement over the years. This work is dedicated to my family in thanks of their support.

PREFACE

The work presented here is a sandwich style thesis. There is a general introduction to the concepts relating to experiments performed. Chapters 2, 3 and 4 constitute manuscripts that have been published (Chapters 2 and 3) or will be submitted for publication in the future (Chapter 4). Preceding each manuscript chapter is a preface detailing the significance to the thesis as a whole and contributions of each author to the work.

All chapters have been reproduced with permission of all co-authors. Irrevocable, non-exclusive licenses have been granted to McMaster University and to the National Library of Canada from all publishers. Copies of permission and licenses have been submitted to the School of Graduate Studies.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 3

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF ABBREVIATIONS

ATP: adenosine tri-phosphate

ANOVA: analysis of variance

AU: arbitrary units

β-HAD: beta-hydroxyacetyl coenzyme A dehydrogenase

CS: citrate synthase

CTX: cardiotoxin

DAG: di-acylglycerols

DAPI: 4,6- diamidino-2-phynylindole

DIO: diet-induced obesity

ECM: extracellular matrix

EDL: extensor digitorum longus

ERK 1/2: extracellular signal-regulated protein kinase 1 and 2

ETC: electron transport chain

GP: gastrocnemius-plantaris complex

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

H&E: hematoxylin and eosin

HFD: high fat diet

IFG: impaired fasting glucose

IGF-1: insulin-like growth factor 1

IGT: impaired glucose tolerance

IL-6: interleukin-6

IL-10: interleukin-10

IMCL: intramyocellular lipids

IPGTT: intraperitoneal glucose tolerance test

IPITT: intraperitoneal insulin tolerance test

IRS-1: insulin receptor substrate 1

LCFA-CoA: long chain fatty acyl CoA

MMP: matrix metalloproteinase

Myf5:   myogenic factor 5

Myh3:   embryonic myosin heavy chain

MyoD: myogenic differentiation

NEFA: non-esterified fatty acid

NFκβ: nuclear factor kappa-light-chain-enhancer of activated B cells

ND: normal diet

OXPHOS: oxidative phosphorylation

PAI-1:   plasminogen activator inhibitor

PFK: phosphofructokinase

PIP3:   phosphatidylinositol (3,4,5)-triphosphate

PI3K: phosphoinositide 3-kinase

PTPN1:   tyrosine-protein phosphatase non-receptor type 1

S6K1:   ribosomal protein S6 kinase beta-1

SC: satellite cell

SDH: succinate dehydrogenase

SOCS-3:   suppressor of cytokine signaling 3

SOL: soleus

STAT3:   signal transducer and activator of transcription 3

TA: tibialis anterior

TAG: tri-acylglycerols

TLR-4:   toll-like receptor-4

TNF-α: tumor necrosis factor alpha

T2DM: type II diabetes mellitus

WAT: white adipose tissue

CHAPTER 1

General introduction and objectives

INTRODUCTION AND OBJECTIVES

An unhealthy lifestyle consisting of a diet high in fat and a lack of exercise contribute to the development of obese and insulin resistant states including Prediabetes, a state of impaired glucose handling, and Type II Diabetes Mellitus (T2DM) which is associated with a wealth of health concerns and co-morbidities (Bavenholm et al. 2003, DeFronzo 1992, Weiss et al. 2003). The contribution of obesity to disease development is significant as excessive body mass accounts for almost 90% of all new cases of T2DM (Hossain, Kawar & El Nahas 2007). Prediabetes has become a growing concern in our society as it is estimated that 4.3 million adults aged 40-74 in Canada will have this disease by 2016 (Colagiuri, R. Fitzgerald, T. Furlong, D. Huot, C. Keeley, M. Krueger, H. Robinson, A. Silverberg, J. Turner, C. 2010). The Canadian Diabetes Association defines Prediabetes as impaired fasting glucose (IFG; 6.1-6.9mmol/L), impaired glucose tolerance (IGT; 7.8-11.0mmol/L 2hr-post 75g oral glucose) or elevated glycated hemoglobin (A1C; 6.0-6.4%) (Goldenberg, Punthakee 2013). While obesity-associated T2DM, and now Prediabetes, was once described as having an adult onset, an increasing number of adolescents are being diagnosed with these diseases (Duncan 2006, Li et al. 2009, Lee et al. 2011). Aerobic and resistance exercise are recommended therapies for obese individuals, with or without Prediabetes, as exercise can lead to reductions in adiposity and body mass as well as improve insulin signaling (Burr et al. 2010, Phielix et al. 2012, Slentz, Houmard & Kraus 2009, Fenicchia et al. 2004, Houmard et al. 2002, Dorsey, Songer 2011). However, many individuals do not incorporate adequate physical activity into their lifestyle (Dorsey, Songer 2011) which emphasizes the need for

improvements to exercise prescriptions and alternative therapies to exercise to attenuate the progression of obese and insulin resistant states towards T2DM. Lifestyle modifications recommended by clinicians to Prediabetic patients include dietary restriction of calories or fat and increased physical activity with the aim of reducing obesity which often results in improved insulin sensitivity (Dorsey, Songer 2011). Thus, dietary excess leading to obesity can be a contributing cause of Prediabetes. Given the complex criteria required in order to use the term Prediabetes, rodent studies utilizing a high-fat diet to induce obese and possibly glucose intolerant states will be referred to herein as diet-induced obesity (DIO).

The physiological changes that occur during the development of DIO are poorly understood despite the pervasiveness of Prediabetes in our society and the significant impact it has on current and future health (Anselmino, Sillano 2012, Tsimihodimos, Florentin & Elisaf 2013). Similar to T2DM, DIO may lead to a number of alterations to muscle health in addition to insulin resistance. A shift in muscle fiber types towards more fast-twitch glycolytic fibers has been noted with T2DM and may also occur with DIO (Toft et al. 1998). Such a fiber type shift could impair the ability to perform prolonged, low-intensity exercise and result in a reduced capacity for lipid oxidation leading to an increase in intramyocellular lipids (IMCLs) which could, in turn, perpetuate muscle insulin resistance.

Given that muscle health, such as fiber type and IMCL content, can significantly impact progression of DIO this review will discuss the current state of knowledge concerning physiological changes in response to DIO with a focus on skeletal muscle

health. Length of DIO utilizing a high-fat diet in mice will determine the severity of disease development, thus characterizing physiological changes following varying lengths of DIO enables characterization of disease development. DIO in mice for ~5-8 weeks results in a number of physiological changes including glucose intolerance and muscle insulin resistance (Turner et al. 2007, Hoeks et al. 2011). Furthermore 16 weeks of DIO results in a similar phenotype to T2DM in humans including impaired oral glucose tolerance, insulin resistance, elevated fasting plasma glucose and insulin, expanded white adipose tissue (WAT) mass, adipocyte hypertrophy, ectopic tissue lipid deposition and increased hepatic and pancreatic mass due to lipids (Fraulob et al. 2010). Importantly a short 3 day HFD also elicits similar results between mice and humans as expression of lipid oxidation genes is enhanced for both species (Boyle et al. 2011, de Wilde et al. 2008). Given the impact that DIO can have on current and future health (Anselmino, Sillano 2012, Tsimihodimos, Florentin & Elisaf 2013, Goodpaster et al. 2010), it is important to understand the factors associated with the development of this disease. Only upon a proper understanding of disease development can therapies and treatments be successfully devised.

Impairments to lipid oxidation have been noted with DIO and are proposed to result in increased IMCLs contributing to insulin resistance (Kewalramani, Bilan & Klip 2010). A number of studies utilizing DIO have demonstrated muscle insulin resistance following approximately 8 weeks making this an ideal time to determine the presence of mechanisms proposed to elicit muscle insulin resistance such as altered lipid oxidation and inflammation (Turner et al. 2007, Hoeks et al. 2011, de Wilde et al. 2008, de Wilde et

4

al. 2009). However, the reported state of lipid oxidation and inflammation at and prior to 8 weeks of DIO are conflicting (Turner et al. 2007, Hoeks et al. 2011, de Wilde et al. 2008, de Wilde et al. 2009). Lipid oxidation status and indicators of lipid oxidation status (termed oxidative potential in this review) are important components of muscle health as they impact the insulin sensitivity of muscle. The oxidative potential of muscle can be determined though morphological features, such as fiber type, and molecular features including oxidative enzymes, proteins and transcripts. Reports of muscle health with Prediabetes in humans and DIO are conflicting but include negative changes such as impaired growth (Peterson, Bryner & Alway 2008), reduced oxidative enzyme activity (He, Watkins & Kelley 2001), and a glycolytic fiber type shift (Lillioja et al. 1987, Tanner et al. 2002). Changes to the oxidative potential of muscle with DIO are important to fully understand as they relate to changes in functional lipid oxidation, can impact the overall health of muscle and can provide information regarding the mechanism(s) by which muscle insulin resistance develops.

The mechanisms by which DIO leads to glucose intolerance and muscle insulin resistance is currently a topic of some debate within the scientific community, thus the following is a general outline of currently proposed mechanisms. DIO leads to increased lipids within the blood and increased storage of lipids in adipose tissue (Kewalramani, Bilan & Klip 2010). This process is accompanied by activation of local and newly attracted adipose macrophages to the M1 pro-inflammatory phenotype; leading to the description of T2DM as a low-grade inflammatory state (Fujisaka et al. 2009). Once adipose tissue becomes insulin resistant, lipolysis is no longer impaired by insulin and

more fatty acids are released into the circulation (Kewalramani, Bilan & Klip 2010). Initially, elevated non-esterified fatty acids (NEFAs) in the blood was thought to induce insulin resistance (Karpe, Dickmann & Frayn 2011). However it is now recognized that ectopic lipid deposition in tissues, such as muscle and liver, is a significant contributor to the development of insulin resistance which has led to the term lipotoxicity (Steinberg 2007, Samuel, Shulman 2012). Randle (Randle et al. 1963) first described lipid mediated inhibition of insulin-stimulated glucose uptake in muscle and proposed that it was due to reduced glycolysis. It is now clear that lipid induces muscle insulin resistance through impairing insulin signalling leading to reduced glucose uptake (Samuel, Shulman 2012, Dresner et al. 1999). IMCLs in muscle will increase with either excess import into the muscle or impaired processing by the mitochondria, both of which have been proposed to occur with obesity (Kewalramani, Bilan & Klip 2010, Hegarty et al. 2002, Cameron-Smith et al. 2003). Once in the muscle, long chain fatty acids are esterified to ceramide (saturated fatty acids only) or long-chain fatty acyl-CoA (LCFA-CoA) which, if not entering the mitochondria, is then attached to glycerol to form mono-, di- (DAGs) or tri-acylglycerols (TAGs) (Samuel, Shulman 2012). While it has been shown that TAGs are fairly inert, DAGs and ceramides significantly contribute to muscle insulin resistance (Samuel, Shulman 2012, Liu et al. 2007). LCFA-CoAs can interact with free carnitine to form acylcarnitines which can be taken up into the mitochondria where they will proceed through β-oxidation (Samuel, Shulman 2012). During each cycle of β-oxidation a two-carbon acetyl-CoA molecule is formed that can continue on to the Krebs cycle where a series of chemical reactions reduces NAD+ to NADH and produces CO_2. Lastly electrons

are donated to the electron transport chain (ETC) to generate energy in the form of ATP. It is unclear whether impairments to lipid oxidation within the mitochondria lead to excess IMCLs and lipid intermediates or are a consequence of muscle insulin resistance (Turner et al. 2007, Hoeks et al. 2011, Koves et al. 2008, Sparks et al. 2005). In addition to lipid intermediates, cytokines can also contribute to glucose intolerance and muscle insulin resistance (Kewalramani, Bilan & Klip 2010, Yu et al. 2011) (Figure 1.1). Increases in pro-inflammatory circulating cytokines, including TNF-α and IL-6, can occur with obesity through activation of M1 pro-inflammatory macrophages in adipose tissue, as previously mentioned, and activation of Kupffer cells in hepatic tissue (Steinberg 2007, Cai et al. 2005, Baffy 2009, Bhargava, Lee 2012, Nieto-Vazquez et al. 2008, Galic, Oakhill & Steinberg 2010). Cytokines produced by tissues, including adipose and hepatic tissues, can be secreted into the bloodstream and contribute to insulin resistance in other insulin sensitive tissues such as muscle (Steinberg 2007, Samuel, Shulman 2012). Although inflammation within muscle has recently been implicated in muscle insulin resistance (Kewalramani, Bilan & Klip 2010), the extent to which inflammation leads to the onset of muscle insulin resistance with DIO is unclear. Thus further investigation into the presence of mechanisms proposed to induce muscle insulin resistance with differing lengths of DIO is warranted. The presence of muscle insulin resistance following 8 weeks of DIO and a noted increase in macrophage content within mouse muscle after 3 weeks of DIO (Hong et al. 2009) makes these ideal time-points to identify changes to muscle health and the presence of mechanisms proposed to elicit muscle insulin resistance with

DIO. Overall, a better understanding is needed of changes to inflammation and lipid handling in insulin sensitive tissues and plasma after 3 weeks and 8 weeks of DIO.

DIO often results in muscle insulin resistance, yet normal insulin signalling is important for muscle growth and maintenance (Turner et al. 2007, Kewalramani, Bilan & Klip 2010, Vander Haar et al. 2007). Similarly the hormone leptin contributes to increased muscle mass and resistance to this adipokine occurs with Prediabetes (Bartell et al. 2011, Sainz et al. 2009, Van Heek et al. 1997, Steinberg et al. 2006a, Maffei et al. 1995). Thus, muscle mass may be affected with DIO which would further exacerbate disease progression as muscle mass is positively associated with insulin sensitivity (Srikanthan, Karlamangla 2011). Disease progression can also be exacerbated with inappropriate recovery of muscle from injury resulting in an increase in sedentary behaviour. A decrement in muscle repair may exist with DIO as individuals with T2DM have impaired wound healing and prolonged recovery following arthroscopic rotator cuff repair (Arya, Pokharia & Tripathi 2011, Clement et al. 2010). Muscle repair in Prediabetic individuals has not been investigated and the few studies conducted thus far investigating the effect of DIO on muscle repair in mice have had conflicting results (Hong et al. 2009, Nguyen, Cheng & Koh 2011, Hu et al. 2010, Woo et al. 2011). An important aspect of muscle repair is the contribution of SCs which become activated, divide (to generate progeny termed myoblasts) and then differentiate and fuse to form new muscle fibers (Hawke, Garry 2001). Decrements to the proper function of SCs can result in inadequate muscle repair as occurs with Duchenne's Muscular Dystrophy (Heslop, Morgan & Partridge 2000, Decary et al. 2000). SCs derived from muscle in mice

with DIO may exhibit alterations to their function as SCs from a genetic model of obesity, the obese Zucker rat, display impaired proliferation (Peterson, Bryner & Alway 2008).

The overall objective of this thesis was to improve our understanding of muscle health in the Prediabetic state in young adult mice. Mice (10 weeks of age) were given a normal diet (ND) or a HFD (60% kcal fat) for 3 or 8 weeks. Disease progression was assessed at both time-points as was muscle health including morphological, metabolic and contractile measurements. In order to observe the association between mechanisms proposed to induce muscle insulin resistance and the natural progression of disease development with DIO, fatty acid oxidation and inflammation were assessed after both diet lengths in the whole body and muscle. Finally, the ability of HFD muscle to undergo repair in an insulin resistant state was assessed using cardiotoxin (CTX) intramuscular injection following 8 weeks of HFD, a time-point that should result in muscle insulin resistance.

The specific objectives of this research were to:

1. Comprehensively assess the impact that 8 weeks of HFD consumption has on the health of mouse skeletal muscle; including mass, fiber type composition, contractile function, lipid metabolism, lipid storage and insulin sensitivity in postural and non-postural muscles.

2. Investigate disease development and changes to muscle health (similar to Objective 1) after only 3 weeks of HFD. In addition, determine changes to lipid storage, lipid metabolism and inflammatory state in insulin sensitive tissues (adipose tissue, hepatic tissue, muscle) and plasma.

3. Determine the impact of HFD consumption on the capacity of skeletal muscle to repair following injury including the effects on the muscle satellite cell population.

This review is concerned with the HFD mouse model in so far as its usefulness to investigating the development of early obesity and insulin resistance. While the introduction reviewed the generally accepted and proposed mechanisms by which DIO leads to glucose intolerance and muscle insulin resistance, the following sections will focus specifically on the impact of DIO on skeletal muscle health.

Changes to muscle health with DIO.

Changes to muscle health can be advantageous or detrimental to obese diabetic states. For the purposes of this review of the literature muscle health is divided into assessments of whole muscle (mass and contractile function), oxidative potential (fiber type, oxidative enzymes, oxidative proteins and oxidative transcripts) and metabolism (insulin stimulated glucose uptake and lipid oxidation). The following is a summary of the current literature on muscle health in obese diabetic states with a focus on DIO in mice. When appropriate, potential mechanisms to explain changes to skeletal muscle health will be discussed.

Muscle mass and contractile function.

Muscle is an insulin-sensitive organ involved in blood glucose removal; a function that is blunted with Prediabetes and T2DM (Weiss et al. 2003, Li et al. 2009, Defronzo 2009). Muscle mass, relative to body mass, is positively associated with insulin sensitivity and low relative muscle mass increases risk of Prediabetes and T2DM in humans (Srikanthan, Karlamangla 2011). The detrimental outcome of low relative muscle mass can be improved through resistance exercise which leads to improved muscle insulin resistance (Burr et al. 2010). Growth or maintenance of muscle mass may be impaired with DIO as two important hormonal regulators of muscle mass, insulin and leptin, become dysfunctional (Kewalramani, Bilan & Klip 2010, Galic, Oakhill & Steinberg 2010, Van Heek et al. 1997, Steinberg et al. 2006a, Maffei et al. 1995, Sugioka et al. 2012). Although insulin enhances protein synthesis and reduces proteolysis, when vasodilation to muscle is adequate, thus encouraging an increase in muscle mass the

11

development of insulin resistance in obese states would reduce these effects (Guillet, Boirie 2005, Timmerman et al. 2010). Leptin is an adipokine, meaning that it is a hormone produced in adipose tissue, that normally activates PI3K and ERK1/2 signalling and reduces myostatin thus promoting increases in muscle mass (Kellerer et al. 1997, Maroni, Bendinelli & Piccoletti 2003, Maroni, Bendinelli & Piccoletti 2005). Although administration of leptin has led to increased muscle mass in mice deficient in the hormone (Sainz et al. 2009, Hamrick et al. 2010) and mice exhibit elevated circulating leptin with DIO, normal muscle growth in DIO mice may be blunted due to the development of peripheral leptin resistance (Galic, Oakhill & Steinberg 2010, Van Heek et al. 1997, Sugioka et al. 2012). Indeed impaired muscle mass occurs in genetic mouse models of obesity with impaired leptin signalling, and this has been partly attributed to increased protein breakdown (Nguyen, Cheng & Koh 2011, Trostler et al. 1979, Kemp et al. 2009). Another adipokine that can regulate muscle mass is adiponectin, as it prevents lipid-induced proteolysis in muscle cells (Zhou et al. 2007). Adiponectin levels are negatively associated with obesity and T2DM in humans and are reduced in male, but not female, DIO mice (Galic, Oakhill & Steinberg 2010, Hu, Liang & Spiegelman 1996, Nickelson et al. 2012, Hotta et al. 2000). Thus, obese diabetic states could lead to increased muscle proteolysis and decreased muscle mass due to reduced adiponectin levels. In addition to the aforementioned hormones, other factors known to negatively impact muscle mass have been found elevated with Prediabetes or obesity (Lyons, Haring & Biga 2010, Lee et al. 2011, Gorzelniak et al. 2002, Allen et al. 2008) including TNF-α which leads to NF-$\kappa\beta$ mediated atrophy of myotubes *in vitro* (Li, Reid 2000), angiotensin-II which leads to

atrophy of muscle by a reduction in IGF-1 (Brink et al. 2001), and myostatin which leads to reductions in muscle mass via increased transcription of atrophy related genes and decreased transcription of anabolic genes (Elkina et al. 2011). Elevated circulating levels of TNF-α and angiotensin-II are proposed to be the mechanisms by which muscle mass is reduced after 16 weeks of DIO in rats (Sishi et al. 2011). Muscle growth is also impaired in some DIO mouse studies (Turner et al. 2007, Woo et al. 2011) although reports are limited. Interestingly secretion of adipokines associated with T2DM development may be hereditary and pre-dispose children and adolescents to develop Prediabetes and eventually T2DM (Al-Daghri et al. 2011).

Interpretation of muscle mass with DIO should include assessment of fiber type and contractile function as an increased fiber area of glycolytic fibers results in a reduced, rather than an increased, peak tetanic force in adiponectin knock-out mice (Krause et al. 2008). Muscle strength may be reduced with DIO as older individuals with T2DM have greater muscle mass than their healthy counterparts, yet have less muscle strength (Park et al. 2006). In addition upper body strength, as indicated by grip strength, in individuals with T2DM or metabolic syndrome is lower compared to healthy individuals (Sayer et al. 2007, Sayer et al. 2005). The fatigability of muscle determines muscular endurance and a decrement to fatigability could impact daily living. Given the blood profile of Prediabetic individuals it is suggested that muscle growth would be impaired, yet the extent to which this occurs with varying length of DIO in mice is unclear. In addition to muscle mass the health of muscle is also determined by strength, endurance and fatigability which may all be impaired with DIO.

Oxidative Potential

Inadequate capacity for lipid oxidation in muscle is proposed as a contributing factor to muscle insulin resistance occurring with DIO. The lipid oxidation capabilities of muscle are implied from the oxidative potential of muscle which can be assessed through analysis of fiber type, enzymes, transcripts and proteins. Although a glycolytic fiber type shift has been reported in muscle from Prediabetic individuals (Lillioja et al. 1987, Tanner et al. 2002) other reports of changes to muscle fiber types in muscle of Prediabetic individuals are unable to be simply classified as oxidative or glycolytic. This is demonstrated by a report of both a glycolytic fiber type shift and an increased area of oxidative fibers in the same muscle (Toft et al. 1998). Yet another report noted both an increased area of glycolytic and oxidative muscle fibers (Larsson et al. 1999). Changes to fiber types in obese mouse models are also conflicting as an oxidative fiber type shift occurred in a genetic model (Kemp et al. 2009), yet DIO had no effect on fiber area or fiber type in the EDL muscle (Turpin et al. 2009). In the latter study all the fibers of the oxidative soleus muscle increased in area (Turpin et al. 2009) demonstrating that muscle type examined is important to note when interpreting results as different muscle types exhibit different responses. While there is evidence of a reduction in mitochondrial content and enzymes in muscle from Prediabetic and T2DM individuals (Ritov et al. 2005, Simoneau, Kelley 1997), reports of changes to mitochondrial content with DIO in mice vary. Impaired oxidative potential is implied after 6 weeks of DIO by reductions to indicators of mitochondrial biogenesis, oxidative phosphorylation (OXPHOS) gene expression and protein content (Sparks et al. 2005). However oxidative potential has been

demonstrated to be elevated with DIO following 4 and 8 weeks as well as only 3 days of diet intervention despite no significant gain in body mass (de Wilde et al. 2008, de Wilde et al. 2009). Specifically elevated transcripts related to lipid catabolism and oxidation were noted after 3 days, slow myosin heavy chain and OXPHOS protein content were elevated after 4 weeks and increased classes of genes involved in lipid oxidation were present after 8 weeks (de Wilde et al. 2008, de Wilde et al. 2009). The presence of conflicting reports in the literature concerning changes to the oxidative potential of muscle from DIO mice is likely due to differences in diet composition and length of diet. Thus, a time-line assessment of changes to the oxidative potential of muscle with DIO would help clarify changes to muscle health during development of obesity related muscle insulin resistance and aid with future treatments for Prediabetes which could involve reversing or preventing changes to muscle health as muscle exhibits a great deal of plasticity (Schiaffino, Sandri & Murgia 2007).

Muscle insulin resistance

DIO results in glucose intolerance and insulin resistance in tissues including muscle. The following is a summary of the relationship between muscle insulin resistance and the proposed mechanisms responsible, namely, functional muscle lipid oxidation and inflammation. For clarity of interpretation of the literature, an important distinction must be made between determination of glucose intolerance and muscle insulin resistance. Glucose intolerance can be determined by whole body assessments such as the intraperitoneal glucose tolerance test (IPGTT). An insulin tolerance test or a hyperinsulinemic-euglycemic clamp experiment can help determine muscle insulin

resistance, but to confirm that a reduction in basal glucose disposal rate is due to muscle insulin resistance *ex vivo* assessment of insulin responsive pathways and actions should be performed and may include: phosphorylation state of signalling intermediates, insulin stimulated glucose uptake and insulin stimulated glycogen synthesis (Bonnard et al. 2008, Steinberg et al. 2010, Pimenta et al. 2008).

An increase in IMCLs is one of the first morphological changes to occur with lipid infusion in humans or DIO in mice (Dresner et al. 1999, Turpin et al. 2009) and there is a strong link between IMCLs and Prediabetes or DIO (Weiss et al. 2003, Houmard et al. 2002, Samuel, Shulman 2012, Liu et al. 2007). Furthermore IMCLs can directly and indirectly interfere with insulin signalling within muscle (Karpe, Dickmann & Frayn 2011, Dresner et al. 1999, Galic, Oakhill & Steinberg 2010). Thus, the lipid oxidation capability of muscle has become a point of interest as an impairment in lipid oxidation can lead to a build-up of IMCLs and hence insulin resistance. In addition, the role of inflammation in muscle as a contributing factor to induction of muscle insulin resistance has recently been investigated (Kewalramani, Bilan & Klip 2010). Muscle insulin resistance is a hallmark of Prediabetes development, yet the mechanisms responsible for its induction with DIO are not yet fully understood.

Mechanisms leading to muscle insulin resistance

Reduced lipid oxidation has been associated with insulin resistance in individuals with Prediabetes or T2DM. Boushel (Boushel et al. 2007) reported that lipid oxidation in muscle from T2DM individuals was impaired when assessed in muscle strips yet unaltered when corrected for mitochondrial content suggesting a reduction in

mitochondrial content. However, evidence that lipid oxidation is increased in humans following an increased lipid load has contributed to a reassessment in the literature of the state of lipid oxidation in obese states (Kewalramani, Bilan & Klip 2010, Cameron-Smith et al. 2003). Elevated circulating leptin levels present with Prediabetes (Galic, Oakhill & Steinberg 2010, Sugioka et al. 2012) may lead to initial, but not prolonged, increases in muscle lipid oxidation due to the development of leptin resistance (Galic, Oakhill & Steinberg 2010). Leptin resistance is demonstrated by the reduced ability of leptin to stimulate lipid oxidation in primary myotubes derived from obese human muscle (Steinberg et al. 2006a). Since studies in Prediabetic individuals generally represent established insulin resistance, DIO rodent models provide more detailed information regarding early time-points of Prediabetes development.

DIO in mice results in peripheral leptin resistance following 8 weeks, but not 1 week, of DIO (Lin et al. 2000). Thus, muscle lipid oxidation may be enhanced with DIO at very early time-points, yet would likely be impaired following 8 weeks of DIO. In line with this theory, DIO in rats has been reported to initially elevate and then impair lipid oxidation in an oxidative muscle (Chanseaume et al. 2007). However, no change was found for lipid oxidation in a mixed fiber type muscle at the same time-points demonstrating a fiber type difference (Chanseaume et al. 2007). Since this study did not directly assess muscle insulin resistance, a link between alterations in lipid oxidation and muscle insulin resistance is difficult to define. Similar to rats, DIO in mice has also been reported to enhance lipid oxidation in muscle (Turner et al. 2007). Specifically lipid oxidation in muscle homogenates was enhanced, yet unaltered in isolated mitochondria

suggesting an increase in mitochondrial content (Turner et al. 2007). However this study analyzed muscle insulin resistance in an oxidative muscle and lipid oxidation in a mixed fiber type muscle (Turner et al. 2007). Thus, it is again difficult to conclude that whole muscle lipid oxidation is enhanced with early muscle insulin resistance, especially since lipid oxidation responses differ between oxidative and mixed fiber type muscles (Chanseaume et al. 2007). Lipid oxidation has also been proposed to be elevated at the level of the whole muscle with DIO as Hoeks (Hoeks et al. 2011) reported unaltered lipid oxidation in isolated mitochondria yet enhanced OXPHOS content. Again, neither whole muscle lipid oxidation nor muscle insulin resistance was directly assessed. While there are conflicting lipid oxidation results in the literature for muscle during DIO, some of the confusion appears to stem from assessment techniques as measurements are made using excised whole muscle, muscle strips, muscle homogenates and isolated mitochondria. One apparent explanation for discrepancies in whole muscle lipid oxidation may be that initially lipid oxidation is enhanced with DIO before undergoing an impairment in function as has been demonstrated in rats (Chanseaume et al. 2007). Although the exact relationship of these changes to the development of muscle insulin resistance is yet to be defined. Interestingly dysfunctional lipid oxidation has also been shown to occur with DIO as Koves (Koves et al. 2008) showed excess β-oxidation without a similar increase in Krebs cycle function despite unaltered complete lipid oxidation in both glycolytic and oxidative muscle homogenates. While muscle insulin resistance was not directly assessed it was suggested that an increase in incomplete to complete lipid oxidation can induce muscle insulin resistance. Thus, the role of muscle lipid oxidation in the development of

insulin resistance may be more nuanced than simply a change in complete lipid oxidation. Nevertheless it is clear that direct assessments of both muscle insulin resistance and whole muscle lipid oxidation with DIO are needed to clarify the relationship between these two important components of muscle health.

A relatively new avenue of investigation into mechanisms responsible for the development of muscle insulin resistance with Prediabetes is inflammation, including the pro-inflammatory cytokines IL-6 and TNF-α (Steinberg et al. 2006b, Kim et al. 2011, Tzeng, Liu & Cheng 2005). IL-6 signals through STAT3 to increase expression of Toll-like receptor-4 (TLR-4) in non-obese insulin resistant individuals (Kim et al. 2011) and TNF-α impairs IRS-1 action through IKK and PTPN1 (alias PTP1B) (Nieto-Vazquez et al. 2008). Appropriately, TLR-4 null mice are protected from DIO and reduced function of TLR-4 decreases lipid induced insulin resistance in myotubes (Radin et al. 2008). Compared to insulin sensitive patients, those with Prediabetes or T2DM exhibit elevated transcripts of IL-6 and TNF-α in muscle and in media from primary myoblast cultures (Saghizadeh et al. 1996). Thus, skeletal muscle is able to secrete IL-6 and TNF-α. Pro-inflammatory macrophages can also secrete IL-6 and TNF-α and macrophages have been identified in mouse muscle following three (Hong et al. 2009) and twenty (Nguyen et al. 2007) weeks of DIO. However, Steinberg (Steinberg et al. 2010) found no elevation in serum IL-6 or TNF-α content following twelve weeks of DIO in mice. Thus, changes to cytokines with DIO are unclear. Inflammatory signaling in muscle cells is also induced by saturated fatty acids such as palmitate resulting in increased expression of *Tnf-α* and *Il-6* (Jove et al. 2006, Jove et al. 2005). In addition the transcription of other pro-inflammatory

factors such as suppressor of cytokine signalling 3 (*Socs3*), protein tyrosine phosphatase non-receptor type 1 (*Ptpn1*) and toll-like receptor-4 (*Tlr4*) have been reported elevated in insulin resistant muscle in response to either circulating cytokines or lipids and have been shown to contribute to muscle insulin resistance (Nieto-Vazquez et al. 2008, Kim et al. 2011, Kim et al. 2011, Radin et al. 2008, Rieusset et al. 2004, Nieto-Vazquez et al. 2008, Nieto-Vazquez et al. 2007, Ueki, Kondo & Kahn 2004, Reyna et al. 2008). Importantly, there is some evidence that elevated levels of anti-inflammatory cytokines, such as IL-10, can mitigate the development of muscle insulin resistance with DIO (Hong et al. 2009). Many investigations into mechanisms responsible for muscle insulin resistance use diabetic models with established insulin resistance, yet the extent to which these mechanisms are present when insulin resistance initially develops is unclear. This highlights the importance of thoroughly assessing inflammatory state in different tissues during DIO and the association with metabolic consequences of DIO including muscle insulin resistance and glucose intolerance.

Repair of muscle with DIO

Movement along the continuum of DIO to T2DM is enhanced with reduced physical activity and delayed or reversed with increased physical activity (Bavenholm et al. 2003, Burr et al. 2010, Goodpaster et al. 2010). The efficiency with which muscle recovers from injury following DIO could impact type and frequency of exercise prescription as well as return to daily physical activities following serious injury or surgery. Wound healing and recovery from arthroscopic surgery are adversely affected with T2DM (Arya, Pokharia & Tripathi 2011, Clement et al. 2010), yet muscle repair

with DIO in mice has had conflicting results (Hong et al. 2009, Nguyen, Cheng & Koh 2011, Hu et al. 2010, Woo et al. 2011). Repair of muscle following injury is a complex process involving an early degenerative phase followed by a reparative phase (Charge, Rudnicki 2004). There is a large degree of overlapping processes including necrosis and phagocytosis of damaged myofibers, satellite cell (SC) activation/proliferation and de novo myofiber formation (d'Albis et al. 1988, Hawke et al. 2003, Arnold et al. 2007). Decrements to the proper function of SCs can result in inadequate muscle repair as occurs with Duchenne's Muscular Dystrophy or altered expression of transcription factors and cell cycle regulatory genes including p21 (Heslop, Morgan & Partridge 2000, Decary et al. 2000, Hawke et al. 2003, Hawke, Jiang & Garry 2003) (Figure 1.2). The following is a review of current knowledge surrounding muscle repair and the contribution of the SC in muscle from healthy and diseased (ie. DIO) rodents.

Morphological response to injury and alterations with DIO

Experimental muscle injury is often induced using the snake venom cardiotoxin (CTX) which causes widespread damage to muscle (Hawke et al. 2003, Czerwinska et al. 2012, Couteaux, Mira & d'Albis 1988). In comparison, alternative methods of injury include a crush or cold injury, which are also intense but localized, and exercise which is more widespread but very mild (Woo et al. 2011, Tatsumi et al. 1998, Parise, McKinnell & Rudnicki 2008). Following CTX injection, muscle undergoes a degenerative phase of necrosis and phagocytosis marked by invasion of inflammatory cells, destruction of muscle fibers and activation of muscle satellite cells (SCs) to contribute to repairing degraded muscle fibers (Tiidus 2008) (Figure 1.2). The inflammatory response is rapid

21

such that within one day leukocytes and neutrophils are present which release cytokines that further the inflammatory response by attracting monocytes including macrophages (Couteaux, Mira & d'Albis 1988, Garry et al. 1997). A small population of resident macrophages within muscle may be involved in perpetuating the pro-inflammatory response upon injury which enhances SC proliferation, while a switch to an anti-inflammatory macrophage phenotype enhances myoblast differentiation (Arnold et al. 2007, Brigitte et al. 2010). In addition, SCs activated following injury can attract macrophages to the site of injury (Charge, Rudnicki 2004). Both snake venom, through membrane depolarization leading to myolysis (Czerwinska et al. 2012), and macrophages (Couteaux, Mira & d'Albis 1988) contribute to necrosis and phagocytosis of muscle fibers. Necrosis can be identified by macrophages surrounding a fiber followed by their infiltration into the fiber and loss of the sarcolemma (Couteaux, Mira & d'Albis 1988). Although fibers are degenerated and their plasma membranes gone, the basal lamina remains as it guides new fiber growth which is evidenced by the presence of multiple centrally nucleated myofibers within one basal lamina sheath during early repair (Czerwinska et al. 2012, Couteaux, Mira & d'Albis 1988). By three days post-injury, there are many de novo myofibers although inflammation is not significantly reduced until five days (Hawke et al. 2003, Czerwinska et al. 2012). New myofibers initially express a developmental forms of myosin heavy chain (e.g. embryonic myosin heavy chain, Myh3) followed by expression of the mature forms of myosin heavy chain, with fast isoforms being expressed as early as four days after injury (Czerwinska et al. 2012, Couteaux, Mira & d'Albis 1988, Yan et al. 2003, Krause et al. 2011). Ten days following

injury the majority of muscle fibers have undergone repair and have begun to mature as evidenced by increased fiber area and loss of Myh3 content (Krause et al. 2011). Thus, a greater amount of Myh3 content ten days following injury is indicative of delayed repair as is a greater amount of myonecrosis and excess fibrosis (Hawke et al. 2003, Krause et al. 2011). Fibrosis, including collagens, may also enhance repair as collagen type IV, normally located in the basement membrane, enhances the ability of myoblasts to form myotubes in vitro (Maley, Davies & Grounds 1995) while collagen type I, normally located in the ECM, has been associated with accelerated muscle repair resulting from low-level laser therapy (de Souza et al. 2011). Following crush injury collagen types IV and I are found in the regenerating region at 5 and 7 days respectively (de Souza et al. 2011, Grounds et al. 1998). Since the normal process of muscle degeneration and repair following injury is well characterized decrements to muscle repair with DIO may be determined through comparison with otherwise healthy regenerating muscle.

Attenuated repair is reported following 3 weeks (Woo et al. 2011) or 8 months (Hu et al. 2010) but not a 12 weeks (Nguyen, Cheng & Koh 2011) of DIO. The 12 week DIO study may have found no reduction in muscle repair due to the diet intervention beginning at 4 weeks of age (Nguyen, Cheng & Koh 2011). At this early age there is rapid muscle growth as demonstrated by there being an approximate threefold increase in mean fiber volume of mouse EDL muscle from post-natal day twenty-one (p21; ~ three weeks of age) to p56 (~ eight weeks of age) (White et al. 2010). Thus rapid muscle growth may delay the onset of muscle insulin resistance due to increased energy demands on the muscle (Fenicchia et al. 2004, Craig, Everhart & Brown 1989). An excess of

collagen in regenerating muscle has been proposed to result in reduced muscle repair with

DIO (Hu et al. 2010). Excess collagen occurs during muscle repair following prolonged

DIO (8 months) (Hu et al. 2010). The extent to which excess collagen accumulates during

repair following a shorter diet intervention is unknown. Excess collagen in muscle can

result from a dysfunctional inflammatory response as occurs with the fibrinolytic pathway

in T1DM muscle (Krause et al. 2011). Increased TGF-β has also been linked to increased

collagen content (Jiang et al. 2007). PAI-1 is elevated with Prediabetes in humans (Xu et

al. 2011) yet the downstream proteins inhibited by PAI-1 termed matrix

metalloproteinases (MMPs), which degrade collagens, are elevated in muscle with DIO

(Biga et al. 2013). Increased MMP content in muscle with DIO may also result in

increased TGF-β content as MMPs can activate TGF-beta through proteolytic cleavage

(Yu, Stamenkovic 2000). Another mechanism regulating collagen content is elevated

blood glucose levels (Berria et al. 2006) which have been reported to be both elevated (de

Wilde et al. 2008) and unaltered with short-term DIO despite impaired glucose tolerance

(Turner et al. 2007). Thus, it is unclear how collagen content in muscle is affected by

DIO. Reduced protein turnover has been linked to reduced muscle repair with prolonged

DIO as elevated levels of PTEN (protein phosphatase and tensin homolog deleted from

chromosome ten) were noted which impair phosphatidylinositol 3,4,5-triphosphate (PIP_3)

which, when activated by phosphorylation, signals through Akt to increase muscle mass

partly though activation of ribosomal protein S6 kinase (S6K1) (Hu et al. 2010, Lai et al.

