Muscle Growth and Function in Mouse Models of Type 2 Diabetes Mellitus

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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Abstract

Type 2 diabetes mellitus is associated with an accelerated muscle loss during aging, decreased muscle function, and increased disability. To better define the mechanisms causing this muscle deterioration in Type 2 diabetes, we assessed muscle weight, exercise capacity, and protein expression in db/db and TallyHo mice at ages in which they exhibited prediabetes and overt diabetes. Maximum running speeds and muscle weights were already reduced before signs of overt diabetes in db/db mice when compared to lean controls and more severely reduced in the overtly diabetic db/db mice. In contrast to db/db mice, TallyHo muscle size dramatically increased and maximum running speed was maintained during the progression from prediabetes to overt diabetes. Analysis of mechanisms that may contribute to decreased muscle weight in db/db mice demonstrated that insulin-dependent phosphorylation of enzymes that promote protein synthesis was severely blunted in db/db muscle. In addition, muscle from db/db mice with prediabetes (6 week old) and diabetes (12 week old) db/db exhibited an increase in established markers of proteasomal protein degradation: MuRF-1, Atrogin-1, and the level of polyubiquitinated proteins. Chronic treadmill training of db/db mice improved glucose tolerance, exercise capacity, and reduced markers of protein degradation, but only mildly increased muscle weight. The differences in muscle phenotype between these models of type 2 diabetes suggest that insulin resistance and chronic hyperglycemia alone are
insufficient to rapidly decrease muscle size and function and that the effects of diabetes on muscle growth and function are model-dependent.
Dedication

This dissertation is dedicated to my Dad, and to all of those who, like him, deal courageously with the challenges of muscle atrophy.
Acknowledgments

The ultimate goal in many scientific experiments is to determine the mechanism of how a biological process occurs. I have seen repeatedly that no signaling pathway acts alone; multiple pathways and systems work together to achieve the desired effect. Similarly, none of what I have learned or accomplished in research or life has been possible alone. I appreciate the regular input and feedback of my research mentor, Dr. Periasamy, and his lack of fear in critiquing our own ideas and work. In addition, I am grateful for Dr. Santosh Maurya, who I was fortunate to be able to work closely with. I am grateful for his willingness to discuss ideas, troubleshoot, and perform experiments, especially in our study of proteasomal protein degradation. I was also fortunate to have a supportive environment of fellow graduate and postdoctoral students that were each kind to give their support, feedback, friendship, time for proofreading, and to share insight into experiments and life: Naresh Bal, Sana Shaikh, Danesh Sopariwala, Sanjaya Sahoo, Anuradha Kalyanasundaram, Leslie Rowland, and Meghna Pant. In addition, key experiments would not have been possible without the help from several key collaborators and their mentors: Dr. Hassan Talukder (Langendorff perfused heart experiments) and Dr. Justin Dials/Dr. Steve Devor/Dr. Steve Roof/Dr. Mark Ziolo (exercise studies). I would be remiss to not acknowledge the support of my committee
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Chapter 1: Muscle in diabetes

1.1 General Introduction

As humans age, it is common for skeletal muscle mass and function to deteriorate (Park et al., 2009). When muscle loss progresses to a pathologic extreme, it is termed “sarcopenia” and contributes to physical disability (Goodpaster et al., 2006), increased risk for injury, and prolonged hospitalizations (Singh et al., 2009). Type 2 diabetes mellitus (T2DM) is associated with an accelerated loss of muscle mass and function in the elderly (Park et al., 2007), leading to increased prevalence of clinical sarcopenia among those with T2DM. This decrease in muscle function may contribute several comorbidities associated with diabetes: increased risk (400%) for major falls (Maurer et al., 2005), increased prevalence and severity of physical disability (Gregg et al., 2000; Park et al., 2007; De Rekeneire et al., 2003; Ryerson et al., 2003), and prolonged hospitalizations (Koproski et al., 1997).

Despite the clear association between diabetes and muscle loss, the onset, progression, and mechanisms of muscle loss in diabetes are unclear. The purpose of this dissertation is to better define the onset and progression of muscle loss in diabetes and to better describe the underlying mechanisms, with the long-term objective of identifying pathways for therapeutic intervention. To give context to the experimental studies, this introduction will summarize the current body of knowledge on diabetes, muscle growth,
and muscle loss as distinct processes, review what is known about muscle loss due to diabetes, and then focus on key gaps in knowledge in the study of diabetes and muscle.

### 1.2 Diabetes mellitus

Diabetes mellitus is a disease in which the body is unable to maintain normal blood glucose levels due primarily to either insulin deficiency (Type 1 diabetes, 5-10% of patients), or insulin resistance (type 2 diabetes, 90-95% of patients). (Centers for Disease Control and Prevention, 2011) Diabetes mellitus is significant to human health because of 1) increased prevalence and severity of comorbidities in diabetes, 2) a 2-fold increased risk of death in diabetes and 3) increasing prevalence of diabetes in many countries. Indeed, diabetes mellitus is a growing epidemic that has been diagnosed in 23.6 million people in the United States and is increasingly prevalent among not only adults (Figure 1.1), but also children and adolescents (from 1-2% of new onset pediatric diabetes in 1990 to 8-45% in 2005). (American Diabetes Association, 2000; Fagot-Campagna et al., 2000; Hotu et al., 2004)

Type 2 diabetes detrimentally affects many organs and tissues throughout the body by causing decreased glucose metabolism, accumulation of toxic lipid intermediates, protein glycosylation, advanced glycation end products (AGE) mitochondrial stress, and increased production of reactive oxygen species (Ahmed, 2005; Brownlee, 2001; Chinen et al., 2007; Kersten et al., 2000; Park et al., 2008). For example, diabetes-associated tissue damage causes injury and dysfunction of the cardiovascular, nervous, renal, and immune systems in type 2 diabetes, causing a number of comorbidities that decrease both quality and duration of life (Table 1.1).
The complexity of multi-organ and multi-system changes that occur simultaneously during the development and progression of type 2 diabetes present a difficult challenge to researchers trying to establish cause-effect relationships between specific signaling pathways and tissue damage. This difficulty in isolating cause-effect mechanistic relationships in diabetes-associated organ disease is a key limiting factor in developing novel therapeutic approaches for diabetes.

1.2.1 Therapy and Cost of Diabetes Mellitus

Treatment of diabetes focuses on increasing insulin signaling and controlling blood glucose levels, as monitored by fasting blood glucose levels, glucose tolerance tests, and/or glycated hemoglobin (HbA1C%). (American Diabetes Association, 2012) Insulin signaling is increased by administration of insulin and/or insulin sensitizing medication. Blood glucose is further controlled by exercise-induced glucose uptake, reduced glycemic load via altered diet, and drugs that decrease intestinal glucose absorption (e.g., acarbose) or gluconeogenesis (e.g., metformin) (Clissold and Edwards, 1988; Kim et al., 2008). Lowering blood glucose levels (below 110 mg/dL (fasting) and below 140 mg/dL (2 hr glucose tolerance test), and HbA1C% < 7.0%) has been strongly associated with increased duration of life and reduction of comorbidities of diabetes. (American Diabetes Association, 2012)
1.2.2 Continued Need for Research in T2DM

Despite the number of therapeutic tools that ameliorate and reduce the comorbidities of diabetes, therapy is not completely effective. For example, even rigorous treatment of patients with T2DM to reduce blood sugar levels to a target blood HbA\(_1C\) of 7.0\% - 7.5\% only partially ameliorated the increased risk of death due to cardiovascular disease in T2DM, and further lowering actually increased mortality in the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial (The Action to Control Cardiovascular Risk in Diabetes Study Group, 2008). This data suggests that some complications of diabetes are not only caused by hyperglycemia, but are caused by other variable affected by diabetes (such as vasodilation, hormonal signaling, and oxidative stress). Indeed, diabetes mellitus remains a key contributor to morbidity and mortality in the United States and in many countries throughout the world. The total annual cost of diabetes in the United States was estimated in 2012 to be $245 billion, with $176 billion attributed to direct medical costs and $69 billion to reduced productivity (i.e. absenteeism, disability, early mortality). (American Diabetes Association, 2013) These costs are associated with a directly diabetes-related increase in health care expenses of $7,900 per year cost per person with diabetes. (Association, 2013) The continued health impact of diabetes despite the use of current treatment options indicates that additional research is needed to address treatment of diabetes beyond just glucose control. This need is clearly illustrated by the finding that rigorous, “ideal” glucose control in patients with T2DM only partially reduces the increased risk of cardiovascular death. Thus, further research to discover how diabetes causes end-organ
disease, identify responsible signaling pathways, and develop novel therapies capable of preventing and/or reversing diabetes-related organ dysfunction is necessary to reduce the impact of diabetes on human health.

1.2.3 Glucose metabolism in health and disease

The specific focus of this dissertation is how T2DM affects skeletal muscle growth and function. As detailed later in this chapter, T2DM impairs muscle metabolism, reduces muscle function, and accelerates muscle loss in the elderly, contributing to disability and injury. Because skeletal muscle plays a dominant role in insulin-dependent glucose uptake (~80-90%) and muscle growth and function are both closely linked to energy availability and metabolism, a discussion of the role of muscle in glucose metabolism in individuals with intact insulin signaling, compared to individuals with diabetes, is an appropriate starting point to understand the impact of diabetes on skeletal muscle (DeFronzo et al., 1981).

1.2.3.1 Glucose handling in healthy individuals after a meal

In healthy individuals, carbohydrate digestion leads to increased blood glucose levels. In the pancreas, transmembrane GLUT2 glucose transporters allow for passage of blood glucose into the beta cells of the islets of Langerhans, which allows pancreatic monitoring of blood glucose levels. Accumulation of intracellular glucose in pancreatic beta cells increases the intracellular ATP/ADP ratio, causing closure of ATP-sensitive K⁺ channels, depolarization of the cell membrane, and the opening of voltage-sensitive Ca²⁺ channels. Ca²⁺ influx triggers endocrine secretion of stored insulin and promotes the
synthesis of additional insulin. (Seino et al., 2011) Insulin then circulates in the blood throughout the body and binds to the integral membrane insulin receptor on many tissues (Federici et al., 1997; Pullen et al., 1976).

Insulin receptor binding and subsequent autophosphorylation leads, via a signaling cascade, to Glut4 glucose transporter translocation to the membrane, which allows for increased glucose uptake. (Cushman and Wardzala, 1980; Suzuki and Kono, 1980) When glucose levels lower again, insulin secretion is reduced and glucagon secretion (from pancreatic islet alpha cells) is increased as necessary to promote glycogenolysis, gluconeogenesis, and fat oxidation in order to maintain stable blood glucose levels. When these mechanisms function properly, blood glucose levels after fasting are maintained at less than 100-125 mg/dL and less than 200 mg/dL after an oral glucose tolerance test (American Diabetes Association, 2012).

1.2.3.2 Glucose handling in type 1 diabetes after a meal

In individuals with type 1 diabetes, pancreatic dysfunction results in deficient insulin secretion by the pancreas. Insulin secretory dysfunction in type 1 diabetes has multiple etiologies including: autoimmune, genetic (30-50%), infectious, chemical, iatrogenic, cancer, and diet. When blood glucose increases after food consumption in individuals with type 1 diabetes, insufficient insulin is secreted by the pancreas. Although the insulin receptor and insulin-dependent signaling are normally sensitive, decreased insulin secretion results in decreased peripheral glucose uptake and increased hyperglycemia. Diet modification and insulin replacement therapy are used to reduce glucose load and hyperglycemia, respectively. Type 1 diabetes accounts for 5-10% of
cases of diabetes mellitus and onset typically occurs in younger individuals (<40 years old).

1.2.3.3 **Glucose handling in type 2 diabetes after a meal**

In individuals with type 2 diabetes, the primary defect in glucose metabolism is tissue-level resistance to insulin signaling. At the initial stages of type 2 prediabetes, upon food consumption and increased blood glucose levels, the pancreas is intact and secretes insulin normally. Insulin circulates throughout the body and binds to insulin receptors normally, but insulin receptor activation and/or downstream insulin signaling are blunted. Reduced insulin signaling results in decreased glucose uptake (relative to the insulin levels). Increasing levels of insulin are secreted by the pancreas to compensate for insulin resistance and to normalize blood glucose. Chronic hyperglycemia (and overt type 2 diabetes) develops when the pancreas eventually becomes incapable of secreting enough insulin to compensate for the insulin resistance. This may occur when the insulin resistance exceeds the capacity of the pancreas, or commonly when the pancreatic islets begin to degenerate and atrophy after chronic overuse. type 2 diabetes accounts for 90-95% of cases of diabetes mellitus and has been traditionally considered an adult-onset disease (40+ years of age). However, a disturbing increase in prevalence of insulin resistance and type 2 diabetes among younger individuals, even as adolescents, has recently become apparent. Most studies of diabetes focus on the typical older population of 50-80 year olds; relatively little is known about the pathogenesis and effects of T2DM on the long-term growth, development, and health of the young-onset T2DM population.
1.2.3.4 Role of muscle in glucose uptake

In healthy individuals, skeletal muscle accounts for ~40% of total body weight, 80-90% of insulin dependent glucose disposal, and 20-30% of resting oxygen consumption.(DeFronzo et al., 1981; Janssen et al., 2000; Zurlo et al., 1990) Therefore, it is logical that skeletal muscle insulin resistance impairs whole-body glucose intolerance. Indeed, conditions that cause muscle disuse (e.g., bed rest, paralysis, or immobilization) are sufficient to rapidly cause insulin resistance and glucose intolerance.(Duckworth et al., 1980; Lipman et al., 1972; Stuart et al., 1988) Moreover, previous studies (DeFronzo and Tripathy, 2009) have strongly suggested that muscle insulin resistance is the primary defect in the pathogenesis of type 2 diabetes. Their conclusion is drawn based upon familial cases of T2DM, in which tissue specific glucose uptake during euglycemic-hyperinsulinemic clamps is monitored in youth before, during, and after the onset of T2DM. In these studies, skeletal muscle was chronologically the first tissue to show reduced glucose uptake, suggesting that skeletal muscle is the causal tissue for T2DM. However, the muscle-specific insulin-receptor knockout (MIRKO) mouse model failed to exhibit altered glucose homeostasis(Mauvais-Jarvis et al., 2000), suggesting that adaptive responses to deficient muscle insulin signaling may compensate for deficient muscle insulin signaling. Thus, the ability of muscle insulin resistance to initiate progression to whole body insulin resistance, glucose intolerance, and T2DM remains controversial.

Despite the controversial role of muscle in the initial pathogenesis of diabetes, it is apparent that muscle of patients with T2DM is metabolically responsive to insulin,
often resistant to insulin, and exhibits altered metabolism. Although it is unclear how altered metabolism in diabetes and deficient insulin signaling affect muscle growth and contractile function, there are many cases in which metabolic signaling modulates growth signaling. For example, several insulin- and metabolism-responsive intracellular signaling nodes (e.g., Akt, mTOR, and AMPK) play dual roles in regulating both metabolism, protein synthesis, and protein degradation. Further details on these pathways in healthy and insulin resistant muscle can be found in Chapter 3.

1.2.3.5 Role of non-muscle tissues in glucose uptake

Despite the dominant role of muscle in insulin-dependent glucose uptake (muscle accounts for 80-90% of total insulin-dependent glucose uptake) (DeFronzo et al., 1981), it is appropriate to acknowledge that glucose metabolism, particularly in the non-fed state, is also regulated by the liver and adipose tissue. Indeed, recent work in the liver and white adipose tissue highlights the important roles of these non-muscle tissues in regulating blood glucose. Liver-specific and adipose-specific mutations have been shown to be sufficient to cause overt type 2 diabetes in animal models. Therefore, despite the dominant role of muscle in insulin-dependent glucose uptake, it appears that muscle, liver, or adipose tissue alone can independently cause insulin resistance in specific conditions. Given the synergistic nature of muscle, liver, adipose, and brain tissue in maintaining glucose homeostasis, it is likely that these tissues all contribute jointly during the development of type 2 diabetes in humans.
1.3 **Skeletal muscle is both a contractile and metabolic organ**

Skeletal muscle contractile function plays crucial roles in ambulation, respiration, maintaining posture, thermogenesis, and in performing acts of daily living. In the course of performing these important contractile functions, energy) is utilized by muscle to fuel actin/myosin cross-bridge cycling, ion gradient maintenance, and other intracellular functions. Given that skeletal muscle accounts for ~40% of body weight in healthy individuals, the net energy consumption of skeletal muscle is a major factor in whole body energy consumption and metabolism. Indeed, radioisotope studies suggest that skeletal muscle accounts for 80-90% of insulin dependent and 30-40% of insulin-independent glucose uptake and utilization in healthy individuals. Therefore, it is feasible that changes in skeletal muscle energy utilization and substrate (carbohydrate vs. lipid) preference should affect whole-body metabolism. Conversely, the high rate of energy utilization by muscle makes muscle sensitive to changes in whole body metabolism (e.g., insulin resistance); altered whole body metabolism should impact skeletal muscle metabolism, function, and growth.

1.3.1 **Skeletal Muscle Growth**

Skeletal muscle growth is a carefully regulated process that is an integrated response to a variety of external inputs (e.g., metabolic (amino acids, ATP availability via AMPK status), anabolic (testosterone, insulin, growth hormone/insulin-like-growth-factor-1), muscle use). To understand how diabetes may impact muscle growth, maintenance and function, we will review what controls muscle growth, development, and atrophy.
1.3.1.1 Muscle development

During embryogenesis, middle mesodermal cells of the paraxial mesoderm differentiate into myoblasts. Depending on the local cytokine environment, myoblasts can proliferate, differentiate into myocytes and fuse to form contractile multinucleated skeletal muscle fibers, or dedifferentiate into satellite cells. Satellite cells persist throughout adulthood in skeletal muscle and can proliferate and then differentiate to support muscle repair and regenerate muscle (Bentzinger et al., 2012; Cornelison and Wold, 1997). Muscle fibers acquire characteristics specific to different fiber types based on the local signaling environment (Matsuoka and Inoue, 2008). In addition, muscle fiber type is influenced developmentally and postnatally by neural stimulation pattern; chronic low frequency neural stimulation leads to slow fiber type formation, whereas high frequency stimulation promotes fast fiber type formation (Salmons and Sréter, 1976). Muscle mass increases during development until maturity both by increased myoblast fusion to form additional muscle fibers, as well as by increased myofiber volume.

1.3.1.2 Postnatal muscle growth, maintenance, and hypertrophy

Postnatal muscle growth occurs by myofiber hypertrophy and by de novo fiber generation from satellite-to-myoblast differentiation and myoblast fusion. Postnatal muscle growth is promoted by many factors including testosterone, growth hormone, insulin, amino acid availability and muscle use. Muscle growth is limited by myostatin, insulin-deficiency, energy availability, and disuse. In humans, muscle mass typically increases or is maintained until ~25-50 years of age, after which muscle mass generally
declines. Muscle use promotes muscle growth and maintenance is a key determinant of the onset and rate of muscle loss with age.

Increased muscle use, such as exercise, promotes adaptive skeletal muscle hypertrophy. Exercise is associated with satellite cell proliferation and differentiation. (Cermak et al., 2013; Kadi et al., 2005) The hypertrophic response of skeletal muscle to exercise depends on the type of exercise. Endurance training also promotes increased slow/oxidative fiber content. High load resistance training causes a shift towards more fast/glycolytic fiber types.

The dominant mechanism for mature muscle growth is myofiber hypertrophy via increased cross-sectional area. As discussed previously, many upstream factors (metabolic, anabolic, and use) are integrated to promote myofiber hypertrophy. From a reductionist approach, these hypertrophic inputs generally promote muscle growth by similar mechanisms: positive net muscle protein balance through increased protein synthesis, and decreased protein degradation.

1.3.2 Skeletal Muscle Atrophy

Loss of muscle mass is termed muscle atrophy. Muscle atrophy is a common condition with multiple etiologies that decreases muscle function and can cause acute, chronic, or life-threatening disability (Figure 1.2). Acute atrophy can be caused by muscle denervation or disuse (e.g., limb immobilization, coma, bed rest), severe metabolic disturbances (e.g., untreated type 1 diabetes mellitus, caloric deprivation, and severe burns). Chronic muscle atrophy is associated with many diseases (e.g., AIDS,
cancer, heart failure, chronic obstructive pulmonary disease, renal disease, elevated glucocorticoids, diabetes mellitus, and aging).

1.3.2.1 Mechanisms of muscle atrophy

Despite the different etiologies of the many conditions that promote muscle loss, the common underlying mechanism for all muscle atrophy is net protein loss. Net protein loss is determined by the balance of protein synthesis and protein degradation. Protein synthesis and degradation rates are controlled by many pathways (hormone-dependent, nutrient sensitive, and muscle use-dependent pathways) that converge to control net protein balance. Because of the number and complexity of these upstream pathways, the biochemical pathways linking most clinical conditions to muscle atrophy are poorly defined. This poor understanding of specific upstream mechanisms for muscle loss in distinct atrophic conditions limits the ability to therapeutically target muscle atrophy in a disease-specific manner.

To overcome the challenge of defining the distinct upstream mechanisms in the many different types of muscle atrophy, recent progress in research has been found by studying the common downstream pathways shared by multiple types of muscle atrophy which regulate muscle protein synthesis and degradation. The general translational hypothesis is that therapeutic approaches that dominantly affect downstream protein synthesis and/or protein degradation can be then applied to multiple types of muscle atrophy. This approach has led to novel therapeutic approaches such as adeno-associated virus (AAV) transgenic myostatin inhibition, AAV transgenic myr-Akt expression, and the development of proteasome inhibitors. Unfortunately, these approaches are still in
clinical trials and present considerable risk of adverse side-effects. Thus, even if effective, these transgenic approaches will only be clinically feasible in severe, life-threatening cases of muscle loss (e.g., Duchenne’s muscular dystrophy, spinal muscular dystrophy). Further research is needed to develop interventions which are amenable for use in the majority of cases of muscle atrophy, which are not acutely life-threatening, but reduce the functional quality of life.

### 1.3.2.2 Sarcopenia: Muscle atrophy of aging

In healthy individuals, skeletal muscle mass and function typically increase or are maintained until ~40-50 years of ages, after which a gradual age-related loss of muscle mass and strength begins. This “natural” trend of muscle loss is exacerbated by additional atrophic stimuli, such as cancer, renal failure, congestive cardiomyopathy, disuse, immobilization, denervation, metabolic disorders, and peripheral neuropathies (Figure 1.2). When age-related muscle loss becomes severe and interferes with normal function/living, this muscle loss is medically diagnosed as “sarcopenia”. The term “sarcopenia” is derived from the greek “sarco-“, meaning “flesh”, and “-penia”, meaning “deficient”. Therefore “sarcopenia” literally refers to a deficient amount of muscle mass and/or function.

Sarcopenic muscle loss is of particular clinical concern in the frail older population, in which decreased muscle function and growth may compromise independent function, increase the risk of falls and fractures, and impede recovery from injury or surgery. These risks of musculoskeletal frailty are further compounded by the
increased prevalence of osteoporosis, balance disorders, visual impairment, and mental
dysfunction in elderly individuals.

Diagnosis, Epidemiology, and Treatment of Sarcopenia

The current diagnostic standards with widest acceptance, developed by the
European Working Group on Sarcopenia, require both 1) decreased muscle mass (lean
body mass two standard deviations below a young reference population average) and 2)
low walking gait speed (<0.8m/s in a 4.0m walking test).(Gregg et al., 2002) Based on
varying standards similar to these, the prevalence of sarcopenia in various studies is
estimated to lie between 5-13% among all 60-70 year olds, and 11-50% in all individuals
over 80 years old.(Gregg et al., 2000) The most comprehensive cross-sectional study to
date, the Third National Health and Nutritional Examination Survey, determined that the
prevalence among adults 60 years and older of moderate and severe sarcopenia is 53.1% and
11.2%, respectively, for men, and 21.9% and 9.4%, respectively, for women. Based
on this prevalence, the estimated cost of sarcopenia in the United States in 2000 was 18.5
billion dollars per year.(Janssen et al., 2004) Despite the high prevalence and impact of
sarcopenia, screening is not routine and treatment upon diagnosis is limited to the
prescription of exercise training, improved diet, and increased protein intake.
Compliance with exercise and dietary recommendations is poor and beneficial effects are
modest, limiting the efficacy of current treatment options.

1.4 Muscle loss in humans with T2DM

The effects of diabetes on human skeletal muscle has been best studied in older
adults, in which annual loss of muscle mass is ~26% greater and annual loss of muscle
strength is ~33% greater in individuals with diabetes compared to those without diabetes (Goodpaster et al., 2006; Kim et al., 2010; Park et al., 2007, 2009; Unni et al., 2009). Current treatments of muscle atrophy in diabetes are limited to standard diabetes medications, improved diet, exercise, and hormonal replacement in hypogonadal individuals (Morley, 2008). Thus, T2DM aggravates muscle loss in the elderly, but the degree of overlap between age-related muscle loss and diabetes-related muscle loss is unclear.

1.4.1 Functional disability among individuals with diabetes

Decreased muscle function impairs the ability of an individual to perform activities of daily living, exercise, and predisposes them to loss of balance, falls, and associated fractures. The Health, Aging, and Body Composition Study demonstrated that individuals aged 70-79 years with diabetes have a greater prevalence (53%) of subclinical functional limitation than individuals without diabetes (40%). (De Rekeneire et al., 2003) A broader cross-sectional study including younger age groups (18 yo+) (The National Health Interview Survey) found that increased disability is most distinct in diabetes at young ages, after which the gap between individuals with and without diabetes narrows as more individuals without diabetes become disabled (Figure 1.3). (Ryerson et al., 2003) Annual incidence of at least one functional disability (defined as the inability to climb steps, walk 2-3 blocks, or perform housework, cleaning, or cooking) was more prevalent among women with diabetes (9.8%/year) compared to women without diabetes (4.8%/year). Of note, this study is limited by the exclusion of individuals who were institutionalized or unable to walk without assistance. (Gregg et al.,
2002) A different study investigating similar parameters of functional disability, including both men and women (>=60 years old), revealed self-reported functional disability in 32% of women with diabetes and 15% of men with diabetes compared to 14% of women without diabetes and 8% of men without diabetes. This study also found that diabetes was associated with decreased walking speed. (Gregg et al., 2000)

Despite the clear correlations between diabetes and muscle loss and diabetes and disability, it is unclear to what degree decreased muscle function is responsible for disability in diabetes. Other diabetes-associated conditions, such as obesity, balance disorders, vision impairment, and neuropathies can also contribute to disability in diabetes. Further research is needed to clarify the relative importance of these different mechanisms for disability among individuals with diabetes. For example, within research studies of individuals that report inability to walk 2-3 blocks, classification of the individuals into etiological groups of muscular fatigue, cardiovascular impairment, CNS impairment as the basis for the disability should be included. This would allow for research and clinical interventions to better focus on diabetes-associated conditions that most impair function and cause disability.

Decreased ability to perform basic activities of daily living, coupled with decreased muscle mass and strength among those with diabetes, suggests that more rigorous physical activities such as exercise become more difficult for people with diabetes, potentially contributing to the persistence and cyclical progression of obesity, insulin resistance, and further muscle loss. Further research of how diabetes impacts the capacity of individuals with diabetes to exercise is needed to determine how muscle loss
and disrupted metabolism may impact the ability to exercise. In addition, the response of muscle (increased mass/function) to different types of exercise training is needed to improve the efficacy of exercise in patients with diabetes and sarcopenia.

1.4.2 Increased risk for falls and fractures and worsened prognosis in diabetes

Diabetes is associated with greatly increased incidence of falls in the elderly; a prospective cohort study at a long-term care facility found that the fall incident rate over 299 days was 78% in individuals with diabetes compared with only 30% of individuals without diabetes (p < 0.001). (Maurer et al., 2005; Strotmeyer ES, 2005) As may be expected from this fall data, despite increased bone-mineral density among individuals with type 2 diabetes (Hofbauer et al., 2007), the risk of hip fracture is much greater among individuals with diabetes compared to those without diabetes (RR of 3.3, 95% confidence interval of 1.3-8.2). (Ivers et al., 2001). Among individuals that sustain hip fractures, pre-fracture physical disability, sedentariness, malnutrition, and walking endurance predict the length of acute hospitalization (Singh et al., 2009). Thus, diabetes-associated physical disability and decreased muscle function increases incidence of falls and fractures and prolongs time to recovery..

1.4.3 Mechanisms of muscle loss in sarcopenia and type 2 diabetes

Diabetes and sarcopenia are both clinical syndromes that are diagnosed using measurable markers (hyperglycemia and decreased muscle size/function, respectively) rather than by underlying etiologic mechanisms. Although these diagnostic criteria facilitate disease diagnosis, the lack of definitive mechanism amid the multitude of
diabetes- and sarcopenia-associated conditions impairs our ability to clearly characterize the causal relationships between the two.

For example, by definition, sarcopenia is limited to age-associated muscle atrophy independent of other atrophy-inducing diseases (such as muscular dystrophies, cancer cachexia, burns, and heart failure). This age-linked definition for sarcopenia creates a significant research challenge; the natural course of aging itself is a poorly understood progressive process that entails simultaneous and highly variable changes in hormone signaling, cytokines, metabolism (including insulin resistance), diet, physical activity, and multi-organ function. Thus, because sarcopenia is associated with age, many studies of sarcopenia struggle to differentiate age-associated conditions from factors that cause sarcopenia. Factors that have been linked to sarcopenia include decreased growth hormone, increased insulin resistance, increased TNF-α, increased fat mass, decreased physical activity, decreased estrogen/androgen, increased proteosomal activity, increased IL-6, and decreased protein intake. (Argilés et al., 2005) While it is probable that multiple factors contribute to sarcopenia, the identification of central mechanisms sufficient to cause, and thus control, sarcopenia is necessary to improving the prevention, diagnosis, and treatment of this poorly controlled condition.

Similar to sarcopenia, diabetes is usually an age-associated, with dysfunction and dysregulation of many signaling pathways, organ systems, and systemic changes. Thus, because of the many possible mechanisms involved in each disease, studying the mechanism connecting diabetes to muscle loss cannot begin with assumptions of a specific mechanism (e.g., hyperglycemia or deficient insulin signaling) just by
association. Instead, it is practical to investigate decreased muscle mass beginning with the common factor that is known to be present in muscle loss: net muscle protein loss.

**1.4.3.1 Muscle protein balance in diabetes-related sarcopenia**

From a reductionist approach, changes in muscle mass are caused by changes in muscle protein balance, which are determined by the net effects of protein synthesis and degradation.

1.4.3.1.1 Protein synthesis and Protein Degradation in General Sarcopenia:

In the general population, muscle-specific protein synthesis rates clearly decrease with aging (12) (10) (13). A study of synthetic rates of specific proteins has demonstrated that in aging adults, myosin heavy chain (MHC) and mitochondrial protein synthesis rates decreases with age, whereas sarcoplasmic protein synthetic rates are unchanged. (14) (15) This suggests that either MHC/mitochondrial protein turnover rates are decreased with age, or that lower steady state levels of these proteins are maintained in muscle from aging adults compared to young adults.

1.4.3.1.2 Changes in Protein Synthesis and Degradation in Type 2 Diabetes Mellitus

Radioisotope-based studies in humans using carbohydrate or protein infusion found no difference in infusion-dependent protein synthesis in subjects with type 2 diabetes in response to a similar metabolic load. (Bell et al., 2006; Manders et al., 2008) However, insulin infusion induced less muscle protein synthesis in individuals with type 2 diabetes compared to healthy individuals due to decreased insulin-dependent vasodilation. (Fujita et al., 2006) In contrast, studies of protein degradation show that T2DM is associated
with increased rates of muscle protein degradation (Halvatsiotis et al., 2002) (Bell et al., 2006). Taken together, this data suggests that increased protein degradation is the basis for accelerated muscle loss in T2DM. Beyond this simple mechanism of protein loss in humans with diabetes, our understanding of the etiology of muscle loss in diabetes relies largely on rodent models of diabetes mellitus.

1.4.4 Skeletal muscle growth and maintenance in animal models of diabetes mellitus

1.4.4.1 Muscle loss in Type 1 Diabetes Mellitus

Streptozotocin-induced Type 1 Diabetic (T1DM) mice are the best understood model of muscle atrophy in diabetes, in which pharmacologically-induced insulin deficiency leads to increased muscle proteasomal protein degradation and rapid muscle atrophy. (Pepato et al., 1996) Insulin-dependent signaling pathways are clearly important in this model, as insulin restoration (Price et al., 1996) or phosphatase and tensin homolog (PTEN) ablation (Hu et al., 2007) are sufficient to rescue and prevent muscle atrophy. Blockade of glucocorticoid signaling is also sufficient to prevent muscle atrophy in streptozotocin mice, suggesting a role of glucocorticoids in muscle atrophy in diabetes. (Hu et al., 2009) Although convincing, these studies in T1DM mice are limited because T1DM (primary insulin deficiency) only accounts for 5-10% of individuals with diabetes, with T2DM (primary insulin resistance) accounting for the remaining 90-95% of individual with diabetes mellitus. (Centers for Disease Control and Prevention, 2011)
1.4.4.2 Muscle loss in type 2 diabetes mellitus

Muscle atrophy in rodent models of type 2 diabetes is less consistent compared to models of T1DM. Several mouse models exhibit no change or mild decreases in muscle size (i.e. high fat diet(Sitnick et al., 2009; Turpin et al., 2009; Yokota et al., 2009), muscle insulin receptor knockout\textsuperscript{12}, IRS-1 knockout(Long et al., 2011), and IRS-2 knockout(Long et al., 2011)). In contrast, several models of T2DM demonstrate severely reduced muscle size (leptin deficient (ob/ob)(Kemp et al., 2009; Stickland et al., 1994; Warmington et al., 2000), leptin receptor deficient (db/db)(Wang et al., 2006), muscle specific IRS-1/IRS-2 knockout(Long et al., 2011)). The most detailed rodent studies of type 2 diabetes and muscle atrophy have focused on the ob/ob and db/db mouse models. The molecular basis for differences in muscle phenotypes among models of T2DM is unclear, although these variable phenotypes may provide clues as to the mechanism of muscle loss in T2DM.

1.4.4.2.1 Skeletal muscle growth of the ob/ob mouse

The ob/ob mouse (leptin mutant) is hyperphagic, severely obese(Ingalls et al., 1950), hyperinsulinemic, and hyperglycemic(Tomita et al., 1992). The adult ob/ob mouse has severely reduced weight of fast and mixed skeletal muscles, compared to age-matched wild-type controls (WT), but has normal slow (soleus) muscle weight (Kemp et al., 2009; Stickland et al., 1994; Warmington et al., 2000). The preferential effects on fast muscle in ob/ob mice is emphasized by the marked reduction in cross-sectional area (CSA) and proportion of fast glycolytic fibers, with no change in CSA or proportion of slow oxidative fibers.(Almond and Enser, 1984) Functionally, ob/ob muscle maintains
WT levels of whole muscle and single fiber force production when normalized to CSA, but demonstrates decreased rates of relaxation and premature fatigue during induced tetanus compared to WT (Bruton et al., 2002). Reduced satellite cell proliferation during early postnatal development (Purchas et al., 1985) and in response to chemical injury (Nguyen et al., 2011) has been reported in ob/ob mice. Although leptin administration in adult ob/ob mice normalizes glycemia (Schwartz et al., 1996) and partially restores muscle mass (Sáinz et al., 2009), pair feeding adult ob/ob mice does not improve muscle mass (Sáinz et al., 2009), despite a moderate lowering of blood glucose levels (Schwartz et al., 1996).

1.4.4.2.2 Skeletal muscle growth of the db/db mouse

The db/db mouse (leptin receptor mutant) metabolic phenotype is similar to the ob/ob mouse, presenting with hyperphagia, juvenile onset obesity, and diabetes (Chick et al., 1970). Depending on background genetic strain, db/db mice exhibit pancreatic islet hyperplasia (C57BL/6J (Gapp et al., 1983)) or islet atrophy (C57BL/KsJ (Kawasaki et al., 2005)); pancreatic islet atrophy results in a more severe hyperglycemic phenotype, late-onset weight loss, and a shortened lifespan, compared to ob/ob mice. Db/db mice also exhibit reduced skeletal muscle size, increased muscle protein degradation, elevated proteasome function, and reduced muscle fiber CSA (Wang et al., 2006). Glucose intolerance and reduced muscle size in db/db mouse is partially normalized by chronic rosiglitazone treatment, suggesting a role of metabolism in the reduced muscle size (Wang et al., 2006).

1.4.4.2.3 Leptin signaling and known effects in skeletal muscle
The severe metabolic phenotypes of leptin deficient (ob/ob) and leptin receptor
deficient (db/db) mice raises the question of how leptin signaling interacts with
metabolism and how deficient leptin signaling may influence muscle growth. Leptin is a
16-kDa endocrine and paracrine hormone secreted principally by adipose tissue, but also
by gastric mucosa, fetal membranes, and uterine tissue. (Cammisotto and Bendayan,
2007; Frederich et al., 1995; Klok et al., 2007). Cortisol and insulin stimulate leptin
secretion whereas leptin expression is decreased by beta-adrenergic agonists,
thiazolidinediones, and by increased intracellular cAMP. (Houseknecht et al., 1998)
Leptin receptors are best studied in the hypothalamus, where leptin binding suppresses
appetite by inhibiting appetite stimulants (neuropeptide Y and anandamide), and
increasing synthesis of the appetite suppressant α-MSH. (Houseknecht et al., 1998) In
addition to the hypothalamus, leptin receptors are found in the hippocampus, bone,
hematopoietic cells, lung parenchyma, and muscle. In muscle, leptin signals via JAK-
STAT-nuclear transcription pathways to increase fatty acid oxidation, alter lipid
partitioning, and inhibit glycogen synthesis. (Ceddia, 2001; Liu et al., 1997; Minokoshi et
al., 2002; Muoio et al., 1997) Leptin also modulates insulin-sensitive pathways in muscle
through signaling that rapidly occurs in the cytosol independent of transcriptional effects
of leptin. (Kim et al., 2000) In summary, disruption of leptin signaling may influence
insulin resistance and muscle growth indirectly by hypothalamic signaling increasing
appetite, caloric intake, and obesity, and directly by altering cytosolic signaling and
transcriptional activity in muscle tissue.
1.4.4.2.3 Summary of skeletal muscle findings in T2DM animal models

Taken together, these previous findings suggest that T2DM is associated with fast glycolytic skeletal muscle loss which is partially reversed by interventions that improve metabolism (such as rosiglitazone in db/db mice (Wang et al., 2006), leptin in ob/ob mice (Sáinz et al., 2009), or diabetes treatment in humans (Park et al., 2009)). The ability of insulin to completely rescue muscle atrophy in T1DM mice (Price et al., 1996) suggests that decreased insulin signaling could directly cause muscle loss in T2DM. Canonical insulin signaling promotes protein synthesis (via Akt/mTOR) and inhibits protein degradation (via Akt/FOXO signaling) (Taniguchi et al., 2006); it is logical that insulin resistance could reduce protein synthesis and increase protein degradation. However, it is unclear if decreased insulin signaling in T2DM directly causes muscle loss or if other diabetes-associated factors drive muscle atrophy. Indeed, the onset, progression, biochemical phenotype, and causative signaling pathways for muscle loss in T2DM remain unclear.

1.5 Gaps in Knowledge

As described in this chapter, it is clear that diabetes is associated with accelerated muscle loss and functional decline in the elderly. While much is understood of the diagnosis, treatment, and disease course of diabetes and sarcopenia as individual diseases, little is known about how diabetes affects muscle growth and maintenance. The onset and progression of muscle loss in individuals with T2DM also remain undefined. Moreover, although moderately effective treatment options are well established for diabetes and sarcopenia individually, little is known about how to effectively and safely
prevent and rescue muscle loss in diabetes. Additional research is necessary to determine the basic onset, progression, nature of muscle loss, and efficacy of therapy in muscle loss in type 2 diabetes. The purpose of this dissertation research is to begin to address these important questions in well-studied rodent models of insulin resistance and type 2 diabetes.
## Tables and Figures for Chapter 1

**Table 1.1**: Diabetes mellitus causes or exacerbates dysfunction in many organs

<table>
<thead>
<tr>
<th>Tissue/Organ/Function</th>
<th>Effect of diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>2-4x increased risk of stroke (16% of diabetes-related death certificates)</td>
</tr>
<tr>
<td>Heart</td>
<td>2-4x increase in heart disease death rate (68% of diabetes-related death certificates), 67% of adults with diabetes have hypertension</td>
</tr>
<tr>
<td>Vision</td>
<td>Leading cause of blindness in adults (28.5% of those with diabetes have diabetic retinopathy; 4.4% have severe retinopathy/vision impairment)</td>
</tr>
<tr>
<td>Peripheral Nervous System</td>
<td>60-70% of adults with diabetes have nervous system damage</td>
</tr>
<tr>
<td>Amputation</td>
<td>60% of non-traumatic limb amputees have diabetes; 65,700 diabetic limb amputations/year</td>
</tr>
</tbody>
</table>

Source: (Centers for Disease Control and Prevention, 2011)
Figure 1.1 Diabetes mellitus is increasingly prevalent among the U.S. population (American Diabetes Association, 2011)
Figure 1.2 Etiology of Muscle Atrophy – Insulin resistance is one of many conditions that are associated with muscle atrophy. Many of these atrophy-promoting conditions are prevalent in elderly individuals.
Figure 1.3 Prevalence of a physical disability is increased in those with diabetes, especially at younger ages. (Ryerson et al., 2003)
Chapter 2: The Impact of Type 2 Diabetes Mellitus on Skeletal Muscle Growth, Mass, Maintenance, and Function

The long-term goal of this research is to understand the mechanisms for muscle loss in diabetes and to identify timely and appropriate treatment strategies that can prevent and/or reverse muscle loss in those with diabetes. Two related questions that need to be addressed to achieve these goals are:

1.) How does the loss of muscle mass and function relate chronologically to the onset and progression of the metabolic phenotype in diabetes?

2.) What components (e.g., insulin resistance, chronic hyperglycemia, peripheral neuropathy, decreased muscle use) of the diabetes phenotype are necessary to cause muscle loss?

In this chapter, we first describe what is known about skeletal muscle mass, contractile function, fiber type, and fiber number in humans with diabetes and relevant animal studies. Then, to address these questions, we take advantage of different mouse models of type 2 diabetes to characterize how the transition from “pre-diabetic” to “overtly diabetic” stages affects muscle mass, contractile, and metabolic function.

2.1. Accelerated muscle loss in type 2 diabetes mellitus

The prevalence of muscle loss in diabetes has been well established and characterized in the elderly population in several cross-sectional and prospective
longitudinal clinical studies (Table 2.1). Together, these studies clearly indicate a significant trend towards decreased muscle mass and poor contractile function in insulin resistant individuals. The most comprehensive study to date of the effects of diabetes on muscle, The Health, Aging, and Composition Study (ABC study), found that the rate of loss of lean muscle mass was considerably higher in individuals with untreated diabetes (not yet diagnosed with diabetes at the start of the study) vs. individuals with presumably treated diabetes (already diagnosed at start of the study), suggesting that 1.) altered metabolism plays a large role in muscle loss in diabetes, and 2.) insulin resensitization therapy is sufficient to ameliorate some of the atrophic effects of type 2 diabetes on skeletal muscle. (18)

Changes in muscle fiber characteristics in sarcopenia and diabetes

In addition to decreasing muscle mass, sarcopenia can also decrease muscle function by altering muscle quality (contractile strength relative to cross-sectional area). Skeletal muscle strength and function is determined by a combination of factors: fiber number, fiber cross-sectional area, fiber type, and neural stimulation.

2.1.1 Decreased fiber number in sarcopenic and insulin resistant muscle

Muscles in sarcopenic individuals typically have a greatly reduced number of muscle fibers (~50% relative to healthy individuals). In addition, sarcopenic muscle fibers have reduced cross-sectional areas, with more severe reductions in type 2 (20-50% reduction) fiber size relative to Type 1 fibers (1-25% reduction). The broad ranges of these reductions likely reflect the heterogeneity of study populations. (7) Although no human studies to date show how diabetes impacts muscle fiber number, animals studies
using the insulin resistant ob/ob and db/db mouse models demonstrate decreased total fiber number in slow and fast twitch muscle.

2.1.2 Shift from fast glycolytic muscle fibers to slow oxidative fibers in sarcopenic and diabetic muscle

Skeletal muscle functional units consist of various contractile fiber types (slow oxidative, fast oxidative, and fast glycolytic) with distinct functional and metabolic characteristics. Slow oxidative fibers (also called Type 1 fibers) possess a large number of mitochondria, which allow for aerobic oxidation of glucose and lipids to provide ATP for slower fatigue-resistant contractile activity. These slow fibers also express slower isoforms of excitation and contractile proteins (e.g., Sarcoendoplasmic reticulum Ca\(^{2+}\)-ATPase 2a, Troponin I1, Troponin C1, Calsequestrin 2) that limit the maximum speed of contraction. On the other extreme, fast glycolytic fibers (Type 2b fibers) possess relatively few mitochondria and are dependent largely on the breakdown of glucose through glycolysis to meet the energetic demands of contraction and relaxation. As a result, fast glycolytic fibers are relatively fatigue sensitive, yet are capable of anaerobically generating large levels of ATP and lactate to support short bursts of contractile activity. This metabolic phenotype of fast glycolytic fibers is coupled with the expression of faster isoforms of contractile proteins to produce rapid contractions over a relatively short period of time. Fast oxidative fibers (Type 2a fibers) possess an intermediate phenotype between slow oxidative and fast glycolytic fibers, resulting in intermediate metabolic and functional characteristics. The order of insulin sensitivity of these fiber types is slow oxidative > fast oxidative > fast glycolytic, such that a shift in
fiber type distribution can affect whole-muscle insulin sensitivity, glucose uptake, and metabolism. (He et al., 2001; Henriksen et al., 1990; James et al., 1985; Kern et al., 1990) Given that muscle accounts for >80% of whole-body insulin-dependent glucose disposal in healthy individuals, a shift in muscle fiber type and metabolism can reduce whole body glucose tolerance.

Several small cross-sectional studies describe a decrease in the fraction of oxidative fibers in people with T2DM, with an increase in glycolytic fibers. (Table 2.2) Due to the fact that skeletal muscle accounts for ~40% of total body weight, changes in muscle fiber types can have a drastic effect on whole-body metabolism (e.g., insulin resistance). Conversely, changes in whole body glucose and lipid metabolism can selectively impact specific fiber types, based on fiber-type specific metabolism, resulting in changes in total muscle contractile function and fatigue resistance.

### 2.2 Experimental Questions

Despite the wealth of literature confirming that diabetes has an impact on muscle mass, type, and function, several questions need to be answered to allow for efficient screening, timely diagnosis, and effective treatment of muscle loss in diabetes. These questions include:

1.) When does accelerated muscle loss begin in people with diabetes?

2.) How does muscle loss in diabetes correlate with onset and progression of insulin resistance to overt hyperglycemia?
3.) What component(s) of the phenotype of diabetes (e.g., hyperglycemia vs. insulin resistance vs. decreased use vs elevated glucocorticoids) is(are) necessary for muscle loss in type 2 diabetes?

4.) What are the effects of diabetes therapies on muscle loss in diabetes?

To address these gaps in knowledge, we chose to take advantage of the db/db and TallyHo mouse models of T2DM and compare how muscle weight and function changes during the onset and progression of the phenotype in diabetes. The db/db mouse model (homozygous mutant of leptin receptor) was used because it has a well-studied severe T2DM phenotype (fasting hyperglycemia by 8 weeks of age) with known decreased muscle mass at 9 weeks of age (Almond et al., 1988; Chick et al., 1970; Gapp et al., 1983). The less-studied TallyHo mouse model was included because it is a polygenic model of overt T2DM with intact leptin signaling (Kim and Saxton, 2012; Kim et al., 2006; Stewart et al., 2010). Moreover, TallyHo mice develop diabetes at a mature age (fasting hyperglycemia by ~15 weeks of age), allowing us to study the impact of insulin resistance and hyperglycemia on mature muscle, which is more clinically relevant compared to the juvenile onset of diabetes in db/db mice. We also studied how a common treatment for diabetes and muscle loss – chronic exercise – affects muscle size, glucose tolerance, and markers of muscle protein synthesis and degradation in db/db mice. These studies allowed us to test several specific hypotheses:
2.3 Experimental hypotheses

_Hypothesis 1:_ We specifically hypothesized that insulin resistance alone, prior to the onset of overt type 2 diabetes would be sufficient to cause muscle loss. The rationale for this hypothesis was based on previous literature indicating that absolute insulin deficiency has been shown in models of T1DM to be sufficient to cause rapid muscle atrophy and insulin replacement therapy is sufficient to rescue the muscle loss.

_Hypothesis 2:_ We also hypothesized that chronic exercise would be insufficient to rescue the reduced muscle mass in adult db/db mice. The rational for this hypothesis is that muscle growth is controlled jointly by hormone signaling, muscle use, and muscle metabolic state (e.g., mTOR regulation of protein synthesis is controlled by leucine, AMPK, insulin, and growth hormone); we believed that the altered metabolic state in diabetes would blunt exercise-dependent hypertrophy.

2.4 Research Design and Methods

2.4.1 Animals

Male db/db (BKS.Cg-Dock7m +/- Leprdb/J), age-matched WT controls (C57BLKS/J), and TallyHo (TallyHo/JngJ) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Because the TallyHo strain was originally created at Jackson Labs by selective crossbreeding of multiple strains of mice, no accurate control strain of mice exists to compare to TallyHo mice. To overcome this challenge, eight week old TallyHo mice were studied to establish a prediabetes baseline to compare to the 22-24 week old TallyHo mice with overt diabetes. Obesity was induced in select WT
C57BLKS/J mice by providing a high fat diet (HFD, 4.73 kcal/g, 45% kCal from fat; D12451, Research Diet Inc., New Brunswick, NJ, USA) from the age of 8 weeks until their sacrifice at 20 weeks old, whereas all other mice were fed standard chow diet (3.0 kcal/g, 4.25% kCal from fat), as described previously. (Bal et al., 2012) Male mice only were used due to the relatively homogeneous metabolic phenotype of males compared to females in the db/db and TallyHo mouse models. The mice were maintained on a 12:12 h light:dark cycle with ad libitum access to food and water, except when fasted (described below). Mice were euthanized by CO₂ exposure. All animal experiments were approved by the The Ohio State University IACUC.

2.4.2 Glucose tolerance test

After an overnight fast (db/db mice) or 6 hour fast (TallyHo mice), fasting blood glucose levels were measured using tail blood in a TRUEtrack® blood glucose monitoring kit (Walgreens, Columbus, OH). The maximum limit of the glucometer was 600 mg/dL; glucose levels greater than 600 mg/dL (reading as “HI”) were treated as 600 mg/dL for facility of quantitative data comparison. An injection of glucose (2 g/kg, intraperitoneal, Sigma-Aldrich), dissolved in Dulbecco’s phosphate-buffered saline (DPBS, Invitrogen, Carlsbad, CA), was given to each mouse and blood glucose measurements were obtained at 30, 60, 90, 120, and 240 minutes post injection. Glucose tolerance tests were compared by calculating the integrated area under the curve (AUC) of the blood glucose level for the 240 minutes of the test.
2.4.3 Measurement of body size and muscle weight

Body weight was determined in a fed state. To determine muscle weight, after euthanasia by CO₂ inhalation, muscles were carefully dissected, and tendons were removed to the beginning edge of muscle tissue. Muscles were briefly immersed in DPBS, dabbed on tissue paper to remove excess fluid, weighed on a digital analytical balance, and then frozen in liquid nitrogen.

2.4.4 VO₂max treadmill test

Maximum cardiopulmonary function and running speeds were assessed using a Metabolic Modular Treadmill (Columbus Instruments, Columbus, OH) as described previously (Stølen et al., 2009), with several modifications to adapt to the poor exercise capacity of the db/db mice, as follows. Acclimation- Mice were acclimated to the treadmill 1-3 days prior to testing by being placed on an unmoving treadmill for 3 minutes, after which the shock grid was activated (3 Hz and 1.5 mA) and the treadmill was engaged to a walking speed of 6 m/min for 5 minutes. VO₂max test- Mice were placed on the treadmill (0° incline entire experiment) and the shock grid was activated. The treadmill speeds were then increased until exhaustion as follows: (speed, duration) - (0 m/min, 5 min), (6 m/min, 5 min), (7, 8, 9, and 10 m/min, 30s each), (11m/min, 1 min), (12, 13, 14, and 15 m/min, 2 min each), and (+1 m/min, each 1 min thereafter). Exhaustion (endpoint for treadmill cessation) was defined as the point at which mice maintained continuous contact with the shock grid for 5 seconds. VO₂max was determined by the peak oxygen consumption reached during this test when the respiratory
quotient (RER) was >1.0. Maximum running speed was defined as the treadmill speed at which VO$_{2\text{max}}$ was achieved.

### 2.4.5 Grip Strength Test

To test if exercise limitation in db/db mice could be attributed to decrease muscle strength, forelimb grip strength was assessed using a digital Grip Strength Meter (Columbus Instruments, Columbus, OH). Mice were lifted by their tails, allowed to grasp the forelimb pull bar, pulled smoothly away from the transducer in the horizontal plane, and maximum force exerted on the transducer was recorded. This test was repeated in 3 sets of 10 attempts, with a 15 minute rest in between sets; the maximum value recorded from all attempts by each mouse was designated as maximum grip strength.

### 2.4.6 Isoproterenol Langendorff heart function studies of db/db and WT mice

To test if exercise limitations in db/db mice could be attributed to cardiac limitations, 	extit{ex vivo} maximum response to isoproterenol was assessed. Age-matched WT (n=4) and db/db(n=5) mice were anesthetized by intraperitoneal pentobarbital injection (100-200 mg/kg). Upon sedation, the heart was rapidly excised, atria removed, and heart was hung in standard non-paced Langendorff fashion (Talukder et al., 2007), with retrograde aortic perfusion using 37º C Krebs-Henseleit buffer (pH 7.36-7.38) with added 2mM sodium pyruvate (Broadley, 1979). The buffer was oxygenated with 95% O$_2$/5% CO$_2$ from 30 minutes prior to the start of the experiment and until the end of the experiment. An elevate reservoir was used to maintain a constant pressure of the
transfusion buffer as it flowed into the aorta; coronary artery flow rate throughout the experiment was determined by measurement of the flow rate of perfusion buffer into the aorta using a laser doppler blood flowmeter (AD Instruments, Colorado Springs, CO). Left ventricular pressure development was monitored by inserting a fluid-filled low density polyethylene balloon catheter through the mitral valve into the left ventricle. Pressure development within this balloon catheter was monitored using a Physiologic Pressure Transducer (AD Instruments), which was calibrated with a manual manometer prior to each experiment. Appropriate fitting of the balloon catheter within the left ventricle was optimized by adjusting the internal balloon volume such that end-diastolic pressures were 5mmHg. After optimal balloon volume was established, the heart was perfused and allowed to beat for 30 minutes to allow for stabilization of the metabolic and contractile function of the heart, after which “baseline” ex vivo cardiac function was assessed. The response to adrenergic stimuli was then assessed by serially replacing the buffer with KH buffer containing increasing doses of isoproterenol ($10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$ M). Each dose was continued until heart rate peaked ($HR_{max}$, achieve in 10-30 minutes), after which peak values were recorded, and 30 minutes of perfusion with isoproterenol-free KH buffer was used to establish a new baseline functional level before switching to the higher dose. Parameters measured for each buffer condition include: heart rate (HR), $-dP/dt$ (the most negative slope of left ventricular pressure during $HR_{max}$), $+dP/dt$ (the most positive slope of left ventricular pressure during $HR_{max}$), systolic pressure (maximum pressure during $HR_{max}$), and diastolic pressure (minimal pressure during $HR_{max}$).
2.4.7 Voluntary Activity Measurements

Mouse activity levels were measured using measurement of horizontal infrared beam breaks over the course of 24 hours, using an Oxymax Lab Animal Monitoring System at room temperature (CLAMS, Columbus Instruments, Columbus, OH). The mice had ad libitum access to food and water during these measurements.

2.4.8 Chronic exercise

Mice were exercised from 5-13 weeks of age using the interval training method as described previously (Stølen et al., 2009), with slight modifications. Mice were treadmill exercised 80 minutes per day, 5 days per week, for 8 weeks. Training was performed on a 20º incline, alternating 4 minutes high speed and 2 minutes low speed (high = 80-90% of speed at which VO$_2$max was reached, slow = 50% of speed at which VO$_2$max was reached). The VO$_2$max tests for the exercised mice and their controls were performed as described above but with the following changes to speed/incline in order to determine appropriate training speeds for running on an incline: (speed, duration, incline) - (0 m/min, 5 min, 0º), (6 m/min, 5 min, 0º), (6 m/min, 2 min, 20º), (+2m/min, each 2 min thereafter, 20º).

2.4.9 Histology

Muscles (n=2) were gently rinsed in DPBS, dabbed on tissue paper to remove excess moisture, and then stored in 10% (v/v) formalin. Tissue was then paraffin embedded, cut, and stained with hematoxylin/eosin. Images were obtained using a light microscope at 20X magnification (Zeiss Axioskop 40, Oberkochen, Germany).
2.4.10 Acute cold tolerance of iBAT+ and iBAT- db/db mice

Our lab has recently shown that skeletal muscle plays an important direct role in acute cold tolerance. Bal et al., 2012 To test the impact of reduced muscle size on acute thermogenesis, we assessed cold tolerance in db/db mice with either intact or surgically reduced brown adipose tissue (a major source of thermogenesis in mice but not humans). Interscapular brown adipose tissue (~75% of mouse BAT) was surgically removed from a group (n=4) of 12 week old db/db mice (referred to hereafter as ibat(-) db/db mice), as described previously. Bal et al., 2012 A separate group of age-matched db/db mice (n=4) was included with the interscapular brown adipose tissue left intact (referred to as ibat(+) db/db mice). Wireless thermometer transponders were implanted into all mice in the subcutaneous interscapular dorsal region of each mouse. 2-3 days after surgery, mice were introduced into a temperature controlled (6°C) metabolic cage (Comprehensive Lab Animal Monitoring System, Columbus Instruments, Columbus, OH), with ad libitum access to food and water. Oxygen and carbon dioxide consumption were monitored in response to cold, as was core body temperature. Infrared thermography (Fluke Ti32 camera, Fluke, Everett, WA) was used to visualize difference in body temperatures before and after cold challenge. When core body temperature reached below 20°C, mice were removed from the cold environment and replaced in normal cages at room temperature.

2.4.11 Data interpretation

Statistically significant data was identified by use of the two-sided student t-test, $\alpha = 0.05$. 

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2.5 Results

A major objective of this study was to identify what components of the diabetes phenotype are necessary for muscle loss in type 2 diabetes. To this end, we took advantage of the male db/db and TallyHo mouse models, which exhibit overt T2DM at ~8 and ~14 weeks of age, respectively (Wang et al., 2006). We investigated if the decrease in muscle mass and function is related to the severity of the stage of diabetes by comparing mice at approximately prediabetic and overtly diabetic ages.

2.5.1 Progression of metabolic phenotype in db/db mice

First, we confirmed the development of the diabetes phenotype in db/db mice at 6 and 12 weeks of age compared to age-matched WT controls. Total body weight in db/db mice was moderately elevated in 6 week old mice and severely increased in 12 week old mice compared to controls (Figure 2.1A). Fasting blood sugar was mildly increased in 6 week old (149±10mg/dL WT vs. 249±32mg/dL db/db, p<0.05) and severely increased in 12 week old db/db mice compared to WT controls (113±10 mg/dL WT vs. 488±50 mg/dL db/db, p<0.05). Glucose tolerance was moderately impaired in 6 week old db/db mice (AUC 687±47 mg·hr/dL WT vs. 1406±167 mg·hr/dL db/db, p<0.05), but was more severely impaired in 12 week old db/db mice (AUC 679±32 mg·hr/dL WT vs. 2203±102 mg·hr/dL db/db, p<0.05) (Figure 2.1B). Fasted 12 week old db/db mice exhibited elevated blood levels of total cholesterol, HDL, LDL, and triglycerides (Figure 2.1C). These findings are consistent with what has been reported previously (Chick et al., 1970) Based on these findings, we chose to study changes in db/db skeletal and cardiac muscle
in mice with prediabetes (5-6 weeks old) and with “overt diabetes” (12-13 weeks old) states.

2.5.2 Decline of exercise capacity of db/db mice

The functional decline of cardiac and skeletal muscle was determined by assessing maximal oxygen consumption (VO$_{2}$max) during forced treadmill running. No significant impairment in maximum cardiac function (VO$_{2}$max) was observed in 6 week old mice with prediabetes or 12 week old db/db mice with overt diabetes compared to age-matched WT controls (6 wo 2.28±0.01 ml O$_2$/min WT vs. 2.54±0.08 ml O$_2$/min db/db; 12 wo 3.28±0.18 ml O$_2$/min WT vs. 3.04±0.10 ml O$_2$/min db/db, p>0.05) (Figure 2.1D). The maximum running speed was reduced by 34.6% at 6 weeks old and 64.1% at 12 weeks old (6 wo 36±1m/min WT vs 24±1 m/min db/db; 12 wo 38±1m/min WT vs. 13.8±0.9 m/min db/db, p<0.05) (Figure 2.1E).

2.5.3 Decreased forelimb grip strength in db/db mice

Depressed skeletal muscle functional capacity was confirmed by measuring in vivo forelimb grip strength, which was reduced in 12 week old db/db mice by 28% compared to age-matched WT controls (1.27±0.05 N WT vs. 0.92±0.05 N db/db, p<0.05) (Figure 2.1F).

2.5.4 Progression of metabolic phenotype TallyHo mice

The TallyHo male mouse is a leptin-intact, mature-onset, and polygenic (Stewart et al., 2010) model of T2DM that develops reduced insulin-dependent muscle glucose uptake by 6 weeks of age, whole body insulin resistance at ~8 weeks of age, and overt
hyperglycemia at ~16 weeks of age. (Kim et al., 2006) Based on published literature (Kim and Saxton, 2012; Kim et al., 2006; Stewart et al., 2010), we selected 8-9 week old and 22-24 week old TallyHo male mice to compare the muscle phenotype at prediabetic and overtly diabetic stages, respectively. First, the TallyHo metabolic phenotype (body weight, glucose tolerance, and fasting blood glucose) was confirmed to validate the comparison. TallyHo mice gained body weight during this time period (31.6 ± 0.5g 8wo vs. 36.4 ± 0.7g 24 wo). In addition, fasting hyperglycemia was present in prediabetic 8wo mice (287 ± 27 mg/dL fasting blood glucose) and became significantly higher in the overtly diabetic 23 week old mice (379 ± 46 mg/dL, p<0.05). Glucose tolerance of TallyHo mice was already impaired in the prediabetic TallyHo, and more severely impaired in the overtly diabetic TallyHo mice. This gradual mature onset of diabetes agrees with previous findings. (Kim et al., 2006)

2.5.5 Exercise capacity of TallyHo mice

When compared between mice 7 and 22 weeks old, the exercise capacity of TallyHo mice did not decrease with the onset of overt diabetes (Figure 2.2). Specifically, maximum cardiac capacity increased with age between the 7 week old and 22 week old TallyHo mice (3.07±0.02 ml O2/min 7wo TallyHo vs. 3.68±0.08 ml O2/min 22wo TallyHo)(Figure 2.2C). In addition, maximum running speeds achieved during the tests were not significantly different between the age groups (31 ± 1 m/min 7wo TallyHo vs. 29.5 ± 0.6 m/min 22wo TallyHo, p>0.05)(Figure 2.2D).
2.5.6 Muscle weight is reduced significantly in db/db mice but not in the TallyHo mouse model

To understand the poor running speed and grip strength of db/db mice, muscle weight and histology were assessed. Hindlimb muscle size was visibly reduced in 6 week old and 12 week old db/db mice (Figure 2.3a). No increase in centrally located nuclei (which would suggest muscle injury and regeneration) was evident by histology. Wet muscle mass was decreased in predominantly fast-type skeletal muscle (gastrocnemius, tibialis anterior, and extensor digitorum longus) from prediabetic 6 week old mice and more so in overtly diabetic 12 week old mice. Notably, although the disparity between db/db and WT mice muscle weight increased with age, db/db muscle weight increased throughout this period, but at a much lower rate than WT controls. This suggests decreased muscle growth and not muscle loss as the cause of decreased db/db muscle weight. No significant change was evident in tibia length, cardiac weight, or soleus weight (predominantly slow oxidative muscle) compared to WT controls (Table 2.3). On the other hand, skeletal muscle weight increased from 9 weeks of age to 24 weeks of age in TallyHo mice despite glucose intolerance and hyperglycemia (Table 2.3). Comparison of Tallyho muscle weight with “healthy” muscle weight is limited to using young TallyHo mice with prediabetes due to the lack of age-matched healthy controls for TallyHo mice. This lack of mice from a similar background strain is due to the extensive crossbreeding between different strains of mice used to develop this polygenic model. 

(Kim et al., 2006) No evidence of increased centrally located nuclei was observed in TallyHo muscle by histology.
2.5.7 Chronic exercise improves exercise capacity and glucose tolerance in db/db mice

Voluntary physical activity of db/db mice, as quantified by interruption of in-cage horizontal infrared beams, was severely reduced in 12 week old db/db mice compared to WT controls (4.00x10^5 ±1x10^3 beam breaks/day WT vs. 1.84x10^5 ±4x10^3 beam breaks/day db/db, p<0.05) (Figure 2.4A). We hypothesized that decreased muscle activity may be sufficient to cause the observed reduction of muscle weight. To test if increased physical activity could reverse the muscle phenotype we used chronic exercise, which is a treatment commonly prescribed for individuals with reduced muscle size and type 2 diabetes (Albright et al., 2000). Chronic forced treadmill training for 8 weeks increased both maximum running speed and maximum cardiac capacity (VO_2max) (Figures 2.4C and 2.4D). The exercise treatment did not increase the apparent size of hind-limb musculature, and did not cause an increase in centrally located nuclei (Figure 2.6B). Chronic exercise slightly increased the weight of the gastrocnemius muscle compared to sedentary db/db mice, but did not rescue the muscle weight to healthy WT levels. However, the weight of the tibialis anterior muscle was not significantly increased by exercise. (Table 2.3)

2.5.8 Acute cold tolerance is impaired in db/db mice and further impaired by iBAT removal

A measure of cold tolerance is how long can an animal maintain a core body temperature above 20ºC when the animal is exposed to acute cold (4-6 ºC). Previous work in our lab has shown that adult WT mice with or without iBAT only have mild
reductions in core body temperature when exposed to acute cold, and do not reach the
20ºC removal criteria. (Bal et al., 2012) However, 10 week old db/db (iBAT+) mice, when acutely exposed to cold, exhibited rapid temperature loss (Figure 2.5A) with an average time to a body temperature of 20ºC of 266 minutes (Figure 2.5B-C). Db/db mice with the iBAT surgically removed lost body heat even more quickly, with an average time to a body temperature of 20ºC of only 133 minutes (Figure 2.5B-C).

2.5.9 Cardiac function in ex vivo Langendorff db/db heart studies

Heart weight and basal cardiac function were not significantly different in WT and db/db mice (Figure 2.6, Table 2.4). With increasing doses of isoproterenol, heart rates, coronary blood flow rates, and maximum rates of pressure development (+dp/dt) and pressure relaxation (-dp/dt) all increased in both WT and db/db mice. Although differences between WT and db/db mice at each isoproterenol dose were not significantly different, there was a distinct trend of decreased response to isoproterenol in db/db mice. However, this trend of decreased response to isoproterenol may be related to diverging basal cardiac function over the course of the experiment (Figure 2.6 – Heart Rate After Washout).

2.6 Discussion

Diabetes is strongly associated with decreased muscle mass and function in elderly humans. Despite the extensive clinical studies of this association (Chapter 1: Tables 1.1-2), most of these studies have been cross-sectional studies with only one time point, comparing mean values from individuals with diabetes with mean values of individuals
without diabetes. While these studies are powerful due to the high number of patients studied, these studies are poor at describing the progression of muscle loss in diabetes. For example, single time-point differences in mean muscle mass and strength of the elderly do not differentiate between several alternate hypotheses regarding the course of muscle loss in diabetes:

1.) **Advanced diabetes causes muscle atrophy hypothesis**: Muscle loss may occur only in advanced overt diabetes and chronic hyperglycemia (i.e. hyperglycemia or diabetes sequelae causing muscle loss)

2.) **Prediabetes causes muscle atrophy hypothesis**: Muscle loss may begin during prediabetes, before the onset of overt hyperglycemia (i.e. insulin resistance or lipotoxicity causing muscle loss)

3.) **Muscle atrophy precedes insulin resistance hypothesis**: Muscle loss and weakening may occur prior to developing insulin resistance (i.e. normal muscle growth until metabolic insult causes muscle loss)

4.) **Poor muscle growth precedes insulin resistance and contributes to diabetes hypothesis**: Failure of normal muscle growth may be a predisposing factor for type 2 diabetes, leading to the results of poor muscle size associated with diabetes phenotype (i.e. muscle may never grow normally in many people that later develop diabetes).

The key human evidence to date which begins to resolve these hypotheses is the 6 year longitudinal study performed by Park et al, which, using dual-energy x-ray absorptiometry (DEXA) imaging, found that in elderly individuals with diabetes, loss of
muscle mass was accelerated compared to individuals without diabetes. (Table 1.1) While this data indicates that muscle mass/function of people with diabetes continues to diverge from individuals without diabetes during old age, suggesting that muscle loss occurs during diabetes. However, even with this data, it is does not determine when the muscle loss begins, nor how muscle loss relates chronologically and etiologically to the development of the diabetes phenotype.

2.6.1 Early onset of reduced muscle size in db/db mice

In our studies of db/db mice, reduced fast skeletal muscle size and running ability is already present at 5 weeks of age, before the onset of chronic hyperglycemia (Figure 2.1). This indicates that overt diabetes, chronic hyperglycemia, and comorbidities which accompany chronic diabetes (such as diabetic peripheral neuropathy and diabetic nephropathy) are not necessary for decreased muscle weight and function in this model. If the db/db muscle phenotype is representative of human diabetes muscle loss, this early decreased muscle size would suggest that early insulin resistance may be sufficient to cause early muscle atrophy prior to the onset of overt T2DM. Limited studies in young men (n=51, ages 18-35) do indicate a positive correlation between insulin sensitivity and lean body mass study.(Unni et al., 2009) However, further work must be done to establish if early insulin resistance is sufficient to cause muscle loss in humans with prediabetes.

Alternatively, decreased muscle size and function in db/db mice may not be a representative model of muscle loss in diabetes but instead reflect a failure of physiologic skeletal muscle growth. Indeed, the early onset of decreased muscle size in db/db mice
suggests that dysfunctional metabolism (e.g., altered leptin signaling, insulin resistance, and hyperