DIABETIC MYOPATHY

DIABETIC MYOPATHY: CHANGES TO CONTRACTILE FUNCTION, MORPHOLOGY AND REGENERATIVE CAPACITY OF SKELETAL MUSCLE IN A MURINE MODEL OF TYPE 1 DIABETES MELLITUS

By

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ABSTRACT

Type 1 diabetes mellitus (T1DM) is a disease defined by its complications as much as its central pathology. One such complication, diabetic myopathy, has received more attention in recent years as it has become clear that by maintaining a healthy skeletal muscle mass, diabetic individuals are more likely to maintain metabolic control and avoid the health consequences associated with hyperglycemia. While only a limited number of studies have been performed on diabetic human skeletal muscle, the research clearly indicates that a loss of muscular strength and alterations in muscle phenotype are a result of T1DM, occurring within weeks of disease inception. Studies employing rodent models of T1DM have identified several key changes underlying the loss of contractile capacity and the changes to muscle phenotype. The research to date, however, has yet to thoroughly elucidate the mechanisms underlying diabetic myopathy. The goal of the following studies is to gain a more thorough understanding of the effects of T1DM on skeletal muscle contractile capacity, morphology, and regenerative capacity using the C57BL/6J-*Ins2*^{Akita} (*Ins2*$^{WT/C96Y}$) diabetic mouse model. Given the crucial role of muscle repair in maintaining a healthy muscle mass, any deficit observed here could have important implications in the pathophysiology of diabetic myopathy. The results of the following studies indicate that the *Ins2*$^{WT/C96Y}$ mouse undergoes a loss of glycolytic muscle mass and other morphological/phenotypic alterations concomitant with loss of peak contractile force. Furthermore, the regenerative capacity of the muscle following injury is impaired in glycolytic muscle groups, particularly the tibialis anterior (TA). This impairment in regeneration can be, at least partly, attributed to chronic elevation in plasminogen activator inhibitor-1 (PAI-1). Pharmacological inhibition of this hormone improves regeneration of the TA in the *Ins2*$^{WT/C96Y}$

mouse. These data have improved our mechanistic understanding of diabetic myopathy and have clinical implications for the treatment of T1DM.

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PREFACE

This thesis is a “sandwich” style thesis. Chapter 1 is a published review paper that constitutes the general introduction. Chapters 2, 3, 4 have been published or will be submitted for publication as peer-reviewed research papers. A preface of each chapter describes the details of the submitted articles, as well as my contribution to the multiple-authored work.

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1

CHAPTER 3

CHAPTER 4

LIST OF FIGURES

LIST OF ABBREVIATIONS

AGE: advanced glycation endproduct

ANOVA: analysis of variance

ARI: aldose reductase inhibitor

AU: arbitrary units

β-HAD: beta-hydroxyacetyl coenzyme A dehydrogenase

CS: citrate synthase

CTX: cardiotoxin

DAPI: 4,6- diamidino-2-phenylindole

DHEA: dehydroepiandrosterone

ECM: extracellular matrix

EDL: extensor digitorum longus

FAT: fatty acid translocase

GAS: gastrocnemius

GPS: gastrocnemius-plantaris-soleus complex

GAPDH: glyceraldehye-3-phosphate dehydrogenase

GH: growth hormone

H&E: hematoxylin and eosin

IGFBP: insulin-like growth factor binding protein

IMCL: intramyocellular lipids

***Ins2*$^{Akita+/-}$:** Heterozygotic C57BL/6-Ins2Akita/J

***Ins2*$^{WT/C96Y}$:** Heterozygotic C57BL/6-Ins2Akita/J

MMP: matrix metalloproteinase

MyH3: embryonic myosin heavy chain

NEFA: non-esterified fatty acid

PA: plasminogen activator

PAI: plasminogen activator inhibitor

PFK: phosphofructokinase

PPX: partial pancreatectomy

PWC170: sub-maximal work capacity at a heart rate of 170 beats per minute

RPE: rating of perceived exertion

SDH: succinate dehydrogenase

SOL: soleus

SR: sarcoplasmic reticulum

STZ: streptozotocin, or streptozotocin-induced, or streptozotocin-induced diabetic

T1DM: type 1 diabetes mellitus

TA: tibialis anterior

Ub-P: ubiquitin-proteasome

UCP: uncoupling protein

uPA: urokinase plasminogen activator

VO_2max: maximal oxygen consumption

WT: wild type

CHAPTER 1

General introduction and objective

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PREFACE

Diabetic myopathy refers to the pathophysiological changes to skeletal muscle in response to the type 1 diabetes mellitus (T1DM) environment. For many, T1DM onset occurs in childhood and there may be a protracted period before diagnosis occurs. Even following diagnosis, disease management is challenging and, very often, suboptimal. Coincidentally, this is a time when muscle growth rates are at their peak and this growth period is critical in determining the muscle mass that may be attained by adulthood. Furthermore, as skeletal muscle is a primary organ for blood glucose control and physical movement, alterations to the internal milieu during childhood/adolescence could have life-long effects on the ability to control substrate metabolism and maintain one's physical capacity. This introductory chapter is a published review paper and serves as the literature review of this thesis. In this review, diabetic myopathy and its underlying mechanisms are considered from two perspectives: (i) the pediatric population where the disease is untreated (pre-diagnosis) or sub-optimally regulated during a period where muscle growth and development is at a peak; (ii) the adult population where T1DM is managed (sometimes poorly), but muscle growth and development has ceased. Importantly, providing a clear understanding of the characteristics and underlying mechanisms of diabetic myopathy will promote the advancement of more appropriate therapeutic strategies to ultimately improve overall muscle health in all T1DM populations.

INTRODUCTION

Type 1 diabetes mellitus (T1DM) is commonly associated with numerous pathological complications, such as macrovascular disease, neuropathy, nephropathy and retinopathy (Brownlee. 2005). Diabetic skeletal muscle disease or "myopathy", a much less studied complication of poorly controlled diabetes, is also a common clinical condition characterized by a lower muscle mass, weakness, and an overall reduced physical capacity (Andersen et al. 1997; Andersen, Gjerstad, and Jakobsen. 2004; Andersen et al. 1996; Andersen, Schmitz, and Nielsen. 2005).

The ability of skeletal muscle for growth, adaptation and regeneration is truly remarkable. Nowhere is this ability more evident than during childhood where increases in the overall size and strength of muscle are the result of increased protein synthesis and the fusion of muscle progenitor cell progeny to existing muscle fibers (Hawke and Garry. 2001). This adaptability of muscle is not limited solely to hypertrophy. Atrophic stimuli, such as casting or various disease states, result in a decrease in muscle mass, strength and progenitor cell number (Mozdziak, Pulvermacher, and Schultz. 2000; Darr and Schultz. 1989; Wanek and Snow. 2000). It is important to note that atrophic stimuli placed on adolescent muscle results in a rapid and irreversible remodelling process (Mozdziak, Pulvermacher, and Schultz. 2000; Darr and Schultz. 1989). In contrast to adolescent muscle however, removal of the atrophic stimulus from adult skeletal muscle returns it to a pre-atrophy state, including its capacity for myofiber regeneration (Mozdziak, Pulvermacher, and Schultz. 2000; Wanek and Snow. 2000). Two very important points are gleaned from these studies: (i) adolescent muscle and adult muscle may respond

differently to similar stimuli and (ii) atrophic stimuli, such as poorly controlled T1DM, placed on developing muscle may lead to a lifetime of reduced physical and metabolic capacities.

In this review, we will examine the human diabetic myopathic condition in pediatric and adult populations, as well as discuss the current state of knowledge into the mechanisms underlying T1DM myopathy. It is our goal that this review provides clarity regarding the specific morphological, metabolic, and functional alterations to skeletal muscle that occur during the pathogenesis of T1DM. As a distinctly underestimated complication of T1DM, preventing or treating specifically for diabetic myopathy may prove essential for the management of overall disease progression.

DIABETIC MYOPATHY IN HUMANS.

Muscle atrophy and fiber type alterations.

While some studies have measured a loss of muscle mass by non-invasive methods in long-standing diabetic patients (Andersen et al. 1997; Andersen, Gjerstad, and Jakobsen. 2004), reduced muscle fiber size, a correlate of muscle mass, has also been demonstrated in newly diagnosed young males prior to receiving insulin treatment (Jakobsen and Reske-Nielsen. 1986). Furthermore, a group of recently diagnosed patients (duration of T1DM ranging from 1 to 28 weeks) demonstrate fiber atrophy, disruption of Z-lines, and morphological abnormalities in the mitochondria in the absence of morphological indications of neuropathy (Reske-Nielsen, Harmsen, and Vorre. 1977). These are important findings as they indicate that skeletal muscle is acutely sensitive to

T1DM prior to neuropathic complications and further stresses the importance of early detection and diagnosis. In addition to the reported muscle fiber size loss, an increase in the relative percentage of glycolytic/fast-twitch muscle fibers and glycolytic enzymes has been observed in diabetic adult skeletal muscles (Crowther et al. 2003; Fritzsche et al. 2008).

Work, exercise capacity and musculo-skeletal strength.

Measurements of maximal oxygen consumption (VO_2max), peak oxygen consumption and sub-maximal exercise tests (PWC170) have all been utilized in studies to investigate differences between diabetic and age-matched non-diabetic populations (Austin et al. 1993; Baraldi et al. 1992; Gusso et al. 2008; Hagan, Marks, and Warren. 1979; Huttunen et al. 1984; Komatsu et al. 2005; Larsson et al. 1964; Larsson et al. 1962; Niranjan et al. 1997; Poortmans et al. 1986; Rowland et al. 1992; Sterky. 1963; Veves et al. 1997; Nugent et al. 1997; Heyman et al. 2005). As evidenced in Table 1.1, numerous studies report reduced work capacities in children, adolescents and adults with T1DM (compared to their non-diabetic counterparts). Balancing this are some reports where no evidence of impairment in physical performance tasks in diabetic populations is observed. For example, Nugent and colleagues (Nugent et al. 1997) reported no difference in peak O_2 consumption during a progressive incremental exercise test in adult subjects with long-standing diabetes, while Veves et al (Veves et al. 1997) found that only adult diabetics with demonstrated neuropathic complications or sedentary lifestyles demonstrated reduced exercise performance (VO_2max), thereby suggesting that diabetes itself does not directly affect the exercising capacity in trained subjects. It is also

interesting to note that Huttunen and colleagues (Huttunen et al. 1984), who demonstrated that sub-maximal work capacity at a given heart rate (PWC170) in diabetic boys (without detectable neuropathy) was lower than that of non-diabetic boys, also observed no difference between diabetic and non-diabetic girls (Huttunen et al. 1984). While a small, statistically insignificant, difference was observed (89.0 ±27.4 vs 96.4±38.5W diabetic vs non-diabetic respectively), the values were not made relative to body weight and therefore a detectable difference may have been missed. Though a difference between diabetic and non-diabetic boys was observed using absolute values for PWC170, one could speculate that a lack of fitness in both groups of adolescent girls may have masked a detectable difference.

Importantly, impairments in physical work capacity in those with T1DM are typically, but not always, observed concomitantly with poor metabolic control. For example, studies by Poortmans et al. (Poortmans et al. 1986) and Huttunen et al. (Huttunen et al. 1984) found that although submaximal and maximal work load capacities were significantly lower in the diabetic adolescent boys compared to healthy controls, the degree to which physical capacities were affected was inversely related to the level of metabolic control, as measured by HbA1c (Huttunen et al. 1984; Poortmans et al. 1986). What remains to be answered is whether the reduced physical capacity is the consequence of poor metabolic control, or whether the poor metabolic control is the result of reduced physical capacities.

Though the question of ‘pre-study fitness’ is often an uncontrolled variable and has been used to account for differences between study findings, a handful of studies have

specifically examined total physical activity levels and found equivocal results (Raile et al. 1999; Massin et al. 2005; Sarnblad, Ekelund, and Aman. 2005; Moy et al. 1993; Valerio et al. 2007). Daily physical activity levels, and involvement in sports as determined by structured interviewing, is reported to be greater in diabetic youth (Raile et al. 1999), which is supported by the finding that diabetic youth demonstrate longer periods of activity during 24 hour continuous heart rate monitoring (Massin et al. 2005). Conversely, diabetic youth have also been reported to partake in less daily physical activity (Valerio et al. 2007), or demonstrate no statistical difference from non-diabetics when wearing an accelerometer to determine total acceleration/movement throughout the day (Sarnblad, Ekelund, and Aman. 2005). In adults with T1DM, physical activity level was found to be inversely related with mortality risk (Moy et al. 1993) but no comparison to non-diabetics was performed.

Studies investigating muscular endurance with T1DM have been equivocal in their observations with some noting improved performance in adults [Table 1.2; (Andersen. 1998)], while others noted a reduced capacity relative to peak force in young diabetic men and women (Almeida, Riddell, and Cafarelli. 2008). The former study (Andersen. 1998) found no correlation between endurance index of the muscle group and neuropathic symptoms, while the latter (Almeida, Riddell, and Cafarelli. 2008) established a significant correlation (r=0.54, P<0.02) between time to fatigue and motor nerve conduction velocity, indicating a possible neural component to the impaired muscle function. To the best of our knowledge, there are no studies that have examined the effects of T1DM on muscular endurance in pediatric populations.

Though the aforementioned studies investigated work/exercise capacity in T1DM populations, the results of those studies do not specifically speak to the direct effects of T1DM on skeletal muscle as the measurements performed (e.g. PWC170, VO_2max) are an intertwined relationship between skeletal muscle health and cardiovascular health. Given evidence that myocardial function is altered with T1DM as early as the pre-teen years (Baum, Levitsky, and Englander. 1987; Friedman et al. 1982; Riggs and Transue. 1990), one must consider studies where measurement of muscle strength was directly assessed, if one is interested in the effects of T1DM on skeletal muscle specifically (Table 1.2). A number of studies have shown that skeletal muscle maximal force production, either isometric or isotonic, is reduced in adults with T1DM (Andersen et al. 1997; Andersen et al. 1996; Andersen, Schmitz, and Nielsen. 2005; Andersen. 1998; Andreassen et al. 2009a). In middle age and elderly persons with long-standing T1DM, the impairments in force production are highly correlated to the severity of neuropathy symptoms (Andersen et al. 1996; Andersen. 1998). To date, no studies have specifically examined muscular force production in young diabetics.

Further confounding the results from human trials is evidence to suggest that the contractile/exercise capacity of skeletal muscle is affected by the acute glycemic state. For instance, prior high-carbohydrate diet reduced aerobic exercise capacity in diabetic athletes (McKewen et al. 1999) and glucose infusion causing hyperglycemia caused a reduced isometric maximal voluntary contractile force in diabetic patients (Andersen, Schmitz, and Nielsen. 2005). Conversely, it has been demonstrated that glucose supplementation during moderate intensity aerobic exercise relieves the rating of

perceived exertion (RPE) in control subjects with no change to the RPE of diabetic subjects (Riddell et al. 2000).

Intramyocellular lipid is increased in T1DM.

Accumulation of intramyocellular lipids (IMCL) and its associated intermediates (e.g. ceramides) have been linked to a host of pathological complications in skeletal muscle including alterations in insulin sensitivity and signalling (Brownlee. 2005; Muoio and Newgard. 2008; Watt. 2009; Kraegen et al. 2006; Kraegen and Cooney. 2008). Skeletal muscle from insulin-treated, T1DM young adults typically contains more IMCL compared to non-diabetic muscle. This has been demonstrated with NMR spectrometry (Bernroider et al. 2005; Perseghin et al. 2003), computed tomography (Dube et al. 2006) and biochemical assays of the lipid concentration from muscle biopsies (Standl et al. 1980; Ebeling et al. 1998). In general, patients with poorer glycemic control display greater IMCL content than patients in good glycemic control (as determined by HbA1c% or postprandial blood glucose level); in fact, those with good glycemic control did not differ from non-diabetics in terms of IMCL content (Perseghin et al. 2003; Standl et al. 1980). Those in poor control also exhibited greater plasma non-esterified fatty acids and triglycerides (Standl et al. 1980). Conversely, Dube and colleagues demonstrate the IMCL levels of the thigh muscle to be related to daily insulin requirement, not metabolic control *per se* (Dube et al. 2006). Furthermore, IMCL increases appear to occur in a muscle-specific fashion. One study reported increases in IMCL within soleus but not tibialis anterior muscles in young diabetic men (Bernroider et al. 2005), while another study found IMCL to increase in both muscles of diabetic men and women, though the

IMCL levels in the TA were less than half of that observed in the soleus (Perseghin et al. 2003). Similar to the soleus, increases in IMCL have been detected consistently in the thigh (Dube et al. 2006) or vastus lateralis of the quadriceps (Standl et al. 1980; Ebeling et al. 1998). While elevated IMCLs are not a ubiquitous marker of insulin resistance, their increased levels within skeletal muscle are believed to provide a negative influence for optimal muscle health as they can be associated with long chain acyl-CoAs, ceramides and diacyglycerols (Muoio. 2009; Coen et al. 2010). To the best of our knowledge, only one study has examined IMCL levels in T1DM youth and found no differences in soleus IMCL compared to data of a control group of non-diabetic children from a pre-existing database (Ling et al. 2003), thereby suggesting that disease duration might play a role in the accumulation of IMCL.

Hormonal alterations.

The circulating concentrations of several hormones have been demonstrated to be altered with T1DM; many of which can have a profound influence on skeletal muscle health. Though a number of circulating factors with profound metabolic roles have been demonstrated to be altered in T1DM, such as leptin and adiponectin (Galler et al. 2007; Kiess et al. 1998), we have highlighted a few below which have clear roles in skeletal muscle atrophy, growth, and regeneration (see figure 1.1).

Cortisol, a hormone which promotes negative protein turnover, is generally found to be elevated above control values in T1DM adults, particularly if they are not in optimal glycemic control (Chan et al. 2003; Roy, Collier, and Roy. 1990). Though this observation is not consistently reported in younger T1DM subjects (Galassetti et al.

2006b; Sills and Cerny. 1983), cortisol levels have been reported to be higher in adolescents compared to younger children with T1DM (Diabetes Research in Children Network (DirecNet) Study Group et al. 2009) suggesting that it may be related to disease duration. While cortisol may not be consistently higher at rest in young T1DM subjects, there is a greater rise in cortisol levels in young persons with T1DM compared to healthy controls in response to stressors such as exercise (Sills and Cerny. 1983).

Plasminogen activator inhibitor (PAI)-1 is another hormone implicated in the pathogenesis of diabetic complications (Bosnyak et al. 2003; Chen, Zhang, and Wang. 2006; De Taeye, Smith, and Vaughan. 2005; Gruden et al. 1994; Small et al. 1989). Interestingly, elevations in PAI-1 have also been found to be a potent inhibitor of skeletal muscle regeneration following myotrauma (Koh et al. 2005) and, thus, may be one contributing factor to diabetic myopathy. Although little data exists on youth with T1DM, one study has demonstrated that elevations in PAI-1 occur at the time of clinical diagnosis and may reoccur with prolonged disease duration (Zeitler, Thiede, and Muller. 2001).

IL-6, an inflammatory cytokine, has been linked with numerous atrophic states, such as cancer cachexia (Barton. 2001) and sarcopenia (Jensen. 2008) and is elevated in the pediatric T1DM population (Galassetti et al. 2006b; Dogan et al. 2006; Galassetti et al. 2006a; Rosa et al. 2009; Rosa et al. 2008). While considerably less data has examined IL-6 in adult diabetics, it appears that IL-6 remains elevated in young diabetic men (Gordin et al. 2008) suggesting that as disease progression continues, IL-6 levels will continue to remain elevated above controls. Chronic increases in circulating IL-6 have

been shown to have direct deleterious effects on skeletal muscle likely through downregulation of growth factor-mediated intracellular signalling resulting in atrophy (and a preferential loss of myofibrillar protein) (Haddad et al. 2005). While recent evidence has demonstrated that elevations in IL-6 are observed with intense, longer duration exercise, these effects are believed to be a positive factor in modulating the immunological and metabolic responses to exercise and with training will actually result in decreasing basal IL-6 levels (Fischer. 2006). Clearly, future studies are needed to elucidate the direct and indirect role(s) of chronically elevated IL-6 in diabetic myopathy.

IGF-1 is a critical growth factor for skeletal muscle (Barton. 2006; Booth. 2006) and reductions in its expression or circulating concentration is implicated in several diseases typified by catabolism of muscle (Clemmons and Underwood. 1992; Grounds. 2002). IGF-1 is reduced in adolescent (Moyer-Mileur et al. 2008; Wedrychowicz et al. 2005) and adult type 1 diabetics (Jehle et al. 1998) subjects giving rise to the suggestion that the reduced IGF-1 in T1DM is a primary contributor to the atrophy/poor growth of muscle in T1DM. In addition to alterations in IGF-1 expression, the many isoforms of IGF binding proteins (IGFBP), which regulate the levels of 'free' or 'bioavailable' IGF-1, are found to be altered in T1DM in both adolescent and adult diabetics (Moyer-Mileur et al. 2008; Wedrychowicz et al. 2005; Jehle et al. 1998). It is worth noting that these reductions in circulating IGF-1 are in the face of elevated growth hormone (GH) levels suggesting that the GH/IGF-1 axis is defective. While it is interesting to speculate that alterations in the circulating GH/IGF-1 pathway are primary contributors to diabetic myopathy, the validity of this concept is currently unclear. The current state of

knowledge indicates that local (paracrine/autocrine) IGF-1 expression, and not circulating IGF-1, appears to play a more significant role in skeletal muscle hypertrophy (Adams and Haddad. 1996; Adams. 2002).

Taken together, in addition to significant changes in circulating glucose and insulin levels, the hormonal milieu of the T1DM environment is considerably altered. The involvement of these factors independently, and in combination, to the T1DM myopathic state is an area of considerable interest and delineating their contributions will provide a significant advance to the development of therapies for this disease complication.

ANIMAL STUDIES INVESTIGATING DIABETIC MYOPATHY.

Physical capacities.

Studies examining loss of muscle function in animal models provide insights regarding mechanisms behind the loss of contractile capacity often observed in human T1DM trials. Previous studies employing alloxan- or streptozotocin (STZ)-induced diabetic rodents are by far the most numerous, but have demonstrated equivocal findings with respect to force production. Consistent with human studies (Andersen et al. 1996; Andersen. 1998), absolute force is usually found to decrease (Fahim, el-Sabban, and Davidson. 1998; Lesniewski, Miller, and Armstrong. 2003; Sanchez et al. 2005; Vignaud et al. 2007; Cameron, Cotter, and Robertson. 1990; Cotter et al. 1989; Cotter et al. 1993). However, contractile force expressed relative to muscle mass has been found to decrease (Lesniewski, Miller, and Armstrong. 2003; Cameron, Cotter, and Robertson. 1990; Cotter et al. 1989; Cotter et al. 1993; McGuire and MacDermott. 1999; Stephenson,

O'Callaghan, and Stephenson. 1994; Paulus and Grossie. 1983), increase (Vignaud et al. 2007; Paulus and Grossie. 1983; Ganguly et al. 1986; McGuire et al. 2001; Krause et al. 2009), or remain similar to non-diabetic control values (Lesniewski, Miller, and Armstrong. 2003; Sanchez et al. 2005; Cameron, Cotter, and Robertson. 1990; Cotter et al. 1989; Cotter et al. 1993; McGuire and MacDermott. 1999; Stephenson, O'Callaghan, and Stephenson. 1994). The basis for these discrepancies is not clearly understood, though it may involve the variability in duration of untreated diabetes, the broad range of muscle stimulation protocols employed, the muscle group or fiber types studied, or, in the case of pharmacologically-induced diabetic rodents, the dose of drug administered. A relatively common finding is that muscle groups composed predominantly by slow-twitch muscle fibers are more resistant to loss of function than muscle groups composed of fast-twitch fibers. For instance, soleus muscle or isolated slow-twitch fibers of STZ-diabetic rodents tend to exhibit no loss of force production and, sometimes, slight improvements in relative force capacity while the extensor digitorum longus (EDL) or isolated fast-twitch fibers is often reported to have impaired force production (Sanchez et al. 2005; Cameron, Cotter, and Robertson. 1990; Cotter et al. 1989; Cotter et al. 1993; Stephenson, O'Callaghan, and Stephenson. 1994; Paulus and Grossie. 1983; McGuire et al. 2001). It should be noted that this phenomena of selective loss of fast glycolytic fibers is observed in a variety of myopathic conditions (Parker et al. 2009; Serrano et al. 2008; Yu et al. 2008).

The finding of improved relative force production in STZ- or alloxan-diabetic muscle is perplexing (Vignaud et al. 2007; Paulus and Grossie. 1983; Ganguly et al.

1986; McGuire et al. 2001; Krause et al. 2009). A possible mechanism for increased relative force may be that calcium release/uptake dynamics and/or sensitivity have been altered (McGuire and MacDermott. 1999; Ganguly et al. 1986; McGuire et al. 2001). Calcium handling characteristics are altered in the STZ-diabetic muscle such that longer rise and half-relaxation times of single muscle twitches occurs (McGuire and MacDermott. 1999; Paulus and Grossie. 1983; McGuire et al. 2001; Krause et al. 2009; Hasan, Alshuaib, and Fahim. 2002). Furthermore, STZ muscle was previously demonstrated to exhibit hyperactive Ca^{2+} kinetics and sarcoplasmic reticulum (SR) Ca^{2+}-ATPase activity (Ganguly et al. 1986), and augmented contractile force following caffeine and/or concentrated Ca^{2+} exposure (Lesniewski, Miller, and Armstrong. 2003; Ganguly et al. 1986) which may be an effect of SR or plasma membrane instability (Ganguly et al. 1986). However, these characteristics/mechanisms have only been studied in depth in STZ rodents. *Ins2*$^{Akita+/-}$ mice, a genetic model of T1DM characterized by rapid adolescent onset of β-cell dysfunction and hypoinsulinemia, demonstrate no significant alteration in muscle twitch rise or half-relaxation times (Krause et al. 2009), indicating potential model-specific effects (differences between *Ins2*$^{Akita+/-}$ and STZ-diabetic rodents). The few instances of muscle function studies using *Ins2*$^{Akita+/-}$ mice provide results similar to human studies, i.e.: lower absolute peak force compared to controls, but no change in peak force relative to muscle mass (Vignaud et al. 2007; Krause et al. 2009). Given the findings of all human and animal studies, it seems most likely that the loss of peak force production is most related to the overall loss of muscle

mass. While shifts in fiber type may be a contributing factor, it seems unlikely to be a major factor given the magnitude of the shift.

Atrophy or impaired growth of glycolytic muscle fibers.

The reduction in absolute force production in T1DM may be explained by a fiber type-specific atrophy that is commonly observed in animal models of the disease. Typically, slow-twitch, or type I fibers exhibit minimal loss or a slight gain in fiber area, while fast-twitch fibers, especially the fatigable glycolytic (type IIB) fibers exhibit the most severe atrophy (Cotter et al. 1993; Krause et al. 2009; Armstrong, Gollnick, and Ianuzzo. 1975; Armstrong and Ianuzzo. 1977; Klueber and Feczko. 1994; Snow et al. 2005; Medina-Sanchez et al. 1991). Given that the glycolytic fibers produce the most force by way of high myosin ATPase activity and shortening velocity (Schiaffino and Reggiani. 1994), it follows that atrophy of those fibers should lead to a loss of force production at the level of the whole muscle. However, other studies have found an increase in the relative percentage of glycolytic/fast-twitch muscle fibers and glycolytic enzymes in diabetic humans (Crowther et al. 2003; Fritzsche et al. 2008). It could be hypothesized that these findings, in opposition to the more common finding of an oxidative shift observed in rodent models, is caused by multiple factors such as age, disease duration, physical activity levels, diet, and perhaps most importantly, insulin treatment and glycemic control. For example, it is possible that the periodic nature of insulin treatment could cause an influx of glucose into the muscle, stimulating the muscle for expression of genes suitable for glycolytic metabolism in order to better deal with these glucose “spikes”. It should also be noted that these human studies did not measure

fiber area, but rather counted fiber type frequency over a given area of muscle cross section. This measure alone does not necessarily represent a fiber type shift as the fibers that are increasing in frequency (in this case the glycolytic fiber type) may be simultaneously undergoing atrophy and thus, there would be more, smaller fibers quantified per image, as was the case in a study by Snow et al. (Snow et al. 2005).

Intramyocellular lipid and metabolic status.

As mentioned above, IMCL droplets are in greater abundance in the skeletal muscles of adults with T1DM, a finding that appears related to their level of glycemic control and disease duration. In studies reporting IMCL levels in untreated diabetic rodent models, there are conflicting findings. STZ-diabetic rodents, coinciding with what is observed in human studies, display increased IMCL (Krause et al. 2009; Chao et al. 1976), while diabetic *Ins2*$^{Akita(+/-)}$ mouse muscle has been found to be depleted of triglycerides (Hong et al. 2007) or show no change to IMCL content compared to controls (Krause et al. 2009). This discrepancy may be due to differences in oxidative enzyme activities between the models. For instance, lower activity of enzymes involved in lipid metabolism such as citrate synthase (Krause et al. 2009; Noble and Ianuzzo. 1985; Fewell and Moerland. 1995), β-hydroxyacyl CoA dehydrogenase (Krause et al. 2009), cytochrome oxidase (Fewell and Moerland. 1995), and 3-hydroxybutyrate dehydrogenase (Fewell and Moerland. 1995) is found in STZ-diabetic muscle, perhaps allowing accumulation of lipid bodies within STZ-diabetic muscle. Furthermore, a dose-dependent loss of phosphofructokinase (PFK) and succinate dehydrogenase (SDH) activity is observed in STZ-diabetic rats, despite similar elevation of blood glucose at each dose

level (Chen and Ianuzzo. 1982). On the other hand, *Ins2*$^{Akita(+/-)}$ mouse skeletal muscle enzyme activity appears to be similar to non-diabetic controls (Krause et al. 2009) and, in parallel, displays no accumulation of IMCL. Notably, citrate synthase activity in T1DM human skeletal muscle is not reduced compared to non-diabetics (Harmer et al. 2008), consistent with the absence of change in enzyme activities in *Ins2*$^{Akita(+/-)}$ mice. It is possible that no increase or loss of IMCL in untreated *Ins2*$^{Akita(+/-)}$ mice is due to an increased reliance on lipid as an energy substrate.

In addition to lipid metabolism, the rate of lipid transport into the muscle must be considered as well. It has been demonstrated in both *Ins2*$^{Akita(+/-)}$ mice and STZ-treated diabetic rodents that the fatty acid translocase (FAT)/CD36 is increased in skeletal and cardiac muscle, providing increased lipid transport into those tissues (Krause et al. 2009; Bonen et al. 2003; Chen et al. 2006; Luiken et al. 2002) further supporting the hypothesis of increased utilization of lipid stores in *Ins2*$^{Akita(+/-)}$ mice.

While the reasons underlying the discrepancies between rodent models of T1DM is still unclear, STZ-diabetic skeletal muscle also displays decreased ATP (Fewell and Moerland. 1995; Karakelides et al. 2007) and phosphocreatine content (Fewell and Moerland. 1995) and greater expression of uncoupling protein (UCP)-3 (Havel et al. 2000). Taken together with the reductions in enzyme expression/activity described above, these findings would suggest that, unlike the *Ins2*$^{Akita(+/-)}$ mice, STZ-diabetic skeletal muscle is in a reduced energy state despite the abundance of IMCL stores available.

MECHANISMS UNDERLYING DIABETIC SKELETAL MUSCLE ATROPHY.

As previously discussed, there are many possible underlying causes of skeletal muscle atrophy and impaired growth in the T1DM environment. Hyperglycemia and hypoinsulinemia are perhaps the most obvious, while the presence, absence or altered expression of several other hormones may play key roles in reduced muscle growth or increased proteolysis. With long standing T1DM, pathological changes to the motor neurons and the neuromuscular junctions are assuredly contributing to the phenotype as well.

Effects of hyperglycemia on skeletal muscle.

One negative effect of hyperglycemia is that proteins undergo glycation, the process of reduction of sugars resulting in the chemical modification of proteins. Further oxidation reactions lead to advanced glycation end products (AGE), which have been implicated in the pathophysiology of the aging process, are clearly established as contributing to the other T1DM-related complications (Snow et al. 2006; Snow, Fugere, and Thompson. 2007; Alt et al. 2004). Several of these AGE species have been found to be elevated in skeletal muscle protein, although the levels in skeletal muscle are approximately 5% of that observed in skin collagen (Alt et al. 2004). That AGE are found in skeletal muscle protein is interesting because actin and myosin motility is reduced when incubated in glucose, though not in the presence of the glucose-cleaving agent hydroxyalamine hydrochloride, indicating glycation-dependent reductions in myofibrillar protein function (Ramamurthy et al. 2001). Furthermore, it appears that fast twitch/type II myosin fibers are the most affected by AGE, as they have been

demonstrated to have a greater number of AGE-positive proteins when stained immunohistochemically (Snow et al. 2006). This finding is consistent with the aforementioned studies demonstrating that fast twitch/type II myosin fibers commonly exhibit atrophy (Cotter et al. 1993; Krause et al. 2009; Armstrong, Gollnick, and Ianuzzo. 1975; Armstrong and Ianuzzo. 1977; Klueber and Feczko. 1994) and loss of contractile function (Cameron, Cotter, and Robertson. 1990; Cotter et al. 1989; Cotter et al. 1993; Stephenson, O'Callaghan, and Stephenson. 1994; Paulus and Grossie. 1983; McGuire et al. 2001) in various models of T1DM. It also appears that glycation of myosin *in vitro* interferes with proteolysis at certain cleavage sites, as evidenced by the appearance of larger proteolytic myosin fragments in comparison to non-glycated myosin (Ramamurthy et al. 2001).

Another possible mechanism by which hyperglycemia could adversely affect skeletal muscle is through activation of the polyol pathway, leading to tissue damage by reducing cellular defences against oxidative stress (Brownlee. 2005; Ghahary et al. 1991). The key enzyme in this pathway, aldose reductase, is activated in response to hyperglycemia, leading to the accumulation of sorbitol and related metabolites. The activity of this pathway can be suppressed by aldose reductase inhibitor (ARI) treatment in skeletal muscle and nerve of diabetic rodents with positive results (Cameron, Cotter, and Robertson. 1990; Cotter et al. 1993; Cameron et al. 1997). ARI treatment restores lost nerve conduction velocity (Cameron et al. 1997), muscle fiber area (Cotter et al. 1993), and contractile function (Cameron, Cotter, and Robertson. 1990) in STZ-diabetic rats. Short term clinical trials of ARIs have reported modest success in reducing the

severity of neuropathy and retinopathy (Giannoukakis. 2008; Ramirez and Borja. 2008), but its effect on the human myopathic condition has yet to be examined.

Oxidative stress likely contributes to diabetic myopathy by upregulation of atrophy related genes (termed 'atrogenes'), atrogin-1 and MuRF-1, concomitant with a decrease in genes associated with muscle growth (JunD, MyoD, myogenin, MCK, MHCIIB, MLC1-3) in STZ-diabetic rats (Aragno et al. 2004; Mastrocola et al. 2008). Furthermore, Russell et al. (Russell et al. 2009) have demonstrated that a high glucose environment can cause protein degradation and suppress protein synthesis in myotubes *in vitro* (Russell et al. 2009). In that study, protein degradation was associated with the activation of caspase-3/-8, PKR, and p38, as well as PKR-dependent ROS production leading to ubiquitin proteasome pathway activity, while simultaneously suppressing protein synthesis via PKR-dependent eIF2c phosphorylation (Russell et al. 2009). Experiments utilizing dehydroepiandrosterone (DHEA) treatment, an antioxidant steroid, effectively reduced reactive oxygen species (ROS) and restored expression of those mRNA and proteins listed above, while also preventing some of the muscle/body mass loss (Aragno et al. 2004; Mastrocola et al. 2008). Vitamin C supplementation has also been demonstrated to restore half-relaxation time and twitch tension in STZ mice (Hasan, Alshuaib, and Fahim. 2002), presumably through its free radical-scavenging function. It may be speculated that fibers expressing type IIB myosin would be most adversely affected given that those fibers have the greatest potential for ROS generation and are the slowest in terms of ROS scavenging (Anderson and Neufer. 2006). It should be noted that STZ exposure to muscle cells *in vitro* causes ROS production (Johnston et al. 2007),

which could have contributed to the pathological oxidative stress observed in some of these studies (Aragno et al. 2004; Mastrocola et al. 2008).

Protein synthesis and degradation controlled by hormonal alterations.

Numerous studies have examined the effects of insulin and insulin deprivation on the rate of protein synthesis and/or degradation, as well as mechanisms underlying these effects. Several other hormones including glucocorticoids, IGF-1, growth hormone, and PAI-1 also contribute to the state of the diabetic muscle. While the literature indicates that the insulin signalling pathway is capable of activating protein synthesis (Cross et al. 1997; Marzban, Bhanot, and McNeill. 2001; Somwar et al. 1998; Shen et al. 2005; Hajduch et al. 1998; Pain and Garlick. 1974; Pain, Albertse, and Garlick. 1983), insulin supplementation does not consistently increase synthesis *in vivo* (Nair et al. 1995; Chow et al. 2006; Charlton, Balagopal, and Nair. 1997; Bennet et al. 1990; Godil et al. 2005; Charlton and Nair. 1998). Conversely, it is more consistently found that insulin inhibits protein degradation (Charlton and Nair. 1998; Lee et al. 2004; Price et al. 1996). In T1DM, protein degradation has been shown to be upregulated through the canonical ubiquitin-proteasome pathway as the expression of several genes of this pathway is found to increase (Aragno et al. 2004; Mastrocola et al. 2008; Russell et al. 2009; Lee et al. 2004; Price et al. 1996; Lecker et al. 1999; Lecker et al. 2004; Mitch et al. 1999).

The effect of insulin treatment or islet cell transplantation at the level of the whole muscle is a restoration of phenotype – that is, an improved muscle mass, particularly the glycolytic fibers that undergo the most severe atrophy. As mentioned, insulin, in addition to its canonical role in control of glucose uptake, has important effects by activating the

mTOR signalling pathway for protein synthesis and by inhibiting degradation of skeletal muscle protein. Insulin activates Akt/PKB (Cross et al. 1997; Marzban, Bhanot, and McNeill. 2001; Somwar et al. 1998) which provides downstream signalling that results in elevated protein synthesis *in vitro* (Shen et al. 2005; Hajduch et al. 1998). Insulin has been demonstrated to stimulate muscle protein synthesis in young STZ-diabetic rats as well (Pain and Garlick. 1974; Pain, Albertse, and Garlick. 1983). Conversely, insulin treatment has been shown to have no effect on myosin heavy chain (Charlton, Balagopal, and Nair. 1997) or mixed muscle protein synthesis in diabetic (Nair et al. 1995; Bennet et al. 1990) and non-diabetic adult humans (Chow et al. 2006). It has been suggested that inherent differences between studies of growing, chemically-induced diabetic rats compared to fully-grown adult diabetics are the source of these discrepancies regarding the protein synthesis response to insulin treatment (Charlton, Balagopal, and Nair. 1997), though adolescents with T1DM also do not increase protein synthesis in response to insulin (Godil et al. 2005). It is noteworthy that in the soleus (an oxidative muscle composed primarily of type I fibers) of alloxan-induced diabetic rats, the reduction in protein synthesis compared to healthy controls is almost negligible, while muscle groups composed of glycolytic fibers have severe reductions in protein synthesis (Flaim, Copenhaver, and Jefferson. 1980). This is consistent with the common observation of a selective loss of glycolytic muscle in T1DM rodents (Krause et al. 2009; Armstrong, Gollnick, and Ianuzzo. 1975; Armstrong and Ianuzzo. 1977; Klueber and Feczko. 1994; Medina-Sanchez et al. 1991; Snow, Fugere, and Thompson. 2007; Medina-Sanchez et al. 1994). While it is beyond the scope of this review, it should also be noted that muscle-

specific protein synthesis and degradation rates in the absence or presence of insulin in diabetics is different from that observed in the splanchnic region or at the whole body level (Charlton and Nair. 1998). Splanchnic protein synthesis is elevated in the absence of insulin while muscle protein synthesis is unchanged (Charlton and Nair. 1998), indicating important organ- or region-specific differences in protein metabolism in the presence or absence of insulin.

In addition to protein synthesis, activation of Akt by insulin signalling also inhibits BAD, a pro-apoptotic protein, through phosphorylation, thus promoting cell survival (Datta et al. 1997; Galetic et al. 1999). Furthermore, Akt expression and activation (total and phosphorylated protein) are lower (Ekladous et al. 2008; Oku et al. 2001) in white (glycolytic) but not red (oxidative) STZ-induced diabetic muscle compared to healthy controls (Ekladous et al. 2008). Whether these diabetic effects on these signalling components significantly contributes to the loss of glycolytic muscle mass in T1DM is unknown.

Protein degradation is increased in T1DM. It is has been found that increased expression and activity of the ubiquitin-proteasome (Ub-P) pathway is at least partially responsible for the degradation of skeletal muscle protein in type 1 diabetes. Components of the Ub-P pathway are frequently reported to be elevated in T1DM, including ubiquitin, as well as, E2 and E3 ubiquitin ligases (Aragno et al. 2004; Mastrocola et al. 2008; Russell et al. 2009; Lee et al. 2004; Price et al. 1996; Lecker et al. 1999; Lecker et al. 2004; Mitch et al. 1999). Expression and dimerization of the multiple subunits of NF-κB have been demonstrated to be altered in STZ-diabetic muscle as well (Frier, Noble, and

Locke. 2008). It is important to note that other atrophic states also rely on a similar program of proteolysis: experimental uremia, cancer, diabetes, and even extended fasting activate sets of genes very similarly (Lecker et al. 2004; Cai et al. 2004). Insulin is known to activate PI3K and through this signalling protein represses atrogin-1/MAFbx (Lee et al. 2004) and ubiquitin expression (Price et al. 1996).

Interestingly, the plasma concentration of IGF-1 is reduced in T1DM (Moyer-Mileur et al. 2008; Dehoux et al. 2004; Binz, Zapf, and Froesch. 1989), even with insulin treatment (Scheiwiller et al. 1986). However, IGF-1 supplementation reduces the expression of the ubiquitin-ligase components such as atrogin-1, E2 ligase, and ubiquitin (Dehoux et al. 2004). As well, glucocorticoid-induced expression of atrogin-1 is also abolished by IGF-1 supplementation (Dehoux et al. 2004). In fact, growth is partially restored in the STZ-diabetic rat when infused with recombinant human IGF-1 and fully restored in combination with insulin treatment (Scheiwiller et al. 1986). Chronic resistance exercise in partially-pancreatectomized (PPX) diabetic rats partially restores plasma IGF-1 concentration and restores protein synthesis to that of non-diabetic rats (Farrell et al. 1999a; Farrell et al. 1999b). IGF-1 concentration in exercised gastrocnemius increases over two-fold in PPX diabetic rats, while no change is observed in non-diabetic muscle (Farrell et al. 1999b). Furthermore, IGF-1 concentration in both sedentary and exercised PPX diabetic rats exhibits a significant positive correlation with protein synthesis in muscle, while no such correlation was found in non-diabetic rats (Fedele, Lang, and Farrell. 2001).

Further control over the expression of these Ub-P genes comes from glucocorticoids, which are elevated in T1DM (Mitch et al. 1999; Oishi et al. 2004; Oishi, Ohkura, and Ishida. 2006; Chan et al. 2005) and reduced with insulin treatment (Charlton, Balagopal, and Nair. 1997; Mitch et al. 1999). Adrenalectomy of STZ-diabetic animals returns corticosterone levels to that of non-diabetic controls (Mitch et al. 1999; Oishi, Ohkura, and Ishida. 2006) and normalizes expression of the Ub-P genes (Mitch et al. 1999). Myostatin, a protein with potent inhibitory effects on muscle growth, is upregulated in STZ-diabetic muscle (Chen et al. 2009; Wieteska-Skrzeczynska et al. 2009), and is known to be regulated by glucocorticoid levels (Ma et al. 2003).

Chronic elevation of IL-6 levels has been associated with growth defects (De Benedetti et al. 1997; De Benedetti et al. 2001), loss of muscle mass/strength in the elderly (Visser et al. 2002), and the initiation of skeletal muscle proteolysis (Goodman. 1994). It may be through interactions with growth factors that chronic IL-6 elevation exerts effects on tissue growth. For example, circulating IGF-1 levels are reduced in IL-6 treated mice (De Benedetti et al. 1997). Recent studies have demonstrated that IL-6 is elevated in type 1 diabetics (Galassetti et al. 2006b; Dogan et al. 2006; Rosa et al. 2009; Rosa et al. 2008) and that the glycemic state influences IL-6 levels (Rosa et al. 2009; Galassetti et al. 2006c). It is currently unclear whether the elevations in IL-6 are responsible, either directly, or indirectly by reducing IGF-1 levels, for loss of muscle mass/growth in T1DM. It is also possible that IL-6 elevations may exert effects indirectly on skeletal muscle by affecting glucose homeostasis in other tissues (Kanemaki et al. 1998).

Regeneration following injury.

Repair of skeletal muscle following injury, a process that is critical for the maintenance of a healthy muscle mass, is impaired in the T1DM environment (Vignaud et al. 2007; Gulati and Swamy. 1991). Gulati and Swamy (Gulati and Swamy. 1991) transplanted EDL muscles from STZ-diabetic rats into non-diabetic hosts and vice versa, demonstrating poor regeneration in the diabetic host regardless of the source of the muscle (diabetic or non-diabetic). Furthermore, diabetic EDL transplanted into the non-diabetic host regenerated equivalently to non-diabetic muscles transplanted into non-diabetic hosts (Gulati and Swamy. 1991). This study eloquently demonstrates that the diabetic environment (altered hormonal milieu and hyperglycemia) is the primary cause of poor muscle regeneration following injury.

The overall health of skeletal muscle is also affected by recovery from repeated microtrauma resultant from routine contractile activity and macrotrauma from less frequent injuries; thus, such impairments in the repair process as illustrated by the Gulati study (Gulati and Swamy. 1991) may contribute to the poor muscle growth in T1DM. This may be very important given that it has also been demonstrated that diabetic muscle is more susceptible to contraction-induced damage (Copray et al. 2000).

The hormonal alterations described in the previous section likely influence the rate of regeneration due to their influence on muscle protein synthesis and degradation. A hormone which may also play a role in the injury recovery process is PAI-1, which slows the regeneration process in skeletal muscle following injury (Koh et al. 2005; Suelves et al. 2005) and is found to be elevated in STZ-diabetic rats (Oishi et al. 2004; Oishi,

Ohkura, and Ishida. 2006). Furthermore, downstream protein effectors of the PAI-1 cascade regulate muscle satellite cell activity, providing an additional mechanism of controlling skeletal muscle growth (Fibbi et al. 2001; Fibbi et al. 2002). Interestingly, PAI-1 expression is positively regulated, in part, by glucocorticoid levels which are elevated in T1DM (Oishi et al. 2004; Oishi, Ohkura, and Ishida. 2006; van Zonneveld, Curriden, and Loskutoff. 1988). It is currently unknown if PAI-1 elevations in T1DM significantly impair the regeneration process. It should also be noted that satellite cells from STZ-diabetic rats have an impaired capacity for differentiation (Brannon et al. 1989) which may contribute to poor regeneration following injury (Hawke and Garry. 2001), although this finding may also be due to STZ-specific effects on the satellite cells (Johnston et al. 2007).

Neuropathy.

Studies of adult subjects with long standing T1DM have found a loss of muscle strength in neuropathic, but not non-neuropathic states (Andersen et al. 1997), as well as a loss of muscle volume in distal musculature (Andersen, Gjerstad, and Jakobsen. 2004). Furthermore, a significantly greater number of neuromuscular junctions with signs of degeneration are found after 8 weeks of STZ-induced diabetes (Fahim, el-Sabban, and Davidson. 1998), synaptic delay time is increased by 4 weeks of STZ-induced diabetes (Fahim, el-Sabban, and Davidson. 1998), while motor unit number estimate is reduced by 2 weeks of STZ-induced diabetes (Souayah et al. 2009). Interestingly, a common finding in muscle denervation studies is loss of glycolytic/fast twitch fiber area and/or number of fibers, while oxidative/slow twitch fibers do not atrophy to the same degree if at all

(Bobinac et al. 2000; Ohira et al. 2006; Niederle and Mayr. 1978), similar to what has been frequently described in diabetic myopathy studies (Krause et al. 2009; Armstrong, Gollnick, and Ianuzzo. 1975; Armstrong and Ianuzzo. 1977; Klueber and Feczko. 1994; Chao et al. 1976). Conversely, in newly diagnosed human diabetics (1 to 28 weeks of diabetes), no degeneration of motor end plates was found although myopathic signs such as atrophy, greater presence of IMCL, and sarcomeric disruption were already detectable in the muscle (Reske-Nielsen, Harmsen, and Vorre. 1977), indicating that myopathy occurs independently of diabetic neuropathy. Because diabetic neuropathy worsens over time (Andreassen et al. 2009b), it is likely that neurogenic atrophy occurs in long-standing diabetics to a much greater degree than in diabetic youth (Reske-Nielsen et al. 1970; Reske-Nielsen et al. 1970).

Diabetic amyotrophy is described as a specific type of neuropathy where relatively sudden onset of pain and muscle weakness in the pelvic and thigh muscles occurs, symmetrically or asymmetrically, and is usually accompanied by loss of sensation (Chokroverty et al. 1977; Taylor and Dunne. 2004). It is mentioned here as it may be confused with the peripheral neuropathy affecting limb musculature as we have described above.

Diabetic muscle infarction.

There are a number of case studies of isolated incidents involving spontaneous idiopathic muscle infarction (Kapur, Brunet, and McKendry. 2004; Trujillo-Santos. 2003). These reports indicate that muscle infarction is an underdiagnosed, although very rare, complication in long-term diabetics but should be differentiated from diabetic

myopathy as defined by the studies reviewed here. It is hypothesized that microvascular complications can lead to spontaneous infarction, however, the exact cause is largely unknown (Kapur, Brunet, and McKendry. 2004; Trujillo-Santos. 2003).

CLINICAL CONSIDERATIONS

Historically, healthcare providers have not typically concerned themselves with the assessment of skeletal muscle mass or function in patients with T1DM. In fact, muscle wasting has traditionally been considered a condition of long-standing diabetes, particularly in those showing evidence of other co-morbidities (e.g. neuropathy). In this review, we have established that adolescents with T1DM can have impaired muscle growth, morphology and function as a result of either insufficient muscle accrual and/or increased muscle protein breakdown. In fact, a lower muscle mass and associated muscle weakness will likely be apparent at the time of diagnosis given the extended period of hyperglycemia/hypoinsulinemia that precedes the diagnosis. However, without previous measures for baseline, this is difficult for the healthcare provider to diagnose. In any event, it is well supported that lower muscle mass usually develops within the first few years of T1DM, particularly if metabolic control is suboptimal. Increasing skeletal muscle mass and insulin sensitivity through regular exercise in youth can improve metabolic control (Robertson et al. 2009), and reciprocally, this exercise-mediated enhancement in metabolic control may be critical in helping to maintain a normal skeletal muscle mass and function in T1DM patients.

While maintaining a healthy skeletal muscle mass is more challenging for those with T1DM, its vital role in managing blood glucose levels (not to mention the numerous other health and psychosocial benefits) makes it an ideal target for therapeutic strategies aimed at reducing the rate of progression of diabetic complications, such as nephropathy and cardiovascular disease. The ISPAD Clinical Practice Consensus Guidelines (Robertson et al. 2009) provide a good reference point for the healthcare provider to begin to appreciate the benefits of daily physical activity and also the clinical considerations associated with prescribing these activities in the T1DM patient.

CONCLUSIONS

The goal of this review was to characterize diabetic myopathy as is observed in the limited number of human studies and in the multitude of diabetic animal studies. It is most commonly found that a loss of peak contractile force occurs, likely a result of the atrophy or impaired growth of the glycolytic muscle fibers. The loss of muscle mass can be attributed to many factors, including hyperglycemia, hypoinsulinemia, and alterations to other key hormones such as glucocorticoids. It appears that at least a slight reduction in the rate of protein synthesis occurs, with a much greater increase to muscle proteolysis, which occur secondary to changes in the hormonal milieu. Impaired nerve function likely contributes to the severity of diabetic myopathy with increasing disease duration though the current evidence suggests that diabetic myopathy exists in the absence of observable neuropathic complications. Given the multitude of factors highlighted in this review, there is currently no overarching hypothesis that can account for all aspects of diabetic

myopathy. Understanding the temporal expression of these factors (and their relative contribution to the myopathic phenotype) during disease progression will allow us to elucidate their role in the pathophysiological changes that are occurring in type 1 diabetic skeletal muscle.

TABLES AND FIGURES

Table 1.1. Aerobic measures in T1DM human subjects

Subject Characteristics					Method & Findings						
# of Subjects	Age (years)	Sex	Matched for activity	T1DM duration (years)	Exercise Test/Mode	CO	VO_2max or VO2peak	PWC170	Heart rate at given exercise work load(s)	Other	Reference
27 C, 22 D	10 to 18	F	No	1 to 14	Cycle ergometry	–	–	↔	↑	training ↑ PWC170 and ↓ HR during exercise at a given work load in D	Larsson et al 1962
94 C, 84 D	6.3 to 18.8	M & F	No	1 to 15.1	Cycle ergometry	–	–	↓	–	performance loss is greater in diabetic boys; relationship between performance and HbA1c levels, as well as age	Hottunen et al 1984
17 C, 9 D - HbA1c<8.5%, 8 D - HbA1c>8.5%	16.4 (avg)	M & F	No	7.8 (avg)	Cycle ergometry	–	↓	–	–	VO2max neg. correlates to HbA1c levels; max workload ↓	Poortmans et al 1986
47 C, 33 D	9.2 to 15.7	M & F	No	5.0 (avg)	Treadmill running	–	↓	–	↑	↓ O_2-pulse (O_2 uptake/HR)	Baraldi et al 1992
18 C, 59 D	9.5 to 19.3	M & F	No	–	Cycle ergometry	–	↓ in boys, ↔ in girls	–	–	VO2max neg. correlates to HbA1c levels	Austin et al 1993
46 C, 72 D	9 to 20	M & F	No	4.87 (avg)	Treadmill running	–	↓	–	–	↓ time to exhaustion and HRpeak during incremental exercise test	Komatsu et al 2005
10 C, 12 D	12 to 18	F	No	6.1 (avg)	Cycle ergometry	↓	↓	–	–	↓ stroke volume	Gusso et al 2008
123 C, 129 D	7 to 20	M & F	Yes	–	Cycle ergometry	–	–	–	↑	HR response diverged more with increased age	Sterky. 1963
6 C, 6 D	15 to 19	M	Yes	4 to 12	Cycle ergometry	–	↓	↓	↑	D and C exhibit similar improvement with training	Larsson et al 1964
18 C, 17 D	8.5 to 13	M	Yes	1 to 9	Cycle ergometry	–	–	↔	–	–	Heyman et al. 2005
10 C, 10 D	11 to 15	M	No	<1 to 12	Treadmill running	–	↔	–	↔	no relationship between aerobic capacity and HbA1c levels	Hagan et al. 1979
11 C, 11 D	9.3 to 16.2	M	Yes	0.5 to 13	Cycle ergometry	↔	↔	–	↔	–	Rowland et al. 1992
8 C, 8 D	21 to 46	M & F	No	>10	Cycle ergometry	↔	↔	–	–	–	Nugent et al. 1997
23 C, 23 NNPD, 7 NPD	21 to 48	M & F	Yes	1 to 29	Treadmill running and cycle ergometry	–	↔ in NNPD, ↓ in NPD	–	–	VO2max neg. correlates to HbA1c levels	Veves et al 1997
14 C, 9 NGD, 9 HGD	40 NGD, 31 C, 38 HGD (avg)	M & F	No	21 (avg for both NGD & HGD)	Cycle ergometry	–	↓ in NGD & HGD	–	–	↓ peak work load in HGD only	Niranjan et al. 1997

D: diabetic; C: control; NP:neuropathic; NNP:non-neuropathic; NGD:normoglycemic; HGD: hyperglycemic

Table 1.2. Contractile capacity and morphological abnormalities of diabetic skeletal muscle.

Subject Characteristics					Function			Morphology			
# of Subjects	Age (years)	Sex	Activity matched	T1DM Duration	Assessment	Change	Notes	Method of assessment	Change	Notes	Reference
16 D	23 to 37	M & F	—	7 to 31 yrs	Clinical assessment of muscle function	2 subjects displayed clinical weakness	—	Histology; motor nerves and end plates; muscle biopsy	Bended axons, abnormal end plates, and sprouting consistently observed	Uniform atrophy of muscle fibers is observed	Reske-Nielsen et al. 1970
16 C, 16 D (8 NNP, 8 NP)	31 to 64	—	No	20 to 35 yrs	Isokinetic peak torque of dorsal/plantar flexion of ankle; ext/flex of knee; NP symptoms scored	↓torque at ankle and knee in NP compared to NNP	—	MRI; thigh, lower leg; muscle volume	↓ muscle volumes in NP compared to NNP and C	↓ muscle volume is worse going from proximal to distal	Andersen et al. 1997
26 C, 26 D (15 NNP, 11 NP)	42 to 76	M & F	No	26 to 48 yrs	Follow-up study to (2, 3) - 12.6 years elapsed; Torque at knee and ankle	↓ peak torque in all subjects since previous studies as a result of aging; NP subjects exhibit significantly greater ↓	Torque correlates with muscle volume	MRI; foot, leg; muscle volume	↓ muscle volumes in NP compared to NNP and C	Neg. correlation between NP score and muscle volume	Andreassen et al. 2009
*58 C, 58 D (13 [NNP], 19 NP but asymptomatic, 24 symptomatic NP	31 to 64	M & F	Yes	20 to 47 yrs	Isokinetic peak torque of dorsal/plantar flexion of ankle; ext/flex of knee and wrist; NP symptoms scored	↓ torque at ankle and knee, ↔ torque at wrist	Torque loss at ankle and knee neg. correlates with neuropathy symptom severity	—	—	—	Andersen et al. 1996
44 C, 44 D	46 (avg)	M & F	No	20 to 47 yrs	Isokinetic peak torque, endurance of dorsal/plantar flexion of ankle; ext/flex of knee; NP symptoms were scored	↑ endurance, ↓ peak torque	Peak torque, but not endurance index, correlates with neuropathy score	—	—	—	Andersen 1998
10 C, 10 D	27.5 (avg)	M & F	No	7.8 yrs (avg)	Isometric force, endurance of knee extensors; motor nerve conduction velocity	↓ time to fatigue, ↓ motor nerve conduction velocity (MNCV)	MNCV neg. correlates with glycemic control; MNCV correlates with time to fatigue	—	—	—	Almeida et al. 2008
9 D	16 to 32	M & F	—	< 1 month	—	—	—	Histology; motor nerves and end plates; muscle biopsy	Bended axons, abnormal end plates, and sprouting are observed	Abnormalities not as severe as in older patients of previous study; abnormal muscle fibers observed	Reske-Nielsen et al. 1970
29 D	16 to 43	M & F	—	1 week to 32 years	—	—	—	Light and electron microscopy; muscle biopsy	↓ fiber diameter due to myofilament loss; sarcomere destruction	Lipid bodies greater in recently diagnosed; long-term group worse atrophy; basal lamina thickening, motor end plate degeneration	Reske-Nielsen et al. 1977
4 C, 6 D (3 D no insulin treatment; 3 D insulin treated <1 week)	23.4 (avg)	M	Yes	< 1 week since diagnosis - diabetic period prior unknown	—	—	—	Mean fiber diameter, nbialis anterior muscle biopsy	↓ fiber diameter	↓ still significant when fiber diameter relative to body mass	Jakobsen et al. 1986
23 C, 23 D (15 NNP, 8 NP)	27 to 62	M & F	No	16 to 38 yrs	—	—	—	MRI; foot; muscle volume	↓ muscle volumes in NP compared to NNP and C	Neg. correlation between NP score and muscle volume	Andersen et al. 2004
10 C, 7 D	24.9 (avg)	M & F	Yes	-	—	—	—	Morphology,enzyme activity; vastus lateralis muscle biopsy	↓ type I and IIa %, ↑ IIx %; ↑ GPDH activity; ↓ NOS activity; ↔ SDH activity	Fiber area not determined	Frizzsche et al. 2008

* denotes study using IDDM subjects (ie type 1 and 2 diabetic subjects grouped together)
D: diabetic; C: control; NP:neuropathic; NNP:non-neuropathic;

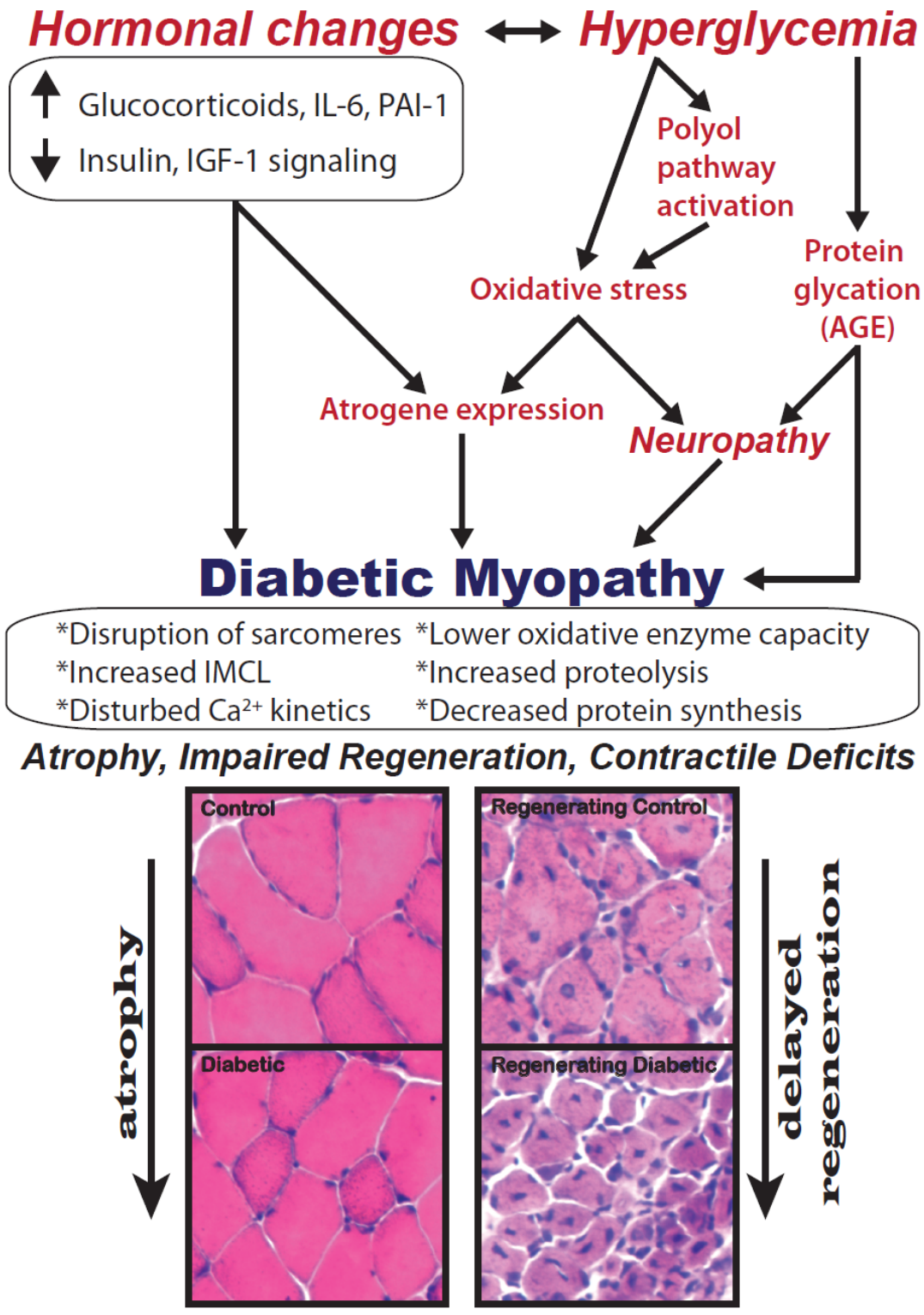

Figure 1.1. Proposed mechanisms underlying diabetic myopathy. Changes in blood glucose and circulating hormone levels are believed to be the major contributors to the myopathic

condition. These alterations in hormonal and glycemic status lead to other pathophysiological changes (e.g.: protein glycation) which negatively influence muscle health, ultimately leading to detrimental alterations in muscle phenotype, regenerative capacity, and contractile characteristics. While there are numerous cellular and molecular maladaptations occurring within the muscle, these are manifested as muscle atrophy and impaired regenerative capacity as illustrated by the hematoxylin and eosin (H&E) stained muscle sections displayed. Representative H&E images demonstrate control (non-diabetic) and diabetic skeletal muscle at 8 weeks of diabetes (left column), and 10 days following a cardiotoxin-induced injury (right column) in C57BL/6 and *Ins2*$^{Akita(+/-)}$ mice. Note reductions in muscle fiber size as indices of muscle atrophy and attenuated regeneration.

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CHAPTER 2

Diabetic myopathy differs between *Ins2*$^{Akita+/-}$ and streptozotocin-induced type 1 diabetic models.

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PREFACE

Significance to thesis

The primary goal of this study was to characterize diabetic myopathy in terms of functional capacity and morphology of skeletal muscle, and to relate the findings to the diabetic animal model being employed. Numerous studies have described some aspect of diabetic myopathy by studying the streptozotocin-induced diabetic rodent. As described in the literature review, serious concerns must be entertained regarding the use of this model in certain studies due to various toxic effects of streptozotocin in several tissues other than the pancreatic β-cell, including skeletal muscle. Hence, we chose the streptozotocin-induced diabetic mouse and the diabetic *Ins2*$^{Akita+/-}$ mouse to study as a model of adolescent T1DM. While there are a few T1DM mouse models available, the streptozotocin-induced and the *Ins2*$^{Akita+/-}$ mouse models represent mouse models where adolescent diabetes can be studied. The other genetic mouse models develop the diabetic phenotype after the adolescent period and would not have been an appropriate model for our future studies investigating the effects of uncontrolled diabetes on skeletal muscle growth. By studying alternative models of T1DM, model-specific characteristics may be elucidated and, therefore, help identify which model is a more ideal representation of the human disease.

Authors' contributions

Matthew P. Krause contributed to the design of the study, performed all experiments and data collection, performed and 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.

Michael C. Riddell contributed to the design of the study, assisted in experiments, and worked on refining drafts of the manuscript and the revisions based on editorial review.

Carly S. Gordon assisted in experiments and microscope image analysis.

S. Abdullah Imam performed microscope image analysis and assisted in animal care.

Enzo Cafarelli worked on refining drafts of the manuscript and the revisions based on editorial review.

Thomas J. Hawke contributed to the design of the study, assisted in experiments, and worked on refining drafts of the manuscript and the revisions based on editorial review.

ABSTRACT

Mechanistic studies examining the effects of type 1 diabetes mellitus (T1DM) on skeletal muscle have largely relied on streptozotocin-induced diabetic (STZ) rodents. Unfortunately, characterization of diabetic myopathy in this model is confounded by the effects of streptozotocin on skeletal muscle independent of the diabetic phenotype. Here we define adolescent diabetic myopathy in a novel, genetic model of T1DM, *Ins2*$^{Akita+/-}$ mice and contrast these findings with STZ mice. Eight weeks of diabetes resulted in significantly reduced gastrocnemius/plantaris/soleus mass (Control: 0.16±0.02g; *Ins2*$^{Akita+/-}$: 0.12±0.01g; STZ: 0.12±0.02g) and IIB/D fiber area in *Ins2*$^{Akita+/-}$ (1294±282μm^2) and STZ (1768±489μm^2) compared to Control (2241±432μm^2). Conversely, STZ type I fibers (1535±496μm^2) were significantly larger than *Ins2*$^{Akita+/-}$ (915±227μm^2) but not Control (1152±257μm^2). Intramyocellular lipid increased in STZ (122.9±3.6% of Control) but not *Ins2*$^{Akita+/-}$ likely resultant from depressed citrate synthase (Control: 6.2±3.1μmol·s^{-1}·g^{-1}; *Ins2*$^{Akita+/-}$: 5.2±2.1μmol·s^{-1}·g^{-1}; STZ: 2.8±1.6μmol·s^{-1}·g^{-1}) and β-HAD (Control: 4.2±1.9nmol·s^{-1}·g^{-1}; *Ins2*$^{Akita+/-}$: 5.0±1.7nmol·s^{-1}·g^{-1}; STZ: 2.7±1.7nmol·s^{-1}·g^{-1}) enzyme activity in STZ muscle. *In situ* muscle stimulation revealed lower absolute peak tetanic force in *Ins2*$^{Akita+/-}$ (70.2±8.2% of Control) while STZ exhibited an insignificant decrease (87.6±7.9% of Control). Corrected for muscle mass, no force loss was observed in *Ins2*$^{Akita+/-}$, while STZ was significantly elevated versus Control and *Ins2*$^{Akita+/-}$. These results demonstrate that atrophy and specific fiber type loss in *Ins2*$^{Akita+/-}$ muscle did not affect contractile properties (relative to muscle mass). Furthermore, we demonstrate distinctive contractile, metabolic and phenotypic properties in STZ versus *Ins2*$^{Akita+/-}$ diabetic muscle despite similarity in hyperglycemia/hypoinsulinemia; raising concerns of our current state of knowledge regarding the effects of T1DM on skeletal muscle.

INTRODUCTION

The capacity of skeletal muscle for growth, adaptation and regeneration is truly remarkable. Nowhere is this ability more evident than during childhood/adolescence where increases in the overall size (hypertrophy) and strength of the muscle are the result of increased protein synthesis and fusion of satellite cell progeny (termed myoblasts) to existing muscle fibers (Hawke. 2005). Naturally, this adaptability of muscle is not limited solely to hypertrophic stimuli. Atrophic stimuli, such as casting, weightlessness and various pathological situations, result in a decrease in muscle mass, strength and satellite cell number (Darr and Schultz. 1989; Mozdziak, Pulvermacher, and Schultz. 2000; Wanek and Snow. 2000) . It is important to note that, in contrast to adult muscle, atrophic stimuli placed on adolescent muscle results in a rapid and irreversible remodelling process involving a decrease in satellite cell content, impaired proliferative capacity and myonuclear accretion (Darr and Schultz. 1989; Mozdziak, Pulvermacher, and Schultz. 2000; Wanek and Snow. 2000).

One of the most common clinical conditions in which the capacity of muscle growth and performance may be dramatically altered is type 1 diabetes mellitus (T1DM). Unfortunately, T1DM diagnosis often occurs in childhood or early adolescence, when management of glycemic control is often suboptimal (Hamilton and Daneman. 2002). Though the effects of T1DM on human adolescent muscle phenotype and function have yet to be fully elucidated, the end-result is an overall reduction in muscle mass and physical performance variables referred to as diabetic myopathy (Riddell and Iscoe. 2006; Almeida, Riddell, and Cafarelli. 2008). Studies of adult T1DM muscle performance indicate a reduction in peak strength (Andersen et al. 1996) but improved fatigue resistance (Andersen. 1998). To date, there are limited studies examining the effect of T1DM on the skeletal muscle phenotype in adolescent or adult humans. Interestingly, of

the studies conducted, it has been shown that adult type 1 diabetic muscle exhibits an increase in glycolytic metabolism (Crowther et al. 2003) and a shift in fiber type towards more glycolytic or fast twitch muscle fibers (Fritzsche et al. 2008), which is contrary to what is typically observed in animal studies; a shift towards more oxidative fiber type composition (Armstrong, Gollnick, and Ianuzzo. 1975; Klueber and Feczko. 1994). A shift in fiber type composition is suggestive of possible alterations to substrate metabolism, though human studies measuring intramyocellular lipid (IMCL) have demonstrated no change (Bernroider et al. 2005) or, similar to animal studies (Klueber and Feczko. 1994; Chao et al. 1976), an increased IMCL content (Perseghin et al. 2003).

Much of our current knowledge regarding diabetic myopathy is the result of studies performed using adult streptozotocin-induced diabetic rodents. Streptozotocin is a glucosamine-nitrosourea compound taken up by pancreatic beta-cells causing DNA damage followed by cell death, rendering the animal insulin deficient (Bennett and Pegg. 1981; Junod et al. 1969; Elsner et al. 2000). The basic indices of skeletal muscle phenotype and function, such as fiber type composition, fiber size, IMCL, capillary-to-fiber ratio and contractile parameters, have all been demonstrated to be altered in the streptozotocin-induced diabetic model (Armstrong, Gollnick, and Ianuzzo. 1975; Klueber and Feczko. 1994; Chao et al. 1976; Armstrong and Ianuzzo. 1977; Aragno et al. 2004; Copray et al. 2000; Sexton, Poole, and Mathieu-Costello. 1994). However, we have recently demonstrated that streptozotocin, independent of hyperglycemia/hypoinsulinemia, impairs body weight gain and attenuates muscle fiber growth *in vivo*, likely as a result of a ROS-mediated G2/M phase cell cycle arrest in myoblasts (Johnston et al. 2007). Thus, it remains unclear if changes observed in streptozotocin-treated skeletal muscle (that were previously attributed to the T1DM) were in fact the result of a direct action of

streptozotocin. Clearly, a new non-streptozotocin model of diabetes is urgently needed to study the potentially deleterious complications of hyperglycemia/hypoinsulinemia to skeletal muscle.

Heterozygotic *Ins2*^{Akita} mice (*Ins2*$^{Akita+/-}$) become spontaneously diabetic due to a mutation in one allele of the Insulin-2 gene resulting in reduced levels of circulating insulin beginning during mouse adolescence (3-4 weeks old) (Kruczek and Gruca. 1990; Lopez, White, and Randall. 2001; Yoshioka et al. 1997). With concerns raised regarding the use of streptozotocin for the study of diabetic skeletal muscle (Johnston et al. 2007), we investigated the *Ins2*$^{Akita+/-}$ mouse as an alternative, non-pharmacological, model for the study of diabetic myopathy. We hypothesized that although both streptozotocin-induced (STZ) and *Ins2*$^{Akita+/-}$ diabetic models exhibit characteristic hyperglycemia and hypoinsulinemia, there would be a number of functional and phenotypic variables that would be different between them. Consistent with this hypothesis, our findings indicate that STZ diabetic mice, compared to *Ins2*$^{Akita+/-}$ mice, display: (i) a more drastic attenuation in weight gain, (ii) elevated plasma non-esterified fatty acid (NEFA) levels, (iii) elevated intramyocellular lipid (IMCL) levels, (iv) reduced muscle lipid and oxidative enzyme activity, (v) a paradoxical maintenance of absolute peak muscle contractile force, and (vi) altered muscle twitch rise and relaxation times.

Not only is skeletal muscle responsible for our physical capacities for movement, it is also the largest organ for glucose disposal. Thus, understanding the effects of T1DM on skeletal muscle in growth and development is of paramount importance if we are to properly and accurately develop therapeutic strategies to combat this devastating disease. Taken together, our results raise concerns regarding the current state of knowledge on the effects of T1DM on skeletal muscle, which is largely based on STZ-induced diabetic muscle, and suggest that non-pharmacological models should be used to confirm and validate previous findings.

METHODS

Animal Characteristics.

Age-matched male *Ins2*$^{Akita+/-}$ and C57Bl/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). *Ins2*$^{Akita+/-}$ mice became hyperglycemic (> 15 mM) over a period of 2-3 days at 4 weeks of age. Correspondingly, at 4 weeks of age, C57Bl/6 mice were randomly assigned into streptozotocin-treated (STZ) or Control groups. The STZ group were administered a single intraperitoneal injection of 120 mg/kg streptozotocin (Sigma-Aldrich, Oakville, ON) dissolved in sterile saline and became diabetic within 2-3 days or were excluded from the study. In total, 10 Control, 10 *Ins2*$^{Akita+/-}$, and 9 STZ mice were studied for an 8 week period. The animal room was maintained at 21°C, 50% humidity and a 12h/12h light-dark cycle and all mice had access to standard diet and water *ad libitum*. Mice had no access to running wheels, however, enrichment was provided in the form of nesting material and cardboard tubing. Though not specifically measured, it appeared that *Ins2*$^{Akita+/-}$ and STZ mice were less active than control mice, particularly in the final weeks of the experimental period, consistent with that observed by others (Hong et al. 2007). Blood glucose was monitored daily [OneTouch Ultra glucometer; maximum 35 mmol/L; Johnson & Johnson)] in the diabetic groups until glycemic values peaked and stabilized; thereafter blood glucose was measured less frequently. Body mass was measured weekly with animals in the fed state (09 00h) and once in the fasted state just before in situ muscle measurements (see below). At 4 weeks of diabetes, fasted plasma samples were obtained to determine glucose and non-esterified fatty acid concentrations. At 5.5 weeks of diabetes, food and water consumption were determined over a 24 hour period. All experiments were approved by the York University Animal Care Committee in accordance with Canadian Council for Animal Care guidelines.

Muscle Stimulation.

Following the 8 week experimental period, mice were fasted overnight and sedated with a ketamine/xylazine IP injection (ketamine: 150 mg/kg; xylazine: 10 mg/kg) prior to surgery. Supplemental doses were unnecessary as the mice were unresponsive to pinch reflex tests for the duration of the protocol. The surgical procedure and muscle stimulation protocol were performed as described previously with minor modifications (Krause et al. 2008). Optimal voltage was determined by generating single twitch contractions at increasing voltages until no increase in single-twitch force production was observed. Optimal muscle length was determined in a similar manner. Specifically, muscle length was manipulated and single-twitch force production was observed. The length and voltage at which a single twitch produced the greatest force were used throughout the entire stimulation protocol. The average voltage used was 30V. The pulse duration was set to 0.1ms for all twitch and tetanic contractions. The direct muscle stimulation protocol consisted of a force-frequency curve to determine peak tetanic force followed by a 2 minute low-frequency (2 Hz) stimulation period to determine fatigue resistance. Twitch contractions were performed before and after the stimulation protocol. The force-frequency curve was determined by stimulating the muscle with trains 1 second in duration separated by 9 seconds of rest with the pulse frequency beginning at 20 Hz and increased by 10 Hz per train until no further increase in force was observed. The greatest force achieved was considered the absolute peak tetanic force. Direct muscle stimulation was chosen over sciatic nerve stimulation so as to directly assess the functional capacity of the muscle and remove potential confounding effects of impaired nerve/neuromuscular junction function (Andersen. 1998; Fahim, el-Sabban, and Davidson. 1998; Andersen et al. 1997; Andersen, Gjerstad, and Jakobsen. 2004). Twitch amplitude, rise time and half-relaxation time were determined before

and after the stimulation protocol. Rise time was defined as the time elapsed from the base to the peak of a single twitch. Half-relaxation time was defined as the time elapsed from the peak of a single twitch to the point of the twitch amplitude returning halfway to baseline. All muscle function data were collected through an AD Instruments Bridge Amp and Powerlab 4/30, and analyzed with Chart5 PowerLab software.

Tissue Collection and Blood analyses.

Following the muscle stimulation protocol, the animal was euthanized via decapitation and tissues were immediately collected and either snap-frozen in liquid nitrogen or mounted on cork using mounting medium and quick-frozen in liquid nitrogen-cooled isopentane. Blood samples were collected and centrifuged at 16000*g* for 2 minutes to separate plasma. Plasma samples were analyzed for non-esterified fatty acids (NEFA; HR Series NEFA-HR2 kit; Wako Diagnostics, Richmond, VA), glucose (YSI-2300 Stat-Plus glucose/lactate analyzer, Interscience, Toronto, ON), and insulin concentrations (90080 Ultra Sensitive Mouse Insulin ELISA Kit , Crystal Chem Inc., IL).

Enzyme Activity Assays.

3-β-hydroxyacyl coenzyme-A dehydrogenase (β-HAD) and citrate synthase (CS) activity were determined as described previously (Carter et al. 2001) on soleus and white gastrocnemius muscle. Sample activities were normalized via Bradford-determined protein concentration (Bradford. 1976).

Western Blot Analysis.

Homogenates of soleus and white gastrocnemius muscles were prepared as described previously (Melling et al. 2007) . Equal amounts of each protein sample (Bradford. 1976) were loaded, resolved by SDS-PAGE, transferred to PVDF membranes and probed using antibodies

for FAT/CD36 (Santa Cruz, CA), cytochrome C (courtesy of Dr. Hood, York University (Stevens, Nishio, and Hood. 1995)), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; AbCam, MA). Primary antibodies were detected using the species-appropriate HRP-conjugated secondary antibodies and signals visualized on film using chemiluminescent reagent (Amersham). Target band optical intensity was quantified using Scion Image software (Scion Corporation) and presented as arbitrary units (AU).

Histochemical Analyses.

Metachromatic fiber type staining was performed on gastrocnemius/plantaris cryosections (10 µm) using a modified Ogilvie and Feeback protocol (Krause et al. 2008; Ogilvie and Feeback. 1990). The red gastrocnemius, white gastrocnemius, and plantaris regions were identified on each section and a representative image of each muscle region was acquired for analysis. Approximately 200 fibers were counted per microscope image per animal to determine fiber type composition. Fiber area was assessed on ~25 fibers/image for each fiber type using Scion Image (n=9 in each group).

Intramyocellular lipid (IMCL) content was determined by Oil-Red-O staining tibialis anterior sections (Koopman, Schaart, and Hesselink. 2001). Optical intensity of IMCL droplet staining was assessed in the red region of the tibialis anterior as previously described (Krause et al. 2008) with Scion Image (~38 fibers/image were quantified; Control n=9, *Ins2*$^{Akita+/-}$ n=7, STZ n=9).

Muscle capillary-to-fiber ratio was assessed by the Lead ATPase stain as previously described (Krause et al. 2008; Rosenblatt et al. 1987) using the same muscle regions examined for fiber type and fiber area. An average of 377 capillaries and 196 fibers were manually

counted in each microscope image and expressed as a capillary:fiber ratio (Control n=7, *Ins2*$^{Akita+/-}$ n=8, STZ n=8).

All images were acquired with a Nikon Eclipse 90*i* microscope and Q-Imaging MicroPublisher 3.3 RTV camera with Q-Capture software.

Data Analyses.

All statistical analyses were performed with GraphPad Prism 5 software. Differences between Control, *Ins2*$^{Akita+/-}$, and STZ groups were determined by one-way (area under fatigue curve, body and tissue masses, body growth rate, fasting plasma glucose and non-esterified fatty acid concentrations, food and water consumption, white gastrocnemius fiber area, Western blot optical intensities, enzyme activities) or two-way (capillary-to-fiber ratio, fiber type composition, red gastrocnemius and plantaris fiber areas, 2-minute fatigue curve, all pre- versus post-fatigue contraction measures) ANOVA followed by Bonferroni post-hoc tests. P values less than 0.05 were considered significant. All data presented are mean ± standard error.

RESULTS

Animal Characteristics.

Assessment of fed body mass throughout the experiment revealed that Control mice gained an average of 0.86 ± 0.07 g/wk, demonstrating that they were in the adolescent phase of development (Fig. 2.1A). *Ins2*$^{Akita+/-}$ mouse fed body mass did not significantly differ from Control until the 7th week of diabetes, while STZ mouse body mass became significantly different from both Control and *Ins2*$^{Akita+/-}$ mice at 3 weeks of diabetes (Fig. 2.1A). Assessment of fasted body mass following the 8 week experimental period revealed no difference between STZ and *Ins2*$^{Akita+/-}$ mice, while both weighed significantly less than control (Fig. 2.1B).

Surprisingly, only *Ins2*$^{Akita+/-}$ mice exhibited hyperphagia (Fig. 2.1C) while polydipsia was markedly elevated in both diabetic groups (Fig. 2.1D).

Both diabetic models exhibited significantly lower absolute muscle, heart, and epididymal fat mass compared to Control mice (Fig. 2.1E-F). In addition to the severe reduction in epididymal fat mass, other fat depots (subcutaneous and perirenal) were virtually undetectable in both diabetic models (data not shown). When tissue masses were expressed relative to body mass, a relative fat mass loss was also observed (Fig. 2.1H). Both diabetic models exhibited a significant decrease in relative muscle mass, indicating a specific loss of skeletal muscle mass (i.e. diabetic myopathy). STZ mice have a significantly lower relative heart mass compared to *Ins2*$^{Akita+/-}$ mice though the cause of this is unknown.

Ins2$^{Akita+/-}$ and STZ mice had similar fed blood glucose profiles throughout the 8-week diabetic period though STZ mice were slower to reach peak blood glucose values (Fig. 2.2A). STZ and *Ins2*$^{Akita+/-}$ mice had similar plasma glucose values following an overnight fast (Fig. 2.2B). Fasting plasma insulin was not statistically different between any group (Control: 0.214±0.022 ng/ml; *Ins2*$^{Akita+/-}$: 0.173±0.019 ng/ml; STZ: 0.175±0.022 ng/ml), consistent with previous diabetes studies using STZ or *Ins2*$^{Akita+/-}$ mice in the fasted state (Yoshioka et al. 1997; Chan et al. 2005b; Chan et al. 2005a; Hong et al. 2007).

Muscle Phenotype.

In red gastrocnemius, a significant loss of type IIB/D fibers was observed in *Ins2*$^{Akita+/-}$ compared to Control mice, while no significant fiber type loss was observed in STZ mice (Fig. 2.3A). Determination of fiber area in this muscle revealed a significantly lower type IIB/D fiber size in both diabetic models compared to Control, while *Ins2*$^{Akita+/-}$ also displayed smaller type IIA fibers compared to Control (Fig. 2.3B). Notably, STZ muscle exhibited significantly larger

type I fibers compared to *Ins2*$^{Akita+/-}$ muscle. In the plantaris, a muscle generally devoid of type I fibers, no significant fiber type percentage shift occurred (Control: 0.7±1.8% type I, 38.7±8.7% type IIA, 60.6±8.8% type IIB/D; *Ins2*$^{Akita+/-}$: 0.2±0.4% type I, 40.0±4.1% type IIA, 59.8±3.9% type IIB/D; STZ: 5.4±5.0% type I, 33.8±8.2% type IIA, 60.8±9.0% type IIB/D). However, a significant reduction in type IIB/D fiber area in both diabetic models versus Control was observed. STZ also exhibited a reduced type IIA fiber area in the plantaris muscle (Control: 1256±244μm^2 type IIA, 2177±379μm^2 IIB/D; *Ins2*$^{Akita+/-}$: 1003±239μm^2 type IIA, 1551±390μm^2 type IIB/D; STZ: 834±131μm^2 type IIA, 1320±104μm^2 type IIB/D). Examination of white gastrocnemius fiber area also demonstrated smaller IIB/D fibers in STZ and *Ins2*$^{Akita+/-}$ compared to Control (Control: 2456±359μm^2; *Ins2*$^{Akita+/-}$: 1698±266μm^2; STZ: 1836±344μm^2). Taken together, these findings suggest that diabetic skeletal muscles shift away from a glycolytic phenotype.

As alterations in fiber type composition may be indicative of changes to other fiber characteristics, such as lipid storage, we determined IMCL content of individual TA muscle fibers. The optical intensity of Oil-Red-O was significantly elevated in STZ muscle fibers compared to both Control and *Ins2*$^{Akita+/-}$ (Fig. 2.4A). Given this striking change in lipid storage, it was prudent to examine measures of lipid uptake and utilization. Fasting NEFA concentrations were found to be significantly elevated in STZ mice compared to both Control and *Ins2*$^{Akita+/-}$ mice (Fig. 2.4B). FAT/CD36, a protein involved in fatty acid transport into the muscle fiber (Chabowski et al. 2004; Keizer et al. 2004), was significantly elevated in the oxidative soleus of both diabetic models (Fig. 2.4D) but not the largely glycolytic white gastrocnemius (data not shown). While no change in cytochrome C expression in the soleus of either diabetic model was observed (Control: 1.00±0.23 AU; *Ins2*$^{Akita+/-}$: 0.80±0.38 AU; STZ:

0.83±0.42 AU), β-HAD activity was depressed in STZ soleus compared to *Ins2*$^{Akita+/-}$ (Fig. 2.4E) and citrate synthase (CS) activity was depressed in STZ soleus compared to Control (Fig. 2.4F).

A decrease in muscle capillary-to-fiber ratio has been previously demonstrated in STZ muscle (Sexton, Poole, and Mathieu-Costello. 1994), therefore, we were interested to determine if this change occurred in *Ins2*$^{Akita+/-}$ mice. A significant reduction in capillary-to-fiber ratio in both diabetic models compared to Control mice was detected in the plantaris and red gastrocnemius muscles, but not in white gastrocnemius (Fig. 2.5A).

Muscle Function.

To ascertain if the phenotypic and metabolic changes occurring in T1DM muscle resulted in functional impairment, we examined several contractile parameters of the GPS muscle group. At each stimulation frequency tested, *Ins2*$^{Akita+/-}$ mice consistently displayed reduced tetanic contractile force compared to other groups (Fig. 2.6A; significant main effect of mouse model). Examination of absolute peak tetanic force revealed a significant ~29% loss of force in *Ins2*$^{Akita+/-}$ mice compared to Control mice prior to fatigue (Fig. 2.6C). When expressed relative to muscle mass, there was no significant reduction in force suggesting that the cause of reduced absolute force was the decrease in muscle mass (Fig. 2.6D). In contrast to *Ins2*$^{Akita+/-}$ muscle, STZ mice exhibit the greatest relative peak force (Fig. 2.6D; significant main effect of mouse model). No differences in fatigue rates were detected between groups during the low-frequency fatigue protocol (Fig. 2.6B).

To gain insight into muscle twitch characteristics and Ca^{2+} handling capacity of each mouse model, we measured twitch rise time and half-relaxation time. Analysis of twitch rise time revealed a significant difference between STZ and *Ins2*$^{Akita+/-}$ mice both before and

following muscle fatigue (Fig. 2.6G). Half-relaxation time was also found to be elevated in STZ mice following the fatigue protocol compared to both Control and *Ins2*$^{Akita+/-}$ mice (Fig. 2.6H).

DISCUSSION

The goal of the current study was to gain a more comprehensive understanding of diabetic myopathy by studying skeletal muscle from two different rodent models of T1DM; the commonly utilized, pharmacologically-induced, STZ mouse and the *Ins2*$^{Akita+/-}$ mouse (Yoshioka et al. 1997), a genetic model of T1DM. While both models exhibited extreme hyperglycemia throughout the 8 week experimental period and both developed some myopathic conditions, we have identified several novel dissimilarities between these models that require consideration given the divergent modes in which diabetes is initiated. Of particular note, we found that compared with muscle from *Ins2*$^{Akita+/-}$ mice, the muscles from STZ mice have: 1) an increase in IMCL content likely the result of a two-fold increase in circulating NEFA and reduced CS and β-HAD activity, 2) elevated type I fiber area compared to *Ins2*$^{Akita+/-}$ muscle, 3) a paradoxical increase in relative muscle twitch and tetanic force and, following fatigue, a reduced rate of relaxation. These changes do not appear to be the result of differences in hyperglycemia/hypoinsulinemia as both groups had similar exposure to glucose throughout the last 6 weeks of diabetes and fasted insulin levels at the time of harvest were not different between models. Rather, we believe that the pharmacological agent streptozotocin has other effects on muscle that are independent of the typical diabetic phenotype.

As direct effects of streptozotocin on muscle both at the molecular and cellular level have previously been demonstrated (Johnston et al. 2007), we propose that interpretation of previous diabetic myopathy research employing STZ-induced diabetic models should be made cautiously.

The present study used a streptozotocin dose lower than what is commonly used to induce T1DM in mice (120 vs. ~200mg/kg) (Klueber and Feczko. 1994; Fahim, el-Sabban, and Davidson. 1998; Vignaud et al. 2007; Fewell and Moerland. 1995; Lehti et al. 2007) suggesting that, even at lower doses, toxicity may still be a concern. Although rats typically do not require doses as high as mice, the changes to muscle phenotype observed in our STZ mice are congruent with those seen previously in STZ rats (Armstrong, Gollnick, and Ianuzzo. 1975; Chao et al. 1976; Armstrong and Ianuzzo. 1977; Sexton, Poole, and Mathieu-Costello. 1994).

IMCL levels are a result of the balance between lipid availability (and uptake) and metabolism. Here we demonstrate that IMCL content in STZ muscle was elevated compared to *Ins2*$^{Akita+/-}$ and Control muscle (Fig. 2.4A,C) and propose this is the result of elevated NEFA levels (Fig. 2.4B), increased FAT/CD36 protein expression (Fig. 2.4D), and depressed CS and β-HAD activity (Fig. 2.4E,F). Hyperglycemia and hypoinsulinemia have been demonstrated to induce FAT/CD36 expression (Luiken et al. 2002; Chen et al. 2006) resulting in elevated transport of fatty acids across muscle membranes (Luiken et al. 2002) and thereby increasing IMCL levels. While an increase in FAT/CD36 was observed in *Ins2*$^{Akita+/-}$ muscle, no increase in IMCL was observed possibly due to sustained activity of muscle enzymes involved in lipid metabolism (Fig. 2.4E,F). Similar to the present findings, Hong *et al.* (2007) demonstrate normal plasma NEFA levels, as well as lower plasma triglyceride and IMCL levels in *Ins2*$^{Akita+/-}$ at 13 weeks of age. It remains possible that IMCL and plasma NEFA levels were elevated in *Ins2*$^{Akita+/-}$ mice at some point during the 8 week experimental period but had normalized by the time of tissue collection because the muscle's high reliance on this fuel as a source of ATP. Human studies have demonstrated either increased (Perseghin et al. 2003), or unchanged (Bernroider et al. 2005) IMCL levels.

The current study did not assess hepatic lipid content or lipid transport proteins such as CD36 in the liver of *Ins2*$^{Akita+/-}$mice, though others demonstrate low levels of liver triglycerides in *Ins2*$^{Akita+/-}$mice (Hong et al. 2007). Similar to the current study, plasma NEFA was not different from control mice, though plasma triglycerides were lower in *Ins2*$^{Akita+/-}$ mice (Hong et al. 2007). Using STZ-diabetic rats, Luiken *et al*. (2002) demonstrate low palmitate transport across hepatic membranes, despite increased in CD36 expression (Luiken et al. 2002). Whether a similar limitation in transport or increased metabolism of lipids is responsible for decreased liver triglycerides in the *Ins2*$^{Akita+/-}$ liver is unknown.

Differences in fiber type composition and fiber area were also detected in diabetic muscle. *Ins2*$^{Akita+/-}$ red gastrocnemius displayed a loss of fast-glycolytic (type IIB/D) fiber number and an atrophy of all fast (type IIA and IIB/D) fibers, while STZ red gastrocnemius displayed atrophied type IIB/D fibers and hypertrophy of slow oxidative (type I) fibers when compared to *Ins2*$^{Akita+/-}$ (Fig. 2.3A,B). Though the term atrophy has been applied to the reduced fiber area observed in these adolescent diabetic models, it cannot be ruled out that the reduced fiber area is the result of attenuated fiber growth. The findings in our STZ mice are consistent with observations in STZ rodents (Armstrong, Gollnick, and Ianuzzo. 1975; Klueber and Feczko. 1994). The cause of the increase in type I fiber area is currently unknown; though abundant availability of lipid may have promoted the increased expression of type I myosin. Support for this comes from a recent study demonstrating increased type I myosin expression in obese mice during the early stages of metabolic syndrome (de Wilde et al. 2008). It is important to note that human studies demonstrate increased glycolytic fiber type composition (Fritzsche et al. 2008) and increase glycolytic metabolism (Crowther et al. 2003), in opposition to the findings of the current study and others (Armstrong, Gollnick, and Ianuzzo. 1975; Klueber and Feczko. 1994).

This could be reflective of several factors such as age, disease duration, physical activity levels, diet, and likely most importantly, insulin treatment.

Our findings of a decreased capillary-to-fiber ratio, though surprising given the reduction in glycolytic fiber number and fast fiber atrophy, are consistent with previous results (Sexton, Poole, and Mathieu-Costello. 1994). One would hypothesize that capillary-to-fiber ratio is influenced by fiber area and therefore the reduction in fiber area was accompanied by a proportionate decrease in capillary-to-fiber ratio.

Ins2$^{Akita+/-}$ and Control mice both became significantly heavier than STZ mice by 3 weeks of diabetes (Fig. 2.1A). *Ins2*$^{Akita+/-}$ fed body mass did not significantly differ from Control mice until 7 weeks of diabetes, while the STZ mice remained behind in growth throughout the experiment. Essentially, STZ mice failed to grow during adolescence; a period characterized by rapid growth. Previous studies have demonstrated that streptozotocin caused: 1) an immediate loss of body and muscle mass despite aggressive insulin therapy and maintenance of euglycemia (Johnston et al. 2007), 2) G2 cell cycle arrest of muscle progenitor cells (Johnston et al. 2007), and 3) DNA alkylation (Bennett and Pegg. 1981; Brambilla et al. 1987). Thus, it is likely that these direct effects of streptozotocin on skeletal muscle contributed to the 6 week delay in growth in STZ mice. Conversely, *Ins2*$^{Akita+/-}$ mice did not differ from Control mice until 7 weeks of diabetes. The *Ins2*$^{Akita+/-}$ mice also exhibited hyperphagia, which may have helped maintain body mass for the first 6 weeks of diabetes. This is clinically relevant, as it demonstrates that attenuated weight gain may not become apparent until several weeks following disease onset, though accelerated food consumption may be a recognizable early symptom of the disease. Early recognition of T1DM is critical, given that early intervention and aggressive insulin treatment can significantly reduce the severity of T1DM complications and normalize growth

(Keen. 1994). Importantly, it remains to be elucidated whether early intervention attenuates physical disability later in life. As skeletal muscle is the primary organ of glucose disposal and an individual's physical capacities are tightly coupled to their muscle mass, understanding the basic mechanisms underlying diabetic myopathy has tremendous importance to the development of appropriate and successful long-term therapeutic strategies to improve life expectancy, quality of life and reduce overall health-care costs.

Given the notable changes in muscle phenotype, we hypothesized that muscle strength would be reduced following 8 weeks of diabetes. Indeed, a significant decrease in absolute force was observed in *Ins2*$^{Akita+/-}$ mice (Fig. 2.6A,C), with this magnitude of force loss consistent with previous human studies (Andersen et al. 1996; Andersen. 1998). In contrast, however, muscles from STZ mice demonstrated similar absolute peak force to the Control mice despite marked muscle atrophy (Fig. 2.6A,C). In human studies, strength or torque measurements cannot be easily corrected for the mass of the muscle tested, however in the present study, when force is corrected for muscle mass, *Ins2*$^{Akita+/-}$ did not exhibit a force deficit, while STZ mice displayed increased relative force (Fig. 2.6D). Previous studies employing STZ diabetic rodents have demonstrated mixed findings with respect to force production. Consistent with human studies (Andersen et al. 1996; Andersen. 1998), absolute force is usually found to decrease (Fahim, el-Sabban, and Davidson. 1998; Vignaud et al. 2007; Sanchez et al. 2005; Lesniewski, Miller, and Armstrong. 2003). However, studies presenting force data relative to muscle mass reveal decreases (Lesniewski, Miller, and Armstrong. 2003; McGuire and MacDermott. 1999; Stephenson, O'Callaghan, and Stephenson. 1994), increases (Vignaud et al. 2007; McGuire et al. 2001; Ganguly et al. 1986), or no change (Sanchez et al. 2005; Lesniewski, Miller, and Armstrong. 2003; McGuire and MacDermott. 1999; Stephenson, O'Callaghan, and Stephenson.

1994). The basis for these discrepancies is not clearly understood, though it may involve the variability in STZ dosage, duration of untreated diabetes, the muscle group or fiber types studied, or the broad range of muscle stimulation protocols employed. A previous study that approximates the design of the current study demonstrated absolute force loss in *Ins2*$^{Akita+/-}$ and STZ skeletal muscle compared to non-diabetic controls (Vignaud et al. 2007) . However, similar to the present study, when force was expressed relative to muscle mass, STZ mice exhibited greater contractile force compared to non-diabetic controls.

The basis for this improved contractile performance in STZ-treated diabetic mice is surprising given the shift away from fast-glycolytic fiber type composition. A possible explanation for increased relative force in STZ muscle may be that calcium release/uptake dynamics and/or sensitivity have been altered (McGuire and MacDermott. 1999; McGuire et al. 2001; Ganguly et al. 1986). Our examination of muscle calcium handling characteristics revealed that STZ mice exhibited increased twitch force, a trend for longer rise time, and longer half-relaxation time in the post-fatigue period (Fig. 2.3D-F), consistent with previous studies of STZ muscle twitch characteristics (McGuire and MacDermott. 1999; McGuire et al. 2001). Furthermore, STZ muscle was previously demonstrated to exhibit hyperactive Ca^{2+} kinetics and sarcoplasmic reticulum (SR) Ca^{2+}-ATPase activity (Ganguly et al. 1986), and augmented contractile force following caffeine exposure (Lesniewski, Miller, and Armstrong. 2003) which may be an effect of SR or plasma membrane instability (Ganguly et al. 1986). In the present study these alterations to muscle twitch characteristics were observed in the STZ mice but not the *Ins2*$^{Akita+/-}$ mice, indicating that STZ may be directly altering Ca^{2+} handling, irrespective of the diabetic muscle phenotype.

The current study has comprehensively characterized the phenotype and functionality of skeletal muscle from two different models of T1DM. Our focus for future diabetic myopathy research is now on the *Ins2*$^{Akita+/-}$ mouse, which exhibits a fiber type shift away from fast-glycolytic fibers and an increased ability for lipid transport into the muscle though no dyslipidemia following 8 weeks of overt diabetes. Despite marked muscle atrophy, contractile properties were not altered when expressed relative to muscle mass, suggesting that metabolic changes within muscle fibers precede detectable impairments in contractile properties. Little is currently known about human muscle during the early stages of T1DM prior to diagnosis and insulin therapy, thus, it is critical that contributions to this field of research are physiologically relevant and not a result of pharmacological side-effects of the diabetes-inducing agent as has been reported (Johnston et al. 2007). As *Ins2*$^{Akita+/-}$ mice become diabetic due to a spontaneous mutation of the Insulin-2 gene, glycemia and insulinemia have been the only factors identified as altered (Yoshioka et al. 1997; Vignaud et al. 2007). While most of what is understood about muscle phenotype and function in early stage T1DM comes from studies employing STZ-induced diabetic rodents, the current study has illustrated several deviations in muscle phenotype, metabolism and function from the *Ins2*$^{Akita+/-}$ mouse. Given the known toxic effects of STZ on skeletal muscle (Johnston et al. 2007), interpretation of results from previous reports using STZ-induced diabetic models should be made cautiously and future animal studies should consider alternative models of T1DM.

FIGURES

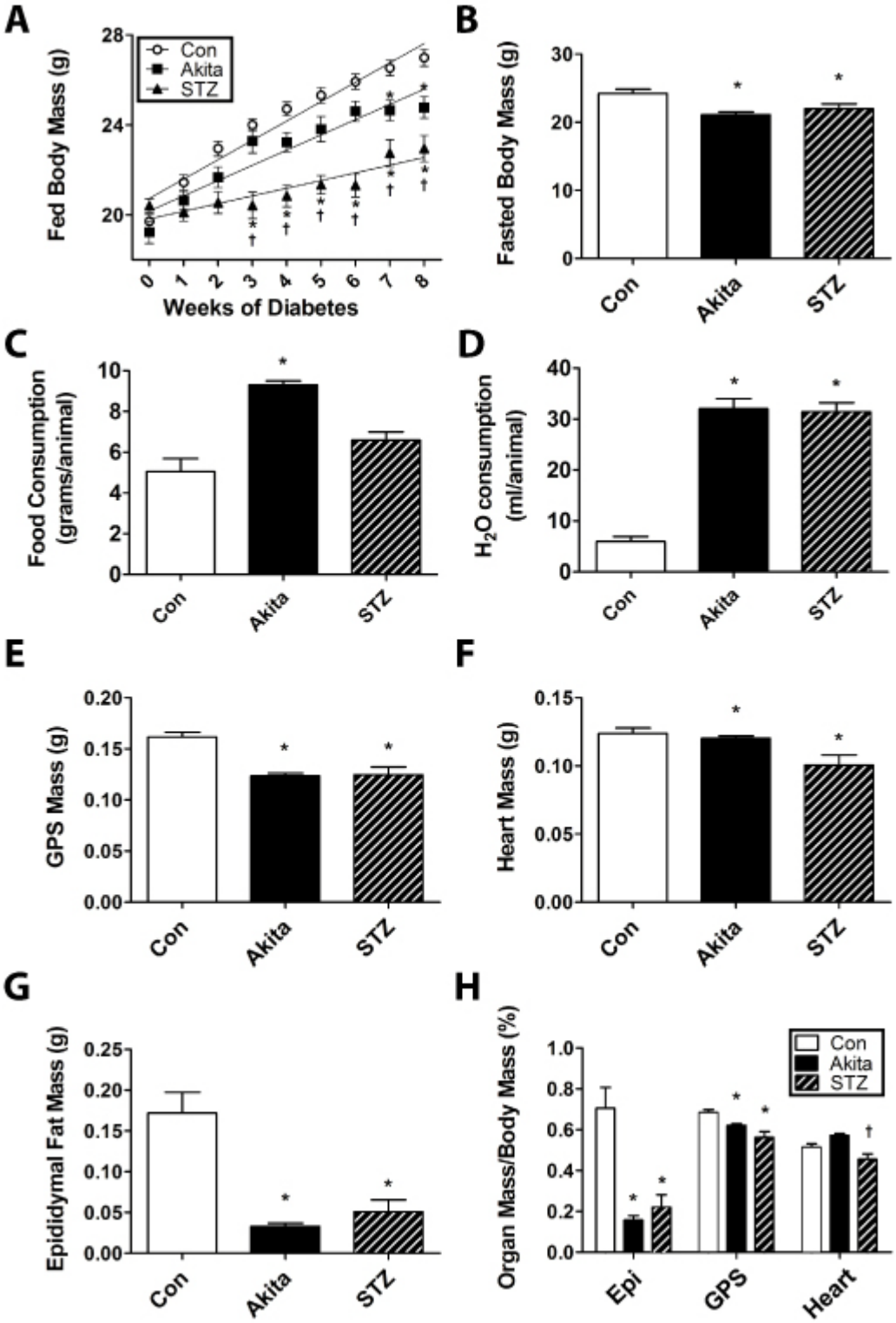

Figure 2.1. Alterations to body and tissue masses in the diabetic state. **A)** Fed body mass measured during 8-week period of diabetes revealed that *Ins2*$^{Akita+/-}$ mice maintain body mass

until the 7th week of diabetes, while STZ mice exhibit a statistically significant growth deficit by the 3rd week. * indicates a significant difference from Control (Con), † indicates a significant difference from *Ins2*$^{Akita+/-}$. Line of best fit, representing the average rate of growth, is illustrated. Control and *Ins2*$^{Akita+/-}$ mice were not significantly different (Control: 0.86 ± 0.07 g/wk; *Ins2*$^{Akita+/-}$: 0.6773 ± 0.04 g/wk). However, STZ mice had a significantly lower growth rate than both Control and *Ins2*$^{Akita+/-}$ (0.3414 ± 0.06 g/wk). **B)** Fasted body mass at 8 weeks of diabetes (~13 weeks of age) demonstrated that *Ins2*$^{Akita+/-}$ and STZ mice weighed significantly less than Control mice. **C)** Only *Ins2*$^{Akita+/-}$ mice demonstrated significantly elevated food consumption, though it is worth noting that the STZ mice were not statistically different either Control or *Ins2*$^{Akita+/-}$ mice. **D)** Water consumption was markedly elevated in both diabetic models. **E)** Gastrocnemius-plantaris-soleus (GPS) complex mass and **F)** heart mass were also significantly decreased in both diabetic models. **G)** Epididymal fat mass was markedly reduced in both *Ins2*$^{Akita+/-}$ and STZ mice compared to Control mice. **H)** Organ masses expressed as a percentage of body mass. Notably, relative heart mass is significantly less in STZ mice compared to *Ins2*$^{Akita+/-}$ mice. (* indicates a significant difference compared to Control group, † indicates a significant difference compared to *Ins2*$^{Akita+/-}$ group).

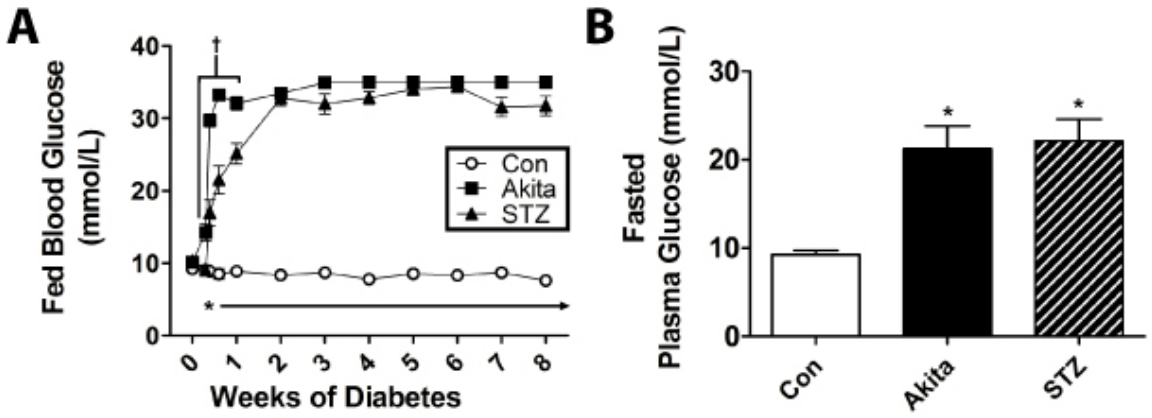

Figure 2.2. Alterations to blood glucose and feeding habits in the diabetic state. **A)** Blood glucose values peaked in *Ins2*$^{Akita+/-}$ mice before STZ mice († indicates a significant difference between STZ and *Ins2*$^{Akita+/-}$ groups), although both diabetic models became significantly hyperglycemic within three days (* indicates a significant difference between Con (Control) and both diabetic groups). **B)** Following an overnight fast, *Ins2*$^{Akita+/-}$ and STZ mice had similar plasma glucose values.

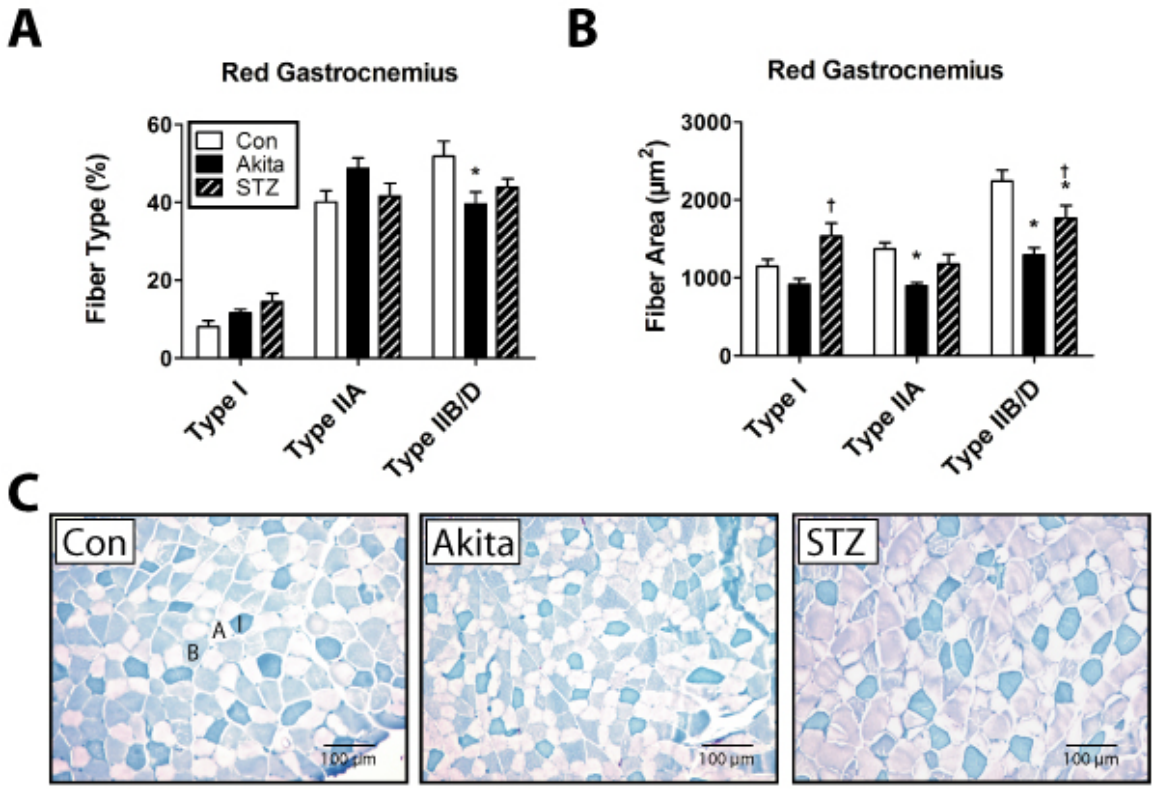

Figure 2.3. Differences in fiber type composition and fiber area are found between diabetic models. **A)** Examination of fiber type percentage of red gastrocnemius revealed a significant interaction between fiber type and mouse model (P=0.0015). A significant decrease in the percentage of type IIB/D fibers in *Ins2*$^{Akita+/-}$ mice compared to Con (Control) mice was detected (indicated by *), while STZ mice did not differ from either group. **B)** Examination of red gastrocnemius fiber area revealed that type I fibers were significantly larger in STZ mice compared to *Ins2*$^{Akita+/-}$ mice, while type IIA fibers were significantly smaller in *Ins2*$^{Akita+/-}$ mice compared to Control mice. *Ins2*$^{Akita+/-}$ mice also had significantly smaller type IIB/D fibers compared to both STZ and Control mice, though STZ IIB/D fibers were also significantly smaller than those of Control (* indicates a significant difference compared to Control group, † indicates a significant difference compared to *Ins2*$^{Akita+/-}$ group). **C)** Representative images of

metachromatic fiber type stains of Control, *Ins2*$^{Akita+/-}$, and STZ mouse red gastrocnemius. Fiber types are indicated (I = type I, A = type IIA, B = type IIB/D). Note the visibly larger type I fibers in the STZ muscle (bar represents 100 μm). (* indicates a significant difference compared to Control group, † indicates a significant difference compared to *Ins2*$^{Akita+/-}$ group.)

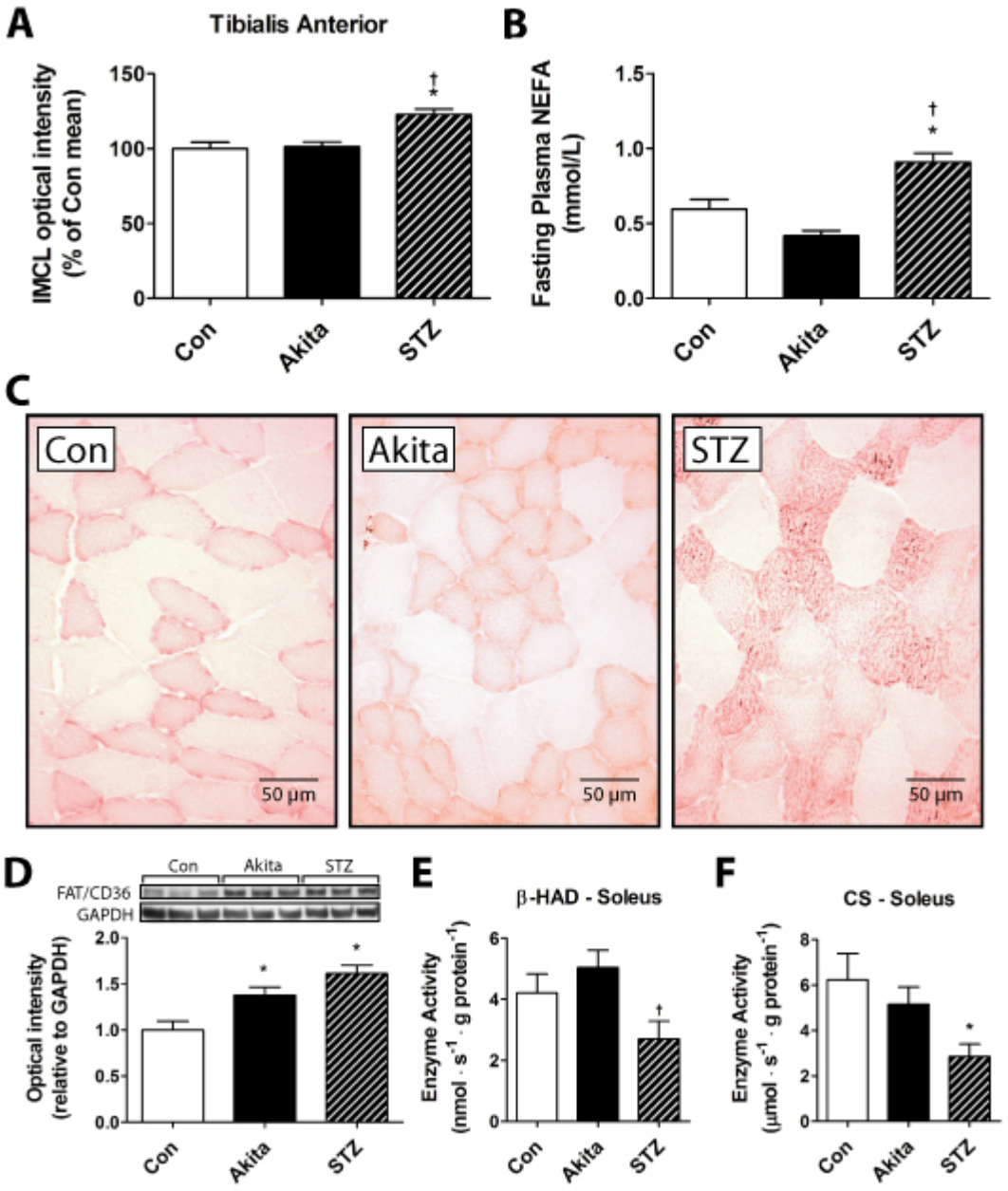

Figure 2.4. Intramyocellular lipid accumulation and elevated plasma NEFA occurs in STZ but not Ins2$^{Akita+/-}$ mice. **A)** Mean optical intensity of intramyocellular lipid (IMCL) stained by oil red O expressed relative to Control mean. STZ mice exhibit elevated IMCL optical intensity compared to both Con (Control) and *Ins2*$^{Akita+/-}$ mice. **B)** STZ mice exhibited a significant increase of non-esterified fatty acids (NEFA) measured in the blood plasma compared to both Control and *Ins2*$^{Akita+/-}$ mice. **C)** Representative images of an oil red O stain of Control,

Ins2$^{Akita+/-}$, and STZ mouse tibialis anterior muscles. (Bar represents 50 μm). **D)** Western blot analysis of FAT/CD36 showed that both diabetic groups had significantly increased FAT/CD36 protein compared to Control. **E)** Activity of the enzyme 3-β-hydroxyacyl coenzyme-A dehydrogenase (β -HAD) was depressed in STZ compared to *Ins2*$^{Akita+/-}$ mice, while citrate synthase (CS) activity **(F)** was lower in STZ compared to Control mice. (* indicates a significant difference compared to Control group, † indicates a significant difference compared to *Ins2*$^{Akita+/-}$ group.)

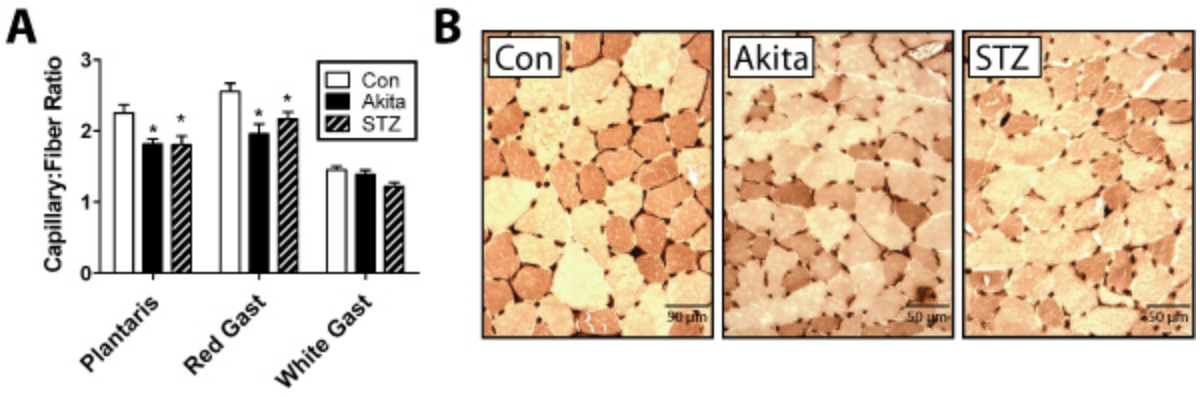

Figure 2.5. Reduced capillary-to-fiber ratio is seen in both diabetic groups. A) Capillary-to-fiber ratio is lower in both *Ins2*$^{Akita+/-}$ and STZ plantaris and red gastrocnemius muscle compared to Con (Control; indicated by *). **B)** Representative images of a lead ATPase stain of Control, *Ins2*$^{Akita+/-}$, and STZ mouse red gastrocnemius. Capillaries appear as dark brown structures in between the muscle fibers (bar represents 50 µm).

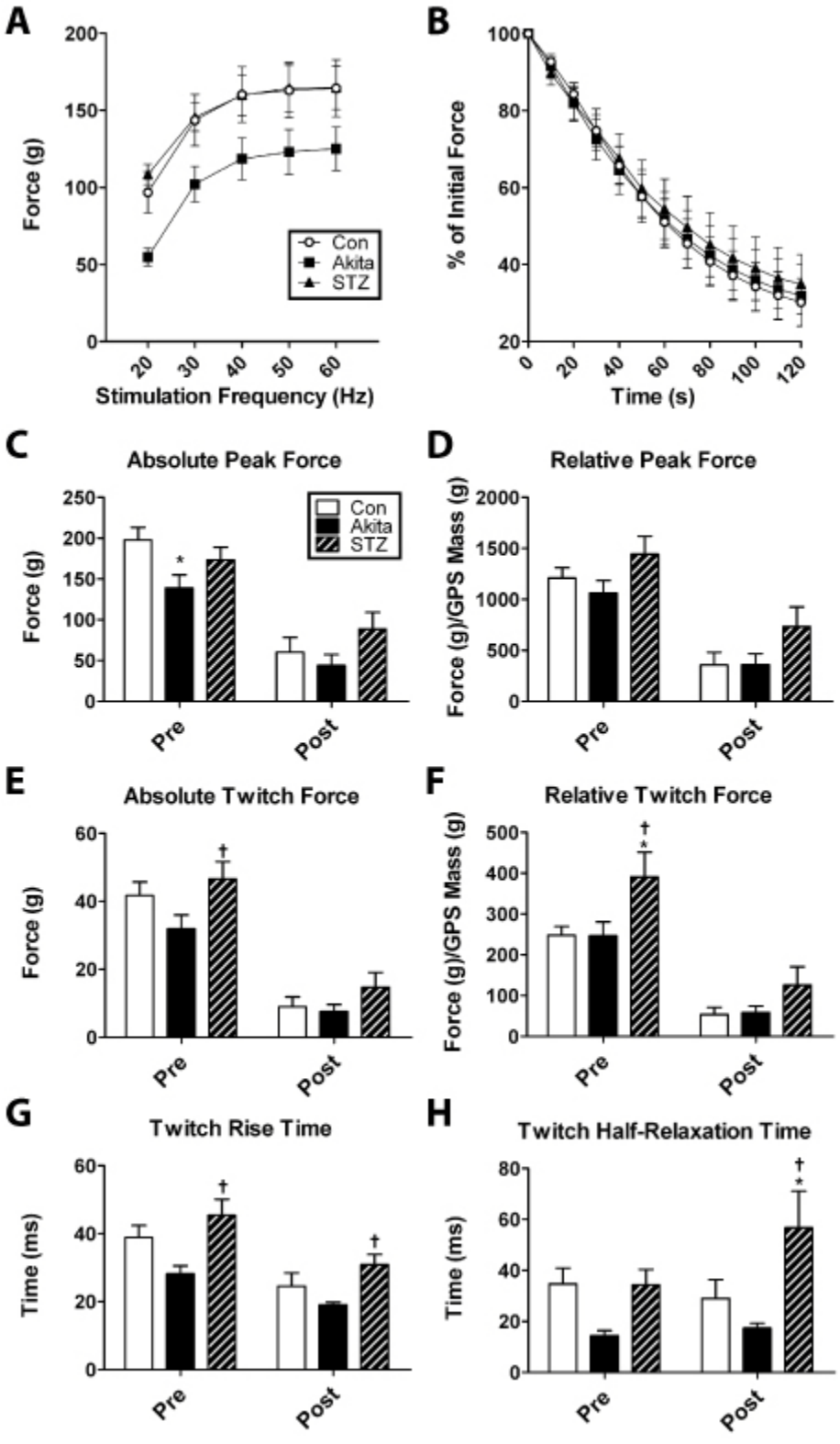

Figure 2.6. In situ muscle stimulation reveals loss of muscle force in Ins2$^{Akita+/-}$ mice proportional to loss of muscle mass and altered contractile properties in STZ mice. A) At

increasing stimulation frequencies, *Ins2*$^{Akita+/-}$ mice display poor peak contractile force compared to other groups ($P<0.05$: main effect of mouse model). **B)** No difference between groups was observed in contractile force during fatigue protocol expressed as a percentage of initial force. **C)** Absolute peak contractile force was found to be significantly lower in *Ins2*$^{Akita+/-}$ mice compared to Con (Control) mice prior to the fatigue protocol (significant difference indicated by *). **D)** Peak contractile force, when corrected to GPS muscle mass, was found to higher in STZ mice ($P<0.05$: main effect of mouse model). **E)** Twitch force was found to be higher in STZ mice compared to *Ins2*$^{Akita+/-}$ prior to fatigue (significant difference indicated by †). **F)** Relative to GPS muscle mass, twitch force was greater in STZ mice compared to both Control and *Ins2*$^{Akita+/-}$ mice prior to fatigue (indicated by * and †). **G)** Twitch rise time was found elevated in STZ compared to *Ins2*$^{Akita+/-}$ mice both before and following fatigue (indicated by †). **H)** In the post fatigue period, STZ mice exhibit longer half-relaxation time than both Control and *Ins2*$^{Akita+/-}$ mice (indicated by * and †).

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CHAPTER 3

Inhibition of Plasminogen Activator Inhibitor-1 Restores Skeletal Muscle Regeneration in Untreated Type 1 Diabetic Mice

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PREFACE

Significance to thesis

The primary goal of this study was to determine a time course of skeletal muscle regeneration in WT and diabetic mice, and to elucidate the mechanism underlying any impaired regeneration. Given that PAI-1 is a known inhibitor of the muscle regeneration process and is chronically elevated in type 1 diabetes mellitus, we inhibited PAI-1 pharmacologically in the diabetic mice to determine if PAI-1 was a primary contributor to the impaired muscle regeneration observed in diabetic mice.

Authors' contributions

Matthew P. Krause contributed to the design of the study, performed all experiments and data collection, performed and supervised microscope image acquisition and 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.

Jasmin Moradi contributed to the design of the study, assisted in animal care, assisted in western blot and immunofluorescent staining, assisted in microscope image analysis, and contributed to the writing of the initial draft of the manuscript.

Aliyah A. Nissar assisted in animal handling/care and refining drafts of the manuscript.

Michael C. Riddell contributed to the design of the study, and worked on refining drafts of the manuscript and the revisions based on editorial review.

Thomas J. Hawke contributed to the design of the study, assisted in experiments, and worked on refining drafts of the manuscript and the revisions based on editorial review.

ABSTRACT

Type 1 diabetes leads to impairments in growth, function, and regenerative capacity of skeletal muscle; however, the underlying mechanisms have not been clearly defined. With the use of *Ins2*$^{WT/C96Y}$ mice (model of adolescent-onset type 1 diabetes), muscle regeneration was characterized in terms of muscle mass, myofiber size (cross-sectional area), and protein expression. Blood plasma was analyzed for glucose, nonesterified fatty acids, insulin, and plasminogen activator inhibitor-1 (PAI-1). PAI-039, an effective inhibitor of PAI-1, was orally administered to determine if PAI-1 was attenuating muscle regeneration in *Ins2*$^{WT/C96Y}$ mice. *Ins2*$^{WT/C96Y}$ mice exposed to 1 or 8 weeks of untreated type 1 diabetes before chemically induced muscle injury display significant impairments in their regenerative capacity as demonstrated by decreased muscle mass, myofiber cross-sectional area, myogenin, and Myh3 expression. PAI-1, a physiologic inhibitor of the fibrinolytic system and primary contributor to other diabetes complications, was more than twofold increased within 2 weeks of diabetes onset and remained elevated throughout the experimental period. Consistent with increased circulating PAI-1, regenerating muscles of diabetic mice exhibited excessive collagen levels at 5 and 10 days postinjury with concomitant decreases in active urokinase plasminogen activator and matrix metalloproteinase-9. Pharmacologic inhibition of PAI-1 with orally administered PAI-039 rescued the early regenerative impairments in noninsulin-treated *Ins2*$^{WT/C96Y}$ mice. Taken together, these data illustrate that the pharmacologic inhibition of elevated PAI-1 restores the early impairments in skeletal muscle repair observed in type 1 diabetes and suggests that early interventional studies targeting PAI-1 may be warranted to ensure optimal growth and repair in adolescent diabetic skeletal muscle.

INTRODUCTION

With type 1 diabetes onset predominantly occurring during youth, a time of critical growth and development, two important issues related to the current study must be considered: 1) atrophic stimuli placed on young, growing muscle results in a rapid and irreversible remodelling process (Mozdziak, Pulvermacher, and Schultz. 2000; Wanek and Snow. 2000; Darr and Schultz. 1989), and 2) populations with pediatric type 1 diabetes consistently display elevated plasminogen activator inhibitor-1 (PAI-1) levels, irrespective of HbA1c (Zeitler, Thiede, and Muller. 2001). Unfortunately, assessment of skeletal muscle health in type 1 diabetes has not been a consideration in the clinical setting because it is assumed that insulin therapy alone is enough to restore normal muscle health by balancing protein synthesis and degradation. However, several studies have demonstrated that insulin treatment does not restore this balance (Bennet et al. 1990; Charlton, Balagopal, and Nair. 1997; Godil et al. 2005; Nair et al. 1995), and the information to date indicates that young patients with diabetes score significantly lower on maximal strength tests (Fricke et al. 2008) and that adolescents newly diagnosed with type 1 diabetes experience reduced muscle fiber size and altered muscle morphology (Jakobsen and Reske-Nielsen. 1986). Studies using appropriate animal models of adolescent type 1 diabetes also demonstrate significant limitations in muscle growth and contractile function (Gordon et al. 2010; Krause et al. 2009; Krause, Riddell, and Hawke. 2011).

For skeletal muscle tissue to stay healthy, it must continuously be maintained, adapt to changing needs, and be capable of repair in instances of overuse, exercise, or trauma. The repair of skeletal muscle is a complex orchestration of events including degeneration, extracellular matrix (ECM) remodelling, and repair/replacement of damaged muscle fibers (Hawke and Garry. 2001). This regenerative process must proceed in an orderly and efficient manner if skeletal

muscle is to be maintained as a healthy, functioning organ. Although it has been reported that the type 1 diabetes environment may affect muscle regeneration after injury (Gulati and Swamy. 1991; Vignaud et al. 2007; Jerkovic et al. 2009), it has been proposed, although never demonstrated, that the lack of insulin's anabolic action is the sole reason for the deficits observed. However, the role of insulin in skeletal muscle repair and regeneration has yet to be established. It is now becoming increasingly evident from studies conducted in various tissues that other factors, such as alterations in circulating PAI-1, may be as important in diabetes complications as hypoinsulinemia/ hyperglycemia (Nicholas et al. 2005; Lyon and Hsueh. 2003; Goldberg. 2009). In skeletal muscle, alterations in PAI-1 levels, an inhibitor of the fibrinolytic system, can have profound effects on ECM remodelling and ultimately delay muscle regeneration after injury (Fibbi et al. 2002; Koh et al. 2005; Naderi et al. 2009; Suelves et al. 2005; Sisson et al. 2009).

In the current study, we sought to determine the temporal pattern of regeneration and elucidate the underlying mechanism(s) resulting in deficits in the regenerative capacity of skeletal muscle in adolescent type 1 diabetes using a genetic murine model of the disease, the *Ins2*$^{WT/C96Y}$ mouse.

METHODS

Animal care.

Male C57BL/6-Ins2^{Akita}/J (hereafter *Ins2*$^{WT/C96Y}$) mice and their wild-type (WT) littermates were purchased at 3 weeks of age from Jackson Laboratory (Bar Harbor, ME). Mice (N = 16/group) were studied over a period of 8 to 13 weeks of untreated type 1 diabetes. A separate group of *Ins2*$^{WT/C96Y}$ and WT mice (N = 3/group) were used for the 1 week of type 1

diabetes regeneration study. *Ins2*$^{WT/C96Y}$ mice become spontaneously diabetic at ~4 weeks of age because of a heterozygous mutation in the Ins2 gene (Yoshioka et al. 1997). Exact onset of diabetes was determined by monitoring blood glucose as previously described (Krause et al. 2009). The *Ins2*$^{WT/C96Y}$ mice were chosen instead of the commonly used streptozotocin-induced diabetic rodent model because of known growth arresting effects of streptozotocin on skeletal muscle (Johnston et al. 2007).

The animal room was maintained at 21°C, 50% humidity, and 12-h/12-h light– dark cycle. All mice had access to standard breeder chow and water ad libitum.

Blood glucose and body mass were measured biweekly (fed state: 1200–1400 h) in the 8-week experimental groups. Blood samples were collected at 2, 4, and 6 weeks of diabetes for analysis of metabolites and hormones. All animal experiments were approved by the McMaster and York University Animal Care Committees in accordance with Canadian Council for Animal Care guidelines.

Skeletal muscle injury.

Skeletal muscle injury was induced with an intramuscular injection of 10 mM cardiotoxin (CTX; Latoxan, France) as previously described (Hawke et al. 2007). Injuries were generated in the left tibialis anterior (TA) and quadriceps muscles of both *Ins2*$^{WT/C96Y}$ and WT mice at 1 and 8 weeks of diabetes. The 1-week group was harvested at 10 days postinjury, whereas the 8-week group was subdivided into four recovery time points: 5, 10, 21, and 35 days.

Tissue collection.

After the specified regeneration period, animals were killed and blood was collected from the thoracic cavity after heart excision. Injured and uninjured TA muscles were coated in

optimum cutting temperature embedding compound and frozen in isopentane cooled by liquid nitrogen, and injured quadriceps muscles were snap-frozen and stored at 280°C.

PAI-039 treatment.

To determine if elevations in circulating PAI-1 were contributing to impaired skeletal muscle regeneration in the diabetic animals, PAI-039, an orally effective inhibitor of active PAI-1 (Elokdah et al. 2004), was administered throughout the regenerative process. An additional group of WT and *Ins2*$^{WT/C96Y}$ mice (N = 4) were treated via oral gavage with vehicle (2% Tween-80 and 0.5% methylcellulose in sterile H2O) or vehicle plus PAI-039 (2 mg/kg; Axon Medchem, the Netherlands), respectively. On the day of CTX injury (at 8 weeks of diabetes), mice were treated with vehicle or vehicle plus PAI-039 at 1100 h, received CTX injury to the TA at 1200 h, and received PAI-039 treatment again at 1500 h. PAI-039 treatment was continued twice daily (1100 and 1500 h) throughout the 5-day regeneration period, at which point the animals were killed and tissues were dissected and stored as described above. Those treatment time points were chosen to best attenuate the peak of PAI-1 activity because of its circadian expression pattern (Oishi et al. 2004). WT mice treated with vehicle (WT + vehicle) demonstrated no significant difference from untreated WT in active urokinase plasminogen activator (uPA), active matrix metalloproteinase (MMP)-9, collagen levels, and Myh3; therefore, these two groups were pooled for comparison with *Ins2*$^{WT/C96Y}$ mice and *Ins2*$^{WT/C96Y}$ mice treated with PAI-039 (*Ins2*$^{WT/C96Y}$ +PAI-039) as illustrated in Fig. 4.3.

Blood analyses.

Heparinized blood plasma was analyzed for insulin and total PAI-1 (MADPK-71 K; Millipore, Billerica, MA) at all collection time points. Plasma was also analyzed for

nonesterified fatty acids with the use of a colorimetric assay (Wako Diagnostics, Richmond, VA).

Western blot analysis.

Snap-frozen quadriceps or TA samples were homogenized, analyzed for protein concentration, electrophoretically separated on acrylamide gels, and transferred to polyvinylidene fluoride membranes as previously described (Hawke et al. 2007). Primary antibodies included Myh3 (Hybridoma Bank F1.652), GAPDH (Abcam 8245, loading control; Cambridge, MA), myogenin (Hybridoma Bank F5D), MMP9 (Abcam 38898), and uPA (Abcam 28230). uPA was analyzed for unbound (active) uPA and uPA bound to PAI-1 as a measure of PAI-1 activity in the muscle (Crandall et al. 2004). Active uPA is found at ~48 kDa, and inactive (PAI-1-bound) uPA is found at ~93 kDa. Appropriate horseradish peroxidase-conjugated secondary antibodies were used and visualized with the addition of chemiluminescent reagent (Amersham, Piscataway, NJ). Images were acquired with a Fusion Fx7 imager (Vilber Lourmat, Eberhardzell, Germany) and analyzed with ImageJ.

Histochemical and immunofluorescent analyses.

Eight-micron skeletal muscle cross-sections were mounted on glass slides and stained as described below.

Hematoxylin–eosin. Hematoxylin–eosin staining was used to determine the average fiber area of uninjured and injured TA. Three images spaced evenly throughout the TA (~1 mm apart) were used for analysis where 25 fibers per image were analyzed for area (75 total fibers per TA). We have previously demonstrated that in this muscle, quantification of this number of fibers provides a representative analysis of fiber area (Krause et al. 2009; Krause et al. 2008).

Picrosirius red. To stain for collagen content, sections were immersed in picrosirius red solution (0.1% w/v Direct Red 80 [Sigma 365548; St. Louis, MO] in a saturated aqueous solution of picric acid [Sigma p6744]) for 1 h. Sections were briefly rinsed in two changes of acidified dH20 (0.5% glacial acetic acid), dehydrated, cleared, and mounted.

Immunofluorescence. Sections were fixed with ice-cold 2% paraformaldehyde, blocked with 10% normal goat serum/1.5% BSA, followed by mouse IgG Block (BMK 2202; Vector Laboratories Inc., Burlingame, CA), and incubated with 1:1 dilution of anti-Myh3 overnight at 4°C. Alexa 488 anti-mouse secondary antibody (A-11001; Invitrogen, Carlsbad, CA) was used for detection, and 4,6- diamidino-2-phenylindole (DAPI) was used to identify nuclei.

Image analysis.

Images obtained with a Nikon 90i-eclipse microscope (Nikon Inc., Melville, NY) were analyzed using NIS Elements software (Nikon, Inc., Melville, NY). Analysis included determination of collagen positive area and Myh3 positive area using signal threshold settings as the detection method. Fiber area was determined manually using NIS Elements software.

Statistical analysis.

For all experiments, the appropriate t test or two-way ANOVA with Bonferroni post hoc analysis was performed between *Ins2*$^{WT/C96Y}$ and WT groups. Two-way ANOVA was run on datasets with dependent variables measured over time, and one-tailed t tests were carried out on data with only single comparisons. One-tailed t tests were justified for these comparisons because differences in a specific direction were hypothesized a priori on the basis of our data and previous reports (Fibbi et al. 2002; Koh et al. 2005; Naderi et al. 2009; Suelves et al. 2005; Sisson et al. 2009). Data are presented as mean ± SEM with $P>0.05$ considered significant. Asterisks denote significant differences identified by t test or Bonferroni post hoc test in pairwise

comparisons, and significant main effect of diabetes or significant interaction between diabetes and time is listed in Figs. 3.1 to 3.3.

RESULTS

Ins2$^{WT/C96Y}$ mice spontaneously developed type 1 diabetes (hypoinsulinemia/hyperglycemia) at 4 weeks of age, which was maintained throughout the study period (Tables 3.1 and 3.2) compared with WT littermates. Relative to WT mice, *Ins2*$^{WT/C96Y}$ mice also displayed decreased body mass gain and developed hyperlipidemia by 6 weeks of untreated diabetes (Tables 3.1 and 3.2), consistent with previous findings (Hong et al. 2007).

Histologic assessment of the uninjured TA served as an index for the effects of type 1 diabetes on skeletal muscle growth. We found that uninjured *Ins2*$^{WT/C96Y}$ muscles displayed no impairment in myofiber cross-sectional area within the first 17 days of type 1 diabetes (Table 3.1), whereas a significant reduction (12%) in myofiber cross-sectional area accrual occurred by 8 weeks of type 1 diabetes that did not significantly worsen with increasing disease duration (up to 13 weeks, Table 3.2). This suggests that impaired growth, rather than progressive atrophy, is responsible for the reduced myofiber area observed in *Ins2*$^{WT/C96Y}$, at least until such time as significant neuropathic complications develop (Andreassen et al. 2009).

In response to muscle damage, deficits in muscle health became considerably more apparent. *Ins2*$^{WT/C96Y}$ muscles exposed to the type 1 diabetes environment for 1 week before injury demonstrated a 21% decrement in myofiber cross-sectional area at the 10-day regeneration mark (Fig. 3.1B). This novel finding suggests that even short-term exposure to type 1 diabetes has profound effects on skeletal muscle's ability to repair after damage.

The regenerative capacity was also impaired at 8 weeks of disease progression because muscle masses and myofiber cross-sectional areas of regenerating *Ins2*$^{WT/C96Y}$ muscles were significantly less than WT muscles from 10 days of regeneration onward (Fig. 3.1C–E). Loss of mass and myofiber area was significant even when expressed as a percentage of either the uninjured, contralateral TA or body mass (Supplementary Fig. 3.1), confirming that the poor regeneration in type 1 diabetes extends beyond the reduced growth rate. These changes in overall mass and myofiber area were preceded by alterations in the protein expression of markers of the regenerative process, myogenin and embryonic myosin heavy chain (Myh3). Myogenin is a myogenic regulatory factor that is expressed during early time points in regeneration and is important for cell-cycle exit of myoblasts and consequent terminal differentiation (Le Grand and Rudnicki. 2007; Smith, Janney, and Allen. 1994). Western blot analysis showed suppressed expression of myogenin in *Ins2*$^{WT/C96Y}$ muscle compared with WT muscle at 5 days postinjury (Fig. 1F). Myh3, a developmental myosin isoform, is expressed transiently during skeletal muscle regeneration (d'Albis et al. 1988) and used as a reference point to assess the process of differentiation (Schiaffino et al. 1986). Similar to myogenin, the protein expression of Myh3 was reduced in *Ins2*$^{WT/C96Y}$ muscle at 5 days of regeneration. Both Western blot and immunofluorescent staining of regenerating muscles demonstrated this expression pattern (Fig. 1G–I). Neither group displayed expression of Myh3 at 21 or 35 days after regeneration, suggesting that the impairments in the regenerative process are within the early phases after injury with diabetes development (<10 days), and after this time, maturation proceeds, albeit delayed.

Skeletal muscle regeneration is a complex process that is heavily dependent, particularly during the early phase, on the optimal functioning of the fibrinolytic system (Fibbi et al. 2002;

Koh et al. 2005; Naderi et al. 2009; Suelves et al. 2005; Sisson et al. 2009). We speculated that impairments in type 1 diabetes muscle regeneration may be due, at least in part, to elevated PAI-1 preventing the activation of uPA and its downstream effectors, the MMPs (e.g., MMP9). Consequently, suppression of the fibrinolytic process would result in attenuation of ECM remodelling, thus creating barriers for infiltration of immune cells and efficient activation and invasion of the myogenic stem cells that are responsible for the formation of new myofibers (Hawke and Garry. 2001). We observed total PAI-1 levels to be more than twofold higher in *Ins2*$^{WT/C96Y}$ mice than in WT mice within 2 weeks of hyperglycemia in the former group, with values remaining elevated with diabetes throughout the experimental period (Fig. 2A and B). Moreover, regenerating *Ins2*$^{WT/C96Y}$ muscle displayed elevations in collagen content at 5 and 10 days of regeneration compared with WT muscle (Fig. 2C and D). Active uPA levels at 5 days of regeneration were decreased by ~35% in *Ins2*$^{WT/C96Y}$ muscle compared with WT muscle (WT: 5978 ± 1362 vs. *Ins2*$^{WT/C96Y}$: 3873 ± 1241; P = 0.15). Although this decrease in active uPA was not statistically significant, active MMP9, the MMP associated with ECM remodelling in skeletal muscle (Zimowska et al. 2008; Kherif et al. 1999), was significantly elevated at 5 days of regeneration in WT but not *Ins2*$^{WT/C96Y}$ muscle, with levels between the two groups similar by 10 days postinjury (Fig. 2E).

With PAI-1 elevated within 2 weeks of type 1 diabetes onset (Fig. 2A), we hypothesized that the deficit in regeneration observed in *Ins2*$^{WT/C96Y}$ mice diabetic for 1 week before CTX injury would also display defective ECM remodelling. As hypothesized, collagen content in regenerating *Ins2*$^{WT/C96Y}$ mice, diabetic for a total of 17 days, was significantly elevated (Fig. 2F), consistent with a role of PAI-1 in the impaired regeneration.

By having identified that increased PAI-1 levels in *Ins2*$^{WT/C96Y}$ mice are associated with early impairments in muscle regeneration, we then determined if these deficits could be restored with pharmacologic inhibition of PAI-1, even in the absence of insulin therapy. Twice-daily oral dosing of PAI-039 (tiplaxtinin), a pharmacologic inhibitor of PAI-1 (Elokdah et al. 2004), effectively increased the amount of active (free) uPA in the injured muscle of 8-week diabetic *Ins2*$^{WT/C96Y}$ mice compared with untreated *Ins2*$^{WT/C96Y}$ mice (Fig. 3A) and increased the ratio of active to inactive uPA (uPA/PAI-1-uPA) (*Ins2*$^{WT/C96Y}$: 1.22 ± 0.13 AU vs. *Ins2*$^{WT/C96Y}$ +PAI-039: 1.74 ± 0.14 AU; P = 0.02). WT mice treated with vehicle alone demonstrated no significant change in active uPA levels (WT: 5978 ± 1362 AU vs. WT + vehicle: 7007 ± 778 AU; P = 0.27) or uPA/PAI-1-uPA (WT: 1.40 ± 0.18 AU vs. WT + vehicle: 1.28 ± 0.09 AU; P = 0.29). The downstream effect of elevated active uPA levels, resultant from PAI-039 treatment, was an increase in active MMP9 (Fig. 3B) and a normalization of collagen content in the regenerating *Ins2*$^{WT/C96Y}$ muscles to the levels observed in WT mice (Fig. 3C and D). The recovery of the fibrinolytic pathway with PAI-039 in *Ins2*$^{WT/C96Y}$ mice restored not only normal ECM remodelling but also Myh3 expression to levels similar to WT regenerating muscles (Fig. 3E and F). Injured TA fiber area demonstrated no significant difference between groups (WT + vehicle: 461 ± 29 mm^2 vs. *Ins2*$^{WT/C96Y}$ +PAI-039: 396 ± 32 mm^2; P = 0.19), whereas TA mass exhibited a small but significant difference (WT + vehicle: 0.0396 ± 0.002 g vs. *Ins2*$^{WT/C96Y}$ +PAI- 039: 0.031 ± 0.002 g; P<0.05). Similarly, no difference was noted in fiber area at 5 days post injury between WT and *Ins2*$^{WT/C96Y}$ mice (Fig. 1E), with a small decrease in muscle mass at that time point (Fig. 1D). The reasons underlying the apparent discrepancy between fiber area and muscle mass in the diabetic mice is unknown; however, it could be speculated that differences in

fibrosis, inflammatory response, or lipid content within the muscles of the various groups could contribute to these observations.

To rule out the possibility that PAI-039 treatment improves glycemic or insulinemic levels, thus improving the diabetic environment in ways other than affecting PAI-1 activity, whole-blood glucose and plasma insulin levels were measured. Blood glucose concentrations remained severely elevated in the *Ins2*$^{WT/C96Y}$ mice treated with PAI-039 (WT + vehicle: 8.9 ± 0.7 mmol/L vs. *Ins2*$^{WT/C96Y}$ +PAI- 039: 35.0 ± 0.0 mmol/L; $P<0.05$), whereas insulin levels remained low (WT + vehicle: 916 ± 74 pg/mL vs. *Ins2*$^{WT/C96Y}$ +PAI-039: 146 ± 29 pg/mL; $P<0.05$).

DISCUSSION

Our results indicate that the type 1 diabetic environment negatively affects the health of skeletal muscle, as defined by impaired growth and poor regenerative capacity. The deficits in regenerative capacity occur rapidly with exposure to type 1 diabetes (within ~2 weeks) and, as we demonstrated, are consistent with elevated PAI-1 and ineffective ECM remodelling.

Maintaining a healthy muscle mass in the type 1 diabetic population has not typically been addressed in the clinical setting. Unfortunately, many studies demonstrate impairments in skeletal muscle health (e.g., impaired morphology, decreased strength, and metabolic capacity) observed early in patients with type 1 diabetes who are receiving insulin therapy, changes that may precede other diabetes complications (Krause, Riddell, and Hawke. 2011). The results presented support these previous findings as we demonstrate that repair from muscle damage is significantly blunted in the diabetic state with as little as 7 days of uncontrolled type 1 diabetes before muscle injury. Furthermore, we also demonstrate that PAI-1 is significantly elevated

within the first 2 weeks of type 1 diabetes onset and that inhibition of this hormone restores the regenerative capacity of type 1 diabetic mice, irrespective of the hypoinsulinemia. Although skeletal muscle is capable of maintaining basic function in the face of extreme stressors, this does not equate to a healthy muscle mass that is functioning optimally. We and others have demonstrated that although basic indices of muscle function may not be significantly impaired, dramatic changes are occurring within the muscle demonstrating compromised health (Gordon et al. 2010; Krause et al. 2009; Shortreed et al. 2009). If we heed lessons from other metabolic disease states (e.g., obesity), as muscle health diminishes, disease severity increases. For example, the muscle wasting that occurs with obesity (sarcopenic obesity) is a serious complication resulting in the expedition of complications within other tissues (Srikanthan, Hevener, and Karlamangla. 2010). Given the importance of skeletal muscle to whole-body fuel metabolism, ensuring that skeletal muscle health is maintained in metabolic disease states is obviously of critical importance.

Type 1 diabetes onset most often occurs during childhood/adolescence, and previous studies have shown that atrophic stimuli (e.g., hindlimb casting) placed on young, growing muscle result in a rapid and irreversible remodelling process, ultimately leading to a failure to achieve its full potential of adult muscle mass (Mozdziak, Pulvermacher, and Schultz. 2000; Wanek and Snow. 2000; Darr and Schultz. 1989). We were interested to determine if type 1 diabetes may prove to be one of these “atrophic environments.” The present findings illustrate that growing skeletal muscle exposed to type 1 diabetes will display a failure to accrue muscle mass/fiber area, and these findings are not the product of progressive atrophy resultant from prolonged type 1 diabetes exposure or neuropathic complications, because no change in muscle mass or fiber area was observed once into adulthood (a further 5 weeks of uncontrolled type 1

diabetes). Although we investigated the uncontrolled diabetic state in a rodent model of diabetes, it is worth considering that there are two situations in which pediatric type 1 diabetic populations may be under this stress: 1) before diagnosis (which may last a period of months) and 2) after diagnosis when glycemic control is difficult and suboptimal (Diabetes Control and Complications Trial Research Group. 1994). Consistent with our results, findings in newly diagnosed juvenile type 1 diabetic humans demonstrate a reduced myofiber area compared with healthy age-matched control subjects (Jakobsen and Reske-Nielsen. 1986).

Repair from muscle damage consists of multiple, overlapping stages (Hawke and Garry. 2001). After the initial injury, an inflammatory phase ensues to remove damaged cells and debris. It is during this early phase that remodelling of the ECM begins, with a dramatic increase in ECM proteins, particularly collagen, which is needed as the structural integrity of the muscle is compromised while damaged muscle fibers undergo phagocytosis. As repair continues, so does ECM remodelling, with the excess of ECM proteins undergoing degradation as nascent myofibers form and mature. Activity of the fibrinolytic system (PAI-1, uPA, MMPs) is critical during this ECM remodelling period (Fibbi et al. 2002; Koh et al. 2005; Naderi et al. 2009; Suelves et al. 2005; Sisson et al. 2009). Newly formed myofibers will initially express immature myosin heavy chain isoforms, such as Myh3 (embryonic myosin heavy chain). As maturation continues, the muscle fibers will increase in size, returning to a preinjury state while replacing the immature contractile proteins with mature isoforms. We show in this study that in response to muscle damage, ECM remodelling is impaired in the type 1 diabetic state, and this is a direct result of elevated PAI-1. The increase in PAI-1 observed in regenerating muscle of type 1 diabetic mice decreased active uPA and MMP9 levels, thereby attenuating ECM turnover (as noted by elevated intramuscular collagen) during the first 10 days postinjury. Restoration of the

fibrinolytic system in type 1 diabetic mice via pharmacologic inhibition of PAI-1 restored active MMP9 expression, returned collagen levels to normative values, and ultimately allowed for nascent muscle fiber growth to occur. Consistent with these findings, mice deficient in uPA exhibit impaired muscle regeneration, whereas PAI-1–deficient mice exhibit augmented muscle repair (Koh et al. 2005; Lluis et al. 2001). It is worth noting that, in the current study, PAI- 039 treatment in *Ins2*$^{WT/C96Y}$ mice resulted in active uPA levels that significantly exceeded that measured in WT mice muscles. Although this may be considered "supraphysiologic," tissues were collected 1 h after PAI-039 administration on the day of harvest, a time when the drug effects were at their peak. With a PAI-039 half-life of 4.1 h (Elokdah et al. 2004), it can be speculated that more time was spent below this elevated level than within it.

Although the evidence to date suggests that insulin administration does not restore function of the fibrinolytic pathway (because insulin therapy does not reduce PAI-1 levels in human pediatric populations) (Zeitler, Thiede, and Muller. 2001), and thus would not improve collagen degradation and de novo myofiber formation, insulin might facilitate the rate of myofiber growth (later stages of regeneration) by modestly improving protein turnover (Bennet et al. 1990; Charlton, Balagopal, and Nair. 1997; Godil et al. 2005; Nair et al. 1995). Future studies are needed to clearly define if PAI-039 treatment, in combination with standard insulin therapy, provides a more optimal regeneration environment. Furthermore, we administered PAI-039 just before the injury; however, future studies should consider the clinically important issue of treatment after injury, in terms of both effectiveness and timeframe. A final clinical consideration of PAI-039 treatment is the long-term effects of its administration if it were to be given prophylactically or for prolonged periods of time. To date, there is limited information on this topic. One study administered PAI-039 for 42 days through addition to the rodent chow and

found an acute protection against radiation-induced intestinal injury but noted no adverse effects of drug treatment (Abderrahmani et al. 2009), whereas a second study provided a 2-month administration of PAI-039 to AngII/ salt–treated mice (Weisberg et al. 2005). In the latter study, PAI-039 was effective in decreasing aortic remodelling with no effect of PAI-039 alone on serum amyloid A levels (an index of systemic inflammation).

Although elevated PAI-1 has been linked to other complications usually associated with diabetes, such as coronary artery disease and nephropathy (Nicholas et al. 2005; Lyon and Hsueh. 2003; Goldberg. 2009), it is somewhat surprising that this is the first time that a clinically relevant inhibition of PAI-1 (through pharmacologic means) has been used to treat a diabetes complication. In fact, to the best of our knowledge, only three other studies have investigated mitigating diabetes complications by altering PAI-1 levels. These studies used streptozotocin-induced diabetes in PAI-1–deficient rodents to investigate the role of PAI-1 in mediating the effects of type 1 diabetes on renal morphology and function (Nicholas et al. 2005; Collins et al. 2006; Lassila et al. 2007). Collectively, these authors found that elevated PAI-1 was contributing to diabetic nephropathy (increased glomerular ECM, decreased glomerular filtration rate) and that amelioration of these symptoms occurred in the PAI-1–deficient background. Given the demonstrated (present study) (Nicholas et al. 2005; Collins et al. 2006; Lassila et al. 2007) and proposed (Lyon and Hsueh. 2003; Goldberg. 2009) linkage of PAI-1 with diabetes complications and the fact that PAI-1 is elevated in populations with pediatric type 1 diabetes regardless of the level of glycemic control (Zeitler, Thiede, and Muller. 2001; Gogitidze Joy et al. 2010; Small et al. 1989), we propose that aggressive therapeutic approaches, including intensive insulin and PAI-1 inhibitor strategies, warrant further investigation for the treatment of young type 1 diabetic patients. This will not only ensure optimal accrual and maintenance of a healthy skeletal muscle

mass but also will reduce the onset and progression of other diabetes complications. Clearly, future studies are needed to definitively demonstrate the causative role of PAI-1 in the impaired muscle regeneration of patients with diabetes, and these studies may also prove valuable in developing therapeutic strategies to ensure the most effective management of other diabetes complications.

TABLES AND FIGURES

Table 3.1. Characteristics of WT and diabetic mice at 8 weeks of diabetes (~13 weeks old).

			Weeks of Diabetes				Diabetes main effect and Interaction with time
Measure	**Group**		**2**	**4**	**6**	**8**	**p-value**
Body Mass (g)	8 weeks + variable time post-CTX	WT	20.1 ± 0.3	23.1 ± 0.4	24.9 ± 0.4	26.2 ± 0.4	DME = 0.0004
		Ins2 $^{WT/C96Y}$	19.1 ± 0.3	21.5 ± 0.3 *	22.9 ± 0.4 *	23.9 ± 0.4 *	Int = 0.0035
Insulin (pg/ml)		WT	789 ± 86	778 ± 175	814 ± 106		DME < 0.0001
		Ins2 $^{WT/C96Y}$	225 ± 24 *	224 ± 35 *	222 ± 56 *		Int = NS
Blood Glucose (mM)		WT	11.3 ± 0.6	9.1 ± 0.3	9.1 ± 0.3	8.2 ± 0.3	DME < 0.0001
		Ins2 $^{WT/C96Y}$	29.1 ± 1.0 *	32.2 ± 0.7 *	32.4 ± 0.7 *	33.1 ± 0.5 *	Int < 0.0001
Non-Esterified Fatty Acids (mM)		WT	0.63 ± 0.06	0.77 ± 0.08	1.10 ± 0.06		DME < 0.0001
		Ins2 $^{WT/C96Y}$	1.05 ± 0.10	0.85 ± 0.07	2.14 ± 0.12 *		Int = 0.0003

Biweekly data for the long-term (8 weeks) type 1 diabetic and WT groups (N = 16). *Ins2*$^{WT/C96Y}$ mice are labelled Ins2. Two-factor ANOVA was run to determine the main effects of diabetes, time, and interaction; P values for diabetes main effect and interaction are listed next to the respective data. Absence of a significant main effect or interaction is indicated as NS. DME, diabetes main effect; Int, interaction with time. *Significant difference at that time point by Bonferroni post hoc comparison.

Table 3.2. Characteristics of WT and diabetic mice during skeletal muscle regeneration after a period of untreated type 1 diabetes.

			Days Post-CTX injury				Diabetes main effect and Interaction with time
Measure	**Group**		**5**	**10**	**21**	**35**	**p-value**
Body Mass (g)	8 weeks + variable time post-CTX	WT	26.8 ± 1.2	26.8 ± 0.9	28.5 ± 0.7	29.9 ± 0.9	DME < 0.0001
		Ins2$^{WT/C96Y}$	25.0 ± 0.6	23.0 ± 0.6 *	24.6 ± 0.5 *	25.5 ± 0.4 *	Int = NS
Insulin (pg/ml)		WT	1294 ± 286	1242 ± 268	1818 ± 461	2413 ± 349	DME < 0.0001
		Ins2$^{WT/C96Y}$	235 ± 23 *	235 ± 41 *	194 ± 115 *	157 ± 53 *	Int = NS
Blood Glucose (mM)		WT	8.2 ± 0.2	9.2 ± 0.4	8.7 ± 0.3	8.6 ± 0.4	DME < 0.0001
		Ins2$^{WT/C96Y}$	31.4 ± 0.7 *	33.2 ± 0.7 *	33.2 ± 0.9 *	32.1 ± 0.6 *	Int = NS
Non-Esterified Fatty Acids (mM)		WT	0.69 ± 0.09	0.56 ± 0.06	0.53 ± 0.08	0.73 ± 0.04	DME < 0.0001
		Ins2$^{WT/C96Y}$	1.27 ± 0.13 *	1.54 ± 0.06 *	1.26 ± 0.19 *	1.97 ± 0.25 *	Int = NS
Uninjured TA mass (grams)		WT	0.047 ± 0.001	0.049 ± 0.002	0.050 ± 0.001	0.054 ± 0.004	DME < 0.0001
		Ins2$^{WT/C96Y}$	0.039 ± 0.002	0.038 ± 0.001 *	0.044 ± 0.002	0.045 ± 0.003 *	Int = NS
Uninjured TA fiber area (μm^2)		WT	2168 ± 135	2349 ± 121	2182 ± 89	2338 ± 229	DME = 0.0007
		Ins2$^{WT/C96Y}$	1905 ± 103	1813 ± 32 *	2074 ± 158	1834 ± 36 *	Int = NS
Uninjured TA fiber area (μm^2)	1 week + 10 days post-CTX	WT		1832 ± 96			
		Ins2$^{WT/C96Y}$		1768 ± 61			

Data for the long-term (8 weeks + variable time post-CTX; N = 16 [4 per time point]) and short-term (1 week + 10 days post-CTX; N = 3) type 1 diabetic and WT groups. *Ins2*$^{WT/C96Y}$ mice are labelled Ins2. Two-factor ANOVA was run to determine the main effects of diabetes, time, and interaction; P values for diabetes main effect and interaction are listed next to the respective data. Absence of a significant main effect or interaction is indicated as NS. DME, diabetes main effect; Int, interaction with time. *Significant difference at that time point by Bonferroni post hoc comparison. For single comparison of short-term groups, t test revealed no significant difference.

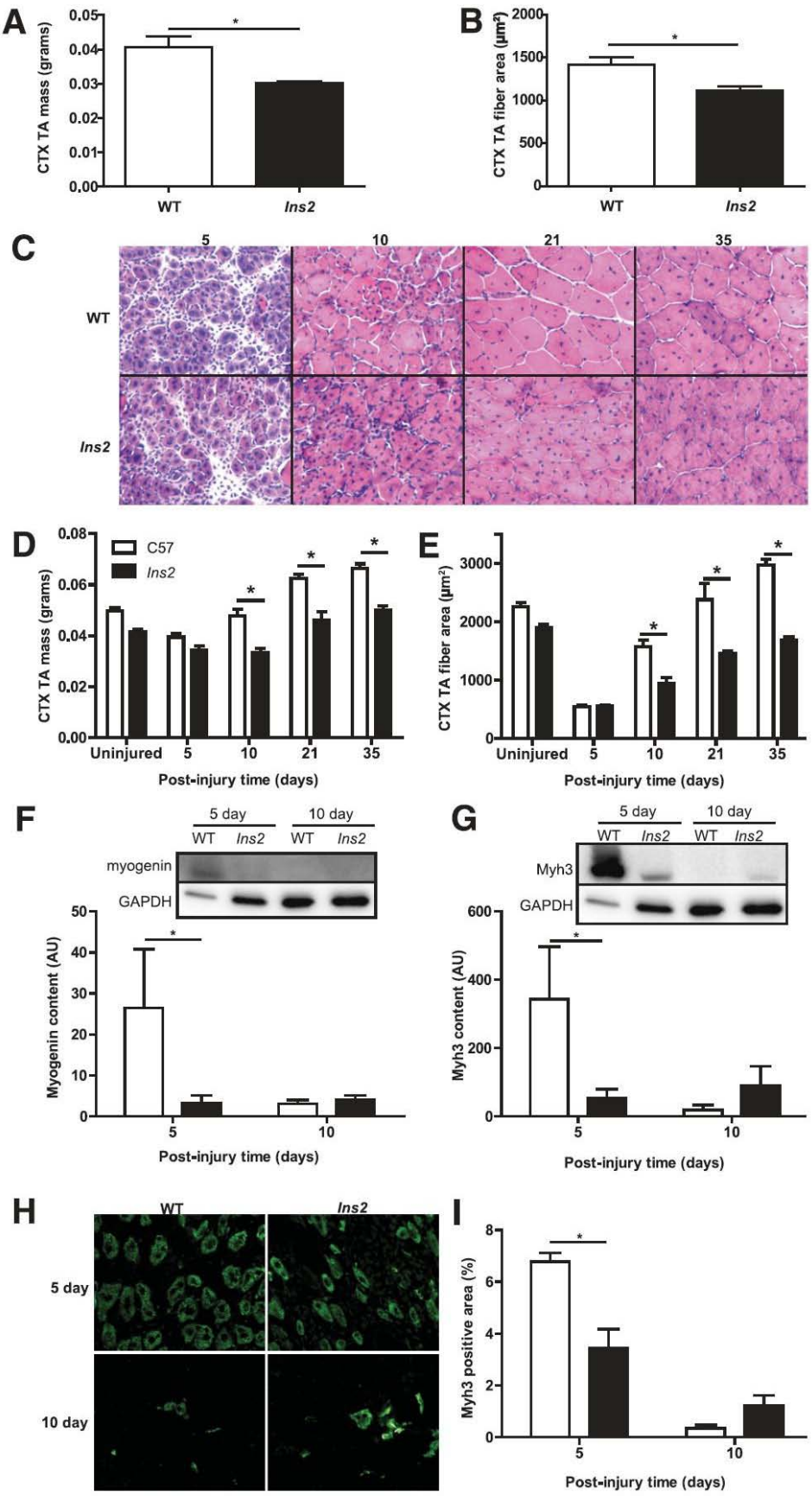

A
CTX TA mass (grams)
0.05
0.04
0.03
0.02
0.01
0.00
*
WT
Ins2
B
CTX TA fiber area (μm²)
2000
1500
1000
500
0
*
WT
Ins2
C
5
10
21
35
WT
Ins2
D
CTX TA mass (grams)
0.08
0.06
0.04
0.02
0.00
C57
Ins2
*
Uninjured
5
10
21
35
Post-injury time (days)
E
CTX TA fiber area (μm²)
3000
2000
1000
0
*
Uninjured
5
10
21
35
Post-injury time (days)
F
5 day
10 day
WT
Ins2
WT
Ins2
myogenin
GAPDH
Myogenin content (AU)
50
40
30
20
10
0
*
5
10
Post-injury time (days)
G
5 day
10 day
WT
Ins2
WT
Ins2
Myh3
GAPDH
Myh3 content (AU)
600
400
200
0
*
5
10
Post-injury time (days)
H
WT
Ins2
5 day
10 day
I
Myh3 positive area (%)
8
6
4
2
0
*
5
10
Post-injury time (days)

Figure. 3.1. Type 1 diabetes impairs regeneration of skeletal muscle after CTX injury. Ten days after injury, TA muscle of the 1-week diabetic *Ins2*$^{WT/C96Y}$ mice demonstrates a significant loss of (A) mass (t test: P = 0.015; N = 3) and (B) myofiber cross-sectional area (t test: P = 0.014; N = 3) compared with WT, indicating impaired regeneration after injury at only 7 days of type 1 diabetes. C: Hematoxylin–eosin staining of TA injured at 8 weeks of type 1 diabetes demonstrates loss of (D) muscle mass (N = 16) and (E) myofiber area (N = 16) compared with WT beginning at 10 days postinjury. The uninjured time point in both panels is the contralateral TA to the 5-day post-CTX muscle and is included to illustrate the decrease in muscle mass and myofiber area associated with the impaired growth of skeletal muscle in the type 1 diabetic state. The values for the uninjured time point are not included in the statistical analysis. A main effect of diabetes (main effect: P < 0.001) is observed in both mass and fiber area with the asterisk (*) denoting specific differences between WT and *Ins2*$^{WT/C96Y}$ as defined by post hoc analysis. Note that the type 1 diabetic muscle does not return to WT mass/fiber size at later time points, but continues to lag in regeneration. Because early expression of myogenic proteins is critical to the early stages of regeneration, (F) myogenin (main effect: P = 0.062, interaction: P = 0.017; N = 8) and (G) embryonic myosin heavy chain. (Myh3) expression (main effect: P = 0.117, interaction: P = 0.009; N = 8) were determined in quadriceps muscle and demonstrate significantly increased expression at 5 days postinjury in WT but not *Ins2*$^{WT/C96Y}$ (labelled Ins2, F and G). H: Immunofluorescent staining of injured TA with anti- Myh3 confirms (I) the lack of Myh3 positive fibers in *Ins2*$^{WT/C96Y}$ compared with WT (main effect: P = 0.013; interaction: P < 0.001; N = 8) at 5 days postinjury. *Differences between groups at specific time points identified by Bonferroni post hoc analysis after 2-way ANOVA (D–G, I). A–I: White bars represent WT, and black bars represent *Ins2*$^{WT/C96Y}$.

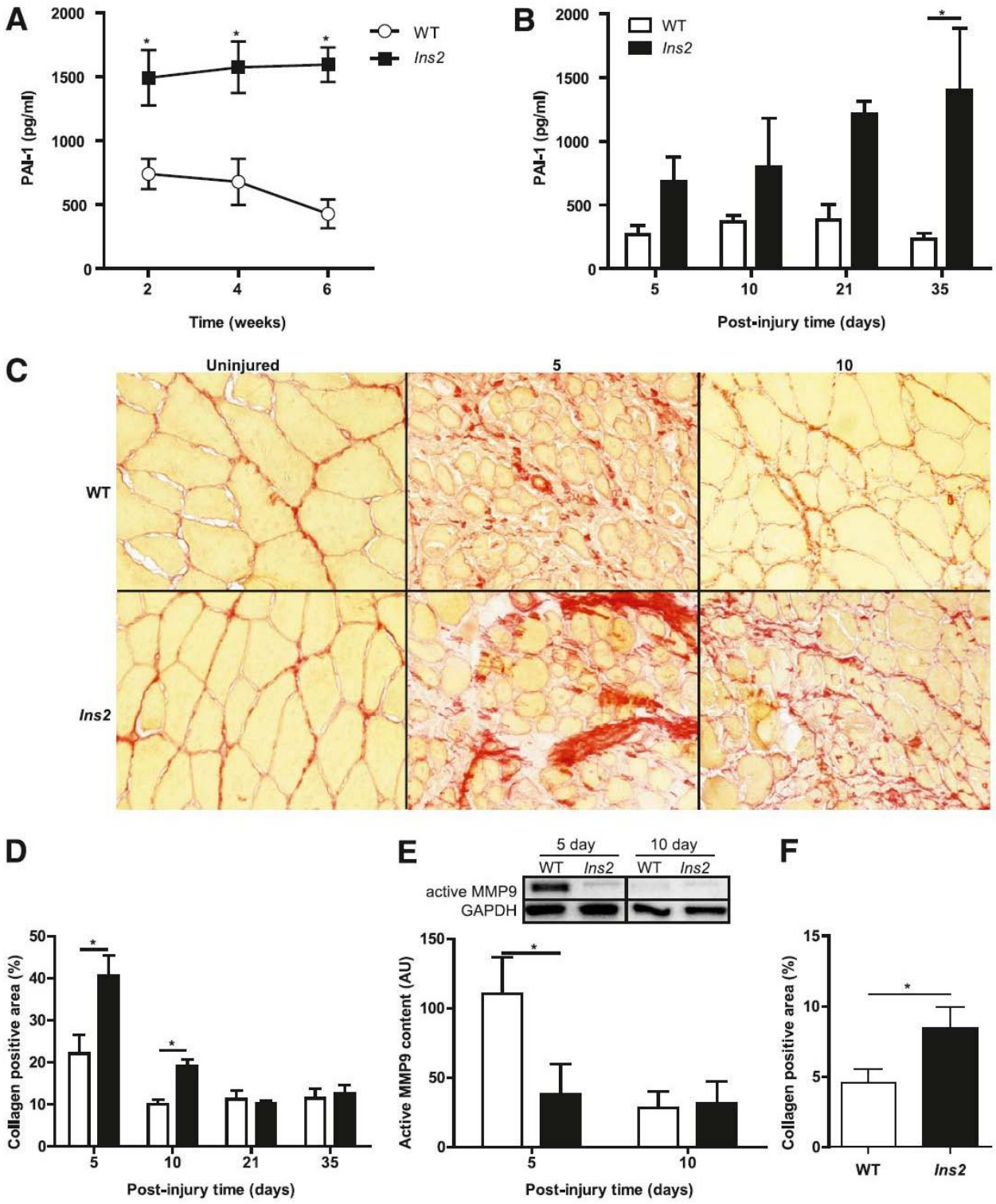


Figure. 3.2. Type 1 diabetes causes elevated PAI-1, suppresses MMP9 activation, and increases collagen content during early regeneration time points. Significantly elevated PAI-

1 levels in *Ins2*$^{WT/C96Y}$ mice compared with WT mice were found in blood plasma collected (A) throughout type 1 diabetes progression (main effect: $P < 0.001$; $N = 16$) and (B) after CTX injury (main effect: $P < 0.001$; $N = 16$). This led to the hypothesis that collagen would be elevated in the *Ins2*$^{WT/C96Y}$ mice because of suppression of the fibrinolytic pathway. C: Picrosirius red staining of injured TA sections revealed increased collagen (red color), which was statistically significant (D, main effect: $P = 0.001$, interaction: $P = 0.004$; $N = 16$). E: MMP9, an important protease in skeletal muscle collagen cleavage, was also found to be significantly repressed in *Ins2*$^{WT/C96Y}$ mice (labelled Ins2, E) at the 5-day time point ($N = 8$). F: The short-term *Ins2*$^{WT/C96Y}$ mice also exhibited increased collagen at 10 days postinjury (t test: $P = 0.047$, $N = 3$). *Differences between groups at specific time points identified by Bonferroni post hoc analysis after 2-way ANOVA (A, B, D, E). A–E: White bars/circles represent WT, and black bars/squares represent *Ins2*$^{WT/C96Y}$.

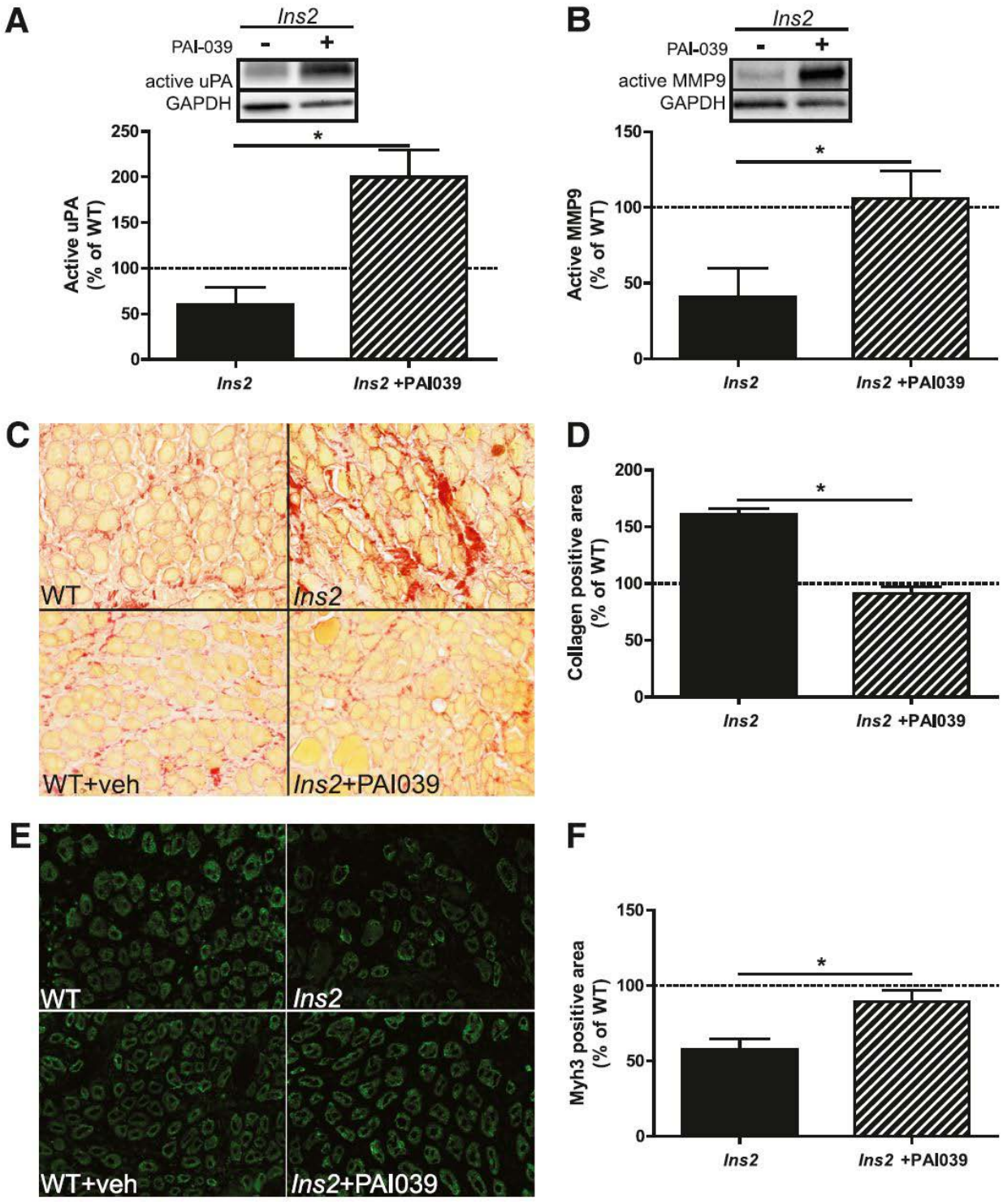
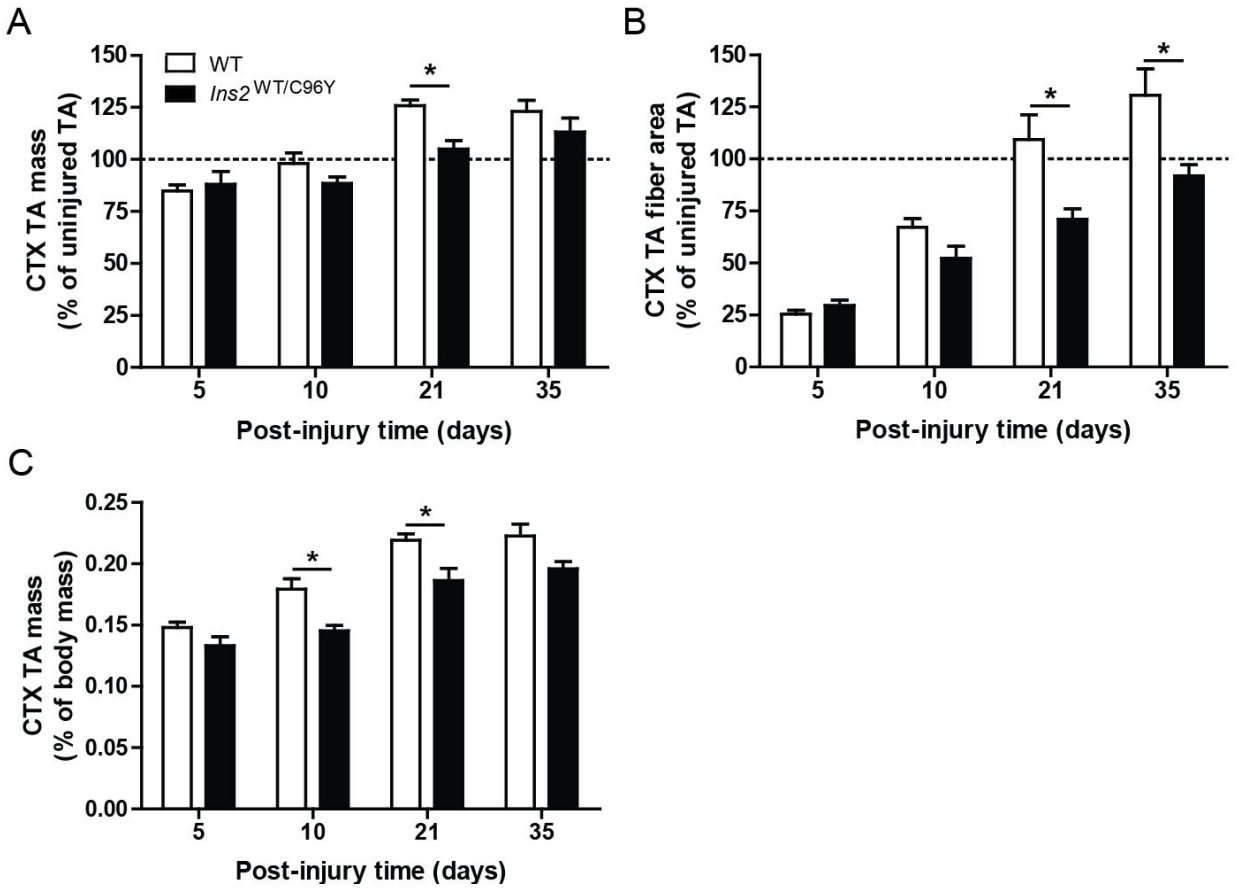


Figure. 3.3. Pharmacologic treatment against PAI-1 improves fibrinolytic pathway activity, collagen degradation, and regeneration at 5 days post-CTX injury in type 1 diabetes. A: Treatment with PAI-039 caused an increase in free uPA in *Ins2*$^{WT/C96Y}$ compared with untreated

Ins2$^{WT/C96Y}$ (t test: P = 0.004; N = 4). B: Similarly, active MMP9 was elevated in PAI-039–treated *Ins2*$^{WT/C96Y}$ (t test: P = 0.025; N = 4). These findings are characteristic of restored fibrinolytic pathway activity, which presumably led to the (C and D) reduced collagen levels (t test: P < 0.001; N = 4) and (E and F) increased Myh3-positive area (t test: P = 0.011; N = 4) observed in PAI-039–treated *Ins2*$^{WT/C96Y}$ compared with untreated *Ins2*$^{WT/C96Y}$ (labelled Ins2, C and E). A–E: Black bars represent *Ins2*$^{WT/C96Y}$, and striped bars represent PAI-039–treated *Ins2*$^{WT/C96Y}$. Data are presented relative to the mean of the WT and WT + vehicle pooled data. *Differences between groups identified by t test.



Supplementary Figure 3.1. Loss of injured skeletal muscle remains significant when expressed relative to uninjured skeletal muscle mass, myofiber area, or body mass in type 1 diabetes. Expressing injured tibialis anterior (TA) mass and fiber area relative to the corresponding value of the uninjured contralateral muscle from the same animal essentially factors out the effect of type 1 diabetes on uninjured muscle/myofiber growth, allowing the true effect of CTX-injury to be observed. A: Relative mass of injured TA demonstrates a deficit in the *Ins2*$^{WT/C96Y}$ mice compared to WT (main effect: P=0.011; N=16). B: Relative myofiber area of injured TA also demonstrates impaired regeneration in the *Ins2*$^{WT/C96Y}$ mice compared to WT (main effect: P<0.001, interaction: P=0.018; N=16). Notably, it appears that the WT myofibers exhibit hypertrophy (exceeding uninjured TA myofiber size), while the *Ins2*$^{WT/C96Y}$ myofibers do not fully return to 100% by 35 days post-injury. C: Mass of injured TA relative to body mass

indicates deficient recovery of skeletal muscle mass following injury, and eliminates the possibility that overall growth following injury might be impaired (main effect: $P<0.001$, $N=16$). (* indicates differences between groups at specific time-points identified by Bonferroni post-hoc analysis following 2 way ANOVA). In all panels, white bars represent WT and black represent *Ins2*$^{WT/C96Y}$.

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CHAPTER 4

Impaired Macrophage Infiltration and Delayed Regeneration Occurs in a Muscle-Specific Fashion in Type 1 Diabetic Mice.

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PREFACE

Significance to thesis

Previous research, including our own work, suggests that soleus muscles of diabetic mice may not exhibit the same degree of pathology observed in other muscles. Thus, the goal of this study was to determine if delayed regeneration in diabetic mouse muscle is found in muscle groups other than the tibialis anterior, and to identify a mechanism by which this may be occurring. It was found that macrophage infiltration into damaged muscle was reduced in diabetic muscle and that this led to slower regeneration. However, this occurred in a muscle specific fashion; the soleus did not exhibit any impairment in the regeneration process. Gaining a better understanding how muscle phenotype can influence regeneration in diabetic mouse muscle will help unravel the underlying causes of diabetic myopathy.

Authors' contributions

Matthew P. Krause contributed to the design of the study, performed all experiments and data collection, performed microscope image acquisition and analysis, performed all statistical analysis, wrote the initial draft of the manuscript and worked on refining later drafts of the manuscript.

Jasmin Moradi contributed to the design of the study and assisted in animal care.

Michael C. Riddell contributed to the design of the study, and worked on refining drafts of the manuscript.

Thomas J. Hawke contributed to the design of the study, assisted in experiments, and worked on refining drafts of the manuscript.

ABSTRACT

Type 1 diabetes mellitus (T1DM) results in several pathological changes to skeletal muscle (collectively termed diabetic myopathy) including regenerative capacity. Recently, elevated PAI-1 in type 1 diabetic C57BL/6-*Ins2*^{Akita}/J (*Ins2*$^{WT/C96Y}$) mice was implicated in attenuating the regeneration of the tibialis anterior (TA) via suppression of the plasminogen activator pathway leading to poor ECM remodelling and delayed regeneration. However, it remained unknown whether impaired regeneration was similar in phenotypically different muscle groups. Male C57BL/6 (WT) and *Ins2*$^{WT/C96Y}$ mice sustained cardiotoxin-induced injury to the TA, gastrocnemius (GAS) and soleus (SOL) and recovered for 5, 10, or 35 days. *Ins2*$^{WT/C96Y}$ TA and GAS demonstrated reduced regenerating fiber area compared to WT, while the SOL exhibited no difference between animal groups. Collagen levels in the TA and GAS were significantly increased post-injury relative to WT, but not in SOL. Regions of degeneration (necrosis) were present until 10 days post-injury in *Ins2*$^{WT/C96Y}$ TA. At this time WT TA were free of necrosis. Necrotic regions of the *Ins2*$^{WT/C96Y}$ TA were found to have a significant reduction in F4/80+ macrophage infiltration compared to WT, while the GAS displayed a trend to reduced macrophage infiltration. *Ins2*$^{WT/C96Y}$ SOL exhibited no delay in eliminating necrotic regions and had no reduction in macrophage infiltration. Furthermore, it was found that the *Ins2*$^{WT/C96Y}$ TA and GAS expressed less embryonic myosin heavy chain. These data suggest that impaired ECM remodelling delays the ability of macrophages to infiltrate the injury muscle, slowing the degenerative phase of muscle repair. Ultimately, attenuation of the regenerative phase occurs in *Ins2*$^{WT/C96Y}$ mice, observed in glycolytic but not oxidative muscles.

INTRODUCTION

Type 1 diabetes mellitus (T1DM) is a disease defined by hyperglycemia and hypoinsulinemia in the absence of exogenous insulin treatment. Unfortunately, exogenous insulin therapy alone does not represent a cure. Rather, it is a patch solution and in the absence of a true cure, it is the complications of diabetes that define the overall health of the affected person. One such complication that has recently received attention is diabetic myopathy (Krause, Riddell, and Hawke. 2011; Krause et al. 2011; Gordon et al. 2010; Krause et al. 2009). The pathophysiology of diabetic myopathy includes immediate and progressive loss of muscle mass and contractile function (Krause, Riddell, and Hawke. 2011; Krause et al. 2011; Krause et al. 2009; Jakobsen and Reske-Nielsen. 1986; Armstrong, Gollnick, and Ianuzzo. 1975; Cotter et al. 1993; Cotter et al. 1989). The evidence to date suggests this is, in part, due to an imbalance between protein synthesis and proteolysis in the diabetic muscle (Bennet et al. 1990; Nair et al. 1995; Lecker et al. 1999) and can be observed as a reduction in muscle mass and myofiber cross-sectional area. While the above situation arises during growth, another series of complications arise in response to muscle injury, where a complex regenerative process is needed to repair/replace damaged myofibers. In this case, a severe impairment in muscle repair is observed in diabetic skeletal muscle; a distinct problem not primarily associated with an imbalance in protein metabolism (Krause et al. 2011; Gulati and Swamy. 1991).

The repair of skeletal muscle is a complex orchestration of events including infiltration of immune cells, degeneration (or repair) of damaged myofibers, extracellular matrix (ECM) remodelling, and formation and subsequent growth of new myofibers (Hawke and Garry. 2001). The activity of plasminogen activators (PA), such as urokinase PA (uPA), are requisite for skeletal muscle regeneration (Novak et al. 2011; Sisson et al. 2009; Bryer et al. 2008; DiPasquale

et al. 2007; Koh et al. 2005), but PA activity is limited by plasminogen activator inhibitor (PAI)-1, a diurnally regulated hormone that is significantly and chronically elevated in both type 1 and type 2 diabetes (Krause et al. 2011; Zeitler, Thiede, and Muller. 2001; Oishi et al. 2004; Oishi, Ohkura, and Ishida. 2006). Recently, our lab demonstrated that PAI-1 is a primary mediator of the delayed regeneration observed in diabetic (*Ins2*$^{WT/C96Y}$) mouse skeletal muscle and pharmacological reductions in PAI-1 activity (through PAI-039) reduced collagen levels and improved muscle regeneration (Krause et al. 2011). PAI-039 treatment increased active uPA and MMP9 levels in *Ins2*$^{WT/C96Y}$ mouse tibialis anterior (TA) muscles, supporting the hypothesis that PAI-1 was suppressing the proteolytic activity of uPA and MMP9 and ultimately impairing the regenerative process.

Other studies investigating the PA system in muscle regeneration indicate that a role for this system may be more directly related to macrophage infiltration and signalling. Bryer et al demonstrate an absence of regeneration following muscle injury in uPA null mice coinciding with little or no macrophage infiltration of the injured muscle (Bryer et al. 2008). Furthermore, macrophage-depleted mice do not regenerate injured muscle, nor do they demonstrate uPA activity in the injured muscle (Novak et al. 2011; Bryer et al. 2008; Koh et al. 2005). Given that PAI-1 suppresses uPA activity and, ultimately muscle regeneration (Krause et al. 2011; Koh et al. 2005) we hypothesized that macrophage infiltration into injured muscle in diabetic *Ins2*$^{WT/C96Y}$mice would be delayed, leading to attenuated muscle regeneration. In rodent studies of type 1 diabetes, muscle groups with a large number of glycolytic myofibers [such as the TA, gastrocnemius (GAS), and extensor digitorum longus (EDL)] appear to suffer the greatest myofiber atrophy while the soleus (SOL) consistently demonstrates resilience to diabetic myopathy (Krause et al. 2009; Armstrong, Gollnick, and Ianuzzo. 1975; Cotter et al. 1993;

Cotter et al. 1989; Medina-Sanchez et al. 1991; Snow et al. 2006; Snow et al. 2005; Sanchez et al. 2005). Thus, we further hypothesized that there would be muscle group-specific differences observed in terms of inflammatory cell infiltration and regenerative capacity with the resilience of the SOL extending to the process of muscle regeneration.

METHODS

Animal Care

Male C57BL/6-*Ins2*^{Akita}/J (hereafter referred to as *Ins2*$^{WT/C96Y}$) mice and their wild-type littermates (WT) were purchased at 3 weeks of age from Jackson Laboratory (Bar Harbor, ME). *Ins2*$^{WT/C96Y}$mice and WT mice (n=12) were studied over a period of up to 8 to 13 weeks of untreated type 1 diabetes. *Ins2*$^{WT/C96Y}$ mice become spontaneously diabetic at approximately 4 weeks of age due to a heterozygous mutation in the *Ins-2* gene. Due to the spontaneity of diabetes inception in this mouse model, the exact onset of diabetes was determined by monitoring blood glucose via tail-nick every second day starting at the 3rd week of age (One Touch Ultra glucometer; maximum 35 mM; Johnson & Johnson). Mice were deemed diabetic upon 2 consecutive blood glucose measurements exhibiting hyperglycemia (>15mM).

The animal room was maintained at 21^{o}C, 50% humidity and 12h/12h light-dark cycle. All mice had access to standard breeder chow and water *ad libitum*. There were no running-wheels in animal cages, but nesting material and plastic housing was provided.

Blood glucose and body mass were measured biweekly with animals in the fed state (1200 – 1400 hours) in the 8 week experimental groups. Blood plasma samples were collected at 2, 4 and 6 weeks of diabetes via tail-nick for analysis of metabolites, hormones and other

cytokines. All animal experiments were approved by the McMaster and York University Animal Care Committees in accordance with Canadian Council for Animal Care guidelines.

Skeletal Muscle Injury

Skeletal muscle injury was induced with an intramuscular injection of 10uM cardiotoxin (CTX; Latoxan, Valence, France) as previously described (Krause et al. 2011). Injuries were generated in the left tibialis anterior (TA) and gastrocnemius-plantaris-soleus (GPS) muscles of both *Ins2*$^{WT/C96Y}$ and WT mice at 8 weeks of diabetes (approximately 12-13 weeks old). These mice were divided into 3 groups to allow different lengths of recovery (5, 10, or 35 days).

Tissue Collection

Following the specified regeneration period, animals were euthanized by cervical dislocation and blood was collected from the thoracic cavity following excision of the heart. The remaining tissues were excised and stored. Injured and uninjured TA and GPS muscles were coated in OCT embedding compound and frozen in isopentane cooled by liquid nitrogen

Blood Analyses

Heparinized blood plasma was analyzed for insulin and total PAI-1 (Multiplex Adipokine assay from Millipore, MADPK-71K) at all collection time points. The multiplex assay was chosen because it enables detection of several hormones with the limited volume of plasma obtainable from mice. Plasma was also analyzed for non-esterified fatty acids (NEFA) with the use of a colorimetric assay (Wako diagnostics).

Histochemical and Immunofluorescent Analyses

Frozen OCT-embedded skeletal muscle was cut into 8μm cross-sections and mounted on untreated glass slides and histochemically or immunofluorescently stained.

H&E staining: Hematoxylin and eosin staining was used to determine average cross-sectional area of uninjured and injured TA myofibers. Three images spaced evenly throughout the TA were used for analysis where 25 fibers per image were analysed for area (75 total fibers per TA). In the soleus (SOL) and gastrocnemius (GAS), between 50-75 fibers were analysed. Our previous studies have demonstrated that using this sample size of myofibers per animal provides an accurate estimate of the whole muscle (Shortreed et al. 2009; Krause et al. 2008).

Picrosirius red staining: To stain for collagen content, freshly cut sections were immersed in picrosirius red solution [0.1% w/v Direct Red 80 (Sigma 365548) in saturated aqueous solution of picric acid (Sigma p6744)] for one hour. Sections were briefly rinsed in two changes of acidified dH_20 (0.5% glacial acetic acid), dehydrated, cleared and mounted. The myofibers appear yellow while the collagen retains the red stain, providing adequate contrast for collagen quantification and is used as a quantifiable method for visualizing collagen in regenerating skeletal muscle (Krause et al. 2011; Hu et al. 2010).

Immunofluorescent Staining: Freshly cut sections fixed with ice-cold 2% PFA were then blocked with blocking buffer (10% normal goat serum, 1.5% bovine serum albumin), followed by mouse IgG Block (Vector, BMK 2202) for 30 min and 1 hour, respectively. To identify Myh3-expressing myofibers, sections were then incubated with 1:1 dilution of Myh3 Ab (Hybridoma Bank, F1.652) in blocking buffer overnight at 4°C. Similarly, F4/80 antigen was used to identify macrophages in injured muscles, using a dilution of 1:200 F4/80 Ab (AbD Serotec, distributed by CedarLane Labs, Burlington, Ontario), however, mouse IgG block was omitted. Primary antibody incubation was followed by a 30 min room temperature incubation of the appropriate secondary antibody (Alexa 594 anti-mouse Ab [Invitrogen, A-11001] or Alexa 594 anti-rat Ab [A-11007]), and 5 min of 1:1000 4,6-diamidino-2-phenylindole (DAPI) to

identify nuclei. To facilitate identification of positively stained myofibers or macrophages, either laminin or type I collagen was stained for using anti-laminin (ab14055, Abcam, Cambridge, MA) or anti-collagen, type I (ab292, Abcam) followed by the appropriate secondary antibody (Alexa 488).

Image Analysis

Images of stained muscle sections were obtained with a Nikon 90i eclipse upright microscope and analyzed using Nikon Elements (NE) software. Analysis included determination of collagen (picrosirius red stain), Myh3, and F4/80 positive area using signal threshold settings as the detection method. For determination of F4/80 cell number, the threshold binary for DAPI stained nuclei was intersected with the F4/80 threshold binary; the number of intersected objects represented the positive cell number. Fiber area in H&E stains was determined manually using NE software as well. TA, GAS, and SOL muscles were analyzed separately to determine muscle group-specific differences. Regenerating and necrotic injured muscle tissue represent different stages of the overall regeneration process, and, thus, were identified in immunofluorescently stained tissue and analyzed separately as illustrated in figures 4.3-4.5, similar to the analyses conducted in previous research (Goetsch et al. 2005).

Statistical Analysis

For all experiments, the appropriate t-test or two-way ANOVA with Bonferroni post-hoc analysis of pairwise comparisons was carried out to classify significant differences ($P<0.05$) between *Ins2*$^{WT/C96Y}$ and WT groups. Two-way ANOVA was run on data sets with dependent variables measured over time, while one-tailed t-tests were carried out on data with only single comparisons. Data are presented as mean ± SEM. An * denotes a significant difference identified by t-test or Bonferroni post-hoc test in pairwise comparisons, while a significant main

effect of diabetes or a significant interaction between diabetes and time are listed within figure legends.

RESULTS

Consistent with previous findings (Krause et al. 2011, Krause et al. 2009, Hong et al. 2007), *Ins2*$^{WT/C96Y}$ mice spontaneously developed hypoinsulinemia and hyperglycemia at ~4 weeks of age, and maintained this diabetic state throughout the experimental time course (Table 4.1). *Ins2*$^{WT/C96Y}$ mice also demonstrated hyperlipidemia, elevated PAI-1 and attenuated body mass accrual by 6 weeks of untreated diabetes (Table 4.1), consistent with observations of T1DM in humans (Zeitler, Thiede, and Muller. 2001; Standl et al. 1980; Rosenfalck et al. 2002) and diabetic rodents (Krause et al. 2011; Oishi et al. 2004; Oishi, Ohkura, and Ishida. 2006; Krause et al. 2008; Hong et al. 2007; Johnston et al. 2007).

Assessment of the uninjured leg of *Ins2*$^{WT/C96Y}$ mice revealed a significant loss of muscle mass in the TA and GPS compared to WT (Table 4.1). Furthermore, fiber area was reduced in *Ins2*$^{WT/C96Y}$ mice compared to WT in H&E stained cross-sections of TA and GAS. Conversely, uninjured SOL of *Ins2*$^{WT/C96Y}$ mice exhibited no difference with WT in myofiber area (Table 4.1), consistent with previous studies (Armstrong, Gollnick, and Ianuzzo. 1975; Cotter et al. 1993; Cotter et al. 1989; Medina-Sanchez et al. 1991; Snow et al. 2006).

In response to cardiotoxin injury, *Ins2*$^{WT/C96Y}$ TA and GAS demonstrated a significant loss of fiber area in regenerating muscle fibers compared to WT (Figure 4.1A-C), even when expressed relative to uninjured fiber area (Figure 4.1E, F). Conversely, SOL did not demonstrate a reduction in regenerating fiber area, in absolute or relative terms (Figure 4.1D,G). Masses of

the injured TA and GPS complex were reduced in *Ins2*$^{WT/C96Y}$ mice compared to WT as well (Figure 4.1H, I), corresponding with the reduced fiber areas in the GAS and TA muscles.

Given this muscle-specific diversity in responses to the diabetic environment, further investigation into the regeneration process of *Ins2*$^{WT/C96Y}$ diabetic muscle was needed. It is well understood that ECM remodelling is a critical component of the regeneration process (Krause et al. 2011; Koh et al. 2005; Goetsch et al. 2011; Koskinen et al. 2001; Koskinen et al. 2002), thus, we determined the expression levels of a primary ECM component, collagen. The injured TA muscles of *Ins2*$^{WT/C96Y}$ mice were found to have elevated collagen expression compared to WT, particularly at 5 days post-injury, as demonstrated by picrosirius red staining (Figure 4.2A, D). While the regenerating GAS exhibits a minor trend (P=0.11), the SOL (P=0.51) muscle does not demonstrate significant differences in collagen expression between *Ins2*$^{WT/C96Y}$ and WT mice (Figure 4.2B, C). The picrosirius red stained TA muscles also clearly demonstrate smaller fiber areas (yellow staining) observable at 10 days post-injury (Figure 4.2D), confirming the analysis of H&E stained tissue sections.

Though the increased expression of collagen during the early phase of regeneration is important to maintain muscle integrity as the myofibers regenerate, we speculated that excessive collagen expression, particularly in the *Ins2*$^{WT/C96Y}$ TA muscles would attenuate the normal regenerative process. More specifically, we hypothesized that excessive collagen expression would delay the degeneration phase; a hypothesis that was tested by evaluating the size of necrotic versus regenerating areas within regenerating *Ins2*$^{WT/C96Y}$ and WT muscles. Sections of TA and GPS were immunofluorescently stained for type I collagen and DAPI (nuclear stain) so as to accurately identify necrotic and regenerating areas in each muscle (Figure 4.3A). Areas with highly disrupted collagen surrounding large, rounded, myofibers were identified as

damaged muscle undergoing necrosis, while areas with smaller, centrally-nucleated myofibers represent areas of regeneration. In each muscle, the sum area of the necrotic regions was determined and expressed as a percentage of the total injured area (necrotic area + regenerating area) in the muscle. The SOL did not have any remaining necrotic regions at 5 days post-injury in WT or *Ins2*$^{WT/C96Y}$ mice, whereas the TA and GAS exhibited varying amounts of necrosis up to 10 days post-injury (Figure 4.3B-D). Specifically, the TA of WT mice demonstrated a larger necrotic region size at 5 days post-injury ($P<0.05$) compared to the *Ins2*$^{WT/C96Y}$ TA. At 10 days post-injury, there was an absence of necrosis in the TA muscle of WT mice but a significant level of necrosis was still measurable in *Ins2*$^{WT/C96Y}$ mice (significant interaction: $P<0.05$. Figure 4.3B). A temporal pattern similar to that observed in the TA was observed in the GAS, although it did not reach statistical significance (Figure 4.3C; $P=0.21$). Quantification of necrotic region size in tissue sections, as depicted in Figure 4.3D, was confirmed in other sections stained immunofluorescently for laminin and DAPI as well as by H&E staining (data not shown).

To determine if the impaired regenerative response observed in *Ins2*$^{WT/C96Y}$ TA and GAS muscles was a direct result of impaired macrophage infiltration secondary to elevated collagen levels we undertook F4/80 immunostaining and quantification within the necrotic and regenerating regions of the injured muscles. In the TA, a significant reduction in F4/80 positive cells was found in the necrotic region (Figure 4.4A). A similar trend was observed in the necrotic region of the GAS, but was not statistically significant (Figure 4.4B; $P=0.09$). In the necrotic regions, macrophages are typically found around the edge of the degenerating myofibers or are found entering the degenerating fibers as they are degraded and phagocytosed (Figure 4.4C). In regenerating regions of the injured muscles, (TA, GAS, SOL) no significant difference

between groups was noted in macrophage number at 5 or 10 days post-injury (Figure 4.4D-G). Interestingly, SOL demonstrates a non-significant trend for elevated macrophage number in the regenerating muscle at 5 and 10 days (main effect; P=0.13).

An important stage of skeletal muscle regeneration involves the de novo formation of myofibers from the fusion of myoblasts. These nascent myofibers will initially express embryonic myosin heavy chain (Myh3) before expressing mature myosin isoforms. In the necrotic region of the TA at 5 days post injury, significantly less Myh3 expression was observed in *Ins2*$^{WT/C96Y}$ mice compared to WT (Figure 4.5A), as well as in the GAS (Figure 4.5B). Myh3 expression in necrotic regions of TA and GAS is illustrated in Figure 4.5C. In regenerating regions, expression of Myh3 observed at 5 days post-injury indicates a reduction in *Ins2*$^{WT/C96Y}$ mice (significant interaction: $P<0.05$). However, at 10 days post-injury there is still significant expression of Myh3 in the *Ins2*$^{WT/C96Y}$ TA (Figure 4.5D) and GAS (Figure 4.5E) compared to WT ($P<0.05$), indicating a delay in regeneration. Consistent with no changes in collagen levels or macrophage infiltration, no delay in Myh3 expression was noted in the *Ins2*$^{WT/C96Y}$ SOL (Figure 4.5F). Size of Myh3-positive cells was also determined in the regenerating region of the three muscle groups and a similar pattern was observed. The GAS demonstrated a delay in the growth of the Myh3-positive cells (interaction: $P<0.05$), while the TA exhibited a trend for a delay (Supplementary Figure 4.1A, B; P=0.078). On the other hand, the SOL did not demonstrate any delay in the growth of Myh3-positive myofibers. These data once again demonstrate a deficit in the regenerative capacity of *Ins2*$^{WT/C96Y}$ mouse TA and GAS, but not the SOL.

DISCUSSION

T1DM has been associated with a number of negative effects on skeletal muscle

including attenuated growth and impaired regeneration (Krause, Riddell, and Hawke. 2011; Krause et al. 2011; Gordon et al. 2010; Krause et al. 2009; Gulati and Swamy. 1991; Vignaud et al. 2007). We recently demonstrated that the TA muscles of *Ins2*$^{WT/C96Y}$ mice exhibited impaired regeneration with as little as 7 days of diabetes exposure prior to injury (Krause et al. 2011). Further, we defined the mechanism for this impairment as a chronic elevation of PAI-1 slowing ECM breakdown through reductions in uPA and MMP9 enzymatic activity. What remained unanswered is: (1) how the PAI-1 -mediated attenuation of collagen turnover may be causing the delay in regeneration and (2) whether this phenomenon was occurring in all muscle groups, as it had been reported previously that the soleus muscle of streptozotocin-induced diabetic mice were largely exempt from the negative effects of type 1 diabetes (Armstrong, Gollnick, and Ianuzzo. 1975; Cotter et al. 1993; Cotter et al. 1989; Medina-Sanchez et al. 1991; Snow et al. 2006).

The findings of the present study reveal that exposure to the type 1 diabetes environment results in impaired skeletal muscle regeneration; a finding that is more pronounced in muscles with a greater glycolytic fiber-type composition. In the *Ins2*$^{WT/C96Y}$ TA, and to a lesser extent the GAS, muscle injury is followed by an excessive collagen accumulation (relative to WT) that reduces macrophage infiltration into the areas of damage, ultimately prolonging the necrotic phase of muscle regeneration. This delay in degeneration translates into an attenuation of the regenerative phase, as illustrated by a temporal delay in the expression of developmental myosin heavy chain isoforms, and ultimately, reduced myofiber sizes. Interestingly, the SOL muscles of *Ins2*$^{WT/C96Y}$ mice did not display any of the impairments noted above, a finding of critical importance as we begin to unravel mechanisms underlying T1DM-mediated complications within skeletal muscle.

PAI-1 has been characterized as a primary suppressor of proteolytic activity in the extracellular space, slowing muscle regeneration down by inhibiting a cascade of protease activation (Koh, etc). PAI-1 directly binds and inhibits uPA, the key plasminogen activator in muscle regeneration, preventing active plasmin from disrupting ECM proteins and from cleaving pro-MMP2 and pro-MMP9 into their active forms. These MMPs, particularly MMP9, are crucial during the early stages of muscle regeneration, functioning to enzymatically cleave protein chains composing the ECM (Mehan et al. 2011; Nishimura et al. 2008; El Fahime et al. 2002). The present study demonstrates impaired collagen breakdown in the *Ins2*$^{WT/C96Y}$ mouse, indicative of PAI-1 mediated suppression of ECM remodelling, consistent with previous studies demonstrating PAI-1 mediated fibrosis in cardiac and skeletal muscle (Zaman et al. 2009; Naderi et al. 2009). The ability to effectively remodel the ECM is of paramount importance for cell infiltration as both macrophages and myoblasts rely on uPA activity in order to migrate to regions of skeletal muscle damage (Novak et al. 2011; Sisson et al. 2009; Bryer et al. 2008; DiPasquale et al. 2007; Koh et al. 2005; Fibbi et al. 2001). Furthermore, MMP9 is also required for myoblast migration, another process critical to efficient injury repair in skeletal muscle (Hawke and Garry. 2001; Nishimura et al. 2008; Lewis et al. 2000). Our lab previously demonstrated that PAI-1 mediated-suppression of ECM remodelling (via uPA and MMP9) caused delayed regeneration in *Ins2*$^{WT/C96Y}$ TA (Krause et al. 2011). The present study builds on these findings by providing data to support that PAI-1 elevations in *Ins2*$^{WT/C96Y}$ mice are associated with impaired macrophage infiltration. Specifically, at 5 days post-injury macrophage number is reduced in necrotic regions of *Ins2*$^{WT/C96Y}$ TA and, to an extent, GAS in comparison with WT. Previous studies confirm that PAI-1 impairs macrophage infiltration via uPA inhibition, thereby suppressing the initiation of injury repair (Novak et al. 2011; Bryer et al.

2008; DiPasquale et al. 2007; Koh et al. 2005; Fibbi et al. 2001). That this is occurring in injured skeletal muscle of diabetic mice is a critical finding because macrophages play multiple roles in the regeneration process. Initially, these cells phagocytose damaged/dying muscle fibers and debris, but by 3-5 days post-injury, macrophages switch to a role of signalling for myoblast fusion and differentiation (Tidball and Villalta. 2010). Our results indicate that reduced macrophage infiltration in the diabetic mouse results in necrosis persistent at 10 days post-injury concurrent with delayed initiation of the regeneration phase indicated by reduced Myh3 expression.

While we believe this cascade of events accurately depicts the deficits in *Ins2*$^{WT/C96Y}$ mouse TA muscle, differential effects were noted between muscles groups in these diabetic mice. Of the muscle groups studied in the current work, the TA demonstrated the most severe impairment in regeneration, while the measures made in the SOL were similar between WT and *Ins2*$^{WT/C96Y}$ mice. The GAS exhibited an intermediate phenotype, with less severe impairments than the TA. The evidence to date, derived from STZ-induced diabetic rodents, supports our findings that the SOL exhibits resilience against diabetic myopathy. Previous studies demonstrate that the SOL, a muscle composed primarily of type I myosin heavy (Burkholder et al. 1994) is resistant to the atrophy or lack of growth induced by T1DM (Armstrong, Gollnick, and Ianuzzo. 1975; Cotter et al. 1993; Cotter et al. 1989). In fact, isolated type I fibers from diabetic rats demonstrate no loss of fiber size or contractile force (Sanchez et al. 2005), contrary to that found in isolated type II fibers (Krause et al. 2009; Cotter et al. 1993; Cotter et al. 1989; Medina-Sanchez et al. 1991; Snow et al. 2006). The results of the current study add further insight into how the SOL may maintain fiber size in the diabetic state. We have demonstrated that in both the WT and the *Ins2*$^{WT/C96Y}$ mice, necrotic areas within the SOL are cleared before 5

days post-injury and there is no difference in Myh3 expression or macrophage infiltration between WT and *Ins2*$^{WT/C96Y}$ mice throughout the early regeneration period. Furthermore, despite the systemic increase in PAI-1, the regenerating SOL muscles of the *Ins2*$^{WT/C96Y}$ mice did not display excessive collagen levels and fiber areas during the regenerative time-course were consistently similar to that observed in the age-matched WT.

Given the results of our previous work (Krause et al. 2011) and the results presented here, chronic elevations in PAI-1 appear to be the primary mediator of the impaired regeneration observed in diabetic mice. What remains to be elucidated therefore is how this systemic rise in PAI-1 can have elicited such dramatic impairments within the TA but have little impact on the regenerative capacity of the SOL. The answer may lie with the intrinsic differences between these muscle groups in the PA pathway. uPA, for example, is highly expressed in SOL but not glycolytic muscle groups (Barlovatz-Meimon et al. 1990), meaning that despite increases in the PAI-1:uPA ratio, free (active) uPA may still be available in the diabetic SOL, affording it the opportunity to activate the proteolytic cascade. As well, following muscle injury or during adolescent growth, a greater inducible-expression and activity of MMP9 is observed in the oxidative muscle, such as the SOL, compared to the glycolytic muscle (Zimowska et al. 2008; Michelin et al. 2009). As discussed, MMP9 activity is critical for muscle regeneration to occur (Mehan et al. 2011; Nishimura et al. 2008; Lewis et al. 2000), and a greater inducible-expression in the diabetic SOL may allow it to proceed through regeneration normally, compared to the glycolytic TA which lacks the same degree of inducible MMP9 expression. Evidence also exists that collagen types I, III and IV, the main constituents of skeletal muscle collagen, undergo a much milder gene expression response in the injured SOL compared to injured glycolytic muscles (Koskinen et al. 2001). This implies that ECM remodelling is less extensive in the SOL

compared to glycolytic muscle groups. Thus, the literature supports the notion that intrinsic differences between muscle groups could be responsible for the observed differential response to injury noted in diabetic muscle groups. However, future studies are clearly necessary to confirm these intrinsic differences in diabetic skeletal muscle.

Another important question raised by the current work is whether the specific effects observed in our work and others (Krause et al. 2009; Armstrong, Gollnick, and Ianuzzo. 1975; Cotter et al. 1993; Cotter et al. 1989; Medina-Sanchez et al. 1991; Snow et al. 2006; Snow et al. 2005; Sanchez et al. 2005) are the result of a muscle phenomenon, at the level of the tissue (including neural and vascular components) or, rather, are direct effects of the fiber-type composition of the muscle. While our findings do not rule out the muscle group-specific hypothesis, the fiber-type composition of the TA is almost exclusively a glycolytic muscle, while the SOL is exclusively oxidative and the GAS is intermediate in fiber-type composition between the TA and SOL (Burkholder et al. 1994). Despite the susceptibility of glycolytic fibers to myopathy in T1DM as indicated by animal studies, it is has been demonstrated that sedentary type 1 diabetic people have a greater proportion of type IIB fibers compared to non-diabetic controls (Fritzsche et al. 2008), though it may be speculated that this is likely a result of their exogenous insulin administration affording survival to the glycolytic fibers. Clearly future studies are needed to investigate this relationship between fiber-type and diabetic myopathy.

Conclusions

In conclusion, our data support the hypothesis that skeletal muscle regeneration in T1DM is characterized by a PAI-1 mediated attenuation of ECM remodelling resultant from an inability for macrophage infiltration. This delay in the degenerative phase slows the regenerative phase and ultimately results in reduced muscle fiber size in the regenerated muscle. Importantly, this

phenomenon does not occur in the oxidative soleus muscle of diabetic mice suggesting that some muscles may exhibit resistance to the diabetic environment. Unraveling the mechanisms affording the oxidative soleus this resistance will be critical in developing therapeutic strategies aimed at maintaining skeletal muscle in T1DM is critical.

TABLES AND FIGURES

Table 4.1. Characteristics of wild type and *Ins2* diabetic mice.

Measure	Group	Time of diabetes					
		6 weeks			9-13 weeks		
Body Mass (g)	WT	24.9	±	0.4	27.7	±	0.1
	Ins2$^{WT/C96Y}$	22.9	±	0.4*	24.5	±	0.1*
Insulin (pg/ml)	WT	814	±	106	1650	±	218
	Ins2$^{WT/C96Y}$	222	±	56*	209	±	24*
Blood Glucose (mM)	WT	9.1	±	0.3	8.7	±	0.2
	Ins2$^{WT/C96Y}$	32.4	±	0.7*	32.2	±	0.4*
Non-Esterified Fatty Acids (mM)	WT	1.10	±	0.06	0.66	±	0.04
	Ins2$^{WT/C96Y}$	2.14	±	0.12*	1.59	±	0.12*
PAI-1 (pg/ml)	WT	429.6	±	112.0	291	±	33
	Ins2$^{WT/C96Y}$	1593.5	±	134.3*	960	±	217*
TA Mass (grams)	WT				0.050	±	0.002
	Ins2$^{WT/C96Y}$				0.041	±	0.002*
GPS Mass (grams)	WT				0.177	±	0.006
	Ins2$^{WT/C96Y}$				0.141	±	0.003*
TA fiber area (μm^2)	WT				2285	±	92
	Ins2$^{WT/C96Y}$				1851	±	36*
GAS fiber area (μm2)	WT				2314	±	102
	Ins2$^{WT/C96Y}$				1926	±	83*
SOL fiber area (μm2)	WT				1154	±	47
	Ins2$^{WT/C96Y}$				1094	±	41

Data collected at 6 weeks of diabetes, or at 5, 10, or 35 days following cardiotoxin muscle injury induced at 8 weeks of diabetes. All measures except for soleus (SOL) fiber area were found to be significantly altered in the diabetic mice. All muscle mass and fiber area measures presented here are from the uninjured muscle; the cardiotoxin injured contralateral leg muscle data are presented in figure 1. NEFA, non-esterified fatty acids; PAI-1, plasminogen activator inhibitor-1; TA, tibialis anterior; GPS, gastrocnemius-plantaris-soleus complex; GAS, gastrocnemius. * denotes significant difference compared to matching wild type (WT) value as assessed by t-test ($P<0.05$).

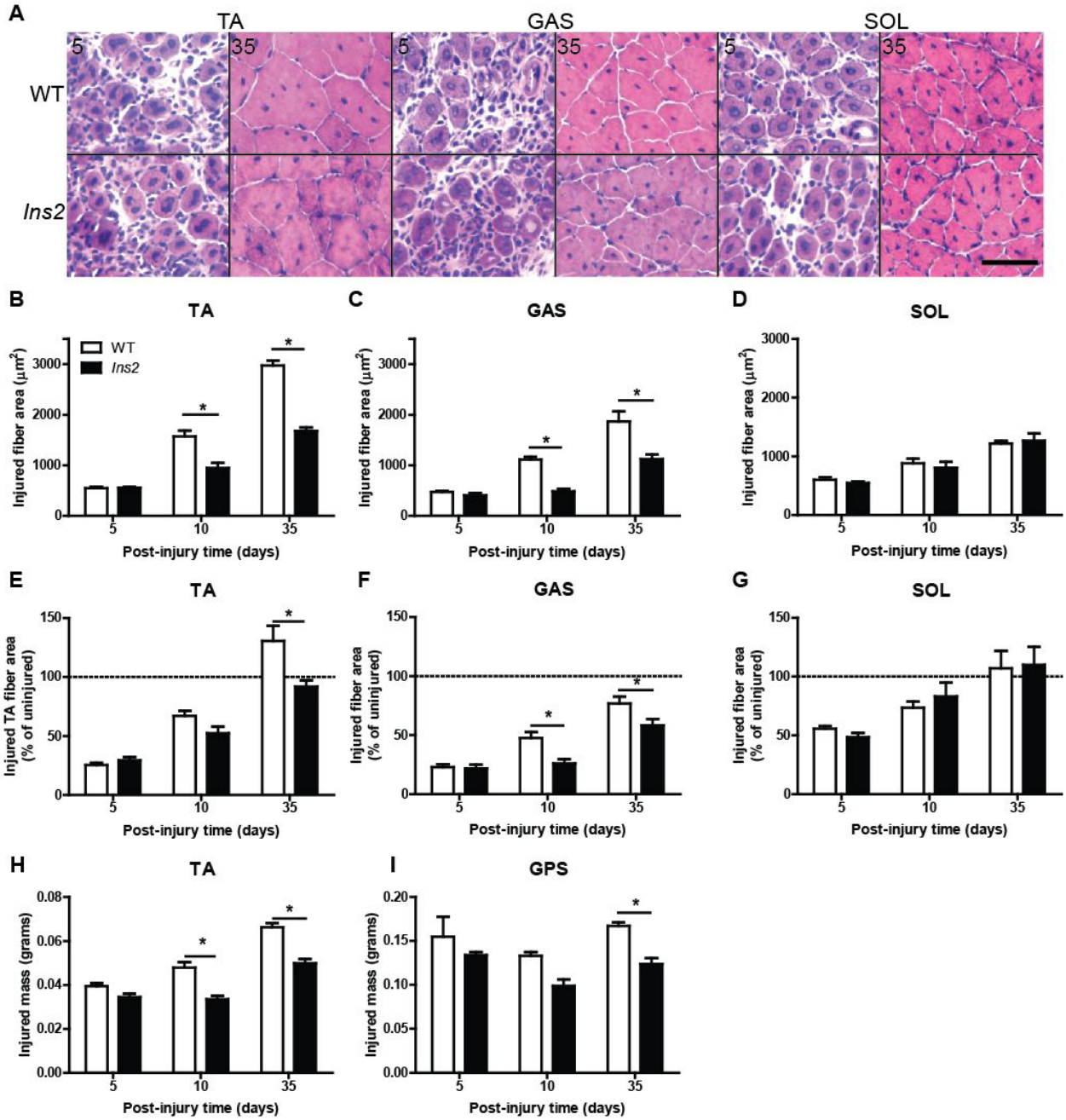


Figure 4.1. Soleus muscle is resilient to impaired regeneration in type 1 diabetes. (A) H&E stained cryosections of tibialis anterior (TA), gastrocnemius (GAS), and soleus (SOL) illustrate decrements in muscle regeneration in diabetic mouse muscles. (B) TA demonstrates the worst impairment in regeneration, as determined by cross sectional fiber area (significant main effect of diabetes and interaction [$P<0.05$]). GAS (C) also demonstrates impaired regeneration

(significant main effect of diabetes and interaction [P<0.05]), while SOL (D) does not. In panels E-F, TA (E), GAS (F), and SOL (G) injured fiber area data are expressed relative to uninjured fiber area. The TA (significant main effect of diabetes and interaction [P<0.05]) and GAS (significant main effect of diabetes [P<0.05]) both demonstrate poor regeneration even when expressed in these relative terms. Muscle mass of the injured TA (H; significant main effect of diabetes and interaction [P<0.05]) and gastrocnemius-plantaris-soleus complex (I; significant main effect of diabetes [P<0.05]) further illustrate poor regeneration. * denotes significant post-hoc analysis differences (P<0.05). Scale bar represents 50um.

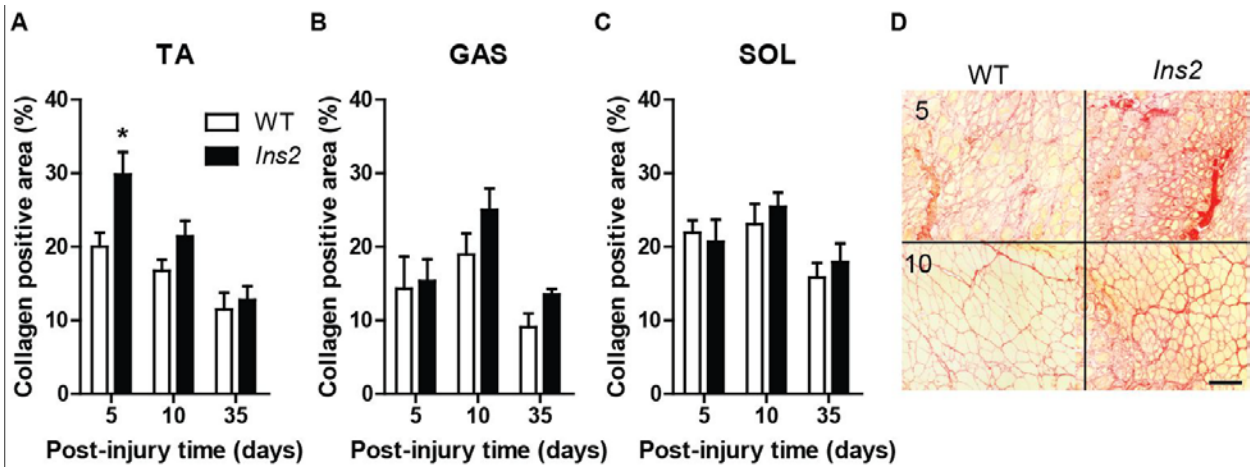


Figure 4.2. Tibialis anterior muscle of *Ins2*$^{WT/C96Y}$ mice demonstrate transient, excessive fibrosis during early regeneration. (A) TA demonstrates elevated collagen during regeneration, particularly at 5 days post-injury (significant main effect of diabetes [P<0.05]), while GAS (B) demonstrates a non-significant trend (main effect of diabetes [P=0.11]). Conversely, SOL (C) does not demonstrate dysregulated collagen expression. * denotes significant post-hoc analysis differences (p<0.05). (D) Representative picrosirius red staining of 5 and 10 day post-injury TA muscle. Note the elevated presence of collagen (red) and smaller size of myofibers (yellow) in *Ins2* TA . Scale bar represents 50um.

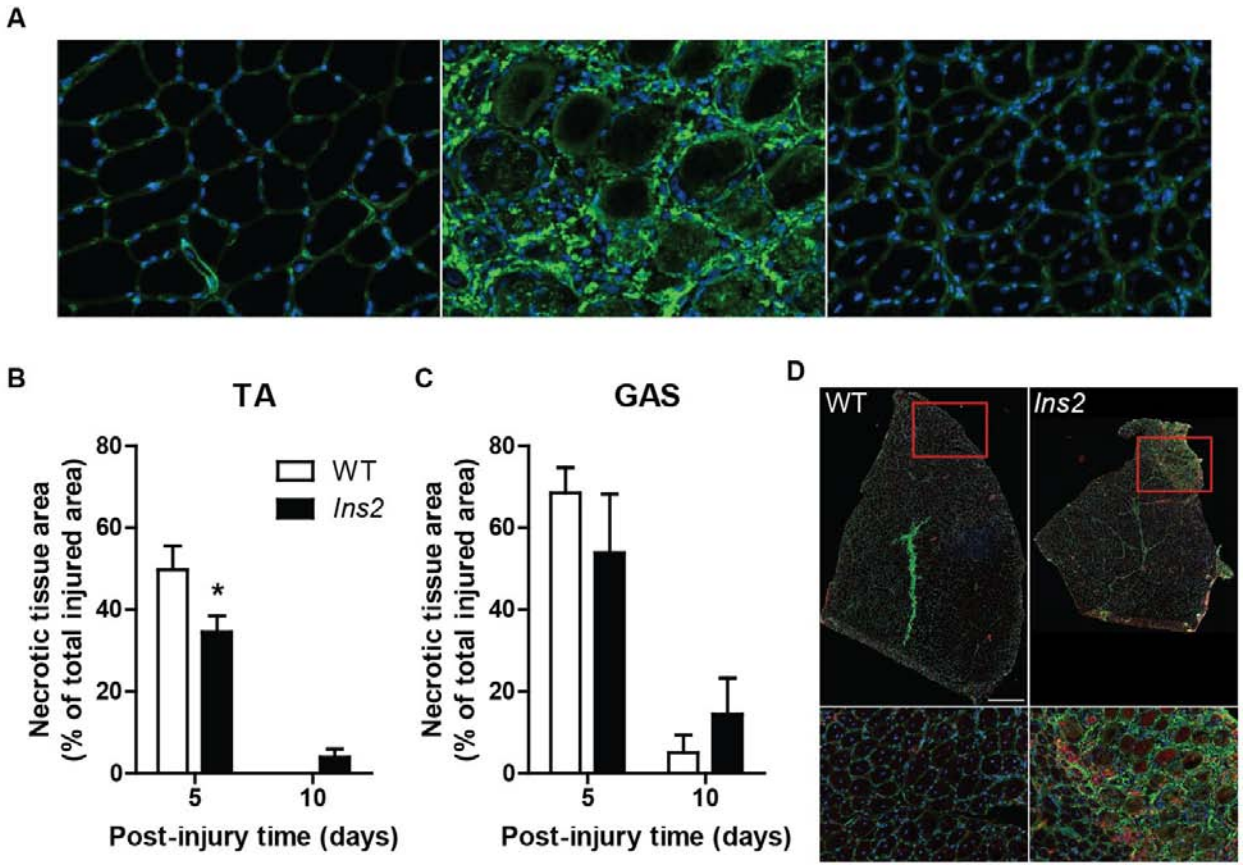


Figure 4.3. Necrosis of muscle fibers persist for throughout muscle regeneration in *Ins2*$^{WT/C96Y}$ tibialis anterior muscle. (A) Uninjured (left), necrotic (center), and actively regenerating (right) regions of skeletal muscle are easily identified in collagen type I immunostain with DAPI counterstain. Note the presence of centrally located nuclei in muscle fibers in regenerating muscle, indicative of new myofiber formation. (B) TA of diabetic mice have clearly defined areas of necrosis remaining at 10 days post-injury (significant interaction [$P<0.05$]; * denotes post-hoc difference). Similarly, GAS (C) follows this pattern although not statistically significant ($P=0.21$). SOL has no areas of necrosis at 5 or 10 days post-injury. (D) Representative image of TA muscle undergoing regeneration at 10 days post-injury in WT and *Ins2*$^{WT/C96Y}$ TA. Note the distinct area of necrosis in the *Ins2* TA. Scale bar represents 500um.

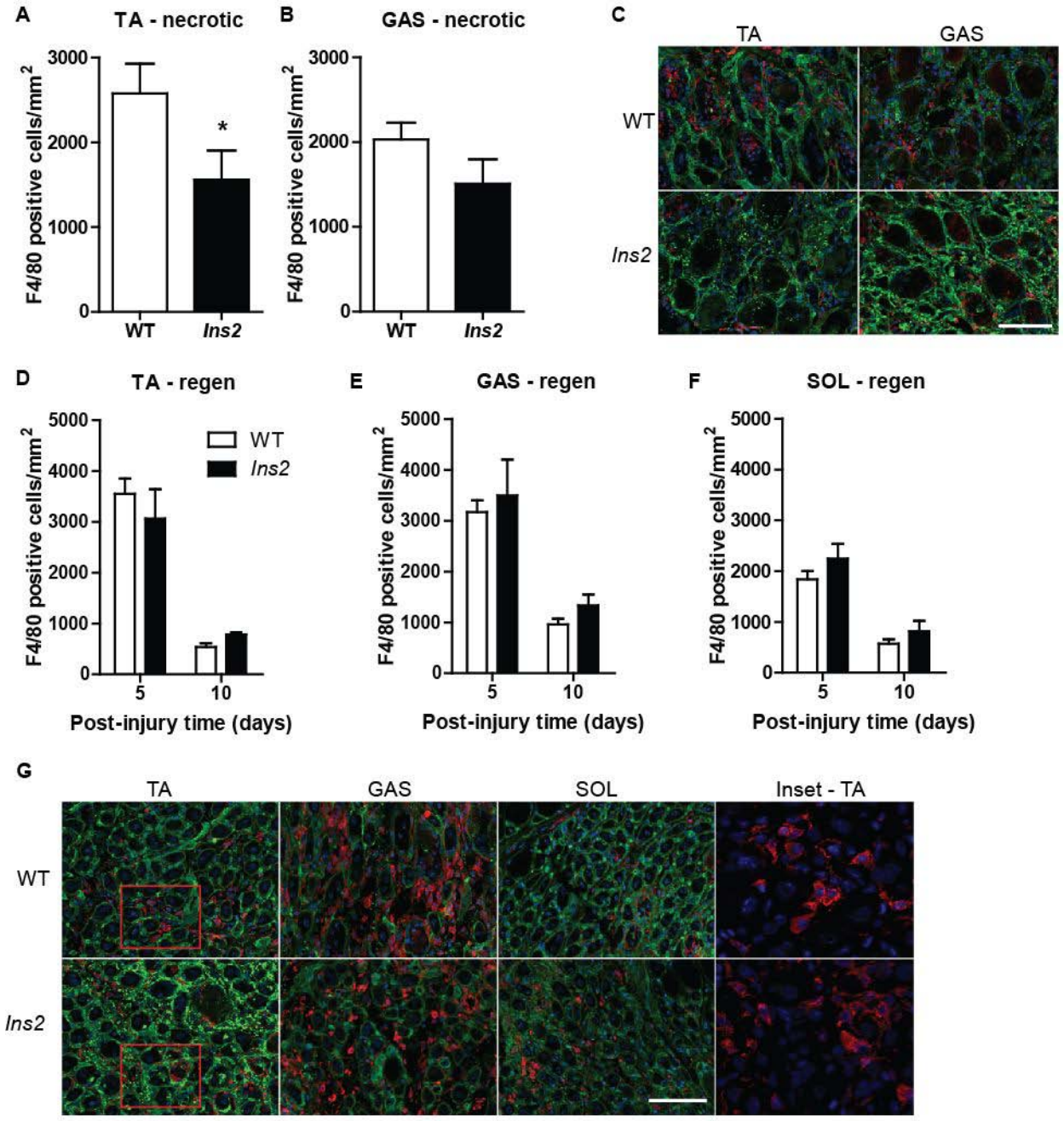


Figure 4.4. Macrophage entry into necrotic muscle is reduced in tibialis anterior of *Ins2*$^{WT/C96Y}$ diabetic mice. (A) The necrotic area of the TA demonstrates a reduced number of macrophages as evidenced by less F4/80 positive cells. * denotes significant difference by t-test

($p<0.05$). (B) A significant attenuation of F4/80 positive cells was not found in necrotic GAS ($P=0.09$). (C) Representative images of necrotic regions of TA and GAS stained immunofluorescently for F4/80 (red) and counterstained for type I collagen (green) and nuclei with DAPI (blue). In actively regenerating areas of the muscles, no significant alteration in macrophage number was found in (D) TA, (E) GAS, or (F) SOL muscles. (G) Representative images of regenerating muscles stained for F4/80 and type I collagen. Inset is provided without green channel to clearly demonstrate F4/80 stain morphology. Scale bar represents 50um in panels C and G.

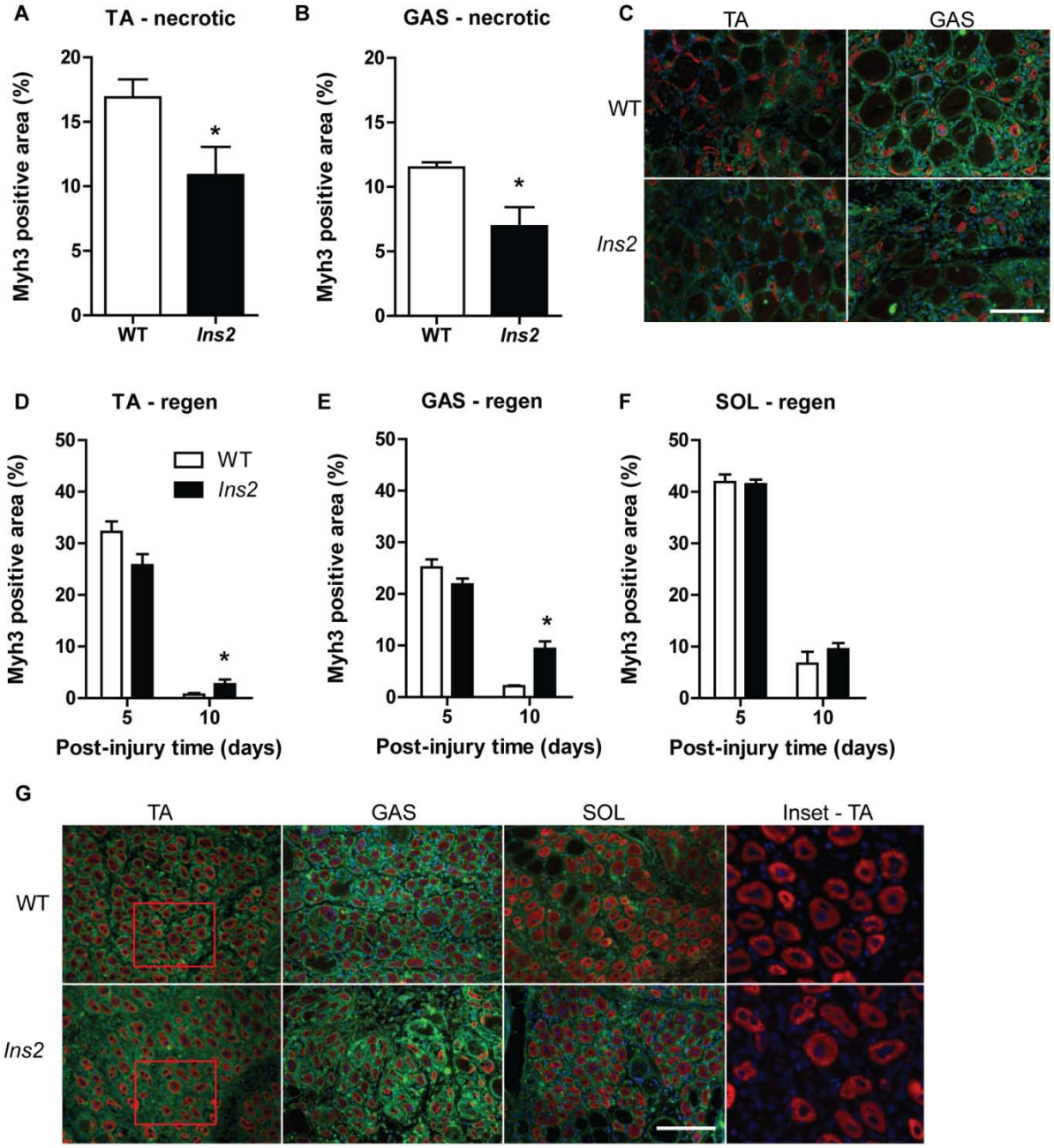
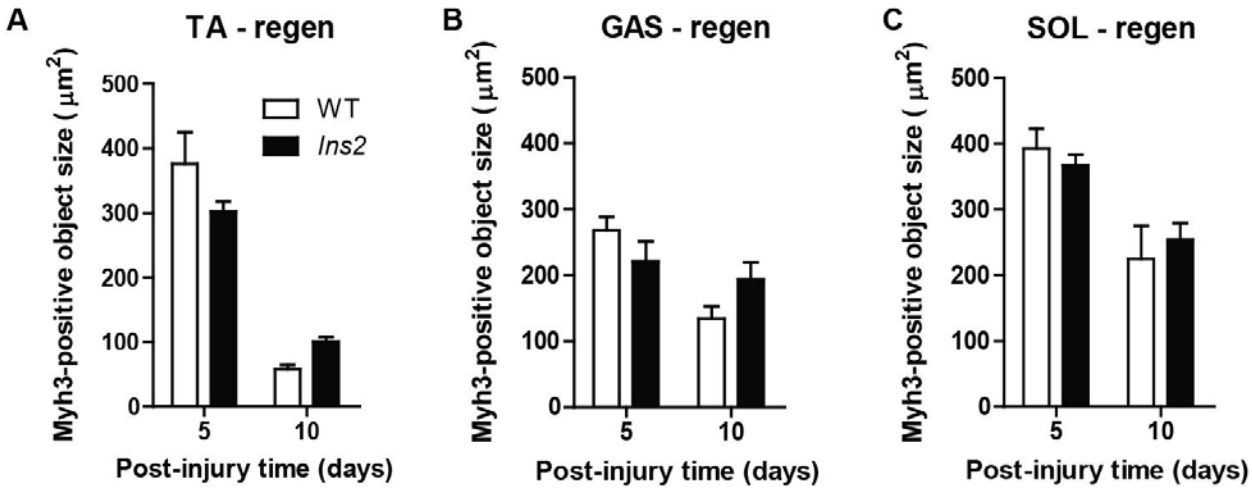


Figure 4.5. Initiation of muscle regeneration is delayed in tibialis anterior and gastrocnemius muscles in type 1 diabetes. Regions of necrotic muscle tissue in (A) TA and (B) GAS demonstrate attenuated response of Myh3-expressing myocytes in diabetic mice. * denotes significant difference detected by t-test ($p<0.05$). (C) Representative image of Myh3 immunofluorescent stained (red) TA and GPS necrotic regions. Counterstaining of laminin

(green) and nuclei with DAPI (blue) was performed. In the actively regenerating regions of the (D) TA and (E) GAS, the attenuation of Myh3 expression was not as severe, and at 10 days post-injury, was found to be significantly elevated in diabetic mice compared to wild type, indicative of delayed regeneration (significant interaction [$P<0.05$]). * indicates significant differences detected by post-hoc testing ($P<0.05$). Conversely, (F) *Ins2*$^{WT/C96Y}$ SOL demonstrates no impairment in establishing Myh3 expression following injury and does not continue to significantly overexpress Myh3 at 10 days post-injury. (G) Representative image of regenerating muscles at 5 days post-injury stained for Myh3 and laminin. Inset is provided without green channel to clearly demonstrate Myh3 stain morphology. Scale bar represents 50um in panels C and G.



Supplementary Figure 4.1. Growth of Myh3-positive cells is delayed in regenerating *Ins2*$^{WT/C96Y}$ tibialis anterior and gastrocnemius but not soleus muscles. The average size of Myh3-positive objects was determined and it was found that the TA (A) exhibited a strong trend for a delay in the growth of Myh3-positive cells (P=0.078), while the delay in growth in the GAS (B) was statistically significant (P<0.05). The SOL exhibited no trend for a delay in growth of Myh3-positive cells (P=0.42).

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CHAPTER 5

General discussion and conclusions

Significance of the studies

Type 1 diabetes mellitus (T1DM) onset typically occurs in childhood, placing a devastating burden on child and family. Diabetic complications can be the most prominent problems that diabetic patients deal with as the disease progresses, thus, developing treatments for diabetic complications may be a more feasible way of improving quality of life in the absence of a true cure. Diabetic myopathy has not conventionally received much clinical consideration despite evidence that skeletal muscle undergoes a negative response nearly immediately upon inception of T1DM (Jakobsen and Reske-Nielsen. 1986; Reske-Nielsen, Harmsen, and Vorre. 1977; Krause et al. 2011) while individuals suffering other disease states with consequential skeletal muscle atrophy, such as cancer cachexia, receive specific treatments (Lecker et al. 2004; Murphy and Lynch. 2009). Perhaps the reason that diabetic myopathy has not received due attention is presently there are no studies in the literature that have attempted to measure the outcome of diabetic myopathy on quality of life. However, in type 2 diabetes mellitus (T2DM) it has recently been demonstrated that independent of obesity, muscle mass is a strong predictor of both susceptibility to T2DM for prediabetics, as well as level of glycosylated hemoglobin, an index of glycemic control, in type 2 diabetics (Srikanthan, Hevener, and Karlamangla. 2010). It is hypothesized that by reducing the size of the glucose sink that glucose disposal would occur less quickly in response to insulin, thereby causing an increased insulin requirement and longer periods of time spent in hyperglycemia (Srikanthan, Hevener, and Karlamangla. 2010). On the other hand, by maintaining a healthy muscle mass, insulin requirements are minimized and glucose disposal improved. Thus, a feasible strategy to improve diabetes management would be to increase muscle mass in diabetics.

Exercise training can help to maintain muscle mass in T1DM and is typically prescribed (Roberston 2009), however, type 1 diabetics have difficulty achieving the same level of fitness gains through training as non-diabetics (Larsson 1964). Furthermore, reduced hand-grip strength is detectable (Fricke et al. 2008) and muscle fiber size is reduced (Jakobsen and Reske-Nielsen. 1986) early in juvenile T1DM demonstrating that skeletal muscle responds quickly to the diabetic environment. Studies on skeletal muscle disuse in young rodents reveal that muscle mass and satellite cell number are reduced even months after the end of the atrophic stimulus (Darr and Schultz. 1989; Mozdziak, Pulvermacher, and Schultz. 2000; Wanek and Snow. 2000). Additionally, the findings presented in this document indicate that the growth rate of skeletal muscle is reduced and the ability to regenerate mature myofibers and muscle mass following injury is severely compromised (Krause et al. 2011; Krause et al. 2009). Taken together, this indicates that young type 1 diabetics are particularly at risk of losing muscle mass or, rather, not attaining their full adult potential muscle mass. The current studies have also identified that reduced glycolytic muscle growth resulted in a proportional deficit in contractile function, and that glycolytic but not oxidative muscle has a reduced regenerative capacity mediated by chronic PAI-1 elevations. These deficits take place in a matter of weeks, indicating that juvenile type 1 diabetics are at risk of losing glycolytic muscle mass particularly during the time period between disease inception and diagnosis. Furthermore, with a reduced regenerative capacity, it is unknown if repeated bouts of muscle damage, such as that encountered in a typical resistance training program, would lead to the desired muscle hypertrophy. Given the importance of skeletal muscle mass to glucose disposal as well as its role in locomotor activity, attenuated growth during the most important muscle growth phase in life could have permanent deleterious effects on the regulation of glucose metabolism and capacity for physical fitness. For these

reasons, it is of paramount importance that strategies are developed for treatment of diabetic myopathy.

PAI-1 is elevated early in T1DM and remains so even with insulin treatment (Zeitler, Thiede, and Muller. 2001; Gogitidze Joy et al. 2010; Small et al. 1989; Auwerx et al. 1988). The key finding of the present muscle regeneration studies is that PAI-1 mediates the early delays in regeneration, which is very interesting in light of the role of PAI-1 in other diabetic complications, namely nephropathy and cardiomyopathy (Bosnyak et al. 2003; Lyon and Hsueh. 2003; Collins et al. 2006; Nicholas et al. 2005; Lassila et al. 2007; Goldberg. 2009). Does this make PAI-1 an ideal target for therapy? It is fascinating to note that diabetic nephropathy is characterized by PAI-1-mediated fibrosis (Collins et al. 2006; Lassila et al. 2007), analogous to the fibrosis observed during early regeneration in the current studies. In fact, elevated PAI-1 predisposes several tissues to fibrosis (Zaman et al. 2009; Kaikita et al. 2001; Eitzman et al. 1996). Currently, pharmacological inhibition of PAI-1 with PAI-039 (tiplaxtinin) and other related compounds remains in the preclinical stage for thrombosis treatment (Elokdah et al. 2004; Hennan et al. 2008; Crandall et al. 2004), however, to the best of our knowledge, we are the first group to demonstrate pharmacological inhibition of PAI-1 to treat a diabetic complication. Nonetheless, PAI-1 remains an exciting possibility for treatment of diabetic complications including myopathy.

Current hypotheses and future experiments

The current study is the first to examine PAI-1 in diabetic myopathy but clearly more studies aimed at elucidating the molecular and cellular mechanisms underlying impaired skeletal muscle regeneration are warranted. Stemming from the present work, it is important to identify alterations to protease expression and activity downstream of urokinase plasminogen activator

(uPA) in resting and injured skeletal muscle groups in the diabetic animals. The role of uPA, plasminogen, and matrix metalloproteinase-9 (MMP9) all have very specific biological roles to play in the context of tissue remodelling but with the exception of the current studies none have been studied in muscle regeneration in T1DM. We currently hypothesize that since uPA and MMP9 are highly expressed in oxidative muscle, (Barlovatz-Meimon et al. 1990; Michelin et al. 2009; Zimowska et al. 2008) they are able to maintain a required level of proteolytic activity to allow muscle regeneration in the soleus (SOL) despite systemically increased PAI-1 expression in the diabetic mouse. A number of studies employing uPA-null mice have established that uPA is an absolute requirement for muscle regeneration to occur (Koh et al. 2005; Bryer et al. 2008; Lluis et al. 2001; Novak et al. 2011; Fibbi et al. 2001). Also noteworthy is that although uPA is expressed by both satellite cells and macrophages in injured skeletal muscle (Bryer et al. 2008; Novak et al. 2011; El Fahime et al. 2002), it appears that uPA expression by macrophages in an otherwise uPA-null mouse model is sufficient for normal skeletal muscle regeneration to proceed following injury (Novak et al. 2011). This is further evidence of the importance of macrophage infiltration during the first few days after muscle injury. In the diabetic state, macrophage infiltration is reduced only in tibialis anterior (TA) muscle, thus, it can be speculated that uPA expression in the TA is insufficient to overcome the amount of local PAI-1 unlike the SOL where uPA expression is much greater. This suppression of uPA would then prevent macrophage infiltration due to inadequate ability to cleave through the extracellular matrix. However, further experiments will need to clarify the source of surplus uPA expression in the SOL since it could be expressed by existing/damaged myofibers, activated satellite cells/myoblasts, or macrophages themselves.

The current studies identified a deficit in an early step in skeletal muscle regeneration that accounts for slow degeneration and delayed regeneration in the diabetic mice. This does not necessarily explain why myofibers, once formed and maturing, accrue fiber area so slowly in the diabetic mice. There is evidence to suggest that skeletal muscle protein metabolism is imbalanced in T1DM through upregulated degradation (Mitch et al. 1999; Lecker et al. 1999) and reduced protein synthesis (Gordon et al. 2010; Nair et al. 1995; Bennet et al. 1990). Interestingly, during hypoinsulinemia, TA and gastrocnemius (GAS) but not SOL muscles demonstrate reduced protein synthesis in diabetic rats, while insulin treatment was found to temporarily stimulate protein synthesis in TA and GAS to near-control levels (Flaim, Copenhaver, and Jefferson. 1980). During muscle regeneration, once the damaged tissue has been degraded by inflammatory and proteolytic processes and nascent myofibers are formed, growth of those myofibers is then largely dependent on protein synthesis exceeding protein degradation (Hawke and Garry. 2001). Thus, it is hypothesized that PAI-1 exerts its negative effects in the first 5-10 days following injury, while altered protein metabolism dynamics account for impaired myofiber growth on a scale of weeks.

Another question stemming from the current studies is what muscle phenotype will result following injury and long term regeneration? Our fiber typing analyses revealed reduced fiber area in glycolytic but not oxidative fiber types as well as a decreased percentage of glycolytic fibers. We speculate that the untreated diabetic environment may be driving a muscle phenotype shift from glycolytic to oxidative. Particularly, the high availability of non-esterified fatty acids (Krause et al. 2011; Krause et al. 2009) and lowered glucose uptake and glycogen availability (Hamilton, Noble, and Ianuzzo. 1984) could influence gene transcription in the muscle (Freyssenet. 2007). When damage has occurred and regeneration begins, newly forming muscle

fibers in a state of development will have greater opportunity to express genes appropriate for existing in said diabetic environment, thus, the choice between oxidative or glycolytic fiber types must be made. On the other hand, evidence shows that oxidative or glycolytic muscle groups will return to its pre-injury fiber type composition due to the characteristics of its resident satellite cells (Kalhovde et al. 2005; Lagord et al. 1998). That is, the satellite cells in SOL tend to give rise to oxidative muscle fibers while those in glycolytic muscle groups tend to generate glycolytic muscle fibers (Kalhovde et al. 2005; Lagord et al. 1998). This raises another tremendous question which is what role does the satellite cell play in the regeneration process in T1DM? Clearly more research is needed to determine the long term effects of muscle regeneration on muscle phenotype, as well as to determine the satellite cells function and contribute to regeneration in T1DM.

Conclusions

The advent of insulin treatment for diabetes is one of the most important medical discoveries of the 20th century. Nearly a century later, it has become clear that a major hurdle in diabetes research will be to improve treatment beyond simply replacing insulin.

> *Monotherapy with insulin has transformed a previously fatal illness into a livable one, but injected hormone has not approached endogenous insulin in either the quality or quantity of life that it makes possible.* (Wang et al. 2010)

While the long term goal of diabetes research must ultimately be to develop a cure, perhaps more realistic short term research goals should be to determine how to best treat for the numerous diabetic complications that are a result of the multitude of alterations to the extracellular environment. Considering that diabetic complications account for the majority of mortality in T1DM (Secrest et al. 2010), treating the complications will have the greatest impact on improving quality and quantity of life. Others have demonstrated that supplementing diabetic

rodents with leptin in the absence of insulin treatment allows the animals to thrive, apparently eliminating hyperglycemia, hyperlipidemia and abolishing diabetic complications (Wang et al. 2010; Yu et al. 2008). Alternatively, diabetic mice deficient in PAI-1 are protected from diabetic nephropathy (Collins et al. 2006). And of course, as this document has demonstrated, severe consequences in skeletal muscle can be relieved with pharmacological treatment against PAI-1 (Krause et al. 2011). It is clear that dysregulated hormones other than insulin can be targeted effectively in diabetic animal models; it is time for this knowledge to translate into clinical advances and improved treatment for those suffering from T1DM.

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