2004, Wang et al. 2009). The reduction in protein turnover was proposed to have led to

the reported reduction in muscle mass. The impact of a shorter DIO paradigm on protein

turnover is unknown. Finally a reduction in SC content has also been proposed to result in reduced muscle repair with DIO (Woo et al. 2011). Overall the literature suggests that muscle repair may be adversely affected with DIO given the possibility for increased collagen content, reduced protein turnover, elevated blood glucose, acquired resistance to insulin and leptin, which as previously mentioned promote muscle mass, and reduced SC content.

Satellite cell response to muscle injury and changes with DIO

Repair following severe muscle injury is primarily performed by satellite cells located within muscle (Hawke, Garry 2001). Normally quiescent in muscle, SCs are activated in response to injury when a bolus release of nitric oxide (NO) causes release of Hepatocyte Growth Factor (HGF) from the ECM allowing HGF to bind with its receptor (c-met) on SCs (Tatsumi et al. 1998, Anderson, Wozniak 2004). Following CTX injury, SCs are activated within 6 hours and begin to replicate their DNA in preparation for division which occurs within approximately 48 hours (Tatsumi et al. 1998, Yan et al. 2003). The initial division of activated SCs results in one daughter cell co-expressing Myf5 and Pax7 (Myf5+/Pax7+), the latter of which is also expressed in quiescence, which will proliferate and contribute to myofiber repair while the other daughter cell is Myf5-/Pax7+ and will return to quiescence (Kuang et al. 2007). In addition to expressing Myf5, proliferating SCs (termed myoblasts) will also express the myogenic regulatory factor MyoD within 24-48 hours of activation (Charge, Rudnicki 2004). As myoblasts differentiate and fuse, myogenin expression appears and expression of MyoD and Myf5 decreases (Charge, Rudnicki 2004). In addition to promoting activation, HGF also

encourages proliferation along with a number of other growth factors and cytokines including insulin-like growth factor (IGF-1), fibroblast growth factor (FGF), IL-6, and leukemia inhibitory factor (LIF) [reviewed in (Charge, Rudnicki 2004, Hawke 2005)] (Figure 1.3). Differentiation is also regulated by growth factors and cytokines with TGF-β negatively influencing both proliferation and differentiation [reviewed in (Charge, Rudnicki 2004, Hawke 2005)]. Importantly pro-inflammatory macrophages present following muscle injury release IL-6, TNF-α and IL-1 which can all influence SC and myoblast function [reviewed in (Tidball, Villalta 2010)] further demonstrating that the milieu of the muscle can affect the ability of SCs to contribute to muscle repair (Hawke 2005).

Muscle from DIO mice may exhibit elevations in pro-inflammatory cytokines such as IL-6 and TNF-α (Hong et al. 2009, Saghizadeh et al. 1996) which are known to affect SC function required for muscle repair (Charge, Rudnicki 2004). Supporting a role for IL-6 in altering SC function in obese insulin resistant states, Scheele (Scheele et al. 2012) demonstrated a reduction in the receptor for IL-6 (IL-6R) in muscle from obese individuals. Given that IL-6 is secreted by pro-inflammatory macrophages following injury and promotes myoblast proliferation (Tidball, Villalta 2010) a decrement in receptor expression with DIO may result in less myoblast proliferation should SCs also exhibit a reduction in IL-6R. Genetic rodent models of obesity with varying degrees of obesity and insulin resistance report unaltered proliferation and differentiation of myoblasts in vitro (Scarda et al. 2010), impaired basal proliferation *in vivo* (Peterson, Bryner & Alway 2008), and reduced content of myoblasts during muscle repair (Nguyen,

Cheng & Koh 2011). While Woo (Woo et al. 2011) noted myoblast content to be less with DIO prior to injury both Hu (Hu et al. 2010) and Nguyen (Nguyen, Cheng & Koh 2011) reported unaltered myoblast content during muscle repair with DIO . Specifically, Hu (Hu et al. 2010) demonstrated whole muscle MyoD expression (mRNA) to be unaltered between diet groups. However, the expression level of MyoD was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which is known to change with metabolic perturbations to muscle including insulin resistant states (Perez, Tupac-Yupanqui & Dunner 2008, Roy et al. 2008). Furthermore, both Hu (Hu et al. 2010) and Nguyen (Nguyen, Cheng & Koh 2011) utilized bromodeoxyuridine (BrdU) incorporation to assess proliferating myoblast content yet BrdU does not solely identify myoblasts since all dividing cells will incorporate this thymidine analogue (including fibroblasts and inflammatory cells) (Thorsson et al. 1998). In addition to proliferation, the ability of myoblasts to differentiate and fuse may be altered with DIO due to alterations to lipid composition as an increased content of polyunsaturated fatty acids (PUFAs) in the myoblast membrane is important for proper fusion (Nakanishi, Hirayama & Kim 2001). Accordingly, differentiation is enhanced by media containing PUFAs and impaired by media containing saturated fatty acids (Lee et al. 2009). Decrements to SC function are difficult to assess *in vivo* due to variability in assessment techniques including BrdU and selected housekeeping genes. In addition if changes to myoblast content are identified *in vivo* it is difficult to state which aspect of myoblast function is altered. Thus, determination of SC and myoblast function *ex vivo* would more clearly identify specific defects associated with DIO. Adequate repair of muscle requires proper activation,

proliferation and differentiation of SCs which may be adversely affected with DIO. Improper repair of muscle following injury in the DIO state could lead to a worsening of muscle health, thus perpetuating the progression towards T2DM.

Conclusion

The purpose of this review was to summarize known physiological changes occurring with DIO in mice. While the literature suggests that DIO leads to significant alterations to muscle health, there are discrepancies which would likely be clarified by a time-course assessment of the relationship of these changes to each other, muscle insulin resistance, glucose intolerance and muscle repair. Thus the overall objective of this thesis was to perform a comprehensive investigation into the early physiological changes, particularly to muscle health, that occur during development of glucose intolerance and muscle insulin resistance in young adult (10 weeks of age) mice over a time-course of DIO (60% kcal fat). An extension of this objective was to investigate the impact of DIO induced changes to muscle health on satellite cell functionality and muscle regeneration.

The ability of muscle to undergo repair, a critical feature of muscle health, has received little attention and the studies undertaken have provided varying results (Nguyen, Cheng & Koh 2011, Hu et al. 2010, Woo et al. 2011). This is despite indications that DIO and insulin resistance adversely affect muscle mass (Van Heek et al. 1997, Sugioka et al. 2012, Guillet, Boirie 2005, Timmerman et al. 2010) and SC functionality partly due to inflammation (Hong et al. 2009, Saghizadeh et al. 1996, Charge, Rudnicki 2004). Thus, it is hypothesized that muscle repair in the DIO state is impaired following a sufficient diet intervention leading to muscle insulin resistance as

occurs with 8 weeks of DIO (Hoeks et al. 2011). Muscle insulin resistance may occur earlier than 8 weeks of DIO as pro-inflammatory cytokines are noted in muscle after only 3 weeks of DIO (Hong et al. 2009) and inflammation can result in muscle insulin resistance (Steinberg et al. 2006b, Kim et al. 2011, Tzeng, Liu & Cheng 2005). Inflammation in insulin sensitive tissues, such as adipose and hepatic tissue, is noted in the literature as a mechanism leading to whole body glucose intolerance (Kewalramani, Bilan & Klip 2010, Steinberg 2007, Samuel, Shulman 2012, Samuel et al. 2004). It is hypothesized that 3 weeks of DIO leads to inflammatory responses throughout insulin sensitive tissues and, given tissue cytokine release, the circulation (Steinberg 2007, Samuel, Shulman 2012). Given the reported association between excess lipid deposition in insulin sensitive tissues and insulin resistance of those tissues (Kewalramani, Bilan & Klip 2010, Samuel, Shulman 2012, Samuel et al. 2004) it is hypothesized that an excess of lipids in insulin sensitive tissues will be found with glucose intolerance and muscle insulin resistance. Since a previous time-line assessment of DIO reported initial enhancement followed by impairment of lipid oxidation in muscle (Chanseaume et al. 2007) and other reports suggest enhanced lipid oxidation in whole muscle (Turner et al. 2007, Hoeks et al. 2011), we hypothesize enhancement of lipid oxidation and the oxidative potential of muscle following 3 weeks of DIO with the possibility for impairment to these indicators of lipid handling and muscle health following 8 weeks of DIO. It is my belief that providing detailed time-line assessments of the changes associated with DIO will further our understanding of Prediabetes development and the impact on skeletal muscle health.
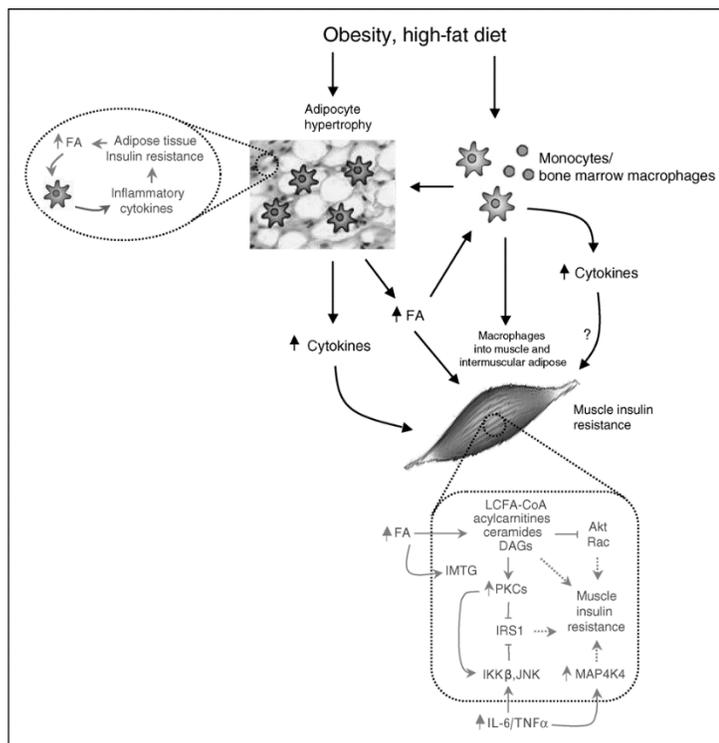
Figure 1.1. Lipotoxicity, cytokines and macrophage inflammatory responses may conspire to cause muscle insulin resistance of glucose uptake.

"High-fat diet leads to obesity due to excess storage of lipid in adipose tissue that elicits increased fatty acid (FA) release from adipocytes, initiation of proinflammatory responses from resident tissue macrophages and recruitment of macrophages from the circulation (as monocytes) [92]. Activated proinflammatory macrophages and stressed adipocyte release TNFα, IL-6 and other cytokines and adipokines to the circulation [92]. Together with FA, theses secreted cytokines impinge on muscle to cause insulin resistance, specifically impaired insulin-stimulated glucose uptake [7]. In addition, the number of adipocytes and macrophages may increase in the muscle tissue between fibre bundles and establish microenvironments of proinflammation and FA release [83]. Insulin resistance of muscle glucose uptake may result from increased accumulation of FA as intramuscular triglyceride (IMTG), long-chain fatty acyl-CoA (LCFA-CoA) and their metabolites diacylglycerols (DAGs), ceramides, and acylcarnitines. Whereas the levels of IMTG and LCFA-CoA positively correlate with obesity and insulin resistance, its cause may lie with the metabolites DAG and ceramide that impair insulin signalling leading to glucose uptake at the level of IRS1 or Akt, respectively, or by additional means (dotted arrow). DAG is thought to act primarily through novel PKC isoforms to enhance IKKβ and JNK which phosphorylate IRS1 on S307 or PKC may directly phosphorylate IRS1 on

30

S1101 reducing signalling to downstream effectors [35, 41]. Ceramides are thought to act through protein phosphatase 2A or PKCζ (not shown) [53,93] and Rac [53]. Cytokines (e.g. TNFα and IL-6) act on muscle cell surface receptors to activate IKKβ and JNK; TNFα can also activate MAP4K4 to induce insulin resistance."

Entire figure including title and description reprinted with permission (Kewalramani, Bilan & Klip 2010).
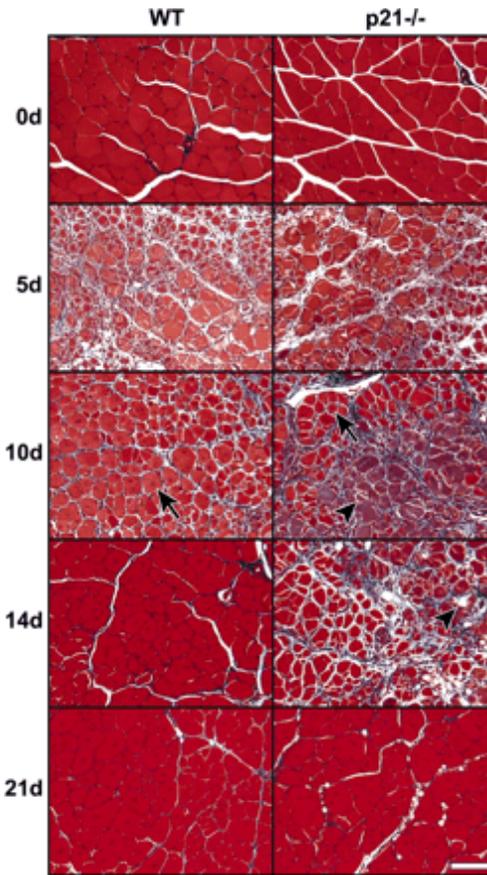
Figure 1.2. Cardiotoxin-induced regeneration is delayed in p21-/- skeletal muscle.

"Cardiotoxin-induced regeneration is delayed in p21-/- skeletal muscle. A: Masson's trichrome staining of regenerating WT and p21-/- skeletal muscle. Cardiotoxin was injected into the tibialis anterior (TA) muscles of WT and p21-/- mice and harvested at 0 days (0d; no injury) or 5, 10, 14, or 21 days (d) after injection. By 5 days of regeneration, the inflammatory response was reduced, the muscle was less edematous, macrophages invaded damaged myofibers, and MPCs were in a highly proliferative state. Numerous newly regenerated myofibers were visible as small basophilic, centronucleated myofibers. No difference between WT and p21-/- skeletal muscle architecture was visible at 5 days postinjury, suggesting that defects associated with the acute phases of regeneration were not responsible for the impaired regeneration in the p21-/- skeletal muscle. At 10 days postinjury, regeneration had largely occurred in regenerating WT skeletal muscle with many newly regenerated myofibers present (centrally located nuclei; arrows). Although some regenerated myofibers were visible in the regenerating p21-/- skeletal muscle, the attenuated regenerative response in the p21-/- skeletal muscle became evident with significant myonecrosis (arrowheads) and fibrosis visible. Regeneration was still visibly abnormal at 14 days postinjury in p21-/-

32

skeletal muscle with areas of myonecrosis apparent, whereas regenerating WT skeletal muscle architecture was essentially indistinguishable from preinjury skeletal muscle architecture. By 21 days postinjury, p21-/- skeletal muscle had regenerated with numerous centrally located nuclei. Scale bar, 100 μm."

Entire figure including title and description reprinted from (Hawke et al. 2003) (no permission required as per RightsLink).
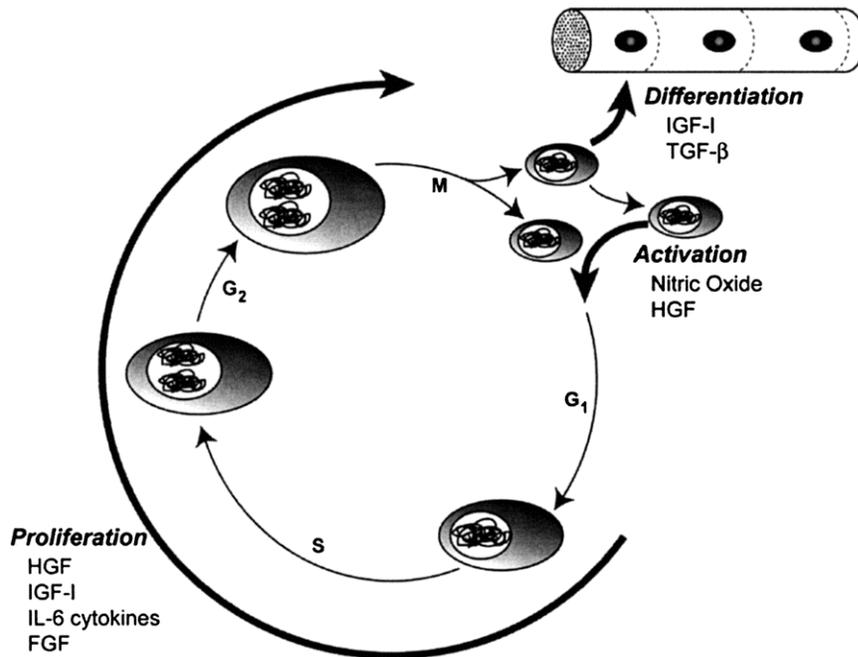
Figure 1.3. Extrinsic cues regulating the cell-cycle progression of muscle progenitor cells.

> *"Extrinsic cues regulating the cell-cycle progression of muscle progenitor cells. In response to stressors such as myotrauma, quiescent myogenic progenitor cells become activated, undergo proliferation, and ultimately differentiate to produce new muscle fibers. These newly regenerated fibers can be identified by their centrally located nuclei. Numerous growth factors have been shown to be important in mediating the progression of the myogenic progenitor cells through these particular phases. This schematic outlines only a few of the growth factors known to modulate myogenic progenitor cell activity. HGF, hepatocyte growth factor; IGF-I, insulin-like growth factor-I; FGF, fibroblast growth factor; TGF-[beta], transforming growth factor-[beta]."*

Entire figure including title and description reprinted with permission (Hawke 2005).

REFERENCE LIST

Al-Daghri, N.M., Al-Attas, O.S., Alokail, M.S., Alkharfy, K.M., Yakout, S.M., Sabico, S.B., Gibson, G.C., Chrousos, G.P. & Kumar, S. 2011, "Parent-offspring transmission of adipocytokine levels and their associations with metabolic traits", *PloS one,* vol. 6, no. 4, pp. e18182.

Allen, D.L., Cleary, A.S., Speaker, K.J., Lindsay, S.F., Uyenishi, J., Reed, J.M., Madden, M.C. & Mehan, R.S. 2008, "Myostatin, activin receptor IIb, and follistatin-like-3 gene expression are altered in adipose tissue and skeletal muscle of obese mice", *American journal of physiology.Endocrinology and metabolism,* vol. 294, no. 5, pp. E918-27.

Anderson, J.E. & Wozniak, A.C. 2004, "Satellite cell activation on fibers: modeling events in vivo--an invited review", *Canadian journal of physiology and pharmacology,* vol. 82, no. 5, pp. 300-310.

Anselmino, M. & Sillano, D. 2012, "Impact of pre-diabetes and diabetes on cardiovascular outcomes", *Current vascular pharmacology,* vol. 10, no. 6, pp. 680-683.

Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., van Rooijen, N., Plonquet, A., Gherardi, R.K. & Chazaud, B. 2007, "Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis", *The Journal of experimental medicine,* vol. 204, no. 5, pp. 1057-1069.

Arya, A.K., Pokharia, D. & Tripathi, K. 2011, "Relationship between oxidative stress and apoptotic markers in lymphocytes of diabetic patients with chronic non healing wound", *Diabetes research and clinical practice,* vol. 94, no. 3, pp. 377-384.

Baffy, G. 2009, "Kupffer cells in non-alcoholic fatty liver disease: the emerging view", *Journal of hepatology,* vol. 51, no. 1, pp. 212-223.

Bartell, S.M., Rayalam, S., Ambati, S., Gaddam, D.R., Hartzell, D.L., Hamrick, M., She, J.X., Della-Fera, M.A. & Baile, C.A. 2011, "Central (ICV) leptin injection increases bone formation, bone mineral density, muscle mass, serum IGF-1, and the expression of osteogenic genes in leptin-deficient ob/ob mice", *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research,* vol. 26, no. 8, pp. 1710-1720.

Bavenholm, P.N., Kuhl, J., Pigon, J., Saha, A.K., Ruderman, N.B. & Efendic, S. 2003, "Insulin resistance in type 2 diabetes: association with truncal obesity, impaired fitness, and atypical malonyl coenzyme A regulation", *The Journal of clinical endocrinology and metabolism,* vol. 88, no. 1, pp. 82-87.

Berria, R., Wang, L., Richardson, D.K., Finlayson, J., Belfort, R., Pratipanawatr, T., De Filippis, E.A., Kashyap, S. & Mandarino, L.J. 2006, "Increased collagen content in insulin-resistant skeletal muscle", *American journal of physiology.Endocrinology and metabolism,* vol. 290, no. 3, pp. E560-5.

Bhargava, P. & Lee, C.H. 2012, "Role and function of macrophages in the metabolic syndrome", *The Biochemical journal,* vol. 442, no. 2, pp. 253-262.

Biga, P.R., Froehlich, J.M., Greenlee, K.J., Galt, N.J., Meyer, B.M. & Christensen, D.J. 2013, "Gelatinases impart susceptibility to high-fat diet-induced obesity in mice", *The Journal of nutritional biochemistry,* vol. 24, no. 8, pp. 1462-1468.

Bonnard, C., Durand, A., Peyrol, S., Chanseaume, E., Chauvin, M.A., Morio, B., Vidal, H. & Rieusset, J. 2008, "Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice", *The Journal of clinical investigation,* vol. 118, no. 2, pp. 789-800.

Boushel, R., Gnaiger, E., Schjerling, P., Skovbro, M., Kraunsoe, R. & Dela, F. 2007, "Patients with type 2 diabetes have normal mitochondrial function in skeletal muscle", *Diabetologia,* vol. 50, no. 4, pp. 790-796.

Boyle, K.E., Canham, J.P., Consitt, L.A., Zheng, D., Koves, T.R., Gavin, T.P., Holbert, D., Neufer, P.D., Ilkayeva, O., Muoio, D.M. & Houmard, J.A. 2011, "A high-fat diet elicits differential responses in genes coordinating oxidative metabolism in skeletal muscle of lean and obese individuals", *The Journal of clinical endocrinology and metabolism,* vol. 96, no. 3, pp. 775-781.

Brigitte, M., Schilte, C., Plonquet, A., Baba-Amer, Y., Henri, A., Charlier, C., Tajbakhsh, S., Albert, M., Gherardi, R.K. & Chretien, F. 2010, "Muscle resident macrophages control the immune cell reaction in a mouse model of notexin-induced myoinjury", *Arthritis and Rheumatism,* vol. 62, no. 1, pp. 268-279.

Brink, M., Price, S.R., Chrast, J., Bailey, J.L., Anwar, A., Mitch, W.E. & Delafontaine, P. 2001, "Angiotensin II induces skeletal muscle wasting through enhanced protein degradation and down-regulates autocrine insulin-like growth factor I", *Endocrinology,* vol. 142, no. 4, pp. 1489-1496.

Burr, J.F., Rowan, C.P., Jamnik, V.K. & Riddell, M.C. 2010, "The role of physical activity in type 2 diabetes prevention: physiological and practical perspectives", *The Physician and sportsmedicine,* vol. 38, no. 1, pp. 72-82.

Cai, D., Yuan, M., Frantz, D.F., Melendez, P.A., Hansen, L., Lee, J. & Shoelson, S.E. 2005, "Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB", *Nature medicine,* vol. 11, no. 2, pp. 183-190.

Cameron-Smith, D., Burke, L.M., Angus, D.J., Tunstall, R.J., Cox, G.R., Bonen, A., Hawley, J.A. & Hargreaves, M. 2003, "A short-term, high-fat diet up-regulates lipid metabolism and gene expression in human skeletal muscle", *The American Journal of Clinical Nutrition,* vol. 77, no. 2, pp. 313-318.

Chanseaume, E., Tardy, A.L., Salles, J., Giraudet, C., Rousset, P., Tissandier, A., Boirie, Y. & Morio, B. 2007, "Chronological approach of diet-induced alterations in muscle mitochondrial functions in rats", *Obesity (Silver Spring, Md.),* vol. 15, no. 1, pp. 50-59.

Charge, S.B. & Rudnicki, M.A. 2004, "Cellular and molecular regulation of muscle regeneration", *Physiological reviews,* vol. 84, no. 1, pp. 209-238.

Clement, N.D., Hallett, A., MacDonald, D., Howie, C. & McBirnie, J. 2010, "Does diabetes affect outcome after arthroscopic repair of the rotator cuff?", *The Journal of bone and joint surgery.British volume,* vol. 92, no. 8, pp. 1112-1117.

Colagiuri, R. Fitzgerald, T. Furlong, D. Huot, C. Keeley, M. Krueger, H. Robinson, A. Silverberg, J. Turner, C. 2010, *Diabetes: Canada at a Tipping Point*.

Couteaux, R., Mira, J.C. & d'Albis, A. 1988, "Regeneration of muscles after cardiotoxin injury. I. Cytological aspects", *Biology of the cell / under the auspices of the European Cell Biology Organization,* vol. 62, no. 2, pp. 171-182.

Craig, B.W., Everhart, J. & Brown, R. 1989, "The influence of high-resistance training on glucose tolerance in young and elderly subjects", *Mechanisms of ageing and development,* vol. 49, no. 2, pp. 147-157.

Czerwinska, A.M., Streminska, W., Ciemerych, M.A. & Grabowska, I. 2012, "Mouse gastrocnemius muscle regeneration after mechanical or cardiotoxin injury", *Folia histochemica et cytobiologica / Polish Academy of Sciences, Polish Histochemical and Cytochemical Society,* vol. 50, no. 1, pp. 144-153.

d'Albis, A., Couteaux, R., Janmot, C., Roulet, A. & Mira, J.C. 1988, "Regeneration after cardiotoxin injury of innervated and denervated slow and fast muscles of mammals. Myosin isoform analysis", *European journal of biochemistry / FEBS,* vol. 174, no. 1, pp. 103-110.

de Souza, T.O., Mesquita, D.A., Ferrari, R.A., Dos Santos Pinto, D.,Jr, Correa, L., Bussadori, S.K., Fernandes, K.P. & Martins, M.D. 2011, "Phototherapy with low-level laser affects the remodeling of types I and III collagen in skeletal muscle repair", *Lasers in medical science,* vol. 26, no. 6, pp. 803-814.

de Wilde, J., Mohren, R., van den Berg, S., Boekschoten, M., Dijk, K.W., de Groot, P., Muller, M., Mariman, E. & Smit, E. 2008, "Short-term high fat-feeding results in morphological and metabolic adaptations in the skeletal muscle of C57BL/6J mice", *Physiological genomics,* vol. 32, no. 3, pp. 360-369.

de Wilde, J., Smit, E., Mohren, R., Boekschoten, M.V., de Groot, P., van den Berg, S.A., Bijland, S., Voshol, P.J., van Dijk, K.W., de Wit, N.W., Bunschoten, A., Schaart, G., Hulshof, M.F. & Mariman, E.C. 2009, "An 8-week high-fat diet induces obesity and insulin resistance with small changes in the muscle transcriptome of C57BL/6J mice", *Journal of nutrigenetics and nutrigenomics,* vol. 2, no. 6, pp. 280-291.

Decary, S., Hamida, C.B., Mouly, V., Barbet, J.P., Hentati, F. & Butler-Browne, G.S. 2000, "Shorter telomeres in dystrophic muscle consistent with extensive regeneration in young children", *Neuromuscular disorders : NMD,* vol. 10, no. 2, pp. 113-120.

Defronzo, R.A. 2009, "Banting Lecture. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus", *Diabetes,* vol. 58, no. 4, pp. 773-795.

DeFronzo, R.A. 1992, "Pathogenesis of type 2 (non-insulin dependent) diabetes mellitus: a balanced overview", *Diabetologia,* vol. 35, no. 4, pp. 389-397.

Dorsey, R. & Songer, T. 2011, "Lifestyle behaviors and physician advice for change among overweight and obese adults with prediabetes and diabetes in the United States, 2006", *Preventing chronic disease,* vol. 8, no. 6, pp. A132.

Dresner, A., Laurent, D., Marcucci, M., Griffin, M.E., Dufour, S., Cline, G.W., Slezak, L.A., Andersen, D.K., Hundal, R.S., Rothman, D.L., Petersen, K.F. & Shulman, G.I. 1999, "Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity", *The Journal of clinical investigation,* vol. 103, no. 2, pp. 253-259.

Duncan, G.E. 2006, "Prevalence of diabetes and impaired fasting glucose levels among US adolescents: National Health and Nutrition Examination Survey, 1999-2002", *Archives of Pediatrics & Adolescent Medicine,* vol. 160, no. 5, pp. 523-528.

Elkina, Y., von Haehling, S., Anker, S.D. & Springer, J. 2011, "The role of myostatin in muscle wasting: an overview", *Journal of cachexia, sarcopenia and muscle,* vol. 2, no. 3, pp. 143-151.

Fenicchia, L.M., Kanaley, J.A., Azevedo, J.L.,Jr, Miller, C.S., Weinstock, R.S., Carhart, R.L. & Ploutz-Snyder, L.L. 2004, "Influence of resistance exercise training on glucose control in women with type 2 diabetes", *Metabolism: clinical and experimental,* vol. 53, no. 3, pp. 284-289.

Fraulob, J.C., Ogg-Diamantino, R., Fernandes-Santos, C., Aguila, M.B. & Mandarim-de-Lacerda, C.A. 2010, "A Mouse Model of Metabolic Syndrome: Insulin Resistance, Fatty Liver and Non-Alcoholic Fatty Pancreas Disease (NAFPD) in C57BL/6 Mice Fed a High Fat Diet", *Journal of clinical biochemistry and nutrition,* vol. 46, no. 3, pp. 212-223.

Fujisaka, S., Usui, I., Bukhari, A., Ikutani, M., Oya, T., Kanatani, Y., Tsuneyama, K., Nagai, Y., Takatsu, K., Urakaze, M., Kobayashi, M. & Tobe, K. 2009, "Regulatory mechanisms for adipose tissue M1 and M2 macrophages in diet-induced obese mice", *Diabetes,* vol. 58, no. 11, pp. 2574-2582.

Galic, S., Oakhill, J.S. & Steinberg, G.R. 2010, "Adipose tissue as an endocrine organ", *Molecular and cellular endocrinology,* vol. 316, no. 2, pp. 129-139.

Garry, D.J., Yang, Q., Bassel-Duby, R. & Williams, R.S. 1997, "Persistent expression of MNF identifies myogenic stem cells in postnatal muscles", *Developmental biology,* vol. 188, no. 2, pp. 280-294.

Goldenberg, R. & Punthakee, Z. 2013, *Canadian Diabetes Association 2013 Clinical Practice Guidelines; Definition, Classification and Diagnosis of Diabetes, Prediabetes and Metabolic Syndrome.*

Goodpaster, B.H., Delany, J.P., Otto, A.D., Kuller, L., Vockley, J., South-Paul, J.E., Thomas, S.B., Brown, J., McTigue, K., Hames, K.C., Lang, W. & Jakicic, J.M. 2010, "Effects of diet and physical activity interventions on weight loss and cardiometabolic risk factors in severely obese adults: a randomized trial", *JAMA : the journal of the American Medical Association,* vol. 304, no. 16, pp. 1795-1802.

Gorzelniak, K., Engeli, S., Janke, J., Luft, F.C. & Sharma, A.M. 2002, "Hormonal regulation of the human adipose-tissue renin-angiotensin system: relationship to obesity and hypertension", *Journal of hypertension,* vol. 20, no. 5, pp. 965-973.

Grounds, M.D., McGeahie, J.K., Davies, M.J., Sorokin, L.M. & Maley, M.A.L. 1998, "The Expression of Extracellular Matrix During Adult Skeletal Muscle regeneration: How the Basement Membrane, Interstitium and Myogenic Cells Collaborate", *Basic Appl Myol,* vol. 8, no. 2, pp. 129.

Guillet, C. & Boirie, Y. 2005, "Insulin resistance: a contributing factor to age-related muscle mass loss?", *Diabetes & metabolism,* vol. 31 Spec No 2, pp. 5S20-5S26.

Hamrick, M.W., Herberg, S., Arounleut, P., He, H.Z., Shiver, A., Qi, R.Q., Zhou, L., Isales, C.M. & Mi, Q.S. 2010, "The adipokine leptin increases skeletal muscle mass

and significantly alters skeletal muscle miRNA expression profile in aged mice", *Biochemical and biophysical research communications,* vol. 400, no. 3, pp. 379-383.

Hawke, T.J. 2005, "Muscle stem cells and exercise training", *Exercise and sport sciences reviews,* vol. 33, no. 2, pp. 63-68.

Hawke, T.J. & Garry, D.J. 2001, "Myogenic satellite cells: physiology to molecular biology", *Journal of applied physiology (Bethesda, Md.: 1985),* vol. 91, no. 2, pp. 534-551.

Hawke, T.J., Jiang, N. & Garry, D.J. 2003, "Absence of p21CIP rescues myogenic progenitor cell proliferative and regenerative capacity in Foxk1 null mice", *The Journal of biological chemistry,* vol. 278, no. 6, pp. 4015-4020.

Hawke, T.J., Meeson, A.P., Jiang, N., Graham, S., Hutcheson, K., DiMaio, J.M. & Garry, D.J. 2003, "P21 is Essential for Normal Myogenic Progenitor Cell Function in Regenerating Skeletal Muscle", *American journal of physiology.Cell physiology,* vol. 285, no. 5, pp. C1019-27.

He, J., Watkins, S. & Kelley, D.E. 2001, "Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fiber type in type 2 diabetes and obesity", *Diabetes,* vol. 50, no. 4, pp. 817-823.

Hegarty, B.D., Cooney, G.J., Kraegen, E.W. & Furler, S.M. 2002, "Increased efficiency of fatty acid uptake contributes to lipid accumulation in skeletal muscle of high fat-fed insulin-resistant rats", *Diabetes,* vol. 51, no. 5, pp. 1477-1484.

Heslop, L., Morgan, J.E. & Partridge, T.A. 2000, "Evidence for a myogenic stem cell that is exhausted in dystrophic muscle", *Journal of cell science,* vol. 113 (Pt 12), no. Pt 12, pp. 2299-2308.

Hoeks, J., Wilde, J., Hulshof, M.F., Berg, S.A., Schaart, G., Dijk, K.W., Smit, E. & Mariman, E.C. 2011, "High fat diet-induced changes in mouse muscle mitochondrial phospholipids do not impair mitochondrial respiration despite insulin resistance", *PloS one,* vol. 6, no. 11, pp. e27274.

Hong, E.G., Ko, H.J., Cho, Y.R., Kim, H.J., Ma, Z., Yu, T.Y., Friedline, R.H., Kurt-Jones, E., Finberg, R., Fischer, M.A., Granger, E.L., Norbury, C.C., Hauschka, S.D., Philbrick, W.M., Lee, C.G., Elias, J.A. & Kim, J.K. 2009, "Interleukin-10 prevents diet-induced insulin resistance by attenuating macrophage and cytokine response in skeletal muscle", *Diabetes,* vol. 58, no. 11, pp. 2525-2535.

Hossain, P., Kawar, B. & El Nahas, M. 2007, "Obesity and diabetes in the developing world--a growing challenge", *The New England journal of medicine,* vol. 356, no. 3, pp. 213-215.

Hotta, K., Funahashi, T., Arita, Y., Takahashi, M., Matsuda, M., Okamoto, Y., Iwahashi, H., Kuriyama, H., Ouchi, N., Maeda, K., Nishida, M., Kihara, S., Sakai, N., Nakajima, T., Hasegawa, K., Muraguchi, M., Ohmoto, Y., Nakamura, T., Yamashita, S., Hanafusa, T. & Matsuzawa, Y. 2000, "Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients", *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 20, no. 6, pp. 1595-1599.

Houmard, J.A., Tanner, C.J., Yu, C., Cunningham, P.G., Pories, W.J., MacDonald, K.G. & Shulman, G.I. 2002, "Effect of weight loss on insulin sensitivity and intramuscular

long-chain fatty acyl-CoAs in morbidly obese subjects", *Diabetes,* vol. 51, no. 10, pp. 2959-2963.

Hu, E., Liang, P. & Spiegelman, B.M. 1996, "AdipoQ is a novel adipose-specific gene dysregulated in obesity", *The Journal of biological chemistry,* vol. 271, no. 18, pp. 10697-10703.

Hu, Z., Wang, H., Lee, I.H., Modi, S., Wang, X., Du, J. & Mitch, W.E. 2010, "PTEN inhibition improves muscle regeneration in mice fed a high-fat diet", *Diabetes,* vol. 59, no. 6, pp. 1312-1320.

Jiang, T., Wang, X.X., Scherzer, P., Wilson, P., Tallman, J., Takahashi, H., Li, J., Iwahashi, M., Sutherland, E., Arend, L. & Levi, M. 2007, "Farnesoid X receptor modulates renal lipid metabolism, fibrosis, and diabetic nephropathy", *Diabetes,* vol. 56, no. 10, pp. 2485-2493.

Jove, M., Planavila, A., Laguna, J.C. & Vazquez-Carrera, M. 2005, "Palmitate-induced interleukin 6 production is mediated by protein kinase C and nuclear-factor kappaB activation and leads to glucose transporter 4 down-regulation in skeletal muscle cells", *Endocrinology,* vol. 146, no. 7, pp. 3087-3095.

Jove, M., Planavila, A., Sanchez, R.M., Merlos, M., Laguna, J.C. & Vazquez-Carrera, M. 2006, "Palmitate induces tumor necrosis factor-alpha expression in C2C12 skeletal muscle cells by a mechanism involving protein kinase C and nuclear factor-kappaB activation", *Endocrinology,* vol. 147, no. 1, pp. 552-561.

Karpe, F., Dickmann, J.R. & Frayn, K.N. 2011, "Fatty acids, obesity, and insulin resistance: time for a reevaluation", *Diabetes,* vol. 60, no. 10, pp. 2441-2449.

Kellerer, M., Koch, M., Metzinger, E., Mushack, J., Capp, E. & Haring, H.U. 1997, "Leptin activates PI-3 kinase in C2C12 myotubes via janus kinase-2 (JAK-2) and insulin receptor substrate-2 (IRS-2) dependent pathways", *Diabetologia,* vol. 40, no. 11, pp. 1358-1362.

Kemp, J.G., Blazev, R., Stephenson, D.G. & Stephenson, G.M. 2009, "Morphological and biochemical alterations of skeletal muscles from the genetically obese (ob/ob) mouse", *International journal of obesity (2005),* vol. 33, no. 8, pp. 831-841.

Kewalramani, G., Bilan, P.J. & Klip, A. 2010, "Muscle insulin resistance: assault by lipids, cytokines and local macrophages", *Current opinion in clinical nutrition and metabolic care,* vol. 13, no. 4, pp. 382-390.

Kim, T.H., Choi, S.E., Ha, E.S., Jung, J.G., Han, S.J., Kim, H.J., Kim, D.J., Kang, Y. & Lee, K.W. 2011, "IL-6 induction of TLR-4 gene expression via STAT3 has an effect on insulin resistance in human skeletal muscle", *Acta Diabetologica, .*

Koves, T.R., Ussher, J.R., Noland, R.C., Slentz, D., Mosedale, M., Ilkayeva, O., Bain, J., Stevens, R., Dyck, J.R., Newgard, C.B., Lopaschuk, G.D. & Muoio, D.M. 2008, "Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance", *Cell metabolism,* vol. 7, no. 1, pp. 45-56.

Krause, M.P., Liu, Y., Vu, V., Chan, L., Xu, A., Riddell, M.C., Sweeney, G. & Hawke, T.J. 2008, "Adiponectin is expressed by skeletal muscle fibers and influences muscle phenotype and function", *American journal of physiology.Cell physiology,* vol. 295, no. 1, pp. C203-12.

Krause, M.P., Moradi, J., Nissar, A.A., Riddell, M.C. & Hawke, T.J. 2011, "Inhibition of plasminogen activator inhibitor-1 restores skeletal muscle regeneration in untreated type 1 diabetic mice", *Diabetes,* vol. 60, no. 7, pp. 1964-1972.

Kuang, S., Kuroda, K., Le Grand, F. & Rudnicki, M.A. 2007, "Asymmetric self-renewal and commitment of satellite stem cells in muscle", *Cell,* vol. 129, no. 5, pp. 999-1010.

Lai, K.M., Gonzalez, M., Poueymirou, W.T., Kline, W.O., Na, E., Zlotchenko, E., Stitt, T.N., Economides, A.N., Yancopoulos, G.D. & Glass, D.J. 2004, "Conditional activation of akt in adult skeletal muscle induces rapid hypertrophy", *Molecular and cellular biology,* vol. 24, no. 21, pp. 9295-9304.

Larsson, H., Daugaard, J.R., Kiens, B., Richter, E.A. & Ahren, B. 1999, "Muscle fiber characteristics in postmenopausal women with normal or impaired glucose tolerance", *Diabetes care,* vol. 22, no. 8, pp. 1330-1338.

Lee, H., Lee, D., Guo, G. & Harris, K.M. 2011, "Trends in body mass index in adolescence and young adulthood in the United States: 1959-2002", *The Journal of adolescent health : official publication of the Society for Adolescent Medicine,* vol. 49, no. 6, pp. 601-608.

Lee, J.H., Tachibana, H., Morinaga, Y., Fujimura, Y. & Yamada, K. 2009, "Modulation of proliferation and differentiation of C2C12 skeletal muscle cells by fatty acids", *Life Sciences,* vol. 84, no. 13-14, pp. 415-420.

Lee, Y.S., Li, P., Huh, J.Y., Hwang, I.J., Lu, M., Kim, J.I., Ham, M., Talukdar, S., Chen, A., Lu, W.J., Bandyopadhyay, G.K., Schwendener, R., Olefsky, J. & Kim, J.B. 2011, "Inflammation is necessary for long-term but not short-term high-fat diet-induced insulin resistance", *Diabetes,* vol. 60, no. 10, pp. 2474-2483.

Li, C., Ford, E.S., Zhao, G. & Mokdad, A.H. 2009, "Prevalence of pre-diabetes and its association with clustering of cardiometabolic risk factors and hyperinsulinemia among U.S. adolescents: National Health and Nutrition Examination Survey 2005-2006", *Diabetes care,* vol. 32, no. 2, pp. 342-347.

Li, Y.P. & Reid, M.B. 2000, "NF-kappaB mediates the protein loss induced by TNF-alpha in differentiated skeletal muscle myotubes", *American journal of physiology.Regulatory, integrative and comparative physiology,* vol. 279, no. 4, pp. R1165-70.

Lillioja, S., Young, A.A., Culter, C.L., Ivy, J.L., Abbott, W.G., Zawadzki, J.K., Yki-Jarvinen, H., Christin, L., Secomb, T.W. & Bogardus, C. 1987, "Skeletal muscle capillary density and fiber type are possible determinants of in vivo insulin resistance in man", *The Journal of clinical investigation,* vol. 80, no. 2, pp. 415-424.

Lin, S., Thomas, T.C., Storlien, L.H. & Huang, X.F. 2000, "Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice", *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity,* vol. 24, no. 5, pp. 639-646.

Liu, L., Zhang, Y., Chen, N., Shi, X., Tsang, B. & Yu, Y.H. 2007, "Upregulation of myocellular DGAT1 augments triglyceride synthesis in skeletal muscle and protects against fat-induced insulin resistance", *The Journal of clinical investigation,* vol. 117, no. 6, pp. 1679-1689.

Lyons, J.A., Haring, J.S. & Biga, P.R. 2010, "Myostatin expression, lymphocyte population, and potential cytokine production correlate with predisposition to high-fat diet induced obesity in mice", *PloS one,* vol. 5, no. 9, pp. e12928.

Maffei, M., Halaas, J., Ravussin, E., Pratley, R.E., Lee, G.H., Zhang, Y., Fei, H., Kim, S., Lallone, R. & Ranganathan, S. 1995, "Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects", *Nature medicine,* vol. 1, no. 11, pp. 1155-1161.

Maley, M.A., Davies, M.J. & Grounds, M.D. 1995, "Extracellular matrix, growth factors, genetics: their influence on cell proliferation and myotube formation in primary cultures of adult mouse skeletal muscle", *Experimental cell research,* vol. 219, no. 1, pp. 169-179.

Maroni, P., Bendinelli, P. & Piccoletti, R. 2005, "Intracellular signal transduction pathways induced by leptin in C2C12 cells", *Cell biology international,* vol. 29, no. 7, pp. 542-550.

Maroni, P., Bendinelli, P. & Piccoletti, R. 2003, "Early intracellular events induced by in vivo leptin treatment in mouse skeletal muscle", *Molecular and cellular endocrinology,* vol. 201, no. 1-2, pp. 109-121.

Nakanishi, M., Hirayama, E. & Kim, J. 2001, "Characterisation of myogenic cell membrane: II. Dynamic changes in membrane lipids during the differentiation of mouse C2 myoblast cells", *Cell biology international,* vol. 25, no. 10, pp. 971-979.

Nguyen, M.H., Cheng, M. & Koh, T.J. 2011, "Impaired muscle regeneration in ob/ob and db/db mice", *TheScientificWorldJournal,* vol. 11, pp. 1525-1535.

Nguyen, M.T., Favelyukis, S., Nguyen, A.K., Reichart, D., Scott, P.A., Jenn, A., Liu-Bryan, R., Glass, C.K., Neels, J.G. & Olefsky, J.M. 2007, "A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways", *The Journal of biological chemistry,* vol. 282, no. 48, pp. 35279-35292.

Nickelson, K.J., Stromsdorfer, K.L., Pickering, R.T., Liu, T.W., Ortinau, L.C., Keating, A.F. & Perfield, J.W.,2nd 2012, "A comparison of inflammatory and oxidative stress markers in adipose tissue from weight-matched obese male and female mice", *Experimental diabetes research,* vol. 2012, pp. 859395.

Nieto-Vazquez, I., Fernandez-Veledo, S., de Alvaro, C. & Lorenzo, M. 2008, "Dual role of interleukin-6 in regulating insulin sensitivity in murine skeletal muscle", *Diabetes,* vol. 57, no. 12, pp. 3211-3221.

Nieto-Vazquez, I., Fernandez-Veledo, S., de Alvaro, C., Rondinone, C.M., Valverde, A.M. & Lorenzo, M. 2007, "Protein-tyrosine phosphatase 1B-deficient myocytes show increased insulin sensitivity and protection against tumor necrosis factor-alpha-induced insulin resistance", *Diabetes,* vol. 56, no. 2, pp. 404-413.

Nieto-Vazquez, I., Fernandez-Veledo, S., Kramer, D.K., Vila-Bedmar, R., Garcia-Guerra, L. & Lorenzo, M. 2008, "Insulin resistance associated to obesity: the link TNF-alpha", *Archives of Physiology and Biochemistry,* vol. 114, no. 3, pp. 183-194.

Parise, G., McKinnell, I.W. & Rudnicki, M.A. 2008, "Muscle satellite cell and atypical myogenic progenitor response following exercise", *Muscle & nerve,* vol. 37, no. 5, pp. 611-619.

Park, S.W., Goodpaster, B.H., Strotmeyer, E.S., de Rekeneire, N., Harris, T.B., Schwartz, A.V., Tylavsky, F.A. & Newman, A.B. 2006, "Decreased muscle strength and quality in older adults with type 2 diabetes: the health, aging, and body composition study", *Diabetes,* vol. 55, no. 6, pp. 1813-1818.

Perez, R., Tupac-Yupanqui, I. & Dunner, S. 2008, "Evaluation of suitable reference genes for gene expression studies in bovine muscular tissue", *BMC molecular biology,* vol. 9, pp. 79-2199-9-79.

Peterson, J.M., Bryner, R.W. & Alway, S.E. 2008, "Satellite cell proliferation is reduced in muscles of obese Zucker rats but restored with loading", *American journal of physiology.Cell physiology,* vol. 295, no. 2, pp. C521-8.

Phielix, E., Meex, R., Ouwens, D.M., Sparks, L., Hoeks, J., Schaart, G., Moonen-Kornips, E., Hesselink, M.K. & Schrauwen, P. 2012, "High oxidative capacity due to chronic exercise training attenuates lipid-induced insulin resistance", *Diabetes,* vol. 61, no. 10, pp. 2472-2478.

Pimenta, A.S., Gaidhu, M.P., Habib, S., So, M., Fediuc, S., Mirpourian, M., Musheev, M., Curi, R. & Ceddia, R.B. 2008, "Prolonged exposure to palmitate impairs fatty acid oxidation despite activation of AMP-activated protein kinase in skeletal muscle cells", *Journal of cellular physiology,* vol. 217, no. 2, pp. 478-485.

Radin, M.S., Sinha, S., Bhatt, B.A., Dedousis, N. & O'Doherty, R.M. 2008, "Inhibition or deletion of the lipopolysaccharide receptor Toll-like receptor-4 confers partial protection against lipid-induced insulin resistance in rodent skeletal muscle", *Diabetologia,* vol. 51, no. 2, pp. 336-346.

RANDLE, P.J., GARLAND, P.B., HALES, C.N. & NEWSHOLME, E.A. 1963, "The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus", *Lancet,* vol. 1, no. 7285, pp. 785-789.

Reyna, S.M., Ghosh, S., Tantiwong, P., Meka, C.S., Eagan, P., Jenkinson, C.P., Cersosimo, E., Defronzo, R.A., Coletta, D.K., Sriwijitkamol, A. & Musi, N. 2008, "Elevated toll-like receptor 4 expression and signaling in muscle from insulin-resistant subjects", *Diabetes,* vol. 57, no. 10, pp. 2595-2602.

Rieusset, J., Bouzakri, K., Chevillotte, E., Ricard, N., Jacquet, D., Bastard, J.P., Laville, M. & Vidal, H. 2004, "Suppressor of cytokine signaling 3 expression and insulin resistance in skeletal muscle of obese and type 2 diabetic patients", *Diabetes,* vol. 53, no. 9, pp. 2232-2241.

Ritov, V.B., Menshikova, E.V., He, J., Ferrell, R.E., Goodpaster, B.H. & Kelley, D.E. 2005, "Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes", *Diabetes,* vol. 54, no. 1, pp. 8-14.

Rossmeisl, M., Rim, J.S., Koza, R.A. & Kozak, L.P. 2003, "Variation in type 2 diabetes--related traits in mouse strains susceptible to diet-induced obesity", *Diabetes,* vol. 52, no. 8, pp. 1958-1966.

Roy, C., Paglialunga, S., Fisette, A., Schrauwen, P., Moonen-Kornips, E., St-Onge, J., Hesselink, M.K., Richard, D., Joanisse, D.R. & Cianflone, K. 2008, "Shift in metabolic fuel in acylation-stimulating protein-deficient mice following a high-fat diet", *American journal of physiology.Endocrinology and metabolism,* vol. 294, no. 6, pp. E1051-9.

Saghizadeh, M., Ong, J.M., Garvey, W.T., Henry, R.R. & Kern, P.A. 1996, "The expression of TNF alpha by human muscle. Relationship to insulin resistance", *The Journal of clinical investigation,* vol. 97, no. 4, pp. 1111-1116.

Sainz, N., Rodriguez, A., Catalan, V., Becerril, S., Ramirez, B., Gomez-Ambrosi, J. & Fruhbeck, G. 2009, "Leptin administration favors muscle mass accretion by decreasing FoxO3a and increasing PGC-1alpha in ob/ob mice", *PloS one,* vol. 4, no. 9, pp. e6808.

Samuel, V.T., Liu, Z.X., Qu, X., Elder, B.D., Bilz, S., Befroy, D., Romanelli, A.J. & Shulman, G.I. 2004, "Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease", *The Journal of biological chemistry,* vol. 279, no. 31, pp. 32345-32353.

Samuel, V.T. & Shulman, G.I. 2012, "Mechanisms for insulin resistance: common threads and missing links", *Cell,* vol. 148, no. 5, pp. 852-871.

Sayer, A.A., Dennison, E.M., Syddall, H.E., Gilbody, H.J., Phillips, D.I. & Cooper, C. 2005, "Type 2 diabetes, muscle strength, and impaired physical function: the tip of the iceberg?", *Diabetes care,* vol. 28, no. 10, pp. 2541-2542.

Sayer, A.A., Syddall, H.E., Dennison, E.M., Martin, H.J., Phillips, D.I., Cooper, C., Byrne, C.D. & Hertfordshire Cohort 2007, "Grip strength and the metabolic syndrome: findings from the Hertfordshire Cohort Study", *QJM : monthly journal of the Association of Physicians,* vol. 100, no. 11, pp. 707-713.

Scarda, A., Franzin, C., Milan, G., Sanna, M., Dal Pra, C., Pagano, C., Boldrin, L., Piccoli, M., Trevellin, E., Granzotto, M., Gamba, P., Federspil, G., De Coppi, P. & Vettor, R. 2010, "Increased adipogenic conversion of muscle satellite cells in obese Zucker rats", *International journal of obesity (2005),* vol. 34, no. 8, pp. 1319-1327.

Scheele, C., Nielsen, S., Kelly, M., Broholm, C., Nielsen, A.R., Taudorf, S., Pedersen, M., Fischer, C.P. & Pedersen, B.K. 2012, "Satellite cells derived from obese humans with type 2 diabetes and differentiated into myocytes in vitro exhibit abnormal response to IL-6", *PloS one,* vol. 7, no. 6, pp. e39657.

Schiaffino, S., Sandri, M. & Murgia, M. 2007, "Activity-dependent signaling pathways controlling muscle diversity and plasticity", *Physiology (Bethesda, Md.),* vol. 22, pp. 269-278.

Simoneau, J.A. & Kelley, D.E. 1997, "Altered glycolytic and oxidative capacities of skeletal muscle contribute to insulin resistance in NIDDM", *Journal of applied physiology (Bethesda, Md.: 1985),* vol. 83, no. 1, pp. 166-171.

Sishi, B., Loos, B., Ellis, B., Smith, W., du Toit, E.F. & Engelbrecht, A.M. 2011, "Diet-induced obesity alters signalling pathways and induces atrophy and apoptosis in skeletal muscle in a prediabetic rat model", *Experimental physiology,* vol. 96, no. 2, pp. 179-193.

Slentz, C.A., Houmard, J.A. & Kraus, W.E. 2009, "Exercise, abdominal obesity, skeletal muscle, and metabolic risk: evidence for a dose response", *Obesity (Silver Spring, Md.),* vol. 17 Suppl 3, pp. S27-33.

Sparks, L.M., Xie, H., Koza, R.A., Mynatt, R., Hulver, M.W., Bray, G.A. & Smith, S.R. 2005, "A high-fat diet coordinately downregulates genes required for mitochondrial

oxidative phosphorylation in skeletal muscle", *Diabetes,* vol. 54, no. 7, pp. 1926-1933.

Srikanthan, P. & Karlamangla, A.S. 2011, "Relative muscle mass is inversely associated with insulin resistance and prediabetes. Findings from the third National Health and Nutrition Examination Survey", *The Journal of clinical endocrinology and metabolism,* vol. 96, no. 9, pp. 2898-2903.

Steinberg, G.R. 2007, "Inflammation in obesity is the common link between defects in fatty acid metabolism and insulin resistance", *Cell cycle (Georgetown, Tex.),* vol. 6, no. 8, pp. 888-894.

Steinberg, G.R., McAinch, A.J., Chen, M.B., O'Brien, P.E., Dixon, J.B., Cameron-Smith, D. & Kemp, B.E. 2006a, "The suppressor of cytokine signaling 3 inhibits leptin activation of AMP-kinase in cultured skeletal muscle of obese humans", *The Journal of clinical endocrinology and metabolism,* vol. 91, no. 9, pp. 3592-3597.

Steinberg, G.R., Michell, B.J., van Denderen, B.J., Watt, M.J., Carey, A.L., Fam, B.C., Andrikopoulos, S., Proietto, J., Gorgun, C.Z., Carling, D., Hotamisligil, G.S., Febbraio, M.A., Kay, T.W. & Kemp, B.E. 2006b, "Tumor necrosis factor alpha-induced skeletal muscle insulin resistance involves suppression of AMP-kinase signaling", *Cell metabolism,* vol. 4, no. 6, pp. 465-474.

Steinberg, G.R., O'Neill, H.M., Dzamko, N.L., Galic, S., Naim, T., Koopman, R., Jorgensen, S.B., Honeyman, J., Hewitt, K., Chen, Z.P., Schertzer, J.D., Scott, J.W., Koentgen, F., Lynch, G.S., Watt, M.J., van Denderen, B.J., Campbell, D.J. & Kemp, B.E. 2010, "Whole body deletion of AMP-activated protein kinase {beta}2 reduces muscle AMPK activity and exercise capacity", *The Journal of biological chemistry,* vol. 285, no. 48, pp. 37198-37209.

Sugioka, Y., Tada, M., Okano, T., Nakamura, H. & Koike, T. 2012, "Acquired leptin resistance by high-fat feeding reduces inflammation from collagen antibody-induced arthritis in mice", *Clinical and experimental rheumatology, .*

Tanner, C.J., Barakat, H.A., Dohm, G.L., Pories, W.J., MacDonald, K.G., Cunningham, P.R., Swanson, M.S. & Houmard, J.A. 2002, "Muscle fiber type is associated with obesity and weight loss", *American journal of physiology.Endocrinology and metabolism,* vol. 282, no. 6, pp. E1191-6.

Tatsumi, R., Anderson, J.E., Nevoret, C.J., Halevy, O. & Allen, R.E. 1998, "HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells", *Developmental biology,* vol. 194, no. 1, pp. 114-128.

Thorsson, O., Rantanen, J., Hurme, T. & Kalimo, H. 1998, "Effects of nonsteroidal antiinflammatory medication on satellite cell proliferation during muscle regeneration", *The American Journal of Sports Medicine,* vol. 26, no. 2, pp. 172-176.

Tidball, J.G. & Villalta, S.A. 2010, "Regulatory interactions between muscle and the immune system during muscle regeneration", *American journal of physiology.Regulatory, integrative and comparative physiology,* vol. 298, no. 5, pp. R1173-87.

Tiidus, P.M. 2008, *Skeletal muscle damage and repair,* Human Kinetics, Champaign, IL.

Timmerman, K.L., Lee, J.L., Dreyer, H.C., Dhanani, S., Glynn, E.L., Fry, C.S., Drummond, M.J., Sheffield-Moore, M., Rasmussen, B.B. & Volpi, E. 2010, "Insulin

stimulates human skeletal muscle protein synthesis via an indirect mechanism involving endothelial-dependent vasodilation and mammalian target of rapamycin complex 1 signaling", *The Journal of clinical endocrinology and metabolism,* vol. 95, no. 8, pp. 3848-3857.

Toft, I., Bonaa, K.H., Lindal, S. & Jenssen, T. 1998, "Insulin kinetics, insulin action, and muscle morphology in lean or slightly overweight persons with impaired glucose tolerance", *Metabolism: clinical and experimental,* vol. 47, no. 7, pp. 848-854.

Trostler, N., Romsos, D.R., Bergen, W.G. & Leveille, G.A. 1979, "Skeletal muscle accretion and turnover in lean and obese (ob/ob) mice", *Metabolism: clinical and experimental,* vol. 28, no. 9, pp. 928-933.

Tsimihodimos, V., Florentin, M. & Elisaf, M.S. 2013, "How should we treat hypertension and dyslipidemia in patients with prediabetes?", *Current pharmaceutical design,* vol. 19, no. 21, pp. 3773-3787.

Turner, N., Bruce, C.R., Beale, S.M., Hoehn, K.L., So, T., Rolph, M.S. & Cooney, G.J. 2007, "Excess lipid availability increases mitochondrial fatty acid oxidative capacity in muscle: evidence against a role for reduced fatty acid oxidation in lipid-induced insulin resistance in rodents", *Diabetes,* vol. 56, no. 8, pp. 2085-2092.

Turpin, S.M., Ryall, J.G., Southgate, R., Darby, I., Hevener, A.L., Febbraio, M.A., Kemp, B.E., Lynch, G.S. & Watt, M.J. 2009, "Examination of 'lipotoxicity' in skeletal muscle of high-fat fed and ob/ob mice", *The Journal of physiology,* vol. 587, no. Pt 7, pp. 1593-1605.

Tzeng, T.F., Liu, I.M. & Cheng, J.T. 2005, "Activation of opioid mu-receptors by loperamide to improve interleukin-6-induced inhibition of insulin signals in myoblast C2C12 cells", *Diabetologia,* vol. 48, no. 7, pp. 1386-1392.

Ueki, K., Kondo, T. & Kahn, C.R. 2004, "Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms", *Molecular and cellular biology,* vol. 24, no. 12, pp. 5434-5446.

Van Heek, M., Compton, D.S., France, C.F., Tedesco, R.P., Fawzi, A.B., Graziano, M.P., Sybertz, E.J., Strader, C.D. & Davis, H.R.,Jr 1997, "Diet-induced obese mice develop peripheral, but not central, resistance to leptin", *The Journal of clinical investigation,* vol. 99, no. 3, pp. 385-390.

Vander Haar, E., Lee, S.I., Bandhakavi, S., Griffin, T.J. & Kim, D.H. 2007, "Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40", *Nature cell biology,* vol. 9, no. 3, pp. 316-323.

Wang, X.H., Du, J., Klein, J.D., Bailey, J.L. & Mitch, W.E. 2009, "Exercise ameliorates chronic kidney disease-induced defects in muscle protein metabolism and progenitor cell function", *Kidney international,* vol. 76, no. 7, pp. 751-759.

Weiss, R., Dufour, S., Taksali, S.E., Tamborlane, W.V., Petersen, K.F., Bonadonna, R.C., Boselli, L., Barbetta, G., Allen, K., Rife, F., Savoye, M., Dziura, J., Sherwin, R., Shulman, G.I. & Caprio, S. 2003, "Prediabetes in obese youth: a syndrome of impaired glucose tolerance, severe insulin resistance, and altered myocellular and abdominal fat partitioning", *Lancet,* vol. 362, no. 9388, pp. 951-957.

White, R.B., Bierinx, A.S., Gnocchi, V.F. & Zammit, P.S. 2010, "Dynamics of muscle fibre growth during postnatal mouse development", *BMC developmental biology,* vol. 10, pp. 21.

Woo, M., Isganaitis, E., Cerletti, M., Fitzpatrick, C., Wagers, A.J., Jimenez-Chillaron, J. & Patti, M.E. 2011, "Early life nutrition modulates muscle stem cell number: implications for muscle mass and repair", *Stem cells and development,* vol. 20, no. 10, pp. 1763-1769.

Xu, L., Jiang, C.Q., Lam, T.H., Bao, B., Cheng, K.K. & Thomas, G.N. 2011, "Plasminogen activator inhibitor-1 and HbA1c defined prediabetes: the Guangzhou Biobank Cohort Study-CVD", *Clinical endocrinology,* vol. 74, no. 4, pp. 528-531.

Yan, Z., Choi, S., Liu, X., Zhang, M., Schageman, J.J., Lee, S.Y., Hart, R., Lin, L., Thurmond, F.A. & Williams, R.S. 2003, "Highly coordinated gene regulation in mouse skeletal muscle regeneration", *The Journal of biological chemistry,* vol. 278, no. 10, pp. 8826-8836.

Yu, J., Shi, L., Wang, H., Bilan, P.J., Yao, Z., Samaan, M.C., He, Q., Klip, A. & Niu, W. 2011, "Conditioned medium from hypoxia-treated adipocytes renders muscle cells insulin resistant", *European journal of cell biology,* vol. 90, no. 12, pp. 1000-1015.

Yu, Q. & Stamenkovic, I. 2000, "Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis", *Genes & development,* vol. 14, no. 2, pp. 163-176.

Zhou, Q., Du, J., Hu, Z., Walsh, K. & Wang, X.H. 2007, "Evidence for adipose-muscle cross talk: opposing regulation of muscle proteolysis by adiponectin and Fatty acids", *Endocrinology,* vol. 148, no. 12, pp. 5696-5705.

CHAPTER 2

Muscle-Specific Adaptations, Impaired Oxidative Capacity and Maintenance of Contractile Function Characterize Diet-Induced Obese Mouse Skeletal Muscle

Authors: Karin E. Shortreed, Matthew P. Krause, Julianna H. Huang, Dili Dhanani, Jasmin Moradi, Rolando B. Ceddia and Thomas J. Hawke

Corresponding Author: Thomas J. Hawke, Dept. of Pathology and Molecular Medicine, McMaster Univ., 4N65, Health Sciences Centre, 1200 Main St. W., Hamilton Ontario, L8N 3Z5, Canada (email:hawke@mcmaster.ca).

PREFACE

Significance to thesis

The primary goal of this study was to perform a comprehensive characterization of changes to muscle physiology (growth, morphology, metabolism and functional capacity) in the diet-induced obese (DIO) mouse. As noted in the literature review, studies employing the DIO mouse model have reported conflicting findings as to the state of muscle health which may be due to variations in studies including muscle type assessed, diet length/composition and assessment techniques. In particular the state of oxidative metabolism in muscle with DIO is debated in the literature. Here we employed a novel single muscle fiber approach to assess oxidative metabolism thus avoiding potential disruption of the muscle and altered trafficking of substrates inside the cell. Other aspects of muscle health were assessed in different muscle types to provide clarity to the literature as to the different physiological responses associated with muscle type following DIO. Assessing many aspects of muscle health in one study will help elucidate the associations between these changes and early muscle insulin resistance which could improve our understanding of the mechanisms behind muscle insulin resistance with DIO.

Authors' contributions

Karin E. Trajcevski contributed to the design of the study, performed all experiments and data collection unless noted below, supervised microscope image

analysis, performed all statistical analysis, wrote the initial draft of the manuscript and worked on refining this draft and the revisions based on editorial review.

Krause MP performed muscle stimulation experiments and analyses and assisted with muscle staining and analyses.

Huang JH, Dhanani D, and Moradi J assisted with performing experiments and some fiber type analyses.

Ceddia RB contributed to the design of the study, assisted with radioactive experiments and worked on revisions of the manuscript based on editorial review.

Hawke TJ contributed to the design of the study, assisted with performing experiments, and worked on refining drafts of the manuscript and the revisions based on editorial review.

ABSTRACT

Background. The effects of diet-induced obesity on skeletal muscle function are largely unknown, particularly as it relates to changes in oxidative metabolism and morphology. **Principal Findings.** Compared to control fed mice, mice fed a high fat diet (HFD; 60% kcal: fat) for 8 weeks displayed increased body mass and insulin resistance without overt fasting hyperglycemia (i.e. pre-diabetic). Histological analysis revealed a greater oxidative potential in the HFD gastrocnemius/plantaris (increased IIA, reduced IIB fiber-type percentages) and soleus (increased I, IIA cross-sectional areas) muscles, but no change in fiber type percentages in tibialis anterior muscles compared to controls. Intramyocellular lipid levels were significantly increased relative to control in HFD gastrocnemius/plantaris, but were similar to control values in the HFD soleus. Using a novel, single muscle fiber approach, impairments in complete palmitate and glucose oxidation (72.8±6.6% and 61.8±9.1% of control, respectively; $p<0.05$) with HFD were detected. These reductions were consistent with measures made using intact extensor digitorum longus and soleus muscles. Compared to controls, no difference in succinate dehydrogenase or citrate synthase enzyme activities were observed between groups in any muscle studied, however, short-chain fatty acyl CoA dehydrogenase (SCHAD) activity was elevated in the HFD soleus, but not tibialis anterior muscles. Despite these morphological and metabolic alterations, no significant difference in peak tetanic force or low-frequency fatigue rates were observed between groups. **Conclusions.** These findings indicate that HFD induces early adaptive responses that occur in a muscle-specific

51

pattern, but are insufficient to prevent impairments in oxidative metabolism with continued high-fat feeding. Moreover, the morphological and metabolic changes which occur with 8 weeks of HFD do not significantly impact muscle contractile properties.

INTRODUCTION

Sedentary behavior and consumption of high-energy diets favor the early development of disease states such as obesity, insulin resistance, and ultimately type 2 diabetes mellitus. These activities have created a serious health crisis in our society. In the United States alone, it is estimated that approximately 57 million people have pre-diabetes (impaired glucose tolerance preceding type 2 diabetes mellitus development). Of this, over 2 million are under the age of 20 years old; an age group that until recently was generally unaffected by these disorders (American Diabetes Association).

As skeletal muscle plays a major role in energy expenditure and insulin-stimulated glucose disposal, understanding changes that occur to this tissue with obesity and pre-diabetes development are critical to elucidating the underlying causes for insulin resistance and type 2 diabetes. Though a number of studies have investigated the effects diet-induced obesity on skeletal muscle oxidative capacity and insulin sensitivity, we are unaware of any that relate these changes with alterations in skeletal muscle morphology and functional capacity. Undertaking a comprehensive analysis in a variety of muscles is particularly important given that the disparities in model used, length and composition of diet intervention, muscles analyzed and analytical techniques have made comparisons between studies very challenging. For example, an increased (Turner et al. 2007), decreased (Chanseaume et al. 2006, Koves et al. 2008, Lionetti et al. 2007) or unchanged (Koves et al. 2008) capacity for oxidative metabolism with high fat diet intervention have all been demonstrated. Furthermore, an increase in oxidative

phosphorylation protein complexes have been measured in the gastrocnemius muscles following 4 weeks of high fat feeding (de Wilde et al. 2008), while a decreased expression of oxidative phosphorylation genes and cytochrome C protein has been demonstrated in the quadriceps muscles in response to 3 weeks of high fat diet consumption (Sparks et al. 2005). It is worth noting that studies assessing oxidative metabolism have investigated changes through the utilization of homogenized muscle, skinned fibers and/or isolated mitochondria. While these techniques are valuable tools for the assessment of specific aspects of mitochondrial oxidative capacity, the disruption of the muscle may eliminate potential impairments in fatty acid uptake, transport, and trafficking inside the cell caused by high fat diet, precluding extrapolation of this data to the intact whole muscle.

Thus, it was the aim of the current study to comprehensively analyze skeletal muscle morphology, metabolism and contractile function in young adult skeletal muscle. Specifically, we assessed skeletal muscle glucose and fatty acid oxidation rates in isolated muscle fibers and in intact oxidative and mixed muscles along with key oxidative enzyme activities, skeletal muscle morphology, and contractile properties in mice fed either a high fat diet (HFD) or standard rodent chow (control) for 8 weeks. Our findings indicate that the skeletal muscle of mice with diet-induced obesity undergoes significant alterations in fiber type, fiber area and intramyocellular lipid (IMCL) levels and that these changes occur in a muscle-specific manner. However, complete glucose and palmitate oxidation rates were decreased in all muscles analyzed, suggesting that elevated IMCL levels and alterations in SCHAD activity do not solely explain the insulin resistance and impaired

oxidative capacities. Moreover, peak tetanic force and overall fatigue rates were maintained despite significant changes in muscle morphology and oxidative capacity.

METHODS

Animals and blood sampling.

All experimental protocols were approved by the York University Animal Care Committee in accordance with Canadian Council for Animal Care guidelines. Male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed in temperature and humidity-controlled facility with a 12/12 h light/dark cycle and had *ad libitum* access to water and food. After an initial acclimatization, animals (10 weeks of age; N = 20 per group) were randomly assigned to either a high fat diet [HFD; TestDiet, cat#58126: energy (kcal/g) from protein (18.3%), fat (60.9%), carbohydrate (20.1%)] or standard mouse chow [LabDiet 5015 Mouse Diet: energy (kcal/g) from protein (20%), fat (25%), carbohydrate (55%)]. Body mass and blood glucose (via tail nick; OneTouch Ultra glucometer; Johnson & Johnson) were assessed in the fed state on biweekly basis. Fasted body weight was assessed at 4 weeks (following an 8 hr fast) and 8 weeks (following a 16 hr fast) of diet intervention. Fasted (8 hr) plasma insulin was assessed at 4 weeks of diet intervention as described below for insulin from the intraperitoneal glucose tolerance test (IPGTT).

An IPGTT was performed on mice fasted overnight (16 hrs) after 7 weeks of diet intervention. Glucose was injected IP (2 g/kg of body weight) and blood glucose was assessed by tail bleeds at 0, 15, 30, 60, 90, 120, 150 min. Plasma was collected by tail bleed at 45 min and later analyzed for insulin, in duplicate, using 5 μl in the Ultra

Sensitive Mouse Insulin ELISA Kit (cat#90080, Crystal Chem, Illinois), according to the manufacturer's instructions.

Experimental procedures.

Following 8 weeks of diet, mice were fasted overnight (16 hrs) and weighed. Subsequently, mice were anaesthetized, the muscle stimulation protocol (described below) was performed and tissues were harvested. Immediately prior to the electrical stimulation protocol, the left and right tibialis anterior (TA), extensor digitorum longus (EDL), peroneus, and soleus muscles were removed from the muscle-stimulation leg (left). EDL and peroneus longus muscles were used for single fiber isolation and soleus was used for intact muscle oxidative capacity, snap frozen or mounted with tissue freezing medium and frozen in isopentane cooled by liquid nitrogen. The TA muscles were snap frozen for future analysis or mounted with tissue freezing medium and frozen in isopentane cooled by liquid nitrogen. Following electrical stimulation, the gastrocnemius/plantaris complex was weighed and then either mounted with tissue freezing medium and frozen in isopentane cooled by liquid nitrogen for subsequent histological analysis or snap frozen in liquid nitrogen for future analysis.

Muscle stimulation protocol.

Prior to surgery, mice were injected with ketamine/xylazine (150 mg/kg: 10 mg/kg) and the left gastrocnemius/plantaris complex was isolated from its distal insertion, attached to a force transducer and optimal voltage and length were determined (Krause et al. 2008, Krause et al. 2009). A force-frequency curve was determined before (pre-) and after (post-) a fatigue protocol consisting of 2 minutes of low-frequency (30 Hz)

stimulations lasting 333 ms in 1 s trains. Pre-fatigue force determination consisted of 1 s stimulation every 30 s at increasing frequencies of 10 Hz starting at 20 Hz (Krause et al. 2008, Krause et al. 2009). Post-fatigue force determination was similarly conducted except that stimulations were spaced 10 s apart, instead of 30 s, in order to ensure that peak force determination was completed prior to recovery from the fatigue protocol as determined by pilot studies. The post-fatigue force-frequency curve provided an assessment of immediate force recovery following the fatigue protocol. All muscle function data were collected through an AD Instruments Bridge Amp and Powerlab 4/30, and analyzed with Chart5 PowerLab software.

Single muscle fiber isolation.

Single skeletal muscle fibers were harvested from the EDL and peroneus longus muscles using a collagenase digestion protocol as previously described (Hawke, Jiang & Garry 2003). Single fibers were collected using glass blown Pasteur pipettes and placed in matrigel-coated 35 mm culture dishes (BD Biosciences, Canada) containing plating media [10% horse serum (Invitrogen, USA), 0.5% chick embryo extract (MP Biomedicals, Ohio) in Dulbecco's Modified Essential Medium (DMEM; Invitrogen, USA)].

Fatty acid and glucose oxidation in single fibers.

Approximately 18 hours following isolation, palmitate and glucose oxidation rates were assessed on groups of single fibers in 35 mm cell culture dishes. The 18 hour incubation period was critical to allow fibers to recover from the isolation procedure, settle onto the matrigel-coated dishes and allow any non-viable fibers to hypercontract

and hence be removed with rinses. After the incubation period, wells were slowly rinsed three times with warm PBS and viable fibers were counted. On average, 15 to 30 viable, healthy fibers from each mouse muscle were used for oxidation rate assessment. Fiber viability was assessed by the presence of cross-striations along the length of fiber and the absence of sarcolemmal damage. Fibers were then serum-starved for 3 hours in low-glucose DMEM supplemented with sodium bicarbonate and pH adjusted. Following starvation fibers were incubated with either [U-^{14}C]glucose (0.2 µCi/ml) + cold glucose (5.5 mM) for the determination of glucose oxidation or [1-^{14}C]palmitic acid (0.15 µCi/ml) + cold palmitate (100 µM) complexed with fat-free BSA + L-carnitine (500 µM) for the determination of palmitate oxidation. Each well was incubated with the palmitate or glucose mixture for 2 hours in a closed system then the CO_2 produced was assessed as previously described (Ceddia, Sweeney 2004, Pimenta et al. 2008).

Whole muscle glucose and fatty acid oxidation and glycogen synthesis.

Following 8 weeks of diet intervention, mice were fasted overnight and anesthetized with ketamine/xylazine (150 mg/kg: 10 mg/kg) prior to removal of soleus and EDL muscles. Palmitate oxidation (N = 4 per muscle group), glucose oxidation (N = 4–6 per muscle group), and glycogen synthesis (N = 4–6 per muscle group) were assessed as previously described (Ceddia, Sweeney 2004, Fediuc, Gaidhu & Ceddia 2006, Ceddia, William & Curi 1999). Briefly, isolated EDL and soleus muscles were quickly extracted and mounted onto thin, stainless steel wire clips to maintain resting length. The incubations were performed immediately after extraction and the muscles were placed in plastic scintillation vials containing 2 ml of gassed (O_2:CO_2 – 95:5%) Krebs-Hanseleit

bicarbonate (KHB) buffer with 4% fat-free BSA and 5.5 mM glucose. The scintillation vials were then sealed with rubber stoppers and gasification (O_2:CO_2 – 95:5%) was continued during all incubations. After pre-incubation, the muscles were transferred to a second set of vials with 1.5 ml of KHB buffer containing either [U-^{14}C]glucose (0.2 µCi/ml) + cold glucose (5.5 mM) for the determination of glucose oxidation and glycogen synthesis or [1-^{14}C]palmitic acid (0.15 µCi/ml) + cold palmitate (0.2 mM) complexed with fat-free BSA + L-carnitine (500 µM) for the determination of palmitate oxidation. Assessment of the effects of insulin on glucose oxidation and glycogen synthesis was performed in the presence of 100 nM of the hormone.

Enzymatic assays.

Determination of citrate synthase (CS) and short chain fatty acyl CoA dehydrogenase (SCHAD) was performed using pulverized TA (~25 mg) or soleus (~5 mg) muscle powder sonicated with 1:20 (w/v) of extraction buffer as previously described (Carter et al. 2001), assayed using a spectrophotometer (BioRad SmartSpecPlus, CA) and normalized to protein concentration determined by Bradford assay (Carter et al. 2001). CS activity was assessed in duplicate or triplicate and measurements were taken every 10 s over a 3 min period. SCHAD activity was assessed using both 5 µl and 10 µl samples to determine optimal volume, measurements were taken every 2 s over a 2.5 min period.

Histochemical analyses.

Fiber type and IMCL were assessed using the metachromatic and Oil-Red-O staining methods respectively (Krause et al. 2009, Hawke, Jiang & Garry 2003,

Koopman, Schaart & Hesselink 2001, Ogilvie, Feeback 1990). Metachromatic stained muscle sections were used to assess fiber type areas for gastrocnemius/plantaris (N = 4, average 187 fibers/muscle) and soleus (N = 3, average 214 fibers/muscle) and fiber type percentages for gastrocnemius/plantaris (N = 4, average 187 fibers/muscle), soleus (N = 3, average 328 fibers/muscle), and TA (N = 4, average 331 fibers/muscle) using Scion Image. Succinate dehydrogenase (SDH) activity was assayed by incubation of muscle sections in medium consisting of 100 mM phosphate buffer (pH 7.6), 1 mM KCN (Sigma, 207810), 6.3 mM EDTA, and 1.22 mM nitroblue tetrazolium (Sigma, N6876). IMCL and SDH intensity were quantified in representative mixed fiber type regions in serial sections of the gastrocnemius/plantaris (IMCL: N = 4 average 187 fibers/muscle; SDH: N = 4, average 187 fibers/muscle) and soleus (IMCL: N = 4–5, average 51 fibers/muscle; SDH: N = 3, average 62 fibers/muscle). To measure SDH activity per fiber, images were converted to grey scale, fibers were encircled in Adobe Photoshop and the mean pixel intensity/optical intensity, graphically represented as arbitrary units (A.U.) relevant to the overall control mean, in the area of interest was recorded. Consequently, the darker the stain per fiber, the more SDH activity, the greater the mean pixel intensity. The same procedure was undertaken to assess IMCL levels using Oil-Red-O staining with the increase in lipid droplets resulting in a greater red color and thus a greater value for mean pixel intensity. All images were acquired with a Nikon Eclipse 90*i* microscope and Q-Imaging MicroPublisher 3.3 RTV camera with Q-Capture software. All control and HFD images for each morphometric analysis were taken at the same exposure with the same microscope settings.

Data analyses.

All statistical analyses were performed with GraphPad Prism 5 software. Differences between groups were determined using the appropriate student t-test, one-way or two-way ANOVA followed by Bonferonni post-hoc tests when appropriate. P values less than 0.05 were considered significant. All data presented are mean±standard error of the mean.

RESULTS

High fat diet induces insulin resistance and excessive weight gain in young adult mice

Young adult mice fed a standard chow diet continued to gain weight during the first 4 weeks of experimental assessment, verifying that these mice were still within the growth and maturation phase of development (Figure 2.1A). Mice consuming a diet with 60% kcal from fat (HFD) are significantly heavier than the standard chow fed controls as early as 4 weeks from the onset of HFD. After 4 weeks of diet intervention, the body weight of control mice stabilized, whereas HFD mice continued to increase, such that by the time of harvest, there was nearly a 40% gain in body mass (Figure 2.1A).

There was an approximate 2.5-fold increase in epididymal fat mass (Figure 2.1B) without any differences in absolute tibialis anterior (TA) or soleus muscle masses between groups (Control TA: 52.2±1.4 mg, Control soleus: 7.7±0.7 mg; HFD TA: 51.7±1.4 mg, HFD soleus: 7.7±0.5 mg).

There was no difference in fed blood glucose levels after 6 weeks of diet intervention (Control: 10.2±0.3 mM; HFD: 11.6±0.7 mM) or fasted blood glucose levels

after 7 weeks of diet intervention (Figure 2.1C, time 0). However HFD mice displayed a significantly reduced capacity for glucose clearance in response to an intraperitoneal glucose tolerance test (IPGTT), indicating insulin resistance (Figure 2.1C). Insulin resistance was further demonstrated by elevated resting plasma insulin levels at 4 weeks of diet intervention and at the 45 minute time point of the IPGTT in HFD mice (Figure 2.1D).

HFD results in a shift towards more and larger oxidative fibers

In HFD gastrocnemius/plantaris muscles, type I and IIA fiber type proportions increased (IIA: 40.3±3.5% vs. 32.9±1.9%, I: 9.80±1.8% vs. 5.5±1.8%, HFD vs. control, respectively), while type IID and IIB fiber types were decreased in number (IID: 20.2±2.1% vs. 23.8±3.9%, IIB: 29.7±1.5% vs. 37.7±2.7%, HFD vs. control, respectively; main effect of interaction, $p<0.05$). Considering that type IIA and IIB fibers represent the most oxidative and glycolytic fiber types in mouse skeletal muscle respectively (Hamalainen, Pette 1993), and that these two fiber types comprise more than 70% of all fibers we quantified in gastrocnemius/plantaris muscles, we specifically compared the change in fiber type percentages between these two fiber types (Figure 2.2A middle panel). HFD caused a significant rise in the percentage of oxidative IIA fibers and a significant decrease in the percentage of glycolytic IIB fibers in the gastrocnemius/plantaris muscles. In the TA and soleus muscles, no change in fiber type percentages were observed between HFD and control groups (Figure 2.2A left and right panels).

Assessment of cross-sectional area per fiber type revealed no differences in the gastrocnemius/plantaris muscles (Figure 2.2B middle panel) between groups. However a significant increase in the cross-sectional area of both type I and IIA muscle fibers (which comprise the entire muscle) was observed in HFD soleus compared to control (Figure 2.2B right panel).

Histological staining for succinate dehydrogenase (SDH) activity in the control muscle was greatest in fibers identified by metachromatic stain as type IIA followed by IID≥I>IIB, respectively. This finding validated the fiber type (metachromatic) staining results. No significant differences in SDH enzyme activity were detected between HFD and control in either the gastrocnemius/plantaris or soleus muscles, regardless of fiber-type (Figure 2.2C middle and right panel).

As expected, the IMCL levels across fiber types were different; with oxidative fiber types displaying greater absolute IMCL levels than glycolytic fiber types. HFD gastrocnemius/plantaris muscles displayed a significant increase in IMCL levels compared to control muscles (Figure 2.2D middle panel). All fiber types within the HFD gastrocnemius/plantaris displayed a similar percent increase (~50–60%) in IMCL levels above that measured in the respective control fiber type. Interestingly IMCL levels relative to cross-sectional area in the soleus muscles were not different between control and HFD regardless of fiber type (Figure 2.2D right panel). The observed increase in HFD soleus muscle cross-sectional area may have allowed for dispersion of IMCLs such that the density of IMCLs was unchanged between diet groups.

Complete palmitate and glucose oxidation rates are impaired in HFD skeletal muscle

Complete glucose and fat oxidation (to CO_2) were assessed using a novel, intact single muscle fiber protocol, as well as the well-established whole skeletal muscle preparation (Ceddia, Sweeney 2004, Pimenta et al. 2008). The whole muscle preparation was used to confirm the palmitate oxidation results in single fibers and by assessing glucose oxidation and glycogen synthesis we could further demonstrate insulin resistance within the intact skeletal muscle of HFD mice. Palmitate oxidation rates in intact fibers from extensor digitorum longus (EDL) and peroneus longus muscles of the HFD were approximately 65% of that measured in control muscle fibers (Figure 2.3A). Using this single fiber technique, we also demonstrated that basal glucose oxidation rates were significantly diminished in single fibers from HFD muscle compared to single fibers from control fed mice (Figure 2.3B).

Similar results to those acquired in intact single fibers were obtained when intact EDL and soleus muscles were used to assess palmitate (Figure 2.4A–B). While whole body insulin resistance was demonstrated by IPGTT and elevated insulin levels at rest and in response to IPGTT, we were interested in validating skeletal muscle insulin resistance and determining if there were muscle-specific differences in insulin resistance in HFD mice. Using intact soleus and EDL muscles, we undertook insulin stimulated glucose oxidation and glycogen synthesis assays (N = 4–6 muscles per group). A consistent blunting of the insulin-stimulated response was observed in all HFD muscles (Figure 2.4C–F). Importantly, this observation is made despite the soleus being a purely oxidative muscle (type I and IIA) and the EDL being a mixture of glycolytic (type IIB)

and oxidative (type IID) fiber types. Note that synthesis rates were higher in soleus compared to EDL muscles as would be expected based on their fiber type distribution (Pimenta et al. 2008).

Metabolic enzyme activities in HFD and control muscles

Citrate synthase (CS) and SCHAD are key enzymes in the TCA (tricarboxylic acid) cycle and β-oxidation pathways, respectively. No difference between HFD and control muscle CS or SCHAD activity was observed in homogenates from the TA muscles (Figure 2.5A–B). In homogenates from the soleus muscles, CS activity was not different between groups, though a significant increase in SCHAD enzyme activity was observed with HFD compared to control (Figure 2.5C–D).

As mentioned previously, an SDH activity stain was undertaken. In fibers identified as type IIB by metachromatic staining, SDH staining was of the lowest intensity, while fibers identified as type IIA by metachromatic staining displayed the most intense SDH staining. Thus, SDH activity staining validated the fiber type data obtained by metachromatic staining. This analysis also allowed us to determine if fiber type specific adaptations to SDH enzymatic activity and mitochondrial content were occurring in HFD versus control muscle. No differences in mean SDH staining intensity were observed between HFD and control muscles in the soleus or gastrocnemius/plantaris muscles, regardless of fiber type (Figure 2.2C).

Muscle contractility is not significantly altered following 8 weeks of high-fat diet

The peak tetanic force pre- and post-fatigue was not significantly different between control and HFD (Figure 2.6A). When the pre-fatigue force-frequency curve was

plotted, an insignificant, though consistently lower force production at each stimulation frequency was observed in the HFD fed mouse compared with control (main effect of diet: P = 0.089; Figure 2.6B top panel). Analysis of the post-fatigue force-frequency curve revealed that the ability to immediately recover force following a low-frequency fatigue protocol was impaired in HFD muscle compared with control (main effect of diet: P<0.05; Figure 2.6B bottom panel). Though a significant level of fatigue was induced in both control and HFD groups, there was no difference between groups in rate or degree of fatigue development (Figure 2.6C).

DISCUSSION

Here we provide novel evidence that the skeletal muscles of mice fed a high fat diet for 8 weeks develop pre-diabetes, undergo muscle-specific morphological and enzymatic adaptations and exhibit impairments in complete glucose and fat oxidation. These changes in muscle morphology and metabolism did not have significant effects on peak tetanic force and low-frequency fatigue rates. These results demonstrate for the first time that skeletal muscle health is impaired in the pre-diabetic state and that despite these significant impairments in oxidative metabolism, there is a maintenance of muscle contractile properties.

A significant muscle-specific shift towards a greater contribution of oxidative fibers was found in mice fed a HFD. In fact, the gastrocnemius/plantaris muscles increased the percentage of oxidative fibers, while the soleus increased fiber cross-sectional area and the TA displayed no fiber type change. This is in line with a recent

study reporting that HFD led to increased soleus muscle fiber areas, but not fiber type proportions and no change in fiber type percentage or area in the EDL (Turpin et al. 2009). The shift in oxidative potential of the soleus and gastrocnemius/plantaris, but not the TA, may be the result of an increased activity in the calf musculature compared to dorsiflexors in relatively sedentary rodents. The shift towards a greater oxidative fiber contribution seen in the present study is also consistent with that observed by others (de Wilde et al. 2008, Farkas et al. 1994, Kemp et al. 2009). Of note, deWilde et al. (de Wilde et al. 2008) found that following 3 and 28 days of high-fat feeding (45% kcal from fat) there was a significant increase in the mRNA and protein expression of slow myosin heavy chain and other markers of the slow oxidative fiber phenotype. While myosin ATPase expression, demonstrated using metachromatic staining, is often correlated with oxidative potential of the muscle, a shift in myosin ATPase expression does not directly indicate there is an increase in oxidative potential. Here we substantiate the shift towards an increased oxidative potential using SDH activity staining. The SDH enzyme catalyzes the conversion of succinate to fumarate in the TCA cycle. It consists of two large subunits which form complex II of the mitochondrial respiratory chain along with two smaller subunits, which attach SDH to the inner mitochondrial membrane. SDH staining has been shown to be extremely useful for detecting variations in the fiber distribution of mitochondria, particularly in states of mitochondrial dysfunction (Sciacco, Bonilla 1996, Tanji, Bonilla 2008).

In our experiments, the mean intensity of SDH staining per fiber type was not different between control and HFD muscles, although soleus muscle had increased fiber

cross-sectional area (Figure 2.2D). If increased fiber areas or a shift towards more oxidative fibers were not associated with a concomitant increase in mitochondrial content, then the mean pixel intensity for SDH would have decreased proportionally. However, since there was a proportional increase in SDH density in our study, we interpret that a relative increase in mitochondrial content has occurred in HFD muscles, indicating a shift towards an increased oxidative potential. This could represent an early positive adaptation to the abundance of lipids and elevated IMCL levels typical of the HFD.

In fact, IMCL levels were increased with HFD in this study, albeit in a fiber type and muscle-group specific manner. There were a number of ways in which an increased IMCL deposition was observed in this study including greater IMCL density (observed in the gastrocnemius/plantaris), increased oxidative muscle fiber area with unchanged IMCL density (observed in the soleus), and increased oxidative muscle fiber percentages (observed in the gastrocnemius/plantaris). Although our findings suggest an early attempt to enhance oxidation in response to high-fat feeding, after 8 weeks of such a diet a downward spiral seemed to develop leading to impaired glucose and lipid oxidation rates. This is consistent with previous observations showing time-dependent alterations in mitochondrial density or structure within the gastrocnemius muscles of mice fed a high-fat/high-sugar (HFHSD) diet (Bonnard et al. 2008). In fact, Bonnard and colleagues (Bonnard et al. 2008) found that significant alterations in mitochondrial density or structure occurred only after 16 weeks of HFHSD. It was further reported that 4 weeks of diet intervention was associated with some metabolic perturbations that could reflect the initiation of deleterious processes in pre-diabetic skeletal muscle. Our study

also provides evidence of early adaptive responses to HFD, which are followed by impairment in complete glucose and lipid oxidation. Furthermore, it is important to note that the changes in muscle morphology and metabolic enzyme activities are occurring in a muscle-specific and time-dependent manner. Therefore, caution is warranted when deriving conclusions based on the analyses of one specific muscle or limited metabolic markers.

In an attempt to address the apparent discrepancy between a shift towards an oxidative muscle phenotype and the reduced lipid and glucose oxidation rates, we assessed the activity of CS and SCHAD in muscles that either demonstrated an increase (soleus) or no change (TA) in oxidative potential in response to HFD. We did not find any significant differences in CS and SCHAD activities in the TA muscles of control and HFD mice. These results were consistent with the lack of change in fiber type proportion in this muscle. Interestingly, we found a significant increase in SCHAD, but not in CS activity, in the HFD soleus. Whether the increase in SCHAD activity in the HFD soleus is the result of the increased cross-sectional area and proportionate increase in mitochondrial content remains to be determined. However, since oxidative muscle fiber types increased in size (soleus) or number (gastrocnemius/plantaris) and a concomitant rise in SDH staining intensity was found, it suggests that 8 weeks of HFD did not decrease mitochondrial content. In fact, we would argue that the uniform increase in staining throughout the fibers is indicative of an increase in SDH content resultant from an overall increase in mitochondrial content and not solely increased SDH activity. Importantly, regardless of whether there was an increase in mitochondrial content or a

fiber type specific increase in oxidative potential of muscles exposed to HFD, a significant impairment in complete glucose and palmitate oxidation was detected in our studies. These observations were made using a novel isolated single fiber technique and validated with the classical, isolated muscle methodology (Ceddia, Sweeney 2004, Pimenta et al. 2008). The isolated single fiber approach facilitates exchange of substrates and gases with the incubation medium and maintains all populations of mitochondria (subsarcolemmal and intermyofibrillar). Furthermore, it preserves the mitochondria and metabolic enzymes in their natural surrounding. Therefore, both approaches allowed us to study the ability of both the skeletal muscle cell and the skeletal muscle tissue from mice fed a HFD to metabolize glucose and fatty acids.

A number of studies have demonstrated that a prolonged exposure to elevated levels of fatty acids lead to insulin resistance and impair skeletal muscle glucose and lipid metabolism. The buildup of harmful lipid metabolites including long-chain fatty acyl CoA, diacylglycerol, and ceramides have been proposed to cause these deleterious metabolic effects in skeletal muscle (Smith et al. 2007, Silveira et al. 2008, Lee et al. 2006). Furthermore, a recent study using rats fed a HFD (45% fat for 12 weeks) demonstrated an increase in incomplete β-oxidation with excess acid-soluble metabolites being produced, indicating depletion of intermediates of the TCA cycle (Koves et al. 2008). These authors also demonstrated the importance of fatty acid entry into the mitochondria, and not simply increased IMCL, to elicit HFD induced insulin resistance (Koves et al. 2008). In this context, our findings that the increase in SCHAD activity was not followed by a proportional increase in CS activity suggest a flux through

β-oxidation that is not proportionally matched by the TCA cycle. Furthermore, the fact that soleus muscles displayed insulin resistance and impaired complete oxidation of lipids and glucose even in the face of elevated SCHAD activity and normal IMCL levels, support the idea that HFD causes insulin resistance through lipotoxicity specifically within mitochondria (Koves et al. 2008, Marin et al. 1994).

Despite significant alterations in muscle morphology and metabolism, no significant impairment in peak tetanic force either pre- or post-fatigue was observed. However when we analyzed the force-frequency curves, we see that at all stimulation frequencies tested, HFD skeletal muscle consistently displayed reduced force production regardless of stimulation frequency in the pre-fatigue state (P = 0.089). We hypothesize that the early adaptations that occurred in response to HFD attenuated muscle force loss and continued exposure to a HFD would ultimately result in significant decreases in contractile force, consistent with that observed in humans (Sayer et al. 2005). The absence of a difference between groups in response to a low-frequency fatigue protocol is likely explained by the low intensity and short duration of this protocol. That is, this submaximal fatigue protocol was insufficient to elicit differences due to changes in oxidative metabolism. Though no change in peak tetanic force post-fatigue was observed, analysis of the force-frequency curve revealed a significant main effect of diet (Figure 2.6B). This finding would imply that following fatiguing contractions, the ability to immediately recover force is attenuated in HFD muscles compared to control muscles. Given the impaired metabolic capacity in the HFD muscles, this result was not unexpected. While it would be premature to extrapolate too much from this result, we

hypothesize that the ability to respond to exercise or exercise- training may be impaired in these pre-diabetic mice.

Overall, the current study provides, for the first time, a comprehensive analysis of skeletal muscle morphology, metabolism and function following 8 weeks of high fat diet consumption. Our findings substantiate the proposal of deWilde and colleagues (de Wilde et al. 2008) that skeletal muscle responds to high fat diet intervention with an early, positive adaptive response. However, with continued high-fat diet exposure, perturbations in gene and protein expression will ultimately result, causing decreased oxidative capacity at a later stage. Taken together, this work advances our understanding of skeletal muscle health prior to the development of type 2 diabetes mellitus and, in part, aids in explaining the variability that has been observed in previous studies investigating pre-diabetic skeletal muscle. Furthermore, these results support the undertaking of early therapeutic interventions in obese, pre-diabetic youth prior to significant long-term effects on muscle growth and function.
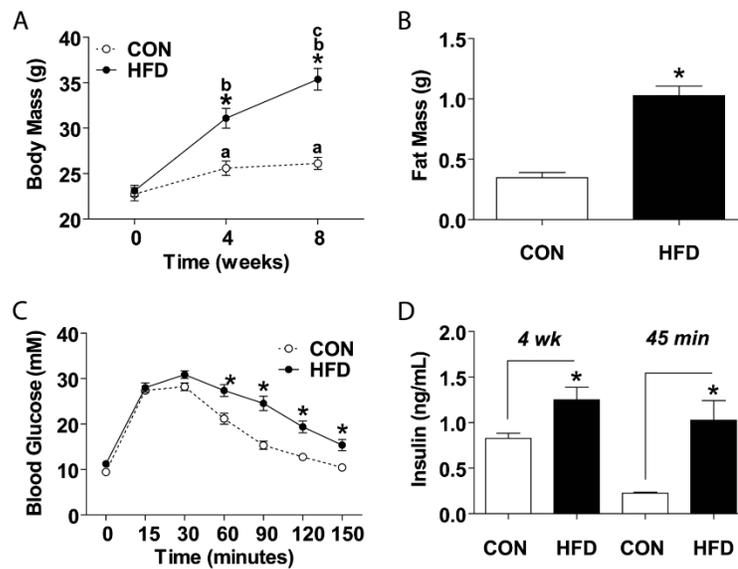
Figure 2.1. Eight weeks of a high fat diet (HFD) elicits pre-diabetes. (A) Fasted body

mass was assessed before experimental diet began and after 4 and 8 weeks (N = 19 CON,

20 HFD). (B) Epididymal fat mass after 8 weeks of diet intervention (N = 19 CON, N =

20 HFD). (C) Intraperitoneal glucose tolerance test (IPGTT) performed after an overnight fast (16 hrs) 1 week before harvest (N = 19 CON, N = 18 HFD). (D) Plasma insulin levels assessed 4 weeks into diet intervention (8 hr fast, N = 10) and at IPGTT 45 minute time-point (16 hr fast, N = 4). Significance is represented by * vs. CON at same time point (A–D), a or b vs. 0 weeks within diet group, and c vs. 4 weeks within diet group, $p < 0.005$.
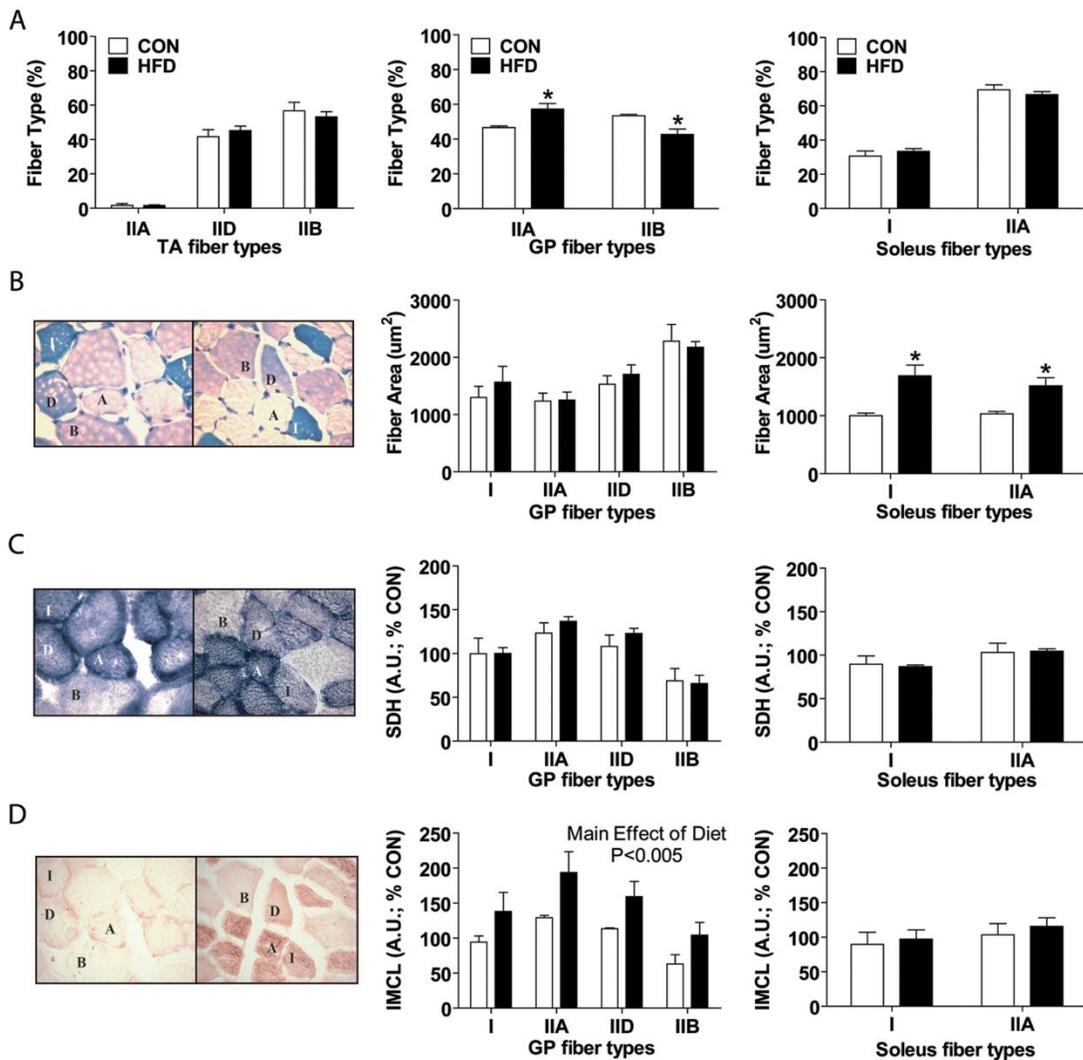
Figure 2.2. Morphometric changes are muscle-specific. Serial cross-sections from control (CON, white bars in all graphs) and high fat diet (HFD, black bars in all graphs) fed mouse muscles [TA (top left only), gastrocnemius/plantaris complex (GP, all middle graphs), and soleus (all right graphs)] were examined for (A) fiber type composition, (B) area, (C) SDH stain intensity, and (D) IMCL stain intensity. Representative images of all stains used, performed on GP muscle cross-sections are shown to the left of graphs B–D with fiber type (type I, and types II- A, D, B) labeled with CON on the left and HFD on the right. (B) Metachromatic fiber type stain was used to assess fiber type. (C) SDH and (D) Oil-Red-O stains are graphically represented by arbitrary units (A.U.) of optical intensity measurements, with greater values for more intense stains and normalized to a percentage of all the control value means for each graph (% CON). All measurements were taken on an average of 51–331 total fibers/animal with a Nikon Eclipse 90i microscope (N = 3–4). Significance is represented by * vs. CON, p<0.005
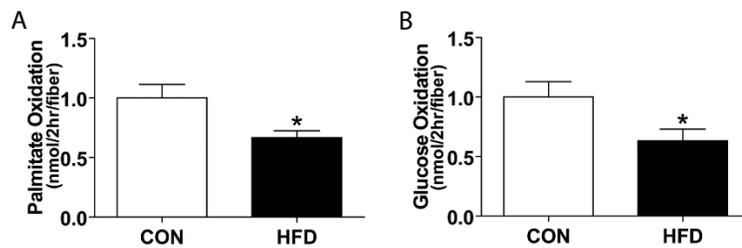
.

Figure 2.3. Impaired palmitate and glucose oxidation in HFD single fibers. (A) Palmitate (N = 19, average of 17 fibers/dish) and (B) glucose (N = 12 CON, N = 10 HFD, average of 23 fibers/dish) oxidation in single fibers derived from EDL and peroneus muscles was similarly impaired in mice fed a high-fat diet (HFD) compared to control

(CON). Values were normalized to control values for each experiment and significance is represented by * vs. CON, p<0.005.
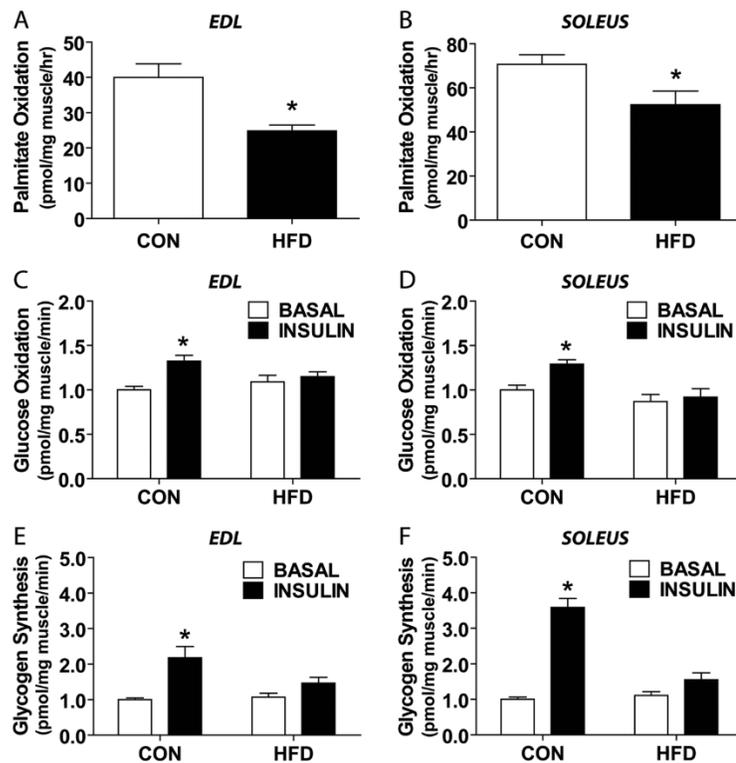
Figure 2.4. Impaired oxidation, glycogen synthesis, and insulin stimulated response in HFD muscle. Palmitate oxidation in whole (A) EDL (N = 4) and (B) soleus (N = 4) muscles is impaired with HFD. Both glucose oxidation in whole (C) EDL and (D) soleus and glycogen synthesis in whole (E) EDL and (F) soleus muscles with HFD demonstrate

a significant blunted response to insulin pre-incubation (INSULIN), vs. no insulin pre-incubation (BASAL), in HFD muscle (N = 4–6, two-way ANOVA with Bonferonni post-tests between insulin conditions within diet). Significance is represented by * vs. CON (A–B), and vs. INSULN (C–F), p<0.005.
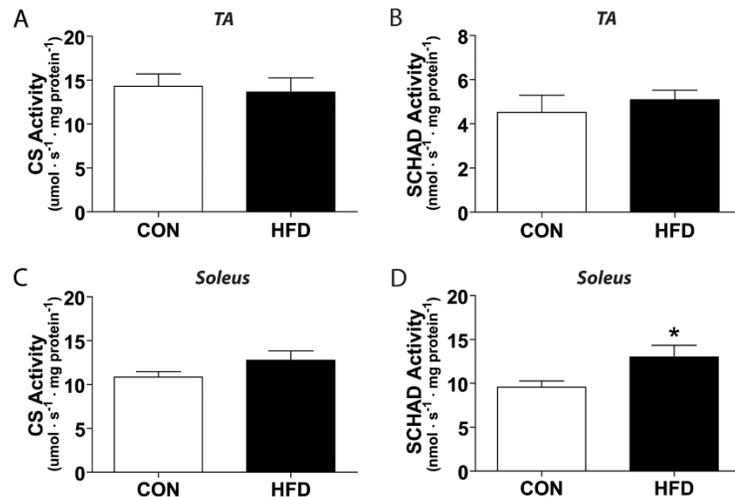
Figure 2.5. Oxidative enzyme alterations are muscle specific. Citrate synthase (CS) activity is unaltered in both (A) TA and (C) soleus muscle between control (CON) and high fat diet (HFD). Short chain 3-β-hydroxyacyl coenzyme-A dehydrogenase (SCHAD)

activity is unaltered in (B) TA muscle, though (D) soleus muscle from HFD mice exhibits

SCHAD activity 136% of CON. Significance is represented by * vs. CON, p<0.005.
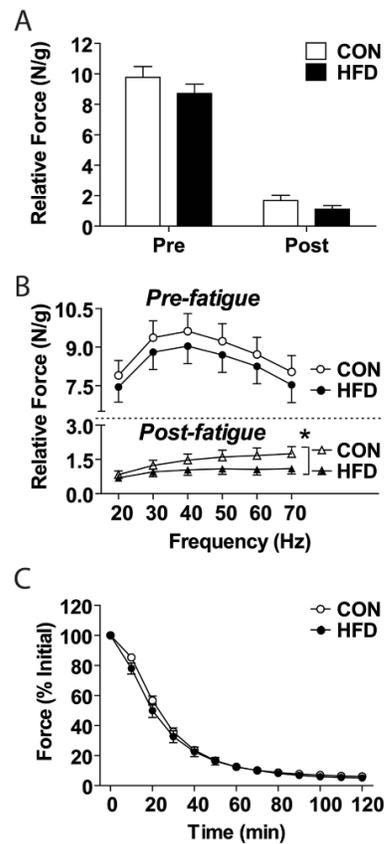
Figure 2.6. *In situ* contractile analysis reveals trend towards force decrements, yet unaltered peak force and fatigue. Relative tetanic force production [in Newtons (N) per gram (g) of wet muscle mass] in the gastrocnemius/plantaris muscle group of high-fat diet (HFD) mice compared to control (CON) was (A) not different before (Pre) or after (Post) the fatigue protocol. (B) There was no difference between diets over all frequencies used to test force production pre-fatigue, however there was a significant main effect of diet post-fatigue. (C) Contractile force, relative to initial (% initial), throughout a 2 minute

low-frequency fatigue protocol was not different between diet groups. Significance is

represented by * vs. CON, p<0.005.

Minor Correction: Correction to Figure 2.6C
In Figure 2.6C, the x-axis is labeled incorrectly. It should read: Time (sec).
REFERENCE LIST

American Diabetes Association. Available: http://www.diabetes.org/diabetes-statistics/prevalence.jsp [2009, 7/13].

Bonnard, C., Durand, A., Peyrol, S., Chanseaume, E., Chauvin, M.A., Morio, B., Vidal, H. & Rieusset, J. 2008, "Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice", *The Journal of clinical investigation,* vol. 118, no. 2, pp. 789-800.

Carter, S.L., Rennie, C.D., Hamilton, S.J. & Tarnopolsky 2001, "Changes in skeletal muscle in males and females following endurance training", *Canadian journal of physiology and pharmacology,* vol. 79, no. 5, pp. 386-392.

Ceddia, R.B. & Sweeney, G. 2004, "Creatine supplementation increases glucose oxidation and AMPK phosphorylation and reduces lactate production in L6 rat skeletal muscle cells", *The Journal of physiology,* vol. 555, no. Pt 2, pp. 409-421.

Ceddia, R.B., William, W.N.,Jr & Curi, R. 1999, "Comparing effects of leptin and insulin on glucose metabolism in skeletal muscle: evidence for an effect of leptin on glucose uptake and decarboxylation", *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity,* vol. 23, no. 1, pp. 75-82.

Chanseaume, E., Malpuech-Brugere, C., Patrac, V., Bielicki, G., Rousset, P., Couturier, K., Salles, J., Renou, J.P., Boirie, Y. & Morio, B. 2006, "Diets high in sugar, fat, and energy induce muscle type-specific adaptations in mitochondrial functions in rats", *The Journal of nutrition,* vol. 136, no. 8, pp. 2194-2200.

de Wilde, J., Mohren, R., van den Berg, S., Boekschoten, M., Dijk, K.W., de Groot, P., Muller, M., Mariman, E. & Smit, E. 2008, "Short-term high fat-feeding results in morphological and metabolic adaptations in the skeletal muscle of C57BL/6J mice", *Physiological genomics,* vol. 32, no. 3, pp. 360-369.

Farkas, G.A., Gosselin, L.E., Zhan, W.Z., Schlenker, E.H. & Sieck, G.C. 1994, "Histochemical and mechanical properties of diaphragm muscle in morbidly obese Zucker rats", *Journal of applied physiology (Bethesda, Md.: 1985),* vol. 77, no. 5, pp. 2250-2259.

Fediuc, S., Gaidhu, M.P. & Ceddia, R.B. 2006, "Regulation of AMP-activated protein kinase and acetyl-CoA carboxylase phosphorylation by palmitate in skeletal muscle cells", *Journal of lipid research,* vol. 47, no. 2, pp. 412-420.

Hamalainen, N. & Pette, D. 1993, "The histochemical profiles of fast fiber types IIB, IID, and IIA in skeletal muscles of mouse, rat, and rabbit", *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society,* vol. 41, no. 5, pp. 733-743.

Hawke, T.J., Jiang, N. & Garry, D.J. 2003, "Absence of p21CIP rescues myogenic progenitor cell proliferative and regenerative capacity in Foxk1 null mice", *The Journal of biological chemistry,* vol. 278, no. 6, pp. 4015-4020.

Kemp, J.G., Blazev, R., Stephenson, D.G. & Stephenson, G.M. 2009, "Morphological and biochemical alterations of skeletal muscles from the genetically obese (ob/ob) mouse", *International journal of obesity (2005),* vol. 33, no. 8, pp. 831-841.

Koopman, R., Schaart, G. & Hesselink, M.K. 2001, "Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids", *Histochemistry and cell biology,* vol. 116, no. 1, pp. 63-68.

Koves, T.R., Ussher, J.R., Noland, R.C., Slentz, D., Mosedale, M., Ilkayeva, O., Bain, J., Stevens, R., Dyck, J.R., Newgard, C.B., Lopaschuk, G.D. & Muoio, D.M. 2008, "Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance", *Cell metabolism,* vol. 7, no. 1, pp. 45-56.

Krause, M.P., Liu, Y., Vu, V., Chan, L., Xu, A., Riddell, M.C., Sweeney, G. & Hawke, T.J. 2008, "Adiponectin is expressed by skeletal muscle fibers and influences muscle phenotype and function", *American journal of physiology.Cell physiology,* vol. 295, no. 1, pp. C203-12.

Krause, M.P., Riddell, M.C., Gordon, C.S., Imam, S.A., Cafarelli, E. & Hawke, T.J. 2009, "Diabetic myopathy differs between Ins2Akita+/- and streptozotocin-induced Type 1 diabetic models", *Journal of applied physiology (Bethesda, Md.: 1985),* vol. 106, no. 5, pp. 1650-1659.

Lee, J.S., Pinnamaneni, S.K., Eo, S.J., Cho, I.H., Pyo, J.H., Kim, C.K., Sinclair, A.J., Febbraio, M.A. & Watt, M.J. 2006, "Saturated, but not n-6 polyunsaturated, fatty acids induce insulin resistance: role of intramuscular accumulation of lipid metabolites", *Journal of applied physiology (Bethesda, Md.: 1985),* vol. 100, no. 5, pp. 1467-1474.

Lionetti, L., Mollica, M.P., Crescenzo, R., D'Andrea, E., Ferraro, M., Bianco, F., Liverini, G. & Iossa, S. 2007, "Skeletal muscle subsarcolemmal mitochondrial dysfunction in high-fat fed rats exhibiting impaired glucose homeostasis", *International journal of obesity (2005),* vol. 31, no. 10, pp. 1596-1604.

Marin, P., Andersson, B., Krotkiewski, M. & Bjorntorp, P. 1994, "Muscle fiber composition and capillary density in women and men with NIDDM", *Diabetes care,* vol. 17, no. 5, pp. 382-386.

Ogilvie, R.W. & Feeback, D.L. 1990, "A metachromatic dye-ATPase method for the simultaneous identification of skeletal muscle fiber types I, IIA, IIB and IIC", *Stain technology,* vol. 65, no. 5, pp. 231-241.

Pimenta, A.S., Gaidhu, M.P., Habib, S., So, M., Fediuc, S., Mirpourian, M., Musheev, M., Curi, R. & Ceddia, R.B. 2008, "Prolonged exposure to palmitate impairs fatty acid oxidation despite activation of AMP-activated protein kinase in skeletal muscle cells", *Journal of cellular physiology,* vol. 217, no. 2, pp. 478-485.

Sayer, A.A., Dennison, E.M., Syddall, H.E., Gilbody, H.J., Phillips, D.I. & Cooper, C. 2005, "Type 2 diabetes, muscle strength, and impaired physical function: the tip of the iceberg?", *Diabetes care,* vol. 28, no. 10, pp. 2541-2542.

Sciacco, M. & Bonilla, E. 1996, "Cytochemistry and immunocytochemistry of mitochondria in tissue sections", *Methods in enzymology,* vol. 264, pp. 509-521.

Silveira, L.R., Fiamoncini, J., Hirabara, S.M., Procopio, J., Cambiaghi, T.D., Pinheiro, C.H., Lopes, L.R. & Curi, R. 2008, "Updating the effects of fatty acids on skeletal muscle", *Journal of cellular physiology,* vol. 217, no. 1, pp. 1-12.

Smith, A.C., Mullen, K.L., Junkin, K.A., Nickerson, J., Chabowski, A., Bonen, A. & Dyck, D.J. 2007, "Metformin and exercise reduce muscle FAT/CD36 and lipid accumulation and blunt the progression of high-fat diet-induced hyperglycemia", *American journal of physiology.Endocrinology and metabolism,* vol. 293, no. 1, pp. E172-81.

Sparks, L.M., Xie, H., Koza, R.A., Mynatt, R., Hulver, M.W., Bray, G.A. & Smith, S.R. 2005, "A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle", *Diabetes,* vol. 54, no. 7, pp. 1926-1933.

Tanji, K. & Bonilla, E. 2008, "Light microscopic methods to visualize mitochondria on tissue sections", *Methods (San Diego, Calif.),* vol. 46, no. 4, pp. 274-280.

Turner, N., Bruce, C.R., Beale, S.M., Hoehn, K.L., So, T., Rolph, M.S. & Cooney, G.J. 2007, "Excess lipid availability increases mitochondrial fatty acid oxidative capacity in muscle: evidence against a role for reduced fatty acid oxidation in lipid-induced insulin resistance in rodents", *Diabetes,* vol. 56, no. 8, pp. 2085-2092.

Turpin, S.M., Ryall, J.G., Southgate, R., Darby, I., Hevener, A.L., Febbraio, M.A., Kemp, B.E., Lynch, G.S. & Watt, M.J. 2009, "Examination of 'lipotoxicity' in skeletal muscle of high-fat fed and ob/ob mice", *The Journal of physiology,* vol. 587, no. Pt 7, pp. 1593-1605.

CHAPTER 3

Enhanced Lipid Oxidation and Maintenance of Muscle Insulin Sensitivity Despite Glucose Intolerance in a Diet-Induced Obesity Mouse Model

Authors: Trajcevski KE, O'Neill HM, Wang DC, Thomas MM, Al-Sajee D, Steinberg GR, Ceddia RB and Hawke TJ.

Corresponding Author: Thomas J. Hawke, Dept. of Pathology and Molecular Medicine, McMaster Univ., 4N65, Health Sciences Centre, 1200 Main St. W., Hamilton Ontario, L8N 3Z5, Canada (email:hawke@mcmaster.ca).

PREFACE

Significance to thesis

The goal of this research was to further investigate associations between characteristics of Prediabetes (glucose intolerance and muscle insulin resistance) and tissue specific changes following DIO compared to normal diet. This work was predominantly focused on 3 weeks of DIO and complements the previous chapter which focused on 8 weeks of DIO. The previous chapter demonstrated a disconnect between impaired oxidative metabolism and an oxidative fiber type shift of which the latter was hypothesized to have occurred earlier in concert with an enhancement in oxidative metabolism. Length of DIO will determine the severity of disease development, thus characterizing physiological changes following varying lengths of DIO enables characterization of disease development. Recent literature has implicated inflammation, in various insulin sensitive tissues including skeletal muscle, and IMCLs as mechanisms leading to muscle insulin resistance. Thus, a secondary goal of this research was to investigate the presence of inflammation during development of muscle insulin resistance. By studying an early time-point of DIO in mice we can begin to understand the early health implications of the unhealthy lifestyle prevalent in our society.

Authors' contributions

Karin E. Trajcevski conceived and designed the experiments, analyzed the data, wrote the manuscript and made revisions based on reviewer comments with assistance as stated below.

O'Neil HM performed radioactive experiments, assisted with insulin/glucose tolerance tests, metabolic cage experiments, data analyses and refining the manuscript.

Wang DC assisted with muscle staining experiments and data analysis.

Thomas MM assisted with animal handling/care, experiments, data analysis, and refining the manuscript.

Al-Sajee D assisted with animal handling/care, experiments, data analysis, and refining the manuscript.

Steinberg GR assisted with reagents for radioactive experiments, metabolic cage equipment, writing the manuscript and revising the manuscript based on editorial review.

Ceddia RB provided insight on study design, assisted with data interpretation, writing the manuscript and revising the manuscript based on editorial review.

Hawke TJ contributed to the design of the study, assisted with performing experiments, and worked on refining drafts of the manuscript and the revisions based on editorial review.

ABSTRACT

Background. Diet-induced obesity is a rising health concern which can lead to the development of glucose intolerance and muscle insulin resistance and, ultimately, type II diabetes mellitus. This research investigates the associations between glucose intolerance or muscle insulin resistance and tissue specific changes during the progression of diet-induced obesity. **Methodology.** C57BL/6J mice were fed a normal or high-fat diet (HFD; 60% kcal fat) for 3 or 8 weeks. Disease progression was monitored by measurements of body/tissue mass changes, glucose and insulin tolerance tests, and *ex vivo* glucose uptake in intact muscles. Lipid metabolism was analyzed using metabolic chambers and *ex vivo* palmitate assays in intact muscles. Skeletal muscle, liver and adipose tissues were analyzed for changes in inflammatory gene expression. Plasma was analyzed for insulin levels and inflammatory proteins. Histological techniques were used on muscle and liver cryosections to assess metabolic and morphological changes. **Principal Findings/Conclusions.** A rapid shift in whole body metabolism towards lipids was observed with HFD. Following 3 weeks of HFD, elevated total lipid oxidation and an oxidative fiber type shift had occurred in the skeletal muscle, which we propose was responsible for delaying intramyocellular lipid accumulation and maintaining muscle's insulin sensitivity. Glucose intolerance was present after three weeks of HFD and was

associated with an enlarged adipose tissue depot, adipose tissue inflammation and excess hepatic lipids, but not hepatic inflammation. Furthermore, HFD did not significantly increase systemic or muscle inflammation after 3 or 8 weeks of HFD suggesting that early diet-induced obesity does not cause inflammation throughout the whole body. Overall these findings indicate skeletal muscle did not contribute to the development of HFD-induced impairments in whole-body glucose tolerance following 3 weeks of HFD.

INTRODUCTION

An unhealthy lifestyle including a high fat diet (HFD) has become common in Western societies and contributes to obese, insulin resistant states such as pre-diabetes, which if left untreated, can progress to Type 2 Diabetes Mellitus (T2DM) (Duncan 2006, Li et al. 2009, Defronzo 2009). It is estimated that 2.4 million Canadians will have T2DM by 2016 (Ohinmaa et al. 2004) with excessive body mass and inactivity accounting for almost 90% of all new cases of T2DM (Hossain, Kawar & El Nahas 2007). Given the importance of skeletal muscle to our overall physical and metabolic capacities (Burr et al. 2010, Goodpaster et al. 2010, Fenicchia et al. 2004, Slentz, Houmard & Kraus 2009, Houmard et al. 2002, Craig, Everhart & Brown 1989, Phielix et al. 2012, Wood, O'Neill 2012, Srikanthan, Karlamangla 2011), improving our understanding of the changes to muscle health during the development of obese, insulin resistant states will advance the development of therapeutic strategies for those individuals.

While the exact cause of muscle insulin resistance with the development of diet-induced obesity is still unknown, one proposed mechanism is elevated delivery of free fatty acids (FFAs) in conjunction with impaired lipid metabolism within skeletal muscle leading to a build-up of intramyocellular lipids (IMCL) and associated lipid derivatives (Kewalramani, Bilan & Klip 2010, Gregor, Hotamisligil 2011, Samuel,

Shulman 2012). Our lab previously reported that mice fed a HFD (60% kcal from fat) for 8 weeks exhibited obesity and muscle insulin resistance accompanied by impaired muscle lipid oxidation and excessive IMCL deposition (Shortreed et al. 2009). Interestingly, despite significant reductions in glucose and palmitate oxidation rates, histological/immunofluorescent analysis of skeletal muscle from HFD fed mice revealed a significant shift towards an oxidative phenotype. This paradoxical observation led us to hypothesize that the shift towards a more oxidative phenotype was an early, adaptive response to the HFD in order to enhance lipid utilization. However, the chronic nature of the diet led to a pathological situation where substrate utilization could not match substrate delivery (Shortreed et al. 2009). Previous reports of rodents fed a similar diet high in fat are equivocal on whether an early adaptive oxidative shift is present (Kewalramani, Bilan & Klip 2010, Bonnard et al. 2008, Turner et al. 2007, Chanseaume et al. 2007, Hancock et al. 2008) and whether muscle lipid oxidation is altered (Kewalramani, Bilan & Klip 2010, Bonnard et al. 2008, Turner et al. 2007). However, differences in rodent models, HFD composition and timing of measurements complicate a direct comparison between these studies.

In addition to alterations in lipid metabolism, inflammation is also linked to insulin resistance development. In particular, inflammation in adipose tissue is being extensively investigated as a mechanism contributing to glucose intolerance and insulin resistance in pre-diabetes and T2DM (Kewalramani, Bilan & Klip 2010, Steinberg 2007, Galic, Oakhill & Steinberg 2010). However, the extent to which inflammation in alternative tissues like liver and skeletal muscle contributes to the initial development of

89

glucose intolerance and tissue specific insulin resistance is unclear as the inflammatory state is often assessed once obesity and insulin resistance is well underway. Furthermore, it is important to directly assess muscle insulin resistance as it is often implied by the presence of glucose intolerance although the two are not always present at the same time during disease development.

The main focus of the present study was on the impact of three weeks of HFD (60% kcal fat) on skeletal muscle morphology, metabolism and insulin resistance development. The overall purpose of our experiments was to determine if there are early positive whole body or muscle-specific adaptations to lipid handling and if changes in inflammatory state and/or lipid metabolism are associated with the development of glucose intolerance and insulin resistance. Our results demonstrate an early elevation in the processing of lipids that we believe leads to the attenuation of muscle insulin resistance following 3 weeks of HFD. Furthermore our data serves to clarify the associations between inflammation, ectopic lipid deposition and glucose intolerance or muscle insulin resistance.

METHODS

Animals and Blood Sampling.

All experimental protocols were approved by the McMaster University Animal Care Committee in accordance with the Canadian Council for Animal Care guidelines. Male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed in a temperature and humidity-controlled facility with a 12/12 h

light/dark cycle and had *ad libitum* access to water and food. Following 1 week acclimatization, animals [10 weeks of age; N = 5–6 per group as noted in Table 3.1 for the first round of experiments (e.g. group 1) lasting 3 weeks (21 days) and 8 weeks (56 days) including 16 hour fasted IPGTT, body and adipose tissue masses, 8 week muscle mass, all histology/cryo-section analysis except liver tissue, muscle qualitative PCR, and plasma collection which was performed in the fed state between 14:00 and 16:00 hrs; N = 8 ND and 10 HFD per group for second round of experiments (e.g. group 2) lasting 3 weeks (21 days) including 6 hour fasted IPGTT and IPITT, CLAMS, 3 week muscle mass, liver histology (Oil-Red-O), adipose and liver tissue quantitative PCR, and *ex vivo* muscle glucose and palmitate experiments] were randomly assigned to either a high fat diet [HFD; TestDiet, cat #58126: energy (kcal/g) from protein (18.3%), fat (60.9%), carbohydrate (20.1%)] or normal diet [LabDiet 5015 Mouse Diet: energy (kcal/g) from protein (20%), fat (25%), carbohydrate (55%)]. Fed state body mass was assessed on a weekly basis between 14:00 and 16:00 hrs. An IPGTT was performed on mice fasted (16 or 6 hr) after 18 days of diet intervention. Glucose was injected IP (2 g/kg of body weight for 16 hour fasted mice or 1 g/kg body weight for 6 hour fasted mice) and blood glucose was assessed by tail bleed at various time-points.

Experimental Procedures.

Group 1 mice were weighed then euthanized by cervical dislocation following 3 or 8 weeks of diet and tissues were harvested and either snap frozen or mounted with tissue freezing medium and frozen in isopentane cooled by liquid nitrogen then frozen in liquid nitrogen. Group 2 mice underwent CLAMS analysis at the beginning of the diet

intervention (N = 8). Mice were placed in the CLAMS and given 24 hours to acclimatize before measurements were taken every 20 minutes. Food intake (see data analysis for calculation) was recorded every 20 min as the difference between weight of ground food removed from the feeder and weight of ground food in the catch tray under the feeder. Mice were weighed and anaesthetized (ketamine/xylazine) prior to dissection of the soleus and EDL muscles for *ex vivo* glucose and palmitate experiments. Euthanization was performed by cervical dislocation and adipose and liver tissues were removed and snap frozen in liquid nitrogen.

Insulin-stimulated 2 Deoxy-D-glucose (2DG) Uptake in Isolated Muscles.

Following 3 weeks of diet intervention, glycolytic EDL and oxidative soleus muscles were removed from anaesthetized (ketamine/xylazine) mice as described in experimental procedures. 2DG uptake (N = 4 ND, 6 HFD per muscle group) was assessed as previously described (Steinberg et al. 2010). Briefly, EDL and soleus muscles were dissected tendon to tendon and quickly placed in glass vials containing 2 ml of pre-gassed (95% O_2:5%CO_2) Krebs-Hanseleit bicarbonate (KHC) buffer (0.1% BSA and 2 mM pyruvate) and pre-incubated for 20 min. Buffer was then replaced with a similar buffer supplemented with −/+ insulin (400 µU/ml) (Actrapid®, Novo Nordisk A/S, Denmark), and muscles were incubated for a further 30 min, before final incubation step whereby 2DG uptake was measured by replacing existing incubation buffer with a similar buffer containing [^{3}H]-2DG (0.5 µCi/ml) (Amersham, GE Healthcare, UK), 2-DG (1 mM) and the extracellular space marker [^{14}C]-mannitol (0.20 µCi/ml) (Amersham, GE Healthcare, U.K.) and incubating for 20 min. Muscle-specific insulin sensitivity, as assessed by

responsiveness to insulin in promoting 2DG uptake, was determined by homogenizing muscles in 300 µl of ice-cold cell lysis buffer (Tris.HCl (20 mM), NaCl (50 mM), dithiothreitol (2 mM), NaF (50 mM), Triton X-100 (1%), sucrose (250 mM), sodium pyrophosphate (5 mM), leupeptin (4 µg/ml), benzmidine (6 mM), phenylmethylslfonylfluoride (500 µM), soybean trysin inhibitor (50 µg/ml), pH 7.4)) and counting radioactivity in 100 µl of muscle lysates by liquid scintillation counting as described previously (Steinberg et al. 2010).

Ex vivo *Palmitate Experiments and Extraction of Muscle Lipids.*

Palmitate oxidation (N = 4 ND, 4 HFD per muscle group, average of duplicates/mouse analyzed) was assessed as previously described (Steinberg et al. 2010). Briefly, soleus and EDL muscles were dissected tendon to tendon and pre-incubated in 2 mL of pre-warmed (30°C) and gassed (95%O_2:5%CO_2) basal buffer (modified Krebs Henseleit buffer with 4% fatty acid-free BSA, 2 mM pyruvate and 0.5 mM palmitate) for 20 min. Incubation media was then replace with a similar buffer containing [^{14}C]palmitic acid (0.5 µCi/mL) for 60 min. Upon removal and freezing of muscles in liquid nitrogen for determination of esterification and oxidative intermediates, complete oxidation of palmitate was assessed by the collection of $^{14}CO_2$ over 90 minutes in 400 µl benzethonium hydroxide following the addition of 1 mL of acetic acid (1 M).

Muscle lipids were extracted from frozen muscle and quantified as previously described (Steinberg, Dyck 2000). Briefly, muscles were weighed, homogenized (chloroform:methanol, 2:1), following a series of centrifugations and the addition of ddH_2O part of the aqueous phase (1 mL) was removed for quantification of oxidation

intermediates and part of the lipophilic phase (650 ul) was removed for lipid quantification. Chloroform was evaporated by N_2, redissolved in 50 μl (chloroform:methanol, 2:1), and spotted onto oven-dried silica gel plates (Fisher Scientific Canada, Missisauga, ON). Lipids were resolved by placing the silica plates in a solvent (60:40:3 heptane-isopropyl ether-acetic acid) containing sealed tank, then plates were air dried, sprayed with dichlorofluorescein dye (0.02% wt/vol in ethanol), visualized with long-wave UV light and bands were scraped into vials for determination of radioactivity by liquid scintillation counting. A cocktail of standards (triacylglycerols and diacylglycerols) was run in a separate lane on the silica plates to ensure correct identification of lipids.

Histochemical Analysis.

Serial sections of GP muscle (8 μm) were cut on a cryostat with one section used for a metachromatic stain to determine fiber type and the other section used for a IIA myosin immunofluorescent (IF) and Oil-Red-O co-stain to confirm fiber type and assess IMCL deposition. Fiber type was assessed in the oxidative region of the GP muscle (N = 4 ND and 3 HFD). IMCL was assessed by fiber type (N = 3–4 ND and 4–5 HFD, minimum 45 fibers per muscle). IIA myosin (2F7, DSHB) and Oil-Red-O was assayed by fixation in 2% PFA followed by regular IF procedure including a MOM blocking kit (Vector Labs), overnight primary antibody incubation (neat, 4°C), Alexa 488 goat anti-mouse secondary (1:1000, 1 hr at room temperature, Invitrogen), Oil-Red-O incubation (30 min) with rinses and mounted with fluoromount (Sigma). SDH activity was assayed as previously described (Shortreed et al. 2009) on a GP muscle x-section in serial to a

section on which a IIA myosin IF was performed as described above without the Oil-Red-O step (N = 4 ND; minimum 15 IIA fibers per muscle). Determination of Oil-Ref-O and SDH content in muscle was performed using Nikon Elements (Nikon). Briefly, fibers of interest were circled and the mean density was recorded. For both stains a darker stained fiber implies greater content of SDH activity or IMCL content and both translate to a greater mean density. Liver was stained with Oil-Red-O and a hematoxilyn counter stain and lipid content was determined by the percentage area that contained lipid (e.g. object area fraction) in the whole liver section. All images were acquired with a Nikon Eclipse 90i microscope.

Western Blotting Analysis.

Muscle lysates (20 µg) were run out on a 4–15% gradient gel (BioRad) at 60V and transferred overnight (4°C, 20V). Primary antibody (OXPHOS rodent WB antibody cocktail; Abcam; 1:1000 dilution) was incubated overnight at 4°C followed by secondary goat anti-mouse HRP (Abcam) and detected with SuperSignal chemiluminescent detection reagent (Thermo Fisher).

mRNA Analysis.

Tissue mRNA was isolated using Trizol reagent (Invitrogen) and converted to cDNA with SSIIRT (Invitrogen). Realtime RT-PCR was performed with cDNA from adipose and liver tissue using on the Rotorgene 3000 (Corbett Research) with assay-on-demand gene expression kits (Applied Biosystems) following the manufacturer's instructions. Expression levels were calculated using the comparative critical threshold (C_t) method. Semi-quantitative RT-PCR was performed with cDNA from muscle as

previously described (Hawke, Jiang & Garry 2003) using the following primer sets: *Socs-3*, TaqMan Mm01249143_g1**;** *Il-6*, 5′-GACAAAGCCAGAGTCCTTCAGAG-3′ 5′-CTAGGTTTGCCGAGTAGATCTC-3′; *Tnf-*, 5′-TCGTAGCAAACCACCAAGTG-3′5′GGAGTAGACAAGGTACAACC-3′; *Tlr-4*, 5′-CACTGTTCTTCTCCTGCCTGAC-3′ 5′-CCTGGGGAAAAACTCTGGATAG-3; *Ptpn1*, 5′-CCCGGCCACCCAAACGCACACT-3′ 5′- GACGCCGCAGACCGCATCCTAAGC-3′.

Plasma Analysis.

Plasma was assessed for 40 inflammatory related proteins using a Proteome Profiler Antibody Array (R&D, Mouse Cytokine Antibody Array, Panel A) as per manufacturer's instructions.

Data Analysis.

Total calories consumed in CLAMS was calculated as follows: food intake (g/day)×9 kcal/g (fat)×ratio of kcal/g fat in diet (0.6 HFD and 0.25 ND)+food intake (g/day)×4 kcal/g (protein and carbohydrate)×ratio of kcal/g in diet (0.4 HFD and 0.75 ND). All statistical analyses were performed with GraphPad Prism 5 software. Differences between groups were determined using the appropriate student t-test or ANOVA followed by the appropriate post-hoc test. P values less than 0.05 were considered significant. All data presented are mean ± standard error of the mean (SEM).

RESULTS

HFD Increases Body Mass and Fat Mass of Mice after 3 and 8 Weeks

HFD increased body mass following 3 (~1.2 fold) or 8 (~1.4 fold) weeks compared to mice on a normal diet (ND; Table 3.1). Epididymal fat pad mass was also elevated with HFD compared to ND after 3 weeks (~ 3.5 fold) and 8 weeks (~ 4 fold) even though ND fat pad mass increased from 3 to 8 weeks which highlights the extent of obesity in the HFD group. Lower limb muscle mass was unaltered after 3 and 8 weeks of HFD (Table 3.S1).

HFD Results in Rapid Increase in Lipid Metabolism

In an effort to understand the progression of changes in lipid oxidation in response to HFD consumption, we quantified the initial whole body metabolic response using the comprehensive laboratory animal monitoring system (CLAMS). Body mass increased in the CLAMS after 4 days of HFD, but not after an initial 4 days of ND (Figure 3.S1). While total calories during the light cycles (Figure 3.1A) increased with HFD, total calories during the dark cycles remained relatively constant (Figure 3.1B). To determine if total calories consumed would differ when corrected for weight gain on HFD, statistics were performed on values from the last day of ND (day -1) and HFD (day 4) relative to body mass at these times. Consistent with initial findings there was still a greater amount of total calories consumed during light cycles following HFD (Day -1:16.65±1.33 kcal/12 hr cycle/body mass (g); Day 4:23.05±1.73 kcal/12 hr cycle/body mass (g); t-test, p<0.05) and no difference during dark cycles (Day -1:38.34±3.81 kcal/12 hr cycle/body mass (g); Day 4:36.25±3.18 kcal/12 hr cycle/body mass(g); t-test, p<0.05). HFD was introduced after a dark cycle (07:00) and a drop in the respiratory exchange ratio (RER) occurred

during the subsequent dark cycle and the second light cycle (Figure 3.1C, D). Light cycle activity level (Figure 3.1E) and exploratory activity (Figure 3.S1) were unaltered with HFD. Dark cycle activity level (Figure 3.1F) declined temporarily during the first three days of HFD and HFD exploratory activity (Figure 3.S1) fluctuated slightly such that there were differences compared to the initial measurement day (day -3) but not the last two days of ND. In summary, upon commencement of HFD, it took 12–24 hours for metabolism to significantly shift towards greater lipid utilization.

Increased Number of Oxidative Fibers and Functional Lipid Oxidation in Muscle Following 3 Weeks of HFD

Our investigation into fatty acid handling in whole muscle demonstrated that the mixed fiber-type EDL muscle (49% fast-glycolytic, 51% fast-oxidative glycolytic), but not the highly oxidative soleus muscle (42% fast-oxidative glycolytic and 58% slow oxidative) (Burkholder et al. 1994) exhibited enhanced total palmitate oxidation after 3 weeks of HFD (Figure 3.2A). The EDL muscle also exhibited less relative esterification than total oxidation with HFD while the soleus did not demonstrate a shift towards utilization of fatty acids, away from storage (Figure 3.2B). There was no difference in palmitate uptake into either EDL or soleus muscle after 3 weeks of HFD (EDL: ND 2.045±0.098, HFD 2.510±0.349 pmol/min/mg; Soleus: ND 4.307±0.482, HFD 3.739±0.352 pmol/min/mg). Both EDL and soleus muscles exhibited a decrease in the relative amount of incomplete (oxidative intermediates, OI) to complete (CO_2) β-oxidation of palmitate with HFD (Figure 3.2C).

Potential mechanisms to explain the increased total palmitate oxidation in mixed fiber-type muscles could include an increase in mitochondrial density or an oxidative fiber-type shift which were investigated in the mixed fiber-type gastrocnemius/plantaris (GP) muscle complex. The density of the oxidative protein succinate dehydrogenase (SDH) in a subset of fast oxidative glycolytic fibers, termed IIA fibers, was not different in the oxidative area of the GP muscle (ND, 1.094±0.067 AU, N = 4; HFD, 1.020±0.148 AU, N = 4, minimum 15 IIA fibers per muscle). An oxidative fiber-type shift began by 3 weeks of HFD as demonstrated by a greater percentage of IIA fibers within the oxidative area of the muscle (Figure 3.2D). Since the GP muscle is predominantly fast-glycolytic (Burkholder et al. 1994), it is not surprising that our detailed assessment of fiber type oxidative shift was only detectable by protein (Western blot) analysis of complexes involved in the electron transport chain (OXPHOS proteins; complexes I–V) after 8 weeks of HFD (Figure 3.2E, F).

Skeletal Muscle is not Insulin Resistant Following 3 Weeks of HFD Despite Glucose Intolerance

Following either an overnight or a 6 hour fast an intraperitoneal glucose tolerance test (IPGTT) was performed. To maximally raise blood glucose, stress the system's responsiveness to a glucose challenge and to assess insulin sensitivity in muscle, a higher dose of glucose was employed following the overnight fast. Glucose tolerance was impaired (Figure 3.3A, B) with HFD after 3 weeks as determined by IPGTT. This occurred despite similar baseline glycaemia between diet groups. During an intraperitoneal insulin tolerance test (IPITT; 6 hr fast), whole-body insulin sensitivity was

not different with HFD (Figure 3.3C). The presence of glucose intolerance in HFD mice (Figure 3.3A, B) could involve a reduced ability of adipose and muscle to uptake glucose in response to insulin secreted by the pancreas. However, as insulin-stimulated glucose uptake in isolated EDL (Figure 3.3D) and soleus (Figure 3.3E) muscles was maintained in HFD fed mice our findings suggest that the glucose intolerance was not due to the presence of skeletal muscle insulin resistance. Thus, glucose intolerance in HFD mice is likely due to a lower responsiveness of the liver to insulin which should translate to a slower lowering of blood glucose during an ITT. The normal ITT result in HFD mice may be a result of initial hyperinsulinemia at baseline although this was not assessed. The lack of muscle insulin resistance coincided with elevated total palmitate oxidation and unaltered IMCL content observed in the mixed fiber-type GP muscle complex (Figure 3.3F). Disease progression was appropriately worsened with time, since plasma insulin levels were not different from control mice following 3 weeks of HFD, but were ~4 fold higher following 8 weeks of HFD (Figure 3.S2).

Increased Inflammation in Adipose Tissue, but Little to no Change in Liver, Muscle or Blood Following 3 Weeks of HFD

Lipid overload and inflammation in adipose tissue and liver is linked to the onset of insulin resistance in these organs (Gregor, Hotamisligil 2011, Samuel, Shulman 2012, Hotamisligil 2003, Moller 2000). The significant adipose tissue expansion observed after 3 weeks of HFD (Table 3.1) was accompanied by an increase in pro-inflammatory markers in this tissue (Figure 3.4A), including the iNOS:arginase ratio, which is indicative of pro-inflammatory M1 macrophages (Ghanim et al. 2004, Weisberg et al.

2003, Xu et al. 2011). While there was a significant increase in hepatic lipid deposition (ND 0.495±0.08%area; HFD 1.211±0.21%area, N = 3–5), pro-inflammatory indices in the liver were unaltered (Figure 3.4B). Inflammatory proteins produced by adipose tissue can be released into the circulation where they can act on alternative insulin sensitive tissues like skeletal muscle. However, there were no alterations to a wide range of pro- and anti-inflammatory factors in the plasma of HFD mice including IL-6, TNF-α, and CCL-2 (Figure 3.S3A). There was also a lack of overt inflammation in skeletal muscle after 3 weeks of HFD as evidenced by unaltered macrophage content, identified by the F4–80 glycoprotein in GP muscle (ND 11.75±1.88 AU; HFD 19.00±6.24 AU, N = 3–4). Moreover, mRNA expression of inflammatory genes including *Socs3*, *Il-6*, *Tnf-α* and *Ptp-n1* in skeletal muscle were found to be unaltered in both tibialis anterior (TA) and soleus muscles (Figure 3.4C, D). Inflammatory signaling within skeletal muscle could also be triggered by circulating fatty acids through toll-like receptor-4 (*Tlr-4*) which was elevated in soleus, but not TA muscles (Figure 3.4E, F), following 3 weeks of HFD.

8 Weeks of HFD Results in Minor Alterations to Inflammatory Signaling in Muscle

Since skeletal muscle insulin resistance has been reported after 8 weeks of our HFD (Shortreed et al. 2009) and pro-inflammatory factors in plasma have been implicated in the development of skeletal muscle insulin resistance (Kewalramani, Bilan & Klip 2010, Samuel, Shulman 2012), inflammatory markers in the plasma and inflammatory signaling in skeletal muscle were investigated at this time-point. There was no difference in plasma inflammatory proteins including Il-6, Tnf-α and Ccl-2 following 8 weeks of HFD (Figure 3.S3B). The majority of inflammatory signaling in muscle was

found to be unaltered with HFD in TA or soleus muscles (Figure 3.5 A, B). However, *Tlr-4* expression was down-regulated in TA muscle and there was a trend (p = 0.07) towards an increase in soleus muscle (Figure 3.5 C, D), which is similar to 3 weeks of HFD.

DISCUSSION

Our results indicate that the early, whole body response to HFD, particularly that of skeletal muscle, is a protective adaptation which provides a buffering period of time before the onset of insulin resistance in muscle after 8 weeks of HFD as previously demonstrated in this model (Shortreed et al. 2009). Specifically, we found that HFD resulted in an early shift towards enhanced whole body and total muscle lipid oxidation and an oxidative fiber-type shift, which we believe attenuated IMCL build-up and prevented muscle insulin resistance following 3 weeks of HFD. Unfortunately, enhanced total lipid oxidation was unable to prevent glucose intolerance which was present after 3 weeks of HFD and associated with adipose tissue inflammation, increased adipose tissue mass and elevated hepatic lipids. Knowing that muscle is insulin resistant after 8 weeks of our HFD treatment (Shortreed et al. 2009), the minimal elevations of inflammatory markers identified in muscle and plasma at, and prior to, this time-point leads us to speculate that inflammation plays a minimal role in the initiation of muscle insulin resistance.

As expected, HFD resulted in a rapid shift towards greater whole body lipid utilization as seen by the reduction in RER within 12–24 hours of HFD commencement. Continued HFD may result in a further reduction of RER as noted in rats fed a HFD (So

et al. 2011). However humans given a HFD experience a rapid reduction in respiratory quotient (RQ) after 24 hours that is not reduced further following 7 days (Schrauwen et al. 2000). The reduction in RER seen with HFD was not solely due to a reduction in activity level as light cycle activity level was not altered and dark cycle activity level was only reduced temporarily. Total calories consumed were unaltered with HFD demonstrating that the composition of the diet, not a caloric increase, was responsible for the change in metabolism. The shift towards increased total lipid oxidation that occurs within hours of switching to HFD continues at 3 weeks as evidenced by the increase in total palmitate oxidation in the mixed fiber-type EDL muscle, but not the highly oxidative soleus muscle. We hypothesize that the soleus does not exhibit an increase in total lipid oxidation due to the high rate of basal (e.g. ND) total lipid oxidation compared to EDL muscle. The rapid increase in IMCLs observed with lipid infusion or HFD (Bachmann et al. 2001) appears to have provided the stimulus for increased muscle fatty acid oxidation in EDL muscle by 3 weeks of HFD which, in turn, helped maintain IMCLs at levels similar to those of ND mice. Conversely we previously demonstrated prolonged HFD (8 weeks) to result in impaired lipid oxidation, excess IMCLs and insulin resistance in muscle (Shortreed et al. 2009).

In line with unaltered IMCL levels in the GP muscle, EDL muscles were insulin-sensitive and exhibited a lower esterification-to-oxidation ratio following 3 weeks of HFD. A high esterification-to-oxidation ratio is observed in obese, insulin resistant states and is thought to contribute to both the build-up of lipids and insulin resistance in muscle (Goodpaster, Kelley 2002, Steinberg et al. 2002). An increase in the ratio of

incomplete to complete fatty acid oxidation has also been proposed to contribute to muscle insulin resistance (Koves et al. 2008). Accordingly, this ratio was decreased for both muscle types in the present study. In the present study whole muscle, as opposed to muscle homogenates, was used to assess palmitate oxidation resulting in the majority of oxidative intermediates, representative of incomplete oxidation, remaining within the muscle and few being released into the surrounding media. Thus, oxidative intermediates (termed ASMs when using homogenates) trapped within muscle were assessed here whereas with homogenates ASMs in the media are determined. Given unaltered palmitate uptake for both muscle types, yet a lower esterification to oxidation ratio for EDL muscle alone, we conclude HFD caused EDL muscle to exhibit a greater rate of fatty acid uptake into mitochondria. There have been differing reports in the literature concerning muscle lipid oxidation with HFD (Shortreed et al. 2009, Turner et al. 2007, Chanseaume et al. 2007, Hoeks et al. 2011, Turner et al. 2009). An initial increase in muscle lipid oxidation has been reported in a few studies (Turner et al. 2007, Chanseaume et al. 2007, de Wilde et al. 2008) which is consistent with our findings after 3 weeks of HFD. Importantly our enhanced lipid oxidation at 3 weeks demonstrates an early change to muscle in response to HFD that may have mitigated the build-up of IMCLs within muscle, which we believe is the mechanism underlying the maintenance of muscle insulin sensitivity after 3 weeks of HFD.

Our reported increase in muscle lipid oxidation was likely due to a rapid decrease in pyruvate dehydrogenase that is known to occur with a low-carbohydrate and high-fat diet (Peters et al. 1998). A prolonged elevation in lipid oxidation could result in a shift in

muscle fiber-types or mitochondrial density. No increase in density of the mitochondrial marker, SDH, was found in oxidative fibers which we previously noted after 8 weeks of HFD (Shortreed et al. 2009). However, an increase in oxidative capacity was evident in muscle after 3 weeks of HFD by an oxidative fiber-type shift within the oxidative area of the GP muscle as was also noted following our previous 8 week HFD study (Shortreed et al. 2009). Although our assessment of fiber-type content is sensitive to early changes in oxidative capacity, our assessment of whole muscle OXPHOS content provides a more global picture of the change in oxidative capacity in the predominantly fast glycolytic GP muscle (Burkholder et al. 1994). Our findings suggest an early adaptive response to HFD within muscle by upregulating lipid oxidation and transitioning to a more oxidative fiber-type. We have now shown that prolonged exposure to HFD (8 weeks) results in further enhancement of the oxidative potential of muscle as demonstrated by an increase in OXPHOS content which complements our previous report of an oxidative fiber type shift after 8 weeks of HFD (Shortreed et al. 2009). However, at the 8 week time-point we previously demonstrated functional lipid oxidation to be impaired with this diet paradigm (Shortreed et al. 2009). Thus, there is an early increase in metabolic machinery that coincides with an increased functional ability to oxidize lipids. While there is a further increase in metabolic machinery with prolonged HFD the functionality is reduced. These studies indicate that measuring the expression of proteins involved in oxidative metabolism alone does not necessarily translate into functional lipid oxidation rates in skeletal muscle, thus both measures should be assessed.

Rapid expansion of adipose tissue upon HFD consumption could be seen as a positive adaptation, since it accommodates a larger supply of circulating FFAs, thereby reducing hyperlipidemia and ectopic lipid deposition in other tissues. HFD for 3 weeks resulted in a significant increase in body mass and epididymal fat pad mass with no change in lower limb muscle mass demonstrating significant adipose tissue expansion. Such a large expansion of adipose tissue could involve the conversion of preadipocytes to adipocytes, known as adipogenesis, or enlargement of existing adipocytes which would constitute hypertrophy. Should adipogenesis have occurred, our observed elevation in adipose tissue *Il-6* expression could be derived from insulin stimulated preadipocytes (LaPensee, Hugo & Ben-Jonathan 2008). In contrast, *Tnf-α* expression was elevated in adipose tissue after 3 weeks of HFD and Tnf-α has been shown to cause transcriptional changes in adipose tissue leading to the prevention of preadipocyte differentiation (Zhang, Chen 2011). Hypertrophy of adipose tissue is also associated with inflammatory cell infiltration and activation of M1 macrophages which are needed for ECM remodeling during adipose tissue expansion (Weisberg et al. 2003, Suganami, Ogawa 2010). We found elevated expression of the F4/80 glycoprotein and the iNOS to arginase ratio demonstrating a greater macrophage content that is of the M1 pro-inflammatory phenotype which secretes Tnf-α, Il-6 and Ccl-2, all of which were found to be upregulated at the mRNA level after 3 weeks of HFD (Galic, Oakhill & Steinberg 2010). Ccl-2 serves to recruit new macrophages, while Il-6 and Tnf-α encourage the inflammatory cycle (Galic, Oakhill & Steinberg 2010). This data serves to support previous reports associating HFD induced adipose tissue mass expansion and adipose

tissue inflammation with glucose intolerance (Galic, Oakhill & Steinberg 2010). Interestingly a recent report demonstrated adipose tissue inflammation was not necessary for development of glucose intolerance following 3 days of HFD although it did contribute following a prolonged (16 week) HFD (Lee et al. 2011). In the present study, glucose intolerance was present following rapid adipose tissue expansion and adipose tissue inflammation despite enhancement of lipid oxidation and maintenance of muscle insulin sensitivity.

While adipose tissue is capable of adaptation to accommodate excess circulating lipids, the liver has minimal capabilities for lipid accommodation. The liver receives a large amount of lipids due to direct dietary supply from the portal vein and other circulating lipids that are not oxidized by muscles and other tissue. In this context, the liver may be more susceptible than skeletal muscles and adipose tissue to lipotoxicity under conditions of lipid oversupply such as when feeding a HFD. It is known that within 3 days of HFD, and possibly sooner, hepatic lipids increase (Lee et al. 2011) and we observed this to be true following 3 weeks of HFD. Increased hepatic lipid content causes insulin resistance resulting in elevated gluconeogenesis and reduced glycogen synthesis in the post-prandial state (Gregor, Hotamisligil 2011, Samuel, Shulman 2012). The liver significantly contributes to glucose uptake during an IPGTT, normally accounting for ~30–40% (DeFronzo, Tripathy 2009). Given our observed increase in hepatic lipids with HFD, early insulin resistance likely occurred in liver and this would contribute to the impaired IPGTT result after 3 weeks of HFD despite insulin sensitivity in muscle. While prolonged HFD consumption has been shown to induce hepatic inflammation, which also

contributes to insulin resistance, no overt evidence for an increase in inflammation was noted in liver suggesting that this organ was at an early stage of insulin resistance (Steinberg 2007, Gao et al. 2007). Large elevations of inflammatory factors were also absent from plasma after 3 or 8 weeks of HFD. This is in line with a recent study in which neither Il-6 nor Tnf-α were elevated in mice following 12 weeks of HFD (Steinberg et al. 2010). We conclude that inflammation in liver and plasma are not directly associated with early glucose intolerance.

Inflammation in muscle has recently been suggested to contribute to muscle insulin resistance (Kewalramani, Bilan & Klip 2010). The only indication of inflammation in muscle was a temporary increase in *Tlr-4* expression after 3 weeks of HFD. Interestingly in addition to cytokines, saturated fatty acids and LPS have been argued to induce an increase in *Tlr-4* expression (Reyna et al. 2008, Radin et al. 2008). While increased muscle *Tlr-4* expression has been shown to contribute to insulin resistance (Reyna et al. 2008), no insulin resistance was present after 3 weeks of HFD when *Tlr-4* was upregulated. Furthermore, our longer 8 week HFD that led to muscle insulin resistance (Shortreed et al. 2009) was only associated with a trend towards upregulation of *Tlr-4* in soleus and a significant downregulation of *Tlr-4* in TA muscle. Thus, while the role of Tlr-4 during early development of muscle insulin resistance warrants further investigation it is clear that inflammation in muscle plays a minimal role in the development of early muscle insulin resistance.

In summary we have demonstrated tissue specific changes to gene expression, metabolism, lipid deposition, and inflammation in C57BL/6J mice fed a HFD which

develop characteristics of Metabolic Syndrome similar to humans and will eventually develop T2DM including elevated fasting blood glucose (Fraulob et al. 2010, Gallou-Kabani et al. 2007). Similar to our results a recent study demonstrated that the pathogenesis of HFD induced glucose intolerance and insulin resistance in mice is characterized by early metabolic and inflammatory changes to adipose and liver tissue (Kleemann et al. 2010). Glucose intolerance was noted with only 1 week of HFD coinciding with our early observation of glucose intolerance (Kleemann et al. 2010). Furthermore, a reduction in transcripts for lipid metabolism was identified in muscle after 6 weeks of HFD which is similar to our previously reported reduction in functional lipid oxidation after 8 weeks of HFD (Shortreed et al. 2009). The present study found that 3 weeks of HFD resulted in early positive adaptations in muscle including enhanced lipid oxidation, an oxidative fiber type shift and maintenance of IMCL levels which would all contribute to maintenance of muscle insulin sensitivity. Unfortunately the positive adaptations within muscle were unable to prevent glucose intolerance which was associated with inflammation in the adipose tissue and the amount of lipids stored in both adipose and hepatic tissues. Only by fully understanding the stages of the onset of a disease can one successfully devise therapies and treatments to delay or prevent its progression. Thus, it is important to understand the underlying etiology of HFD-induced glucose intolerance and muscle insulin resistance. We propose that the adaptations to muscle we observed are advantageous to slowing the development of impaired glucose tolerance. Further studies manipulating HFD-induced adaptations to muscle are required to fully understand their importance. However, our hypothesis is consistent with reports

in which interventions that increase oxidative capacity in skeletal muscle (e.g. exercise) can delay or reverse muscle insulin resistance (Slentz, Houmard & Kraus 2009, Phielix et al. 2012, Wood, O'Neill 2012).

TABLES

	Body Mass (g)		Fat Mass (mg)	
	ND	HFD	ND	HFD
Week 0	25.27±0.35 (N=11)	25.37±0.45 (N=12)	---	–
Week 3	26.60±0.29 (N=6)	31.52±0.77 (N=6)* #	215.14±23.39 (N=6)	766.87±83.66 (N=6)*
Week 8	29.60±0.92 (N=5)#	40.78±2.37 (N=6)* #	309.16±20.11 (N=5) #	1222.88±72.73 (N=6)* #

Table 3.1. Body Composition. Body mass and fat mass for normal diet (ND) and high-fat diet (HFD) mice throughout diet intervention up to 8 weeks. Body mass for mice from both 3 and 8 week time-points was included for the 0 week time-point. Data are means ± SEM. Two-way ANOVAs were performed separately for body mass and fat mass, $p < 0.05$, *vs. ND, #vs. week 0 for body mass or vs. week 3 for fat mass.

	3 weeks		8 weeks	
	TA (mg)	**GP (mg)**	**TA (mg)**	**GP (mg)**
ND	51.11 ± 2.14 (N=8)	169.10 ± 8.15 (N=8)	51.35 ± 1.59 (N=6)	164.90 ± 5.84 (N=6)
HFD	54.15 ± 3.85 (N=10)	171.7 ± 4.89 (N=10)	49.34 ± 1.22 (N=6)	174.20 ± 3.78 (N=6)

Table 3.S1. Muscle mass. Lower limb muscle mass after 3 and 8 weeks of diet intervention. Tibialis anterior (TA), gastrocnemius/plantaris complex (GP), normal diet (ND), high-fat diet (HFD). Values are mean ± SEM in mg, t-test comparing diet groups for each muscle and time-point.
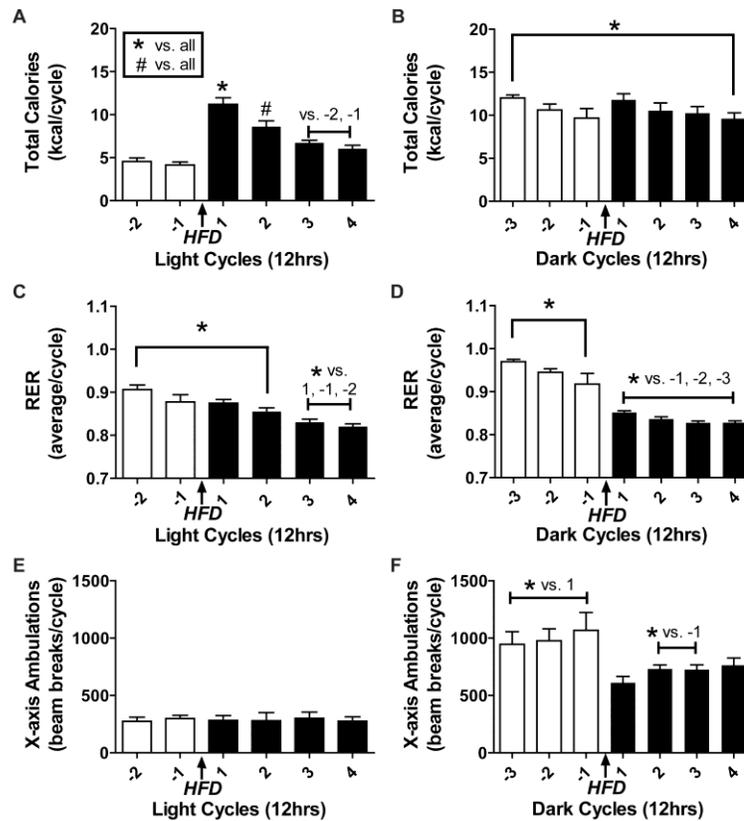
FIGURES

Figure 3.1. Metabolism related changes during HFD transition. Mice in CLAMS were switched from ND to HFD at the beginning of a light cycle. (A) Light cycle and (B) dark cycle calories consumed per 12 hr cycle (N = 7). (C) Light cycle and (D) dark cycle respiratory exchange ratio (RER; $VC0_2$:VO_2). (E) Light and (F) dark cycle average movement (x-ambulations) per 12 hour cycle. Data are mean ± SEM. Statistics: Repeated measures one-way ANOVA with Tukey's multiple comparison test, p<0.05. ND (white bars), HFD (black bars). Average CLAMS measurements per cycle are total (A–B) or means (C–F) of measurements taken every 20 minutes with 1 mouse/CLAMS cage, N = 8 unless otherwise noted. Light cycle = 07:00–19:00, dark cycle = 19:00–07:00.
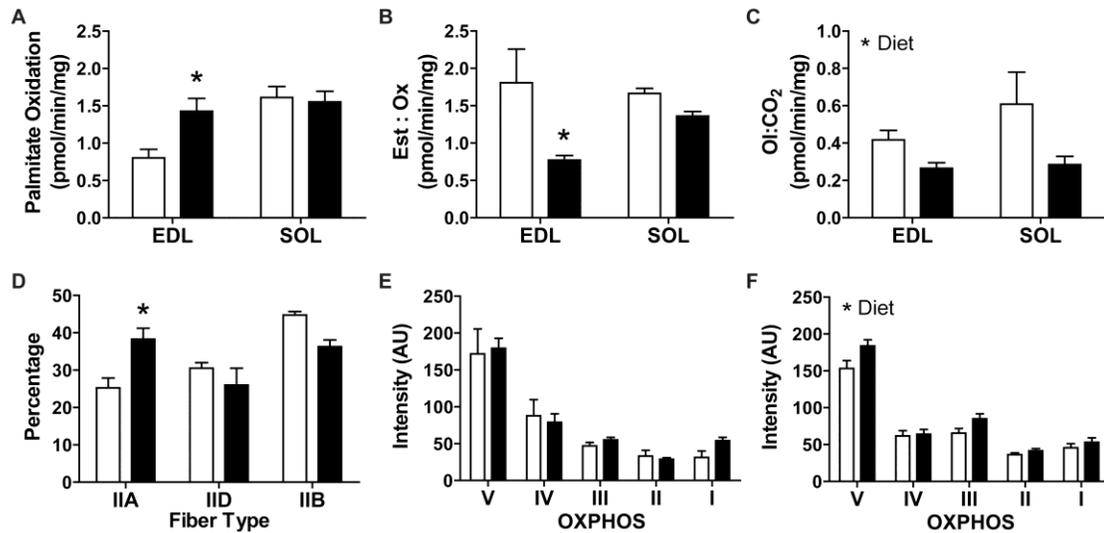
Figure 3.2. Measures of oxidative metabolism in muscle. Following 3 weeks of diet intervention and using palmitate as a substrate EDL and soleus muscle (A) total oxidation [CO_2+ oxidative intermediates (OI)], (B) relative esterification (Est) to TAG and DAG vs. total oxidation (Ox) and (C) incomplete (oxidative intermediates, OI) vs. complete (carbon dioxide, CO_2) oxidation (N = 4 in duplicate) were assessed. (D) Fiber type percentage in the oxidative area of the GP muscle following 3 weeks of HFD. Type I fibers were not included in the analysis due to the minimal content in GP muscle. Protein analysis of complexes involved in the electron transport chain (ETC; complexes I–V) in whole GP muscle homogenates after (E) 3 weeks and (F) 8 weeks of HFD. Data are mean ± SEM. Statistics: (A–F) Two-way ANOVA with Bonferonni post-hoc test, *$p<0.05$. ND, white bars; HFD, black bars. Diacylglycerol (DAG), triacylglycerol (TAG).
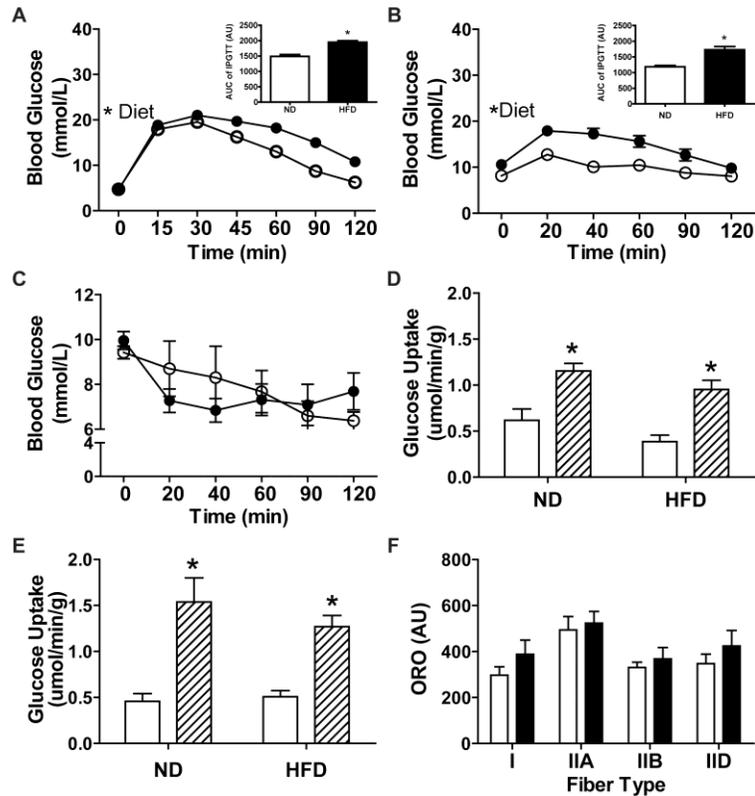
Figure 3.3. Indices of glucose intolerance and muscle insulin resistance following 3 weeks of HFD. IPGTT performed after a (A) 16 hr fast (N = 6) or (B) 6 hour fast (N = 4 ND, 6 HFD) with inset of area under the curve (AUC) graphs. Legend for A–C: ND, open circles; HFD, closed circles. (C) IPITT performed after a 6 hour fast (N = 4 ND, 6 HFD). Insulin stimulated glucose uptake (basal, white bars; insulin, hatched bars) in isolated (D) EDL (N = 4) and (E) soleus muscles (N = 4 ND, 6 HFD). (F) IMCL deposition in the GP muscle complex. Data are mean ± SEM. Statistics: (A–C, F) Two-way ANOVA (repeated measures used for A–C) with Bonferroni post-tests; (D–E) student's t-test, $*p<0.05$. ND, white bars; HFD, black bars unless noted.
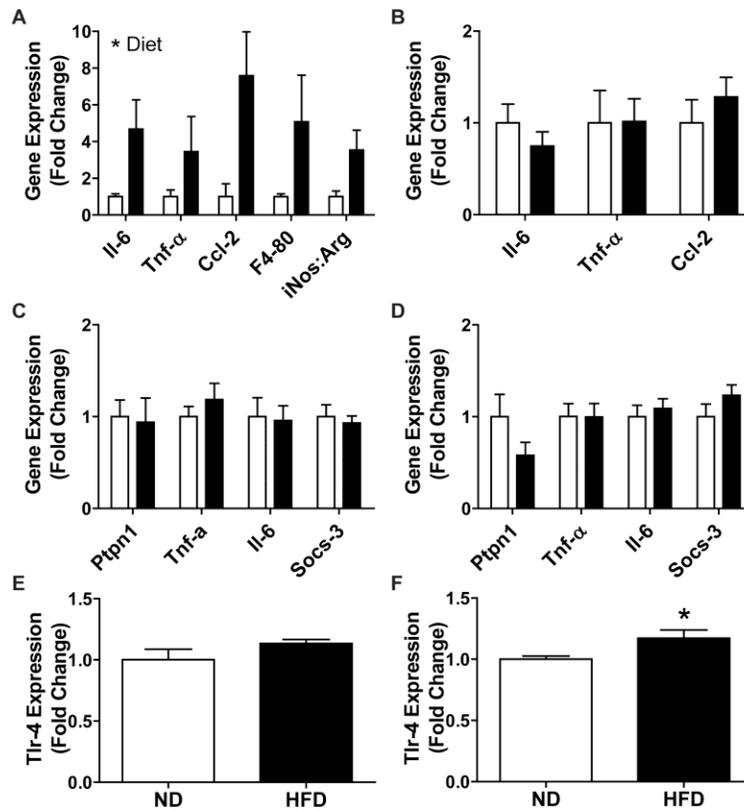
Figure 3.4. Indices of adipose, liver, blood and muscle inflammation following 3 weeks of HFD. Expression of pro-inflammatory indices in (A) adipose tissue (N = 3–6), (B) liver (N = 5–6), (C) TA muscle (N = 6) and (D) soleus muscle (N = 5 ND and 6 HFD). *Tlr-4* gene expression in (E) TA (N = 6) and (F) soleus muscle (N = 5 ND and 6 HFD). Data are means ± SEM. Statistics: (A–D) All ND data was normalized to 1, Two-way ANOVA with Bonferroni post-tests; (E–F) student's t-test, * $p < 0.05$. Values were normalized to β-actin (muscle and adipose tissue) or TATA binding protein (TBP; liver tissue). Normal diet (ND, white bars), high-fat diet (HFD; black bars).
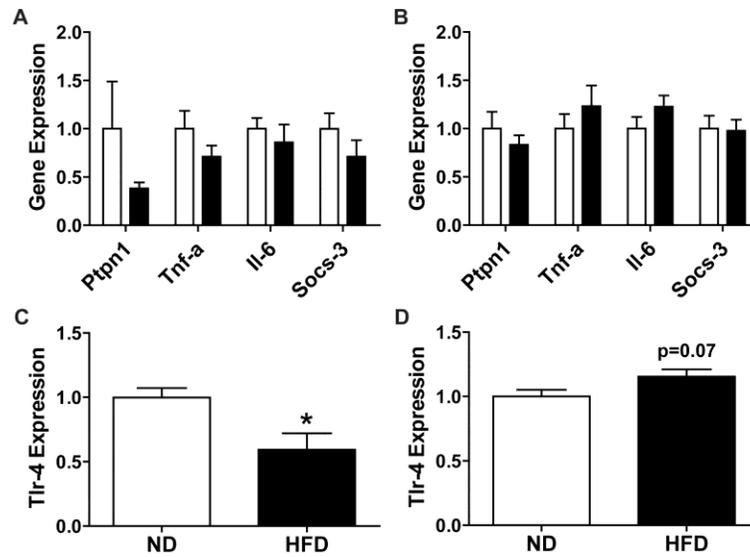
Figure 3.5. Pro-inflammatory transcripts in muscle following 8 weeks of HFD. Relative expression of pro-inflammatory transcripts in (A) TA and (B) soleus muscle after 8 weeks of HFD. Relative expression of *Tlr-4* in (C) TA and (D) soleus muscle after 8 weeks of HFD. Values were corrected for β-actin expression and ND values were normalized to a value of 1. Statistics: (A and B) Two-way ANOVA with Bonferroni post-tests; (C and D) student's t-test, *p<0.05. Normal diet (ND, white bars), high-fat diet (HFD; black bars).
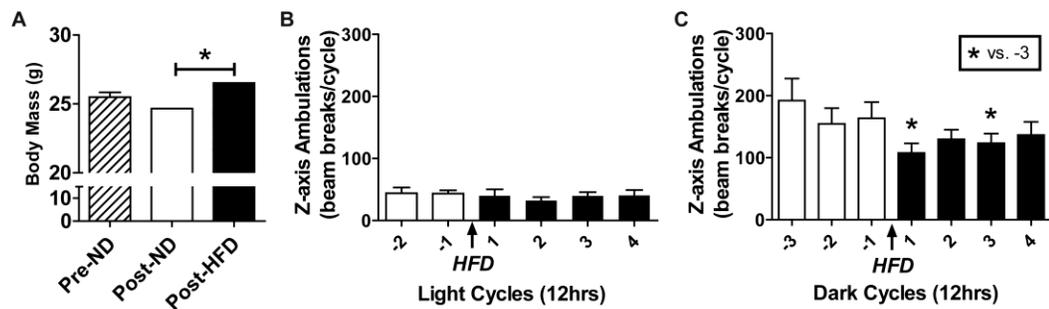
Figure 3.S1. Body mass and exploratory activity upon diet transition. (A) Body mass prior to placement of mice in the CLAMS (Pre-ND), 4 days later before the start of HFD (Post-ND) and after 4 days of HFD (Post-HFD). (B) Light-cycle and (C) dark cycle exploratory activity. Data are mean ± SEM. B–C: Normal diet (white bars), high-fat diet (black bars). Repeated measures one-way ANOVA with Tukey's multiple comparison test, $p<0.05$. Average CLAMS measurements are means of measurements taken every 20 minutes with 1 mouse/CLAMS cage and 8 cages total. Light cycle=07:00–19:00, dark cycle=19:00–07:00.

Figure 3.S2. Plasma insulin during diet protocol. Fed state insulin values prior to and following 3 and 8 weeks of diet intervention. Values are mean ± SEM, two-way ANOVA with Bonferroni post-tests, *P<0.05. Normal diet (white bars), high-fat diet (black bars).
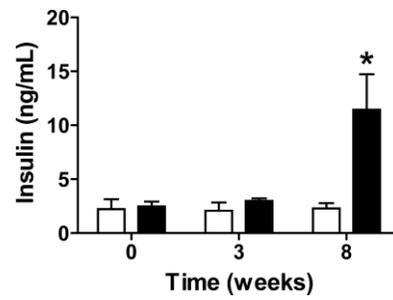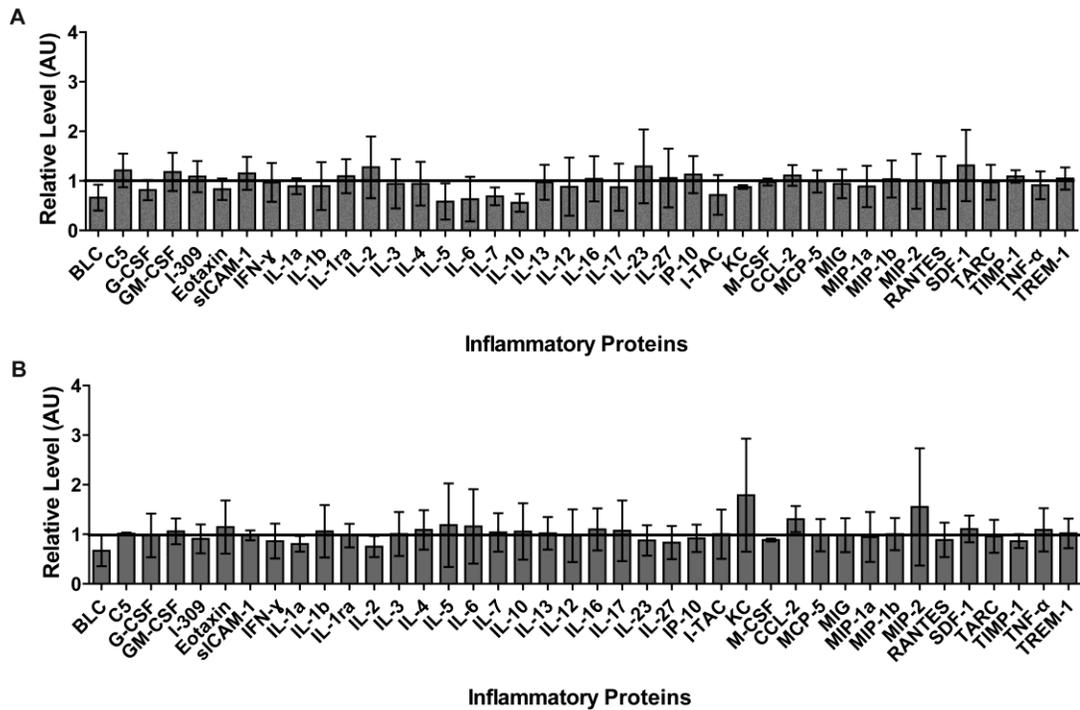
Figure 3.S3. Plasma inflammation indicators. An antibody panel assessing 40 inflammatory proteins was performed on fed state plasma collected during the light cycle after (A) 3 and (B) 8 weeks of diet intervention. ND values (N=3) were normalized to 1 and HFD values (N=3, grey bars) were plotted (mean ± SEM). Inflammatory factors with SEM bars that did not visibly interact with the value of 1 (3-week: BCL, G-CSF, IL-1a, IL-5, IL-7, IL-10, KC; 8 week: BCL, IL-1a, IL-2, M-CSF, TIMP-1) were assessed using a t-test. None of the t-test results were significant (data not shown). In addition IL-6, TNF-α, CCL-2 and IL-10 were assessed using a two-way ANOVA with Bonferroni post-tests, $p < 0.05$. There were no significant results from any of the two-way ANOVAs performed (data not shown).

REFERENCE LIST

Bachmann, O.P., Dahl, D.B., Brechtel, K., Machann, J., Haap, M., Maier, T., Loviscach, M., Stumvoll, M., Claussen, C.D., Schick, F., Haring, H.U. & Jacob, S. 2001, "Effects of intravenous and dietary lipid challenge on intramyocellular lipid content and the relation with insulin sensitivity in humans", *Diabetes,* vol. 50, no. 11, pp. 2579-2584.

Bonnard, C., Durand, A., Peyrol, S., Chanseaume, E., Chauvin, M.A., Morio, B., Vidal, H. & Rieusset, J. 2008, "Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice", *The Journal of clinical investigation,* vol. 118, no. 2, pp. 789-800.

Burkholder, T.J., Fingado, B., Baron, S. & Lieber, R.L. 1994, "Relationship between muscle fiber types and sizes and muscle architectural properties in the mouse hindlimb", *Journal of Morphology,* vol. 221, no. 2, pp. 177-190.

Burr, J.F., Rowan, C.P., Jamnik, V.K. & Riddell, M.C. 2010, "The role of physical activity in type 2 diabetes prevention: physiological and practical perspectives", *The Physician and sportsmedicine,* vol. 38, no. 1, pp. 72-82.

Chanseaume, E., Tardy, A.L., Salles, J., Giraudet, C., Rousset, P., Tissandier, A., Boirie, Y. & Morio, B. 2007, "Chronological approach of diet-induced alterations in muscle mitochondrial functions in rats", *Obesity (Silver Spring, Md.),* vol. 15, no. 1, pp. 50-59.

Craig, B.W., Everhart, J. & Brown, R. 1989, "The influence of high-resistance training on glucose tolerance in young and elderly subjects", *Mechanisms of ageing and development,* vol. 49, no. 2, pp. 147-157.

de Wilde, J., Mohren, R., van den Berg, S., Boekschoten, M., Dijk, K.W., de Groot, P., Muller, M., Mariman, E. & Smit, E. 2008, "Short-term high fat-feeding results in morphological and metabolic adaptations in the skeletal muscle of C57BL/6J mice", *Physiological genomics,* vol. 32, no. 3, pp. 360-369.

Defronzo, R.A. 2009, "Banting Lecture. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus", *Diabetes,* vol. 58, no. 4, pp. 773-795.

DeFronzo, R.A. & Tripathy, D. 2009, "Skeletal muscle insulin resistance is the primary defect in type 2 diabetes", *Diabetes care,* vol. 32 Suppl 2, pp. S157-63.

Duncan, G.E. 2006, "Prevalence of diabetes and impaired fasting glucose levels among US adolescents: National Health and Nutrition Examination Survey, 1999-2002", *Archives of Pediatrics & Adolescent Medicine,* vol. 160, no. 5, pp. 523-528.

Fenicchia, L.M., Kanaley, J.A., Azevedo, J.L.,Jr, Miller, C.S., Weinstock, R.S., Carhart, R.L. & Ploutz-Snyder, L.L. 2004, "Influence of resistance exercise training on glucose control in women with type 2 diabetes", *Metabolism: clinical and experimental,* vol. 53, no. 3, pp. 284-289.

Fraulob, J.C., Ogg-Diamantino, R., Fernandes-Santos, C., Aguila, M.B. & Mandarim-de-Lacerda, C.A. 2010, "A Mouse Model of Metabolic Syndrome: Insulin Resistance, Fatty Liver and Non-Alcoholic Fatty Pancreas Disease (NAFPD) in C57BL/6 Mice

Fed a High Fat Diet", *Journal of clinical biochemistry and nutrition,* vol. 46, no. 3, pp. 212-223.

Galic, S., Oakhill, J.S. & Steinberg, G.R. 2010, "Adipose tissue as an endocrine organ", *Molecular and cellular endocrinology,* vol. 316, no. 2, pp. 129-139.

Gallou-Kabani, C., Vige, A., Gross, M.S., Rabes, J.P., Boileau, C., Larue-Achagiotis, C., Tome, D., Jais, J.P. & Junien, C. 2007, "C57BL/6J and A/J mice fed a high-fat diet delineate components of metabolic syndrome", *Obesity (Silver Spring, Md.),* vol. 15, no. 8, pp. 1996-2005.

Gao, Z., Wang, Z., Zhang, X., Butler, A.A., Zuberi, A., Gawronska-Kozak, B., Lefevre, M., York, D., Ravussin, E., Berthoud, H.R., McGuinness, O., Cefalu, W.T. & Ye, J. 2007, "Inactivation of PKCtheta leads to increased susceptibility to obesity and dietary insulin resistance in mice", *American journal of physiology.Endocrinology and metabolism,* vol. 292, no. 1, pp. E84-91.

Ghanim, H., Aljada, A., Hofmeyer, D., Syed, T., Mohanty, P. & Dandona, P. 2004, "Circulating mononuclear cells in the obese are in a proinflammatory state", *Circulation,* vol. 110, no. 12, pp. 1564-1571.

Goodpaster, B.H., Delany, J.P., Otto, A.D., Kuller, L., Vockley, J., South-Paul, J.E., Thomas, S.B., Brown, J., McTigue, K., Hames, K.C., Lang, W. & Jakicic, J.M. 2010, "Effects of diet and physical activity interventions on weight loss and cardiometabolic risk factors in severely obese adults: a randomized trial", *JAMA : the journal of the American Medical Association,* vol. 304, no. 16, pp. 1795-1802.

Goodpaster, B.H. & Kelley, D.E. 2002, "Skeletal muscle triglyceride: marker or mediator of obesity-induced insulin resistance in type 2 diabetes mellitus?", *Current diabetes reports,* vol. 2, no. 3, pp. 216-222.

Gregor, M.F. & Hotamisligil, G.S. 2011, "Inflammatory mechanisms in obesity", *Annual Review of Immunology,* vol. 29, pp. 415-445.

Hancock, C.R., Han, D.H., Chen, M., Terada, S., Yasuda, T., Wright, D.C. & Holloszy, J.O. 2008, "High-fat diets cause insulin resistance despite an increase in muscle mitochondria", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 105, no. 22, pp. 7815-7820.

Hawke, T.J., Jiang, N. & Garry, D.J. 2003, "Absence of p21CIP rescues myogenic progenitor cell proliferative and regenerative capacity in Foxk1 null mice", *The Journal of biological chemistry,* vol. 278, no. 6, pp. 4015-4020.

Hoeks, J., Wilde, J., Hulshof, M.F., Berg, S.A., Schaart, G., Dijk, K.W., Smit, E. & Mariman, E.C. 2011, "High fat diet-induced changes in mouse muscle mitochondrial phospholipids do not impair mitochondrial respiration despite insulin resistance", *PloS one,* vol. 6, no. 11, pp. e27274.

Hossain, P., Kawar, B. & El Nahas, M. 2007, "Obesity and diabetes in the developing world--a growing challenge", *The New England journal of medicine,* vol. 356, no. 3, pp. 213-215.

Hotamisligil, G.S. 2003, "Inflammatory pathways and insulin action", *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity,* vol. 27 Suppl 3, pp. S53-5.

Houmard, J.A., Tanner, C.J., Yu, C., Cunningham, P.G., Pories, W.J., MacDonald, K.G. & Shulman, G.I. 2002, "Effect of weight loss on insulin sensitivity and intramuscular long-chain fatty acyl-CoAs in morbidly obese subjects", *Diabetes,* vol. 51, no. 10, pp. 2959-2963.

Kewalramani, G., Bilan, P.J. & Klip, A. 2010, "Muscle insulin resistance: assault by lipids, cytokines and local macrophages", *Current opinion in clinical nutrition and metabolic care,* vol. 13, no. 4, pp. 382-390.

Kleemann, R., van Erk, M., Verschuren, L., van den Hoek, A.M., Koek, M., Wielinga, P.Y., Jie, A., Pellis, L., Bobeldijk-Pastorova, I., Kelder, T., Toet, K., Wopereis, S., Cnubben, N., Evelo, C., van Ommen, B. & Kooistra, T. 2010, "Time-resolved and tissue-specific systems analysis of the pathogenesis of insulin resistance", *PloS one,* vol. 5, no. 1, pp. e8817.

Koves, T.R., Ussher, J.R., Noland, R.C., Slentz, D., Mosedale, M., Ilkayeva, O., Bain, J., Stevens, R., Dyck, J.R., Newgard, C.B., Lopaschuk, G.D. & Muoio, D.M. 2008, "Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance", *Cell metabolism,* vol. 7, no. 1, pp. 45-56.

LaPensee, C.R., Hugo, E.R. & Ben-Jonathan, N. 2008, "Insulin stimulates interleukin-6 expression and release in LS14 human adipocytes through multiple signaling pathways", *Endocrinology,* vol. 149, no. 11, pp. 5415-5422.

Lee, Y.S., Li, P., Huh, J.Y., Hwang, I.J., Lu, M., Kim, J.I., Ham, M., Talukdar, S., Chen, A., Lu, W.J., Bandyopadhyay, G.K., Schwendener, R., Olefsky, J. & Kim, J.B. 2011, "Inflammation is necessary for long-term but not short-term high-fat diet-induced insulin resistance", *Diabetes,* vol. 60, no. 10, pp. 2474-2483.

Li, C., Ford, E.S., Zhao, G. & Mokdad, A.H. 2009, "Prevalence of pre-diabetes and its association with clustering of cardiometabolic risk factors and hyperinsulinemia among U.S. adolescents: National Health and Nutrition Examination Survey 2005-2006", *Diabetes care,* vol. 32, no. 2, pp. 342-347.

Moller, D.E. 2000, "Potential role of TNF-alpha in the pathogenesis of insulin resistance and type 2 diabetes", *Trends in endocrinology and metabolism: TEM,* vol. 11, no. 6, pp. 212-217.

Ohinmaa, A., Jacobs, P., Simpson, S. & Johnson, J. 2004, "The Projection of Prevalence and Cost of Diabetes in Canada: 2000 to 2016", *Can J Diabetes,* vol. 28, no. 2.

Peters, S.J., St Amand, T.A., Howlett, R.A., Heigenhauser, G.J. & Spriet, L.L. 1998, "Human skeletal muscle pyruvate dehydrogenase kinase activity increases after a low-carbohydrate diet", *The American Journal of Physiology,* vol. 275, no. 6 Pt 1, pp. E980-6.

Phielix, E., Meex, R., Ouwens, D.M., Sparks, L., Hoeks, J., Schaart, G., Moonen-Kornips, E., Hesselink, M.K. & Schrauwen, P. 2012, "High oxidative capacity due to chronic exercise training attenuates lipid-induced insulin resistance", *Diabetes,* vol. 61, no. 10, pp. 2472-2478.

Radin, M.S., Sinha, S., Bhatt, B.A., Dedousis, N. & O'Doherty, R.M. 2008, "Inhibition or deletion of the lipopolysaccharide receptor Toll-like receptor-4 confers partial protection against lipid-induced insulin resistance in rodent skeletal muscle", *Diabetologia,* vol. 51, no. 2, pp. 336-346.

Reyna, S.M., Ghosh, S., Tantiwong, P., Meka, C.S., Eagan, P., Jenkinson, C.P., Cersosimo, E., Defronzo, R.A., Coletta, D.K., Sriwijitkamol, A. & Musi, N. 2008, "Elevated toll-like receptor 4 expression and signaling in muscle from insulin-resistant subjects", *Diabetes,* vol. 57, no. 10, pp. 2595-2602.

Samuel, V.T. & Shulman, G.I. 2012, "Mechanisms for insulin resistance: common threads and missing links", *Cell,* vol. 148, no. 5, pp. 852-871.

Schrauwen, P., Wagenmakers, A.J., van Marken Lichtenbelt, W.D., Saris, W.H. & Westerterp, K.R. 2000, "Increase in fat oxidation on a high-fat diet is accompanied by an increase in triglyceride-derived fatty acid oxidation", *Diabetes,* vol. 49, no. 4, pp. 640-646.

Shortreed, K.E., Krause, M.P., Huang, J.H., Dhanani, D., Moradi, J., Ceddia, R.B. & Hawke, T.J. 2009, "Muscle-specific adaptations, impaired oxidative capacity and maintenance of contractile function characterize diet-induced obese mouse skeletal muscle", *PloS one,* vol. 4, no. 10, pp. e7293.

Slentz, C.A., Houmard, J.A. & Kraus, W.E. 2009, "Exercise, abdominal obesity, skeletal muscle, and metabolic risk: evidence for a dose response", *Obesity (Silver Spring, Md.),* vol. 17 Suppl 3, pp. S27-33.

So, M., Gaidhu, M.P., Maghdoori, B. & Ceddia, R.B. 2011, "Analysis of time-dependent adaptations in whole-body energy balance in obesity induced by high-fat diet in rats", *Lipids in health and disease,* vol. 10, pp. 99-511X-10-99.

Srikanthan, P. & Karlamangla, A.S. 2011, "Relative muscle mass is inversely associated with insulin resistance and prediabetes. Findings from the third National Health and Nutrition Examination Survey", *The Journal of clinical endocrinology and metabolism,* vol. 96, no. 9, pp. 2898-2903.

Steinberg, G.R. 2007, "Inflammation in obesity is the common link between defects in fatty acid metabolism and insulin resistance", *Cell cycle (Georgetown, Tex.),* vol. 6, no. 8, pp. 888-894.

Steinberg, G.R. & Dyck, D.J. 2000, "Development of leptin resistance in rat soleus muscle in response to high-fat diets", *American journal of physiology.Endocrinology and metabolism,* vol. 279, no. 6, pp. E1374-82.

Steinberg, G.R., O'Neill, H.M., Dzamko, N.L., Galic, S., Naim, T., Koopman, R., Jorgensen, S.B., Honeyman, J., Hewitt, K., Chen, Z.P., Schertzer, J.D., Scott, J.W., Koentgen, F., Lynch, G.S., Watt, M.J., van Denderen, B.J., Campbell, D.J. & Kemp, B.E. 2010, "Whole body deletion of AMP-activated protein kinase {beta}2 reduces muscle AMPK activity and exercise capacity", *The Journal of biological chemistry,* vol. 285, no. 48, pp. 37198-37209.

Steinberg, G.R., Parolin, M.L., Heigenhauser, G.J. & Dyck, D.J. 2002, "Leptin increases FA oxidation in lean but not obese human skeletal muscle: evidence of peripheral leptin resistance", *American journal of physiology.Endocrinology and metabolism,* vol. 283, no. 1, pp. E187-92.

Suganami, T. & Ogawa, Y. 2010, "Adipose tissue macrophages: their role in adipose tissue remodeling", *Journal of leukocyte biology,* vol. 88, no. 1, pp. 33-39.

Turner, N., Bruce, C.R., Beale, S.M., Hoehn, K.L., So, T., Rolph, M.S. & Cooney, G.J. 2007, "Excess lipid availability increases mitochondrial fatty acid oxidative capacity

in muscle: evidence against a role for reduced fatty acid oxidation in lipid-induced insulin resistance in rodents", *Diabetes,* vol. 56, no. 8, pp. 2085-2092.

Turner, N., Hariharan, K., TidAng, J., Frangioudakis, G., Beale, S.M., Wright, L.E., Zeng, X.Y., Leslie, S.J., Li, J.Y., Kraegen, E.W., Cooney, G.J. & Ye, J.M. 2009, "Enhancement of muscle mitochondrial oxidative capacity and alterations in insulin action are lipid species dependent: potent tissue-specific effects of medium-chain fatty acids", *Diabetes,* vol. 58, no. 11, pp. 2547-2554.

Weisberg, S.P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R.L. & Ferrante, A.W.,Jr 2003, "Obesity is associated with macrophage accumulation in adipose tissue", *The Journal of clinical investigation,* vol. 112, no. 12, pp. 1796-1808.

Wood, R.J. & O'Neill, E.C. 2012, "Resistance Training in Type II Diabetes Mellitus: Impact on Areas of Metabolic Dysfunction in Skeletal Muscle and Potential Impact on Bone", *Journal of nutrition and metabolism,* vol. 2012, pp. 268197.

Xu, L., Jiang, C.Q., Lam, T.H., Bao, B., Cheng, K.K. & Thomas, G.N. 2011, "Plasminogen activator inhibitor-1 and HbA1c defined prediabetes: the Guangzhou Biobank Cohort Study-CVD", *Clinical endocrinology,* vol. 74, no. 4, pp. 528-531.

Zhang, Y. & Chen, X. 2011, "Reducing selenoprotein P expression suppresses adipocyte differentiation as a result of increased preadipocyte inflammation", *American journal of physiology.Endocrinology and metabolism,* vol. 300, no. 1, pp. E77-85.

CHAPTER 4

Delayed Muscle Repair and Altered Satellite Cell Function in a Diet-Induced Obesity (DIO) Mouse Model.

Authors: Trajcevski KE, Al-Sajee, D, Wang DC, Thomas MM, Anderson JE, and Hawke TJ.

Corresponding Author: Thomas J. Hawke, Dept. of Pathology and Molecular Medicine, McMaster Univ., 4N65, Health Sciences Centre, 1200 Main St. W., Hamilton Ontario, L8N 3Z5, Canada (email:hawke@mcmaster.ca).

PREFACE

Significance to thesis

There are implications in the literature that muscle repair may be affected with DIO yet few studies have investigated this concept. The primary goal of this study was to assess muscle repair over time following 8 weeks of DIO which led to muscle insulin resistance and other physiological changes within muscle. A secondary goal of this study was to assess primary SC and myoblast functionality. As reviewed in chapter 1 there is a lack of studies investigating SCs derived from DIO mouse muscle despite many suggestions that SC function is impaired with DIO. The ability of muscle to undergo repair is integral to one's ability to exercise and recover from injury or surgery. Given the importance of exercise and muscle health as a whole to future disease outcome for Prediabetic individuals, this research may contribute to improvements of current therapies and exercise prescriptions for Prediabetics.

Authors' contributions

Karin E. Trajcevski conceived and designed the experiments, analyzed the data, wrote the manuscript and made revisions based on reviewer comments with assistance as stated below.

Al-Sajee D assisted with animal handling/care, experiments, data analysis, and refining the manuscript.

Wang DC assisted with muscle staining experiments and data analysis.

Thomas MM assisted with animal handling/care and experiments and refining the manuscript.

Steinberg GR assisted with reagents for radioactive experiments, metabolic cage equipment, writing the manuscript and revising the manuscript based on editorial review.

Anderson JE provided expertise on satellite cell activation experiment design.

Hawke TJ contributed to the design of the study, assisted with performing experiments, and worked on refining drafts of the manuscript and the revisions based on editorial review.

ABSTRACT

Background. The prevalence of obesity is estimated to be 30% in the USA. Often a result of decreased activity and poor diet, obesity predisposes individuals to develop pre-diabetes, insulin resistance and, ultimately, T2DM. A healthy skeletal muscle mass helps reduce the chance of progressing towards T2DM. The capacity for proper muscle repair from injury (e.g. intense exercise, trauma, surgery) is integral to maintaining a healthy muscle mass, yet the capacity for muscle to repair and maintain itself in an obese environment is currently unclear. Furthermore, the functionality of resident muscle progenitors termed satellite cells (SCs) is also unclear in diet-induced obesity (DIO). **Methodology.** C57BL/6J mice were fed a normal or high-fat diet (60% kcal fat) for 8 weeks. Muscle injury was induced by cardiotoxin (CTX) injection into the left tibialis anterior (TA) muscle and gastrocnemius/plantaris (GP) muscle group. Histological and immunofluorescent staining was used to assess the progress of muscle repair. Single myofiber isolation (with and without hepatocyte growth factor supplementation) and immunofluorescence were used to assess SC functionality. **Principal Findings.** Despite similar relative muscle masses of injured muscles between diet groups, muscle repair was delayed (as measured by persistence of necrosis and delayed embryonic myosin heavy chain expression) in GP muscle due to prolonged degeneration and delayed myofiber formation in DIO mice compared to normal diet controls. DIO induced intrinsic impairments in SC activation and proliferation, along with changes in the ability of muscle to release HGF providing potential mechanisms by which muscle repair may be

delayed. In order to maintain a healthy muscle mass, skeletal muscle must be capable of responding to, and regenerating from injury. This study demonstrates that deficits in muscle repair are present in the DIO state and the impairments in the functionality of the muscle satellite cells are likely contributors to the delayed regeneration.

INTRODUCTION

Muscle health is integral to maintaining overall health, including the ability to exercise and regulate blood glucose (Goodpaster et al. 2010, Slentz, Houmard & Kraus 2009, Burr et al. 2010). Improper blood glucose regulation and obesity characterize the disease state of pre-diabetes which can lead to Type II Diabetes and associated co-morbidities (Weiss et al. 2003, Weiss et al. 2004, Bavenholm et al. 2003, DeFronzo 1992). Our lab has previously demonstrated initial early positive adaptations to muscle health with diet-induced obesity (DIO) followed by a decline in muscle health and the development of muscle insulin resistance (Shortreed et al. 2009, Trajcevski et al. 2013). Improvements to muscle health and muscle insulin resistance can be achieved through exercise, though it is known that physical activity will induce varying degrees of muscle damage. This damage to muscle must be repaired to maintain a healthy mass and functionality of the muscle. Thus, understanding the capacity for, and deficits of, muscle to undergo repair with DIO is of paramount importance as exercise-based therapies become more frequently prescribed by health-care providers.

Muscle regeneration following injury is a temporally-sensitive, complex process with necrosis, phagocytosis, satellite cell (SC) activation/proliferation, de novo myofiber formation, and maturation processes overlapping (d'Albis et al. 1988, Hawke et al. 2003, Arnold et al. 2007). A primary contributor to the repair process is the SC population, which are activated from their normally quiescent state in response to injury, wherein they begin to proliferate and ultimately differentiate and fuse to each other or to damaged

myofibers (Hawke, Garry 2001, Charge, Rudnicki 2004). To date, the few studies that have investigated the effect of DIO on muscle repair and SC functionality, though interesting and well-executed, have offered conflicting results; possibly due to variances in diet composition, diet length and injury type (Nguyen, Cheng & Koh 2011, Hu et al. 2010, Woo et al. 2011). Furthermore, these design differences between and within studies make it difficult to extrapolate findings as detailed analyses of many variables (e.g. derangements to metabolism, insulin sensitivity, and fiber type) that may impact the regenerative process in DIO mice remained undefined. Thus, the purpose of this investigation was to provide a more detailed study of muscle repair and satellite cell functionality in a model system which has been consistently used and more thoroughly characterized (Shortreed et al. 2009, Trajcevski et al. 2013).

Here we demonstrate that DIO mice exhibited a protracted time of necrosis and a delay in entering the regenerative phase (as demonstrated by the expression of embryonic myosin heavy chain, Myh3) following muscle injury. Furthermore, in vitro experiments demonstrated that satellite cells from DIO mice were less responsive to hepatocyte growth factor (HGF) signaling and displayed impairments in their proliferative capacity compared to normal diet (ND) fed mice. Crush injury resulted in less HGF release by muscle from DIO mice. When these alterations are placed in the context of literature regarding HGF in obese and insulin resistant states potential mechanisms can be proposed that lead to the delay in regenerative capacity of DIO mice reported here.

METHODS

Experimental procedure.

All experimental protocols were approved by the McMaster University Animal Care Committee in accordance with the Canadian Council for Animal Care guidelines. Male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed in a temperature and humidity-controlled facility with a 12/12 h light/dark cycle and had *ad libitum* access to water and food. Following 1 week acclimatization, animals [10 weeks of age; 1 group was used for CTX experiments including muscle mass and immunofluorescent x-section analysis, N=6; separate groups of animals were used for single fiber (N=2), crushed muscle extract assessments (N=4), and myoblast proliferation (N=6-8)] were randomly assigned to either a high fat diet [HFD; TestDiet, cat #58126: energy (kcal/g) from protein (18.3%), fat (60.9%), carbohydrate (20.1%)] or standard mouse chow [LabDiet 5015 Mouse Diet: energy (kcal/g) from protein (20%), fat (25%), carbohydrate (55%)]. Intramuscular injections of CTX (Latoxan, Valence, France; 10 μM) were performed in left leg GP (50ul in each head of the muscle) and TA (75ul) muscles as previously described (Nissar et al. 2012). Following 5 or 10 days of repair mice were euthanized by CO_2 inhalation followed by cervical dislocation, muscles were excised, covered with tissue freezing medium frozen in cooled isopentane followed by liquid nitrogen and 8μm sections were cut using a cryostat (Leica CM1850) for immunofluorescent (IF) analysis.

Immunofluorescence.

Briefly IF was performed by 2% PFA fixation for 5min at 4 ℃, PBS washes, 1hr RT block (PBS with 10% NGS and 1.5% BSA), PBS washes, primary antibody incubation O/N at 4 ℃ (rabbit anti-dystrophin, ABCAM, 1:200 dilution; rabbit anti-laminin, ABCAM, 1:80 dilution; mouse anti-Myh3, DSHB, used neat; Pax-7, DSHB, neat; MyoD, DAKO, 1:500), PBS washes, appropriate ALEXA secondary antibody (1:1000 dilution), PBS washes, DAPI (5min; 1mg/mL diluted 1:10 000), and mounted with fluoromount (Sigma). Fluorescent images were acquired with a Nikon Inverted Eclipse Ti and analyzed using Nikon Elements software.

Morphometrics and immunofluorescent assessments.

Necrotic fibers were identified by the absence of a distinctive dystrophin ring circling a fiber. Necrotic fibers also exhibit disruption and a reduction in the expression of laminin surrounding them similar to previous reports following injury (Grounds et al. 1998).

Single myofiber isolation and immunofluorescence.

In a set of mice distinct from those given CTX injury single myofibers were obtained by collagenase digestion of EDL and peroneus muscles as previously described (Hawke, Jiang & Garry 2003). Briefly, following collagenase digestion, muscles were triturated with plastic Pasteur pipettes and moved to cell culture dishes with a glass Pasteur pipette. Floating cultures were achieved by coating dishes with 10% normal horse serum prior to addition of plating media [10% normal horse serum, 0.5% chick embryo extract (MP Biomedicals) in low-glucose (1g/L) Dulbecco's modified Eagle's medium (DMEM; Invitrogen)]. Single myofibers were given one of three conditions: basal

(plating media alone), Recombinant Mouse HGF (R &D, 10ng/mL), or DMS (10uM) prior to BrdU being added. Satellite cell activation was determined by performing IF on single myofibers following 24 hour incubation in 5-bromo-2-deoxyuridine (BrdU; Sigma, 10μM) as previously described (Nissar et al. 2012).

Crushed muscle extract and HGF western.

Crushed muscle extract was harvested as previously described (Tatsumi et al. 1998) and HGF content assessed by Western blot. Briefly 100ug protein were run out on an acrylamide gel, transferred to PVDF membrane (BioRad) and primary HGF antibody was incubated O/N at 4℃, secondary HRP linked antibody was incubated for 1 hour at RT and blot was visualized using SuperSignal Chemiluminescent (Thermo Scientific).

Primary myoblasts.

Primary myoblasts were derived from single myofiber cultures attached to a basement membrane matrix (Matrigel; 1:10 dilution) as previously described (Anderson, Wozniak 2004). Following 2 days in plating media single fibers (1/well in a 24-well plate) were removed from culture, plates were washed with PBS and proliferation media (10% FBS, high-glucose media) was added. Myoblast number was counted at 0 hours and after 24 hours in proliferation media.

RESULTS

Relative muscle mass following injury unaltered with DIO.

DIO, following 8 weeks of diet consumption, was previously demonstrated by our lab to result in a state similar to pre-diabetes in humans including: excess body mass,

excess fat mass, glucose intolerance, muscle insulin resistance, and unaltered fasting blood glucose (Shortreed et al. 2009, Trajcevski et al. 2013). Cardiotoxin (CTX) injury was induced in the tibialis anterior (TA) muscle and the gastrocnemius/plantaris (GP) muscle group. Absolute muscle mass of the tibialis anterior (TA) and gastrocnemius/plantaris muscle group (GP) prior to injury was previously demonstrated to be unaltered (Shortreed et al. 2009), and we found no difference in muscle masses of injured (left leg) relative to uninjured (right leg) after 5 or 10 days of injury between diet groups (Figure 4.1).

Prolonged necrosis present in muscle following injury with DIO.

The first steps to proper muscle repair include the removal of damaged cells and infiltrate (necrosis and phagocytosis) to allow for the regenerative/rebuilding phase to begin. Five days following CTX injury, the area of necrosis (defined as the region with damaged muscles lacking a consistent dystrophin expression pattern) was significantly larger in the muscles of DIO mice compared to Normal Diet (ND) (p=0.04; Figure 4.2B) and this difference was still evident ten days post-injury (p=0.08; Figure 4.2C).

Myofiber repair is delayed in muscle with DIO.

Upon observing the protracted presence of necrosis following CTX injury with DIO, we sought to determine if the regenerative phase (i.e. myofiber repair) was also affected. Newly regenerating myofibers, characterized by their centrally-located nuclei, will express developmental isoforms of myosin heavy chain [e.g. embryonic myosin heavy chain (Myh3), Figure 4.3A] before they mature and express adult myosin isoforms (d'Albis et al. 1988, Krause et al. 2011). We observed significantly less Myh3 expressing

fibers in muscle from DIO mice at 5 days post-CTX compared to ND (Figure 4.3B) indicating a delay in regeneration as has been observed with Type 1 diabetes mellitus (Krause et al. 2011). This delay in Myh3 expression at 5 days post-injury resulted in a prolonged presence of Myh3 expression in DIO muscles compared to ND (p=0.07; Figure 4.3C), where most of the regenerating areas in ND muscles had progressed to more mature myosin isoforms by 10 days post-injury. As confirmation of a delay in maturation of Myh3 fibers there were significantly more small Myh3 fibers with DIO at 10 days (Figure 4.3D).

DIO impairs hepatocyte growth factor (HGF) release from damaged skeletal muscle and satellite cell activation is reduced in response to HGF.

Muscle repair can be interrupted by alterations to the SC and it's progeny (myoblasts) including a defect in resting SC content or activation capacity. Immunofluorescent staining of muscle cross-sections with Pax7 demonstrated no difference in TA muscle SC number (ND 1.6 ± 0.23, N=3; DIO 1.2 ± 0.37, N=5; p=0.49. Values are # pax7 positive cells/myonuclei, over 700 nuclei analyzed per animal). Basal activation state, as assessed by the number of MyoD positive cells per resting muscle section, was also not different between groups (ND 0.088 ± 0.077, HFD 0 ± 0, p=0.117; average of 625 nuclei per muscle investigated, N=3).

In vivo SC activation has been demonstrated to occur when HGF binds to its receptor, c-met, on SCs (Anderson, Wozniak 2004). We assessed HGF release from muscle utilizing *an ex* vivo crush injury (Tatsumi et al. 1998) and determined that significantly less HGF was released from crushed muscle extract with DIO (Figure 4.4B).

In vitro experiments suggest that sphingosine-1 phosphate (S1P) may also induce activation of SCs as SIP has been shown to initiate proliferation of reserve cells (myoblasts in G0) (Rapizzi et al. 2008). Interestingly, HGF activates sphingosine kinase 1 (SK1), the enzyme that converts sphingomyelin to S1P (Duan et al. 2006, Hu et al. 2009). We therefore assessed the ability of HGF to enhance SC activation on freshly isolated myofibers from both ND and DIO mice and the involvement of S1P in this process. To perform the latter, myofibers were co-incubated with HGF and DMS (a sphingosine kinase inhibitor to prevent the formation of S1P from sphyingomyelin (Hu et al. 2009). HGF administration successfully activated SCs on ND muscle (Figure 4.4D) as demonstrated by an increase in BrdU nuclei per mm^2 on isolated myofibers, but had no effect on isolated myofibers from DIO muscle (Figure 4.4E). Incubation with DMS partially prevented HGF mediated activation with ND and did not reduce activation below basal values for either diet group (Figure 4.4D).

Myoblast proliferative ability is impaired *ex vivo* and in muscle 5 days after injury with DIO.

Adequate proliferation of myoblasts is essential for myofiber formation and repair from injury, with a peak in myoblast content 2-3 days following CTX injury (Yan et al. 2003, Tidball, Villalta 2010). Myoblasts derived from DIO mouse muscle demonstrated an impaired proliferative capacity in vitro compared to ND myoblasts (Figure 4.5A). An in vivo assessment of myoblasts demonstrates that at 5 days post-injury, DIO muscle trended (p=0.07) toward more MyoD positive myoblasts in the DIO muscle compared to ND (Figure 4.5B). While the increase in MyoD positive cells in regenerating DIO muscle

may seem counter to the in vitro data demonstrating a reduced proliferative capacity, it should be noted that at 5 days post-injury MyoD expressing cells are declining in number as they are differentiating and fusing to regenerate myofibers (Hawke et al. 2003, Charge, Rudnicki 2004). Thus, an increased expression of MyoD within muscle at this time is indicative of a protracted myoblast proliferative phase, a finding consistent with our results of prolonged necrosis and delayed regeneration.

DISCUSSION

A healthy skeletal muscle mass is integral for improving obese and insulin resistant states including pre-diabetes and T2DM. A primary attribute of muscle health is the capacity for maintenance and repair of skeletal muscle. In the present study, we observed a delay in muscle repair as demonstrated by prolonged degeneration and delayed myofiber formation in DIO mice compared to normal diet controls. While a deficit in muscle repair has been noted by some, but not all, investigations involving DIO (Nguyen, Cheng & Koh 2011, Hu et al. 2010, Woo et al. 2011), specific decrements in SC function have previously been unreported. We demonstrate here that DIO induces intrinsic impairments in SC activation and proliferation, along with changes to the milieu of the muscle which may provide potential mechanisms by which DIO delays muscle repair.

Intramuscular injection of cardiotoxin (CTX) results in >80% of the muscle being damaged, allowing for ease of detection of changes to muscle repair pathways. A general marker of muscle repair is relative muscle mass. A reduced mass of regenerating muscle

(relative to tibia length) has been reported 12 days, but not 6 days, post-injury with DIO and this was attributed to altered protein turnover (Hu et al. 2010). The present study noted no difference in regenerating muscle mass (relative to uninjured contralateral leg) between diet groups following 5 or 10 days of repair. Thus we hypothesize that protein turnover would be unaffected during repair with the diet intervention used here. Although regenerating muscle mass was not different from control with DIO, the apparently lower relative muscle mass at 10 days (both ND and DIO) for GP muscle compared to TA muscle led to a focus on GP muscle repair. Detailed indicators of muscle repair include morphometric and SC assessments as following snake venom (CTX or notexin) injury (d'Albis et al. 1988, Arnold et al. 2007, Hirata et al. 2003) there is an early degenerative response including necrosis of myofibers that begins to subside by 4-5 days, when both myoblasts and new myofibers are visible, (Hawke, Jiang & Garry 2003) and is greatly reduced by 10 days when new myofibers have begun to mature (Arnold et al. 2007, Charge, Rudnicki 2004, Hawke, Jiang & Garry 2003). In the present study, assessment of regenerating myofibers was performed using immunofluorescence for Myh3 (a developmental isoform of myosin heavy chain) to avoid inclusion of necrotic or phagocytic fibers which can have internalized nuclei or infiltration of inflammatory cells (Charge, Rudnicki 2004) and may thus be erroneously included when utilizing hematoxylin and eosin (H+E) stained sections. One study reporting no decrement in muscle fiber area with DIO also noted no detriment to muscle repair (Nguyen, Cheng & Koh 2011). However, regenerating fibers were identified through the use of H+E stained sections. In the present study, the appearance of newly regenerated myofibers, as

140

identified using Myh3 staining, was found to be delayed with DIO at 5 days with a trend towards a continued presence at 10 days. Similarly, 3 days of muscle repair following post-natal DIO has led to a reduction of the area containing regenerated myofibers which was attributed to a reduction in uninjured muscle SC content (Woo et al. 2011). Hu (Hu et al. 2010) also demonstrated that long-term DIO led to a shift towards more, smaller myofibers at 6 and 12 days of repair; suggestive of a reduction in the regenerating area. Interestingly Hu (Hu et al. 2010) did not attribute this effect to reduced SC content or myogenicity, but to reduced protein synthesis, increased protein degradation and an excess of collagen. The present study reports an intrinsic (ie. in vitro) impairment in SC myogenicity with DIO. The difference in SC alterations with DIO between the current study and that by Hu (Hu et al. 2010) could be a result of confounding variables in the assessment of *in vivo* SC function or disease progression. Potential confounding variables to the assessment of *in vivo* SC function include whole muscle analysis, choice of housekeeping genes and non-specific BrdU analysis. Specifically, Hu and colleagues (2010) normalized the expression level of MyoD to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which changes with metabolic perturbations to muscle including insulin resistant states (Perez, Tupac-Yupanqui & Dunner 2008, Roy et al. 2008). Furthermore, bromodeoxyuridine (BrdU) incorporation was performed to assess proliferating myoblast content, yet BrdU is a general marker of proliferation and does not solely identify myoblasts; a potential problem in multicellular tissues since all dividing cells will incorporate this thymidine analogue (including fibroblasts and inflammatory cells) (Thorsson et al. 1998). Lastly, the disease state of the

DIO intervention utilized by Hu (Hu et al. 2010) appears to be more severe than the present study as we have previously shown unaltered basal fasting blood glucose levels (Shortreed et al. 2009) unlike the 8 month diet intervention used by Hu (Hu et al. 2010) which found elevated fasting blood glucose levels. This difference is likely due to length of diet intervention. While the *in vivo* contribution of the SC to muscle repair is important to understand, beginning with an *in vitro* approach using primary cells can help unravel the interactions between intrinsic changes to the SC and the environment of the regenerating muscle.

HGF binding to its receptor (c-met) on SCs leads to activation which occurs within 24 to 48 hours of muscle injury followed by myoblast proliferation and new myofiber formation starting after 2-3 days (Hawke, Garry 2001, Charge, Rudnicki 2004). The present study demonstrated DIO to result in a reduction in HGF released upon crush injury and a reduction in the *ex vivo* response of SCs to HGF. HGF provided to myotubes enhances glucose uptake and metabolism, and impairs fatty-acid oxidation (Perdomo et al. 2008). Although glucose uptake and metabolism are impaired in obese insulin resistant states, HGF in adipose tissue and serum are increased with obesity (Rehman et al. 2003). Excess circulating HGF continuously bathing muscle may result in reduced stores of HGF bound to proteoglycan sulfate chains within muscle. Likewise, downregulation of the c-met receptor on SCs may occur. Alternatively, the deficit in HGF activation of SCs with DIO may be due to a problem with the HGF signaling pathway. Here we present cursory evidence that S1P is involved in HGF induced SC activation in control muscle. Future

studies will further investigate the role of S1P in HGF induced SC activation and assess c-met expression on SCs from DIO mice.

Exogenous HGF given to injured muscle is known to inhibit muscle repair, in part by promotion of SC proliferation, which delays differentiation (Miller et al. 2000). As well, high circulating HGF levels are associated with obesity (Rehman et al. 2003). Correspondingly, despite our observed reduction in myoblast proliferation *in vitro*, our results suggest that there is a prolonged presence of myoblasts during repair with DIO although it should be noted that this *in vivo* assessment was performed in TA muscle, not GP muscle. Delayed fusion of myoblasts is supported by the impairment in Myh3 content, an indicator of myoblast differentiation, which occurred at the same time as the elevation in myoblasts. Based on the results reported here and the literature it is hypothesized that one of two scenarios occurs *in vivo* during repair with DIO. One possibility is that SC activation and or proliferation are slow to occur and thus it takes longer for enough myoblasts to accumulate for adequate differentiation. Alternatively, if plasma HGF is elevated and bathing the muscle during repair this may overcome any intrinsic impairments in SC activation and proliferation yet may reduce differentiation.

SC differentiation can also be delayed in response to an increased content of pro-inflammatory (M1) macrophages. The timely conversion of macrophages from M1 to M2 is important to muscle repair as the former contribute to myoblast proliferation while the latter contribute to differentiation (Arnold et al. 2007, Brigitte et al. 2010). The present study noted a larger necrotic area within injured muscle with DIO which suggests a disruption in the normal process of inflammatory cell appearance and conversion from

pro- (M1) to anti-inflammatory (M2) phenotype (Arnold et al. 2007). DIO has been shown to result in an excess of pro-inflammatory (M1) macrophages in uninjured muscle after only 3 weeks of diet (Hong et al. 2009). However, we previously reported no increase in macrophage content with the diet paradigm used here (Trajcevski et al. 2013). Future studies will investigate if M1 macrophages remain in muscle for a longer period of time during repair with DIO than control diet.

Our detailed analysis of muscle repair and alterations to both SC activity and the uninjured muscle milieu has provided us with potential mechanisms that may contribute to our observed delay in muscle repair with DIO. The findings reported here suggest that muscle repair may be reduced with DIO following less severe injury, as can occur during exercise, which may affect the type or amount of exercise prescribed to pre-diabetic individuals. Understanding muscle health with DIO improves our understanding of the progression towards Type II Diabetes Mellitus which is an important public health concern given the growing number of North Americans with obesity and pre-diabetes (Duncan 2006, Li et al. 2009, Lee et al. 2011). Furthermore therapies, including exercise prescription, for pre-diabetes will be more effective with a thorough understanding of changes to muscle health, including response to injury, with disease progression.
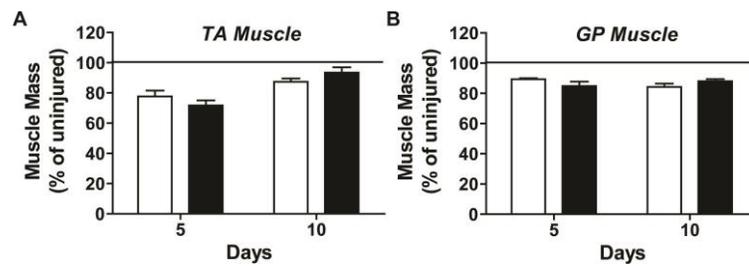
FIGURES

Figure 4.1. **Unaltered injured muscle mass.** Injured muscle mass relative to uninjured contralateral leg 5 or 10 days after CTX injury in the tibialis anterior (TA) muscle and the gastrocnemius/plantaris (GP) muscle complex (N=6).
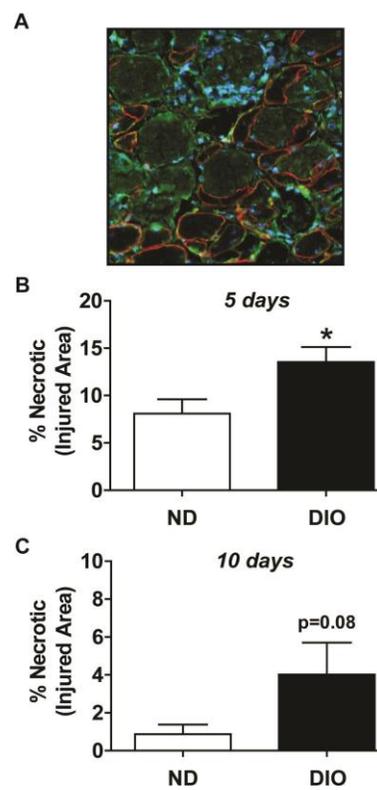
Figure 4.2. Prolonged muscle necrosis. (A) Representative image of ND gastrocnemius/plantaris (GP) muscle 10 days post CTX stained with laminin (green) and dystrophin (red) which both outline healthy fibers (nuclei stained with DAPI, blue).

Arrows indicate necrotic fibers identified by the absence of dystrophin. (B) Percentage area of necrosis in the injured area after 5 days (N=5). (C) Percentage area of necrosis in the injured area after 10 days (N= 6 ND, 5 HFD). Normal diet (ND), diet-induced obesity (DIO). Significance was determined by student's t-test, p<0.05.
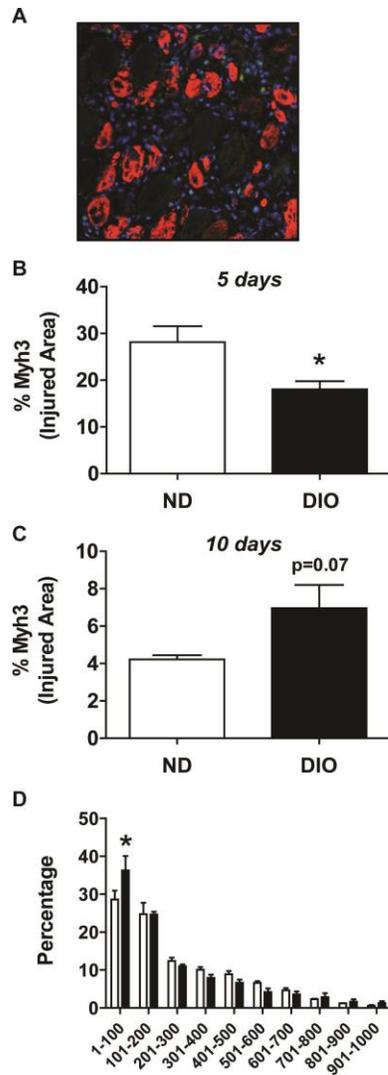
Figure 4.3. Impaired muscle regeneration. Representative image of ND gastrocnemius/plantaris (GP) muscle 5 days after injury stained with Myh3 (red; nuclei stained with DAPI, blue). (B) Percentage area of Myh3 content in the injured area after 5 days (N=5). (C) Percentage area of Myh3 content in the injured area after 10 days (N=4). (D) Percentage distribution for Myh3 positive fiber areas (N=4-5). Normal diet (ND, white bars), diet-induced obesity (DIO, black bars). Significance was determined by (B-C) student's t-test or (D) two-way ANOVA with Bonferroni post-test, $p < 0.05$.
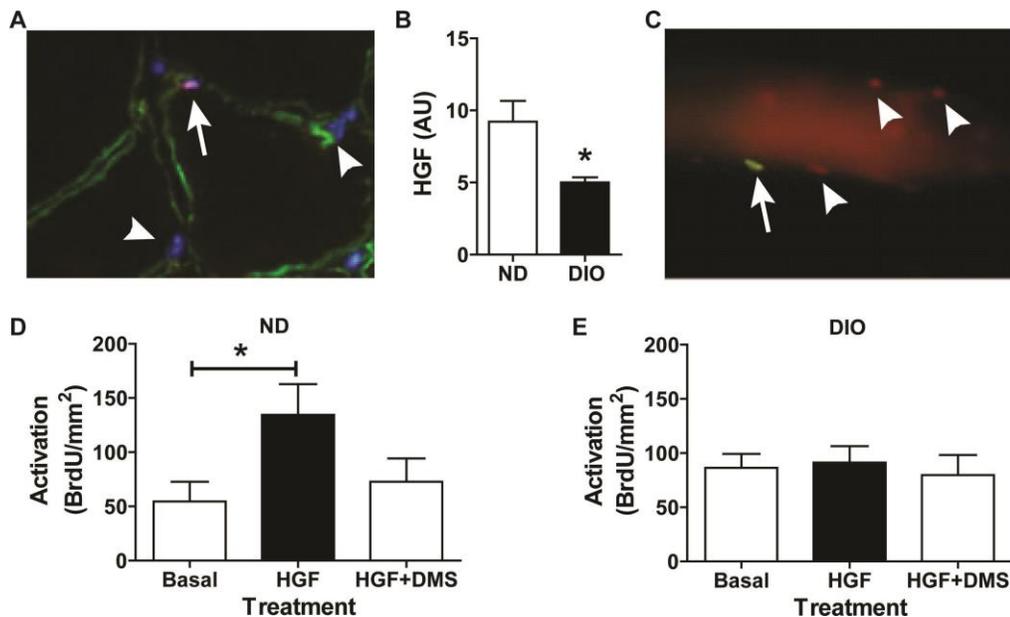
Figure 4.4. Satellite cell content and activation. (A) Representative image of a Pax-7 positive SC (red; nuclei blue) located under the basal lamina (laminin, green) (Hawke, Trajcevski 2012).. (B) Release of active HGF (both α and β chains) relative to the marker of injury myoglobin in crushed muscle extract. (C) Representative image of a single myofiber with an activated, Bromo-deoxy-uridine (BrdU) positive SC (green; nuclei red). (D and E) Activated SCs (BrdU positive) per fiber relative to fiber area (ND, N=9-23

fibers/treatment condition derived from 2 mice; DIO, N=18-31 fibers/treatment condition derived from 2 mice) in response to plating media (Basal), plating media with hepatocyte growth factor (HGF) or plating media with HGF and dimethyl sulphoxide (DMS) in floating cultures of (D) ND and (E) DIO myofibers. Normal diet (ND), diet-induced obesity (DIO). (B) Significance was determined by student's t-test, * vs. ND p<0.05. (D and E) Significance was determined by a one-way ANOVA with Tukey's Multiple Comparison post-test, * p<0.05.
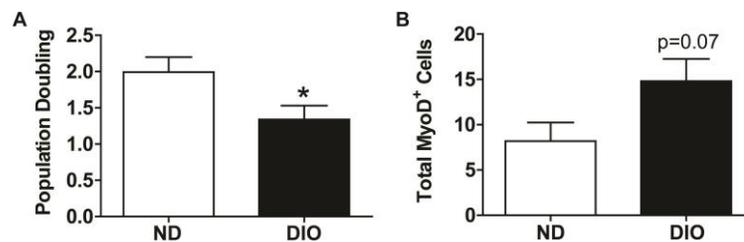

Figure 4.5. Myoblast proliferation. (A) Myoblast proliferation assessed from day 2 to 3 post-harvest in cell culture (N= 6 for ND, 8 for DIO). (B) MyoD positive cells in TA muscle cross-sections at 5 days post-injury (N=5). Significance was determined by student's t-test, * p<0.05.

REFERENCE LIST

Anderson, J.E. & Wozniak, A.C. 2004, "Satellite cell activation on fibers: modeling events in vivo--an invited review", *Canadian journal of physiology and pharmacology,* vol. 82, no. 5, pp. 300-310.

Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., van Rooijen, N., Plonquet, A., Gherardi, R.K. & Chazaud, B. 2007, "Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis", *The Journal of experimental medicine,* vol. 204, no. 5, pp. 1057-1069.

Bavenholm, P.N., Kuhl, J., Pigon, J., Saha, A.K., Ruderman, N.B. & Efendic, S. 2003, "Insulin resistance in type 2 diabetes: association with truncal obesity, impaired fitness, and atypical malonyl coenzyme A regulation", *The Journal of clinical endocrinology and metabolism,* vol. 88, no. 1, pp. 82-87.

Brigitte, M., Schilte, C., Plonquet, A., Baba-Amer, Y., Henri, A., Charlier, C., Tajbakhsh, S., Albert, M., Gherardi, R.K. & Chretien, F. 2010, "Muscle resident macrophages control the immune cell reaction in a mouse model of notexin-induced myoinjury", *Arthritis and Rheumatism,* vol. 62, no. 1, pp. 268-279.

Burr, J.F., Rowan, C.P., Jamnik, V.K. & Riddell, M.C. 2010, "The role of physical activity in type 2 diabetes prevention: physiological and practical perspectives", *The Physician and sportsmedicine,* vol. 38, no. 1, pp. 72-82.

Charge, S.B. & Rudnicki, M.A. 2004, "Cellular and molecular regulation of muscle regeneration", *Physiological reviews,* vol. 84, no. 1, pp. 209-238.

d'Albis, A., Couteaux, R., Janmot, C., Roulet, A. & Mira, J.C. 1988, "Regeneration after cardiotoxin injury of innervated and denervated slow and fast muscles of mammals. Myosin isoform analysis", *European journal of biochemistry / FEBS,* vol. 174, no. 1, pp. 103-110.

DeFronzo, R.A. 1992, "Pathogenesis of type 2 (non-insulin dependent) diabetes mellitus: a balanced overview", *Diabetologia,* vol. 35, no. 4, pp. 389-397.

Duan, H.F., Qu, C.K., Zhang, Q.W., Yu, W.M., Wang, H., Wu, C.T. & Wang, L.S. 2006, "Shp-2 tyrosine phosphatase is required for hepatocyte growth factor-induced activation of sphingosine kinase and migration in embryonic fibroblasts", *Cellular signalling,* vol. 18, no. 11, pp. 2049-2055.

Duncan, G.E. 2006, "Prevalence of diabetes and impaired fasting glucose levels among US adolescents: National Health and Nutrition Examination Survey, 1999-2002", *Archives of Pediatrics & Adolescent Medicine,* vol. 160, no. 5, pp. 523-528.

Goodpaster, B.H., Delany, J.P., Otto, A.D., Kuller, L., Vockley, J., South-Paul, J.E., Thomas, S.B., Brown, J., McTigue, K., Hames, K.C., Lang, W. & Jakicic, J.M. 2010, "Effects of diet and physical activity interventions on weight loss and cardiometabolic risk factors in severely obese adults: a randomized trial", *JAMA : the journal of the American Medical Association,* vol. 304, no. 16, pp. 1795-1802.

Grounds, M.D., McGeahie, J.K., Davies, M.J., Sorokin, L.M. & Maley, M.A.L. 1998, "The Expression of Extracellular Matrix During Adult Skeletal Muscle regeneration: How the Basement Membrane, Interstitium and Myogenic Cells Collaborate", *Basic Appl Myol,* vol. 8, no. 2, pp. 129.

Hawke, T.J. & Garry, D.J. 2001, "Myogenic satellite cells: physiology to molecular biology", *Journal of applied physiology (Bethesda, Md.: 1985),* vol. 91, no. 2, pp. 534-551.

Hawke, T.J., Jiang, N. & Garry, D.J. 2003, "Absence of p21CIP rescues myogenic progenitor cell proliferative and regenerative capacity in Foxk1 null mice", *The Journal of biological chemistry,* vol. 278, no. 6, pp. 4015-4020.

Hawke, T.J., Meeson, A.P., Jiang, N., Graham, S., Hutcheson, K., DiMaio, J.M. & Garry, D.J. 2003, "P21 is Essential for Normal Myogenic Progenitor Cell Function in Regenerating Skeletal Muscle", *American journal of physiology.Cell physiology,* vol. 285, no. 5, pp. C1019-27.

Hawke, T.J. & Trajcevski, K.E. 2012, "Stem Cells, Muscle" in *Encyclopeida of exercise Medicine in Health and disease*, ed. C. Frank Mooren., Springer, , pp. 814-816.

Hirata, A., Masuda, S., Tamura, T., Kai, K., Ojima, K., Fukase, A., Motoyoshi, K., Kamakura, K., Miyagoe-Suzuki, Y. & Takeda, S. 2003, "Expression profiling of cytokines and related genes in regenerating skeletal muscle after cardiotoxin injection: a role for osteopontin", *The American journal of pathology,* vol. 163, no. 1, pp. 203-215.

Hong, E.G., Ko, H.J., Cho, Y.R., Kim, H.J., Ma, Z., Yu, T.Y., Friedline, R.H., Kurt-Jones, E., Finberg, R., Fischer, M.A., Granger, E.L., Norbury, C.C., Hauschka, S.D., Philbrick, W.M., Lee, C.G., Elias, J.A. & Kim, J.K. 2009, "Interleukin-10 prevents diet-induced insulin resistance by attenuating macrophage and cytokine response in skeletal muscle", *Diabetes,* vol. 58, no. 11, pp. 2525-2535.

Hu, W., Bielawski, J., Samad, F., Merrill, A.H.,Jr & Cowart, L.A. 2009, "Palmitate increases sphingosine-1-phosphate in C2C12 myotubes via upregulation of sphingosine kinase message and activity", *Journal of lipid research,* vol. 50, no. 9, pp. 1852-1862.

Hu, Z., Wang, H., Lee, I.H., Modi, S., Wang, X., Du, J. & Mitch, W.E. 2010, "PTEN inhibition improves muscle regeneration in mice fed a high-fat diet", *Diabetes,* vol. 59, no. 6, pp. 1312-1320.

Krause, M.P., Moradi, J., Nissar, A.A., Riddell, M.C. & Hawke, T.J. 2011, "Inhibition of plasminogen activator inhibitor-1 restores skeletal muscle regeneration in untreated type 1 diabetic mice", *Diabetes,* vol. 60, no. 7, pp. 1964-1972.

Lee, H., Lee, D., Guo, G. & Harris, K.M. 2011, "Trends in body mass index in adolescence and young adulthood in the United States: 1959-2002", *The Journal of adolescent health : official publication of the Society for Adolescent Medicine,* vol. 49, no. 6, pp. 601-608.

Li, C., Ford, E.S., Zhao, G. & Mokdad, A.H. 2009, "Prevalence of pre-diabetes and its association with clustering of cardiometabolic risk factors and hyperinsulinemia among U.S. adolescents: National Health and Nutrition Examination Survey 2005-2006", *Diabetes care,* vol. 32, no. 2, pp. 342-347.

Miller, K.J., Thaloor, D., Matteson, S. & Pavlath, G.K. 2000, "Hepatocyte growth factor affects satellite cell activation and differentiation in regenerating skeletal muscle", *American journal of physiology.Cell physiology,* vol. 278, no. 1, pp. C174-81.

Nguyen, M.H., Cheng, M. & Koh, T.J. 2011, "Impaired muscle regeneration in ob/ob and db/db mice", *TheScientificWorldJournal,* vol. 11, pp. 1525-1535.

Nissar, A.A., Zemanek, B., Labatia, R., Atkinson, D.J., van der Ven, P.F., Furst, D.O. & Hawke, T.J. 2012, "Skeletal muscle regeneration is delayed by reduction in Xin expression: consequence of impaired satellite cell activation?", *American journal of physiology.Cell physiology,* vol. 302, no. 1, pp. C220-7.

Perdomo, G., Martinez-Brocca, M.A., Bhatt, B.A., Brown, N.F., O'Doherty, R.M. & Garcia-Ocana, A. 2008, "Hepatocyte growth factor is a novel stimulator of glucose uptake and metabolism in skeletal muscle cells", *The Journal of biological chemistry,* vol. 283, no. 20, pp. 13700-13706.

Perez, R., Tupac-Yupanqui, I. & Dunner, S. 2008, "Evaluation of suitable reference genes for gene expression studies in bovine muscular tissue", *BMC molecular biology,* vol. 9, pp. 79-2199-9-79.

Rapizzi, E., Donati, C., Cencetti, F., Nincheri, P. & Bruni, P. 2008, "Sphingosine 1-phosphate differentially regulates proliferation of C2C12 reserve cells and myoblasts", *Molecular and cellular biochemistry,* vol. 314, no. 1-2, pp. 193-199.

Rehman, J., Considine, R.V., Bovenkerk, J.E., Li, J., Slavens, C.A., Jones, R.M. & March, K.L. 2003, "Obesity is associated with increased levels of circulating hepatocyte growth factor", *Journal of the American College of Cardiology,* vol. 41, no. 8, pp. 1408-1413.

Roy, C., Paglialunga, S., Fisette, A., Schrauwen, P., Moonen-Kornips, E., St-Onge, J., Hesselink, M.K., Richard, D., Joanisse, D.R. & Cianflone, K. 2008, "Shift in metabolic fuel in acylation-stimulating protein-deficient mice following a high-fat diet", *American journal of physiology.Endocrinology and metabolism,* vol. 294, no. 6, pp. E1051-9.

Shortreed, K.E., Krause, M.P., Huang, J.H., Dhanani, D., Moradi, J., Ceddia, R.B. & Hawke, T.J. 2009, "Muscle-specific adaptations, impaired oxidative capacity and

maintenance of contractile function characterize diet-induced obese mouse skeletal muscle", *PloS one,* vol. 4, no. 10, pp. e7293.

Slentz, C.A., Houmard, J.A. & Kraus, W.E. 2009, "Exercise, abdominal obesity, skeletal muscle, and metabolic risk: evidence for a dose response", *Obesity (Silver Spring, Md.),* vol. 17 Suppl 3, pp. S27-33.

Tatsumi, R., Anderson, J.E., Nevoret, C.J., Halevy, O. & Allen, R.E. 1998, "HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells", *Developmental biology,* vol. 194, no. 1, pp. 114-128.

Thorsson, O., Rantanen, J., Hurme, T. & Kalimo, H. 1998, "Effects of nonsteroidal antiinflammatory medication on satellite cell proliferation during muscle regeneration", *The American Journal of Sports Medicine,* vol. 26, no. 2, pp. 172-176.

Tidball, J.G. & Villalta, S.A. 2010, "Regulatory interactions between muscle and the immune system during muscle regeneration", *American journal of physiology.Regulatory, integrative and comparative physiology,* vol. 298, no. 5, pp. R1173-87.

Trajcevski, K.E., O'Neill, H.M., Wang, D.C., Thomas, M.M., Al-Sajee, D., Steinberg, G.R., Ceddia, R.B. & Hawke, T.J. 2013, "Enhanced lipid oxidation and maintenance of muscle insulin sensitivity despite glucose intolerance in a diet-induced obesity mouse model", *PloS one,* vol. 8, no. 8, pp. e71747.

Weiss, R., Dufour, S., Taksali, S.E., Tamborlane, W.V., Petersen, K.F., Bonadonna, R.C., Boselli, L., Barbetta, G., Allen, K., Rife, F., Savoye, M., Dziura, J., Sherwin, R., Shulman, G.I. & Caprio, S. 2003, "Prediabetes in obese youth: a syndrome of impaired glucose tolerance, severe insulin resistance, and altered myocellular and abdominal fat partitioning", *Lancet,* vol. 362, no. 9388, pp. 951-957.

Weiss, R., Dziura, J., Burgert, T.S., Tamborlane, W.V., Taksali, S.E., Yeckel, C.W., Allen, K., Lopes, M., Savoye, M., Morrison, J., Sherwin, R.S. & Caprio, S. 2004, "Obesity and the metabolic syndrome in children and adolescents", *The New England journal of medicine,* vol. 350, no. 23, pp. 2362-2374.

Woo, M., Isganaitis, E., Cerletti, M., Fitzpatrick, C., Wagers, A.J., Jimenez-Chillaron, J. & Patti, M.E. 2011, "Early life nutrition modulates muscle stem cell number: implications for muscle mass and repair", *Stem cells and development,* vol. 20, no. 10, pp. 1763-1769.

Yan, Z., Choi, S., Liu, X., Zhang, M., Schageman, J.J., Lee, S.Y., Hart, R., Lin, L., Thurmond, F.A. & Williams, R.S. 2003, "Highly coordinated gene regulation in mouse skeletal muscle regeneration", *The Journal of biological chemistry,* vol. 278, no. 10, pp. 8826-8836.

CHAPTER 5

General discussion and conclusions

Significance of the studies

Diets high in calories, particularly those high in fat, have become common place contributing to an obesity epidemic and the increasing prevalence of glucose and insulin intolerant states including Prediabetes and Type II Diabetes Mellitus (T2DM). Prediabetes is a relatively new disease classification, compared to T2DM, and as such there has been limited research into adverse physiological changes that were previously only investigated in T2DM (e.g. cardiovascular consequences (Tsimihodimos, Florentin & Elisaf 2013, Anselmino, Sillano 2012). The impact of Prediabetes on muscle health is significant as this large, metabolically active organ becomes insulin resistant and removal of glucose from the bloodstream is adversely affected (Kewalramani, Bilan & Klip 2010). A healthy muscle mass is clearly advantageous to improving the Prediabetic state as exercise is currently recommended as a means of improving muscle insulin sensitivity (Burr et al. 2010, Phielix et al. 2012, Slentz, Houmard & Kraus 2009, Fenicchia et al. 2004, Houmard et al. 2002, Dorsey, Songer 2011). Moreover, muscle mass is an

independent predictor of progression to T2DM for Prediabetics (Srikanthan, Karlamangla 2011). Given that a healthy muscle mass enables exercise and consequently an unhealthy muscle mass may restrict activity and therefore quality of life, adequate characterization of muscle health in the Prediabetic state is required. While drugs used to treat T2DM, such as Metformin, are proving to be useful treatments for Prediabetes (Lily, Godwin 2009), exercise can be more effective than Metformin at reducing progression to T2DM (Knowler et al. 2002, Malin et al. 2012). The beneficial effects of exercise for Prediabetics likely includes reversal of pathological changes to skeletal muscle although this does not appear to have been investigated in depth (Dorsey, Songer 2011, Knowler et al. 2002, Malin et al. 2012). Currently there is little data on the response of skeletal muscle to the Prediabetic state (Corpeleijn et al. 2008, Pagel-Langenickel et al. 2010). Thus, it would be difficult to fully elucidate the beneficial effects of exercise on muscle health. Treatments and therapies to improve outcomes for Prediabetic individuals are integral to a healthy society, yet without a complete understanding of changes to muscle health during disease development, it will be difficult to develop successful therapies and treatments for use when lifestyle modifications fail.

Animal models of obesity and insulin resistance, including the DIO mouse, are useful for expanding our understanding of Prediabetes disease development. Physiological changes characteristic of Prediabetes often present in the DIO mouse, such as glucose intolerance and muscle insulin resistance, can be experimentally induced by a number of exogenous factors including inflammatory cytokines (TNF-α and IL-6), inflammatory signalling proteins (SOCS-3, PTP-N1, and TLR-4), bacteria (endotoxin),

lipid infusion and oral lipid load (Rieusset et al. 2004, Kim et al. 2011, Kim et al. 2011, Nieto-Vazquez et al. 2008, Nieto-Vazquez et al. 2008, Nieto-Vazquez et al. 2007, Ueki, Kondo & Kahn 2004, Reyna et al. 2008, Radin et al. 2008, Nowotny et al. 2013, Carvalho et al. 2012, Frost, Nystrom & Lang 2003). However, the time-course and extent to which these and other mechanisms proposed to elicit glucose intolerance and muscle insulin resistance present with DIO is unclear. Thus, the studies presented here were performed to elucidate potential physiological mechanisms contributing to complications of DIO, in particular changes to skeletal muscle health. Moreover, the impact of DIO induced changes to skeletal muscle heath on muscle regeneration were also investigated since muscle regeneration is an important aspect of exercise mediated improvements to health.

The key findings of the present studies will be discussed here as they pertain to metabolism and regeneration of skeletal muscle. With respect to metabolism, there is an early enhancement in muscle lipid metabolism which appears to initially mitigate the impact of DIO on muscle insulin resistance despite glucose intolerance in the mouse. Interestingly a reduced ability to respond to high-fat feeding with an increase in lipid oxidation in first degree relatives of individuals with T2DM is proposed to result in increased IMCLs, lipid intermediates and oxidative stress, thus placing these individuals at increased risk for T2DM (Samocha-Bonet et al. 2010). Given the apparent importance of adequate lipid metabolism to preventing muscle insulin resistance and the observed decline in mitochondrial function when insulin resistance was present, therapies for Prediabetics should aim to improve mitochondrial function as this appears to be a major component of the pathophysiology of muscle insulin resistance (Pagel-Langenickel et al.

157

2010). In addition the importance of physical activity, which leads to greater lipid oxidation in muscle (Aguer et al. 2013), is emphasized for individuals who have, or are at risk for, Prediabetes. Although the initial enhancement in muscle lipid oxidation reported here was associated with an increase in oxidative fiber content, a continued increase in oxidative fiber content and mitochondrial proteins was not adequate to maintain the early enhancement in lipid oxidation. Interestingly studies that increase muscle mass through a greater content of glycolytic muscle fibers (Guo et al. 2009, Izumiya et al. 2008) demonstrate protection from high-fat diets, possibly through the proposed mechanism of "substrate steal" (Reitman 2002) which states that a larger muscle mass utilizes more glucose from the bloodstream resulting in less glucose available for triglyceride synthesis and subsequent storage in adipose tissue (Guo et al. 2009, Reitman 2002). Thus, although oxidative fiber content and oxidative proteins are indicators of increased lipid oxidation in healthy muscle, as occurs with exercise (Aguer et al. 2013), in the pathological state of DIO increases to oxidative fiber content and oxidative proteins may be markers of a pathological state in which the oxidative machinery, the mitochondria, are becoming dysfunctional. In the present work it can be seen that increases in oxidative fiber content occur with a loss of glycolytic fiber content since the mass of the muscle was not increased with DIO. Given the demonstrated ability of an enhancement in glycolytic fiber content to rescue complications of DIO (Guo et al. 2009, Izumiya et al. 2008), strategies aimed at improving muscle strength, in conjunction with diet and lifestyle changes, may prove a lasting strategy to rescue mitochondrial function, overall muscle health and improve Prediabetes.

The key finding of the present work pertaining to muscle regeneration is that DIO leads to intrinsic impairments in SC functionality and the regenerative ability of muscle. Given that the SC is a primary contributor to growth and repair of skeletal muscle (Hawke, Garry 2001, Charge, Rudnicki 2004) a number of implications can be taken from these findings. Recovery times may be greater for surgical procedures in which skeletal muscle may be atrophied (e.g. hip/knee replacement surgeries) and there may be a longer recovery period following exercise, especially high-intensity exercise. Data presented here suggests there is a longer recovery time immediately following a fatigue protocol (Shortreed et al. 2009). In addition, given the present findings of prolonged necrosis following injury with DIO, one might surmise that this may result in more pain and thus reduce exercise adherence. Exercise adherence in individuals with Prediabetes may also be impacted by higher than average body mass and fat mass (Dishman, Ickes 1981, Gale et al. 1984) as well as genetic variations which are suggested to impact the pleasure-reward system (Herring, Sailors & Bray 2013). Thus the motivation to exercise and the impact of exercise on muscle, both damage induced and recovery period, may be adversely affected in Prediabetic individuals. Clearly more research is needed to fully understand the mental and physiological barriers to exercise for Prediabetic individuals and how these barriers may be overcome.

Current Hypotheses and Future Experiments

The present work is one of only a few that have investigated muscle repair with DIO (Nguyen, Cheng & Koh 2011, Woo et al. 2011, Hu et al. 2010), is the second such work to demonstrate a decrement to repair along with changes to the SC with a relatively

brief diet intervention (Woo et al. 2011), and is the only investigation to assess intrinsic SC functionality. It is hypothesized that *in vivo* repair in the presence of DIO is subject to one of the following scenarios: 1) reduced SC activation and/or proliferation resulting in a delay in acquiring adequate myoblasts for thorough differentiation, or 2) elevated plasma HGF bathing the muscle during repair resulting in a rescue of intrinsic SC impairments to activation and proliferation, although differentiation may be reduced. These theories can be investigated by assessing plasma HGF levels during repair and early time-points of *in vivo* SC activation and proliferation such as 1,2 and 3 days after CTX injury. Given the prolonged necrosis with DIO reported in this work, it would also be prudent to investigate if the degenerative response to injury with DIO is initially greater, thus leading to the observed increase in necrotic area after 5 and 10 days. Alternatively, it may be that if there is a delay in the inflammatory response to injury with DIO, the peak in necrotic area occurs later. There is evidence for both of these scenarios in the literature. Pro-inflammatory (M1) macrophages are essential for phagocytosis of damaged muscle fibers and phagocytosis could be prolonged should conversion to the anti-inflammatory M2 macrophage phenotype not occur (Arnold et al. 2007). Given that DIO has been shown to result in an excess of pro-inflammatory (M1) macrophages in uninjured muscle after only 3 weeks of diet intervention (Hong et al. 2009) future studies will investigate if the DIO mouse model reported here contains an excess or a prolonged presence of M1 macrophages during muscle repair. Contrary to prolonged inflammation contributing to reduced muscle repair, as occurs in T1DM models (Krause et al. 2011), recent research on inducible Heat shock protein 70 (Hsp70) suggests that DIO may lead to attenuated

muscle repair due to a delay in the inflammatory response following injury (Senf et al. 2013). Reductions in Hsp70 are associated with insulin resistant states (Kavanagh et al. 2012) including DIO (Senf et al. 2013). Less available Hsp70 has recently been shown to reduce muscle repair following CTX due to a delayed inflammatory response resulting in attenuation of the degradation response (Senf et al. 2013). Future experiments could clarify the role of necrosis in repair with DIO by assessing the expression of Hsp70 and further assessing necrosis during the repair process.

The noted changes to muscle metabolism in the present study may also have contributed to the observed decrements in SC functionality as lipid oxidation, insulin stimulated glucose uptake and glycogen synthesis are reduced along with proliferative ability in senescing human myoblasts (Nehlin et al. 2011). Exercise can improve aspects of muscle health that are impaired following DIO including decrements to lipid oxidation (Aguer et al. 2013) and insulin sensitivity (Burr et al. 2010). Thus, it is hypothesized that exercise training during DIO would delay the onset of impaired lipid oxidation, insulin resistance and impaired SC proliferation resulting in improved recovery from muscle injury. Furthermore given that IMCLs were noted to be in excess with DIO when muscle was insulin resistant, therapeutic targets to reduce IMCLs, enhance lipid oxidation and thus improve muscle insulin resistance could be gleamed from exercise based studies (Aguer et al. 2013).

Conclusions

Over the years, T2DM complications have been characterized and interventions devised (Lily, Godwin 2009). The fairly recent classification of Prediabetes is beneficial

to patients' health as it emphasizes the seriousness of this early disease state and encourages intervention to prevent disease progression (Lily, Godwin 2009).

> *"Pre-disease is the recognition that the upper limits of normal (what we used to call high normal or borderline) for measurements such as blood pressure and blood glucose might pose a health risk and might be a warning that a patient is progressing toward overt hypertension or diabetes."* (Lily, Godwin 2009)

Metformin, a drug typically used to treat T2DM, is successful at reducing progression of Prediabetes to T2DM over a 3 year period (Lily, Godwin 2009). In addition to pharmacological approaches to improve the outcome of Prediabetes, reduction of a physiological contributor, obesity, through diet, exercise and behavioral modification is successful in the short-term, though long-term results are unknown (Norris et al. 2005). Investigations into the usefulness of pharmacological and lifestyle interventions for the treatment of Prediabetes are to be commended. However, without a thorough understanding of disease development and changes to muscle health, the importance of reversal of physiological changes to muscle will remain unknown. Given the amount of skeletal muscle, relative to other insulin-sensitive tissues, characterizing the health of muscle is important to allow for appropriate and successful long-term interventions.

REFERENCE LIST

Aguer, C., Fiehn, O., Seifert, E.L., Bezaire, V., Meissen, J.K., Daniels, A., Scott, K., Renaud, J.M., Padilla, M., Bickel, D.R., Dysart, M., Adams, S.H. & Harper, M.E. 2013, "Muscle uncoupling protein 3 overexpression mimics endurance training and reduces circulating biomarkers of incomplete beta-oxidation", *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* .

Anselmino, M. & Sillano, D. 2012, "Impact of pre-diabetes and diabetes on cardiovascular outcomes", *Current vascular pharmacology,* vol. 10, no. 6, pp. 680-683.

Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., van Rooijen, N., Plonquet, A., Gherardi, R.K. & Chazaud, B. 2007, "Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis", *The Journal of experimental medicine,* vol. 204, no. 5, pp. 1057-1069.

Burr, J.F., Rowan, C.P., Jamnik, V.K. & Riddell, M.C. 2010, "The role of physical activity in type 2 diabetes prevention: physiological and practical perspectives", *The Physician and sportsmedicine,* vol. 38, no. 1, pp. 72-82.

Carvalho, B.M., Guadagnini, D., Tsukumo, D.M., Schenka, A.A., Latuf-Filho, P., Vassallo, J., Dias, J.C., Kubota, L.T., Carvalheira, J.B. & Saad, M.J. 2012, "Modulation of gut microbiota by antibiotics improves insulin signalling in high-fat fed mice", *Diabetologia,* vol. 55, no. 10, pp. 2823-2834.

Charge, S.B. & Rudnicki, M.A. 2004, "Cellular and molecular regulation of muscle regeneration", *Physiological reviews,* vol. 84, no. 1, pp. 209-238.

Corpeleijn, E., Mensink, M., Kooi, M.E., Roekaerts, P.M., Saris, W.H. & Blaak, E.E. 2008, "Impaired skeletal muscle substrate oxidation in glucose-intolerant men improves after weight loss", *Obesity (Silver Spring, Md.),* vol. 16, no. 5, pp. 1025-1032.

Dishman, R.K. & Ickes, W. 1981, "Self-motivation and adherence to therapeutic exercise", *Journal of Behavioral Medicine,* vol. 4, no. 4, pp. 421-438.

Dorsey, R. & Songer, T. 2011, "Lifestyle behaviors and physician advice for change among overweight and obese adults with prediabetes and diabetes in the United States, 2006", *Preventing chronic disease,* vol. 8, no. 6, pp. A132.

Fenicchia, L.M., Kanaley, J.A., Azevedo, J.L.,Jr, Miller, C.S., Weinstock, R.S., Carhart, R.L. & Ploutz-Snyder, L.L. 2004, "Influence of resistance exercise training on glucose control in women with type 2 diabetes", *Metabolism: clinical and experimental,* vol. 53, no. 3, pp. 284-289.

Frost, R.A., Nystrom, G.J. & Lang, C.H. 2003, "Lipopolysaccharide and proinflammatory cytokines stimulate interleukin-6 expression in C2C12 myoblasts: role of the Jun NH2-terminal kinase", *American journal of physiology.Regulatory, integrative and comparative physiology,* vol. 285, no. 5, pp. R1153-64.

Gale, J.B., Eckhoff, W.T., Mogel, S.F. & Rodnick, J.E. 1984, "Factors related to adherence to an exercise program for healthy adults", *Medicine and science in sports and exercise,* vol. 16, no. 6, pp. 544-549.

Guo, T., Jou, W., Chanturiya, T., Portas, J., Gavrilova, O. & McPherron, A.C. 2009, "Myostatin inhibition in muscle, but not adipose tissue, decreases fat mass and improves insulin sensitivity", *PloS one,* vol. 4, no. 3, pp. e4937.

Hawke, T.J. & Garry, D.J. 2001, "Myogenic satellite cells: physiology to molecular biology", *Journal of applied physiology (Bethesda, Md.: 1985),* vol. 91, no. 2, pp. 534-551.

Herring, M.P., Sailors, M.H. & Bray, M.S. 2013, "Genetic factors in exercise adoption, adherence and obesity", *Obesity reviews : an official journal of the International Association for the Study of Obesity,* .

Hong, E.G., Ko, H.J., Cho, Y.R., Kim, H.J., Ma, Z., Yu, T.Y., Friedline, R.H., Kurt-Jones, E., Finberg, R., Fischer, M.A., Granger, E.L., Norbury, C.C., Hauschka, S.D., Philbrick, W.M., Lee, C.G., Elias, J.A. & Kim, J.K. 2009, "Interleukin-10 prevents diet-induced insulin resistance by attenuating macrophage and cytokine response in skeletal muscle", *Diabetes,* vol. 58, no. 11, pp. 2525-2535.

Houmard, J.A., Tanner, C.J., Yu, C., Cunningham, P.G., Pories, W.J., MacDonald, K.G. & Shulman, G.I. 2002, "Effect of weight loss on insulin sensitivity and intramuscular long-chain fatty acyl-CoAs in morbidly obese subjects", *Diabetes,* vol. 51, no. 10, pp. 2959-2963.

Hu, Z., Wang, H., Lee, I.H., Modi, S., Wang, X., Du, J. & Mitch, W.E. 2010, "PTEN inhibition improves muscle regeneration in mice fed a high-fat diet", *Diabetes,* vol. 59, no. 6, pp. 1312-1320.

Izumiya, Y., Hopkins, T., Morris, C., Sato, K., Zeng, L., Viereck, J., Hamilton, J.A., Ouchi, N., LeBrasseur, N.K. & Walsh, K. 2008, "Fast/Glycolytic muscle fiber

growth reduces fat mass and improves metabolic parameters in obese mice", *Cell metabolism,* vol. 7, no. 2, pp. 159-172.

Kavanagh, K., Wylie, A.T., Chavanne, T.J., Jorgensen, M.J., Voruganti, V.S., Comuzzie, A.G., Kaplan, J.R., McCall, C.E. & Kritchevsky, S.B. 2012, "Aging does not reduce heat shock protein 70 in the absence of chronic insulin resistance", *The journals of gerontology.Series A, Biological sciences and medical sciences,* vol. 67, no. 10, pp. 1014-1021.

Kewalramani, G., Bilan, P.J. & Klip, A. 2010, "Muscle insulin resistance: assault by lipids, cytokines and local macrophages", *Current opinion in clinical nutrition and metabolic care,* vol. 13, no. 4, pp. 382-390.

Kim, T.H., Choi, S.E., Ha, E.S., Jung, J.G., Han, S.J., Kim, H.J., Kim, D.J., Kang, Y. & Lee, K.W. 2011, "IL-6 induction of TLR-4 gene expression via STAT3 has an effect on insulin resistance in human skeletal muscle", *Acta Diabetologica, .*

Knowler, W.C., Barrett-Connor, E., Fowler, S.E., Hamman, R.F., Lachin, J.M., Walker, E.A., Nathan, D.M. & Diabetes Prevention Program Research Group 2002, "Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin", *The New England journal of medicine,* vol. 346, no. 6, pp. 393-403.

Krause, M.P., Moradi, J., Nissar, A.A., Riddell, M.C. & Hawke, T.J. 2011, "Inhibition of plasminogen activator inhibitor-1 restores skeletal muscle regeneration in untreated type 1 diabetic mice", *Diabetes,* vol. 60, no. 7, pp. 1964-1972.

Lily, M. & Godwin, M. 2009, "Treating prediabetes with metformin: systematic review and meta-analysis", *Canadian family physician Medecin de famille canadien,* vol. 55, no. 4, pp. 363-369.

Malin, S.K., Gerber, R., Chipkin, S.R. & Braun, B. 2012, "Independent and combined effects of exercise training and metformin on insulin sensitivity in individuals with prediabetes", *Diabetes care,* vol. 35, no. 1, pp. 131-136.

Nehlin, J.O., Just, M., Rustan, A.C. & Gaster, M. 2011, "Human myotubes from myoblast cultures undergoing senescence exhibit defects in glucose and lipid metabolism", *Biogerontology,* vol. 12, no. 4, pp. 349-365.

Nguyen, M.H., Cheng, M. & Koh, T.J. 2011, "Impaired muscle regeneration in ob/ob and db/db mice", *TheScientificWorldJournal,* vol. 11, pp. 1525-1535.

Nieto-Vazquez, I., Fernandez-Veledo, S., de Alvaro, C. & Lorenzo, M. 2008, "Dual role of interleukin-6 in regulating insulin sensitivity in murine skeletal muscle", *Diabetes,* vol. 57, no. 12, pp. 3211-3221.

Nieto-Vazquez, I., Fernandez-Veledo, S., de Alvaro, C., Rondinone, C.M., Valverde, A.M. & Lorenzo, M. 2007, "Protein-tyrosine phosphatase 1B-deficient myocytes show increased insulin sensitivity and protection against tumor necrosis factor-alpha-induced insulin resistance", *Diabetes,* vol. 56, no. 2, pp. 404-413.

Nieto-Vazquez, I., Fernandez-Veledo, S., Kramer, D.K., Vila-Bedmar, R., Garcia-Guerra, L. & Lorenzo, M. 2008, "Insulin resistance associated to obesity: the link TNF-alpha", *Archives of Physiology and Biochemistry,* vol. 114, no. 3, pp. 183-194.

Norris, S.L., Zhang, X., Avenell, A., Gregg, E., Schmid, C.H. & Lau, J. 2005, "Long-term non-pharmacological weight loss interventions for adults with prediabetes", *The Cochrane database of systematic reviews,* vol. (2), no. 2, pp. CD005270.

Nowotny, B., Zahiragic, L., Krog, D., Nowotny, P.J., Herder, C., Carstensen, M., Yoshimura, T., Szendroedi, J., Phielix, E., Schadewaldt, P., Schloot, N.C., Shulman, G.I. & Roden, M. 2013, "Mechanisms underlying the onset of oral lipid-induced skeletal muscle insulin resistance in humans", *Diabetes, .*

Pagel-Langenickel, I., Bao, J., Pang, L. & Sack, M.N. 2010, "The role of mitochondria in the pathophysiology of skeletal muscle insulin resistance", *Endocrine reviews,* vol. 31, no. 1, pp. 25-51.

Phielix, E., Meex, R., Ouwens, D.M., Sparks, L., Hoeks, J., Schaart, G., Moonen-Kornips, E., Hesselink, M.K. & Schrauwen, P. 2012, "High oxidative capacity due to chronic exercise training attenuates lipid-induced insulin resistance", *Diabetes,* vol. 61, no. 10, pp. 2472-2478.

Radin, M.S., Sinha, S., Bhatt, B.A., Dedousis, N. & O'Doherty, R.M. 2008, "Inhibition or deletion of the lipopolysaccharide receptor Toll-like receptor-4 confers partial protection against lipid-induced insulin resistance in rodent skeletal muscle", *Diabetologia,* vol. 51, no. 2, pp. 336-346.

Reitman, M.L. 2002, "Metabolic lessons from genetically lean mice", *Annual Review of Nutrition,* vol. 22, pp. 459-482.

Reyna, S.M., Ghosh, S., Tantiwong, P., Meka, C.S., Eagan, P., Jenkinson, C.P., Cersosimo, E., Defronzo, R.A., Coletta, D.K., Sriwijitkamol, A. & Musi, N. 2008, "Elevated toll-like receptor 4 expression and signaling in muscle from insulin-resistant subjects", *Diabetes,* vol. 57, no. 10, pp. 2595-2602.

Rieusset, J., Bouzakri, K., Chevillotte, E., Ricard, N., Jacquet, D., Bastard, J.P., Laville, M. & Vidal, H. 2004, "Suppressor of cytokine signaling 3 expression and insulin resistance in skeletal muscle of obese and type 2 diabetic patients", *Diabetes,* vol. 53, no. 9, pp. 2232-2241.

Samocha-Bonet, D., Heilbronn, L.K., Lichtenberg, D. & Campbell, L.V. 2010, "Does skeletal muscle oxidative stress initiate insulin resistance in genetically predisposed individuals?", *Trends in endocrinology and metabolism: TEM,* vol. 21, no. 2, pp. 83-88.

Senf, S.M., Howard, T.M., Ahn, B., Ferreira, L.F. & Judge, A.R. 2013, "Loss of the inducible Hsp70 delays the inflammatory response to skeletal muscle injury and severely impairs muscle regeneration", *PloS one,* vol. 8, no. 4, pp. e62687.

Shortreed, K.E., Krause, M.P., Huang, J.H., Dhanani, D., Moradi, J., Ceddia, R.B. & Hawke, T.J. 2009, "Muscle-specific adaptations, impaired oxidative capacity and maintenance of contractile function characterize diet-induced obese mouse skeletal muscle", *PloS one,* vol. 4, no. 10, pp. e7293.

Slentz, C.A., Houmard, J.A. & Kraus, W.E. 2009, "Exercise, abdominal obesity, skeletal muscle, and metabolic risk: evidence for a dose response", *Obesity (Silver Spring, Md.),* vol. 17 Suppl 3, pp. S27-33.

Srikanthan, P. & Karlamangla, A.S. 2011, "Relative muscle mass is inversely associated with insulin resistance and prediabetes. Findings from the third National Health and Nutrition Examination Survey", *The Journal of clinical endocrinology and metabolism,* vol. 96, no. 9, pp. 2898-2903.

Tsimihodimos, V., Florentin, M. & Elisaf, M.S. 2013, "How should we treat hypertension and dyslipidemia in patients with prediabetes?", *Current pharmaceutical design,* vol. 19, no. 21, pp. 3773-3787.

Ueki, K., Kondo, T. & Kahn, C.R. 2004, "Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms", *Molecular and cellular biology,* vol. 24, no. 12, pp. 5434-5446.

Woo, M., Isganaitis, E., Cerletti, M., Fitzpatrick, C., Wagers, A.J., Jimenez-Chillaron, J. & Patti, M.E. 2011, "Early life nutrition modulates muscle stem cell number: implications for muscle mass and repair", *Stem cells and development,* vol. 20, no. 10, pp. 1763-1769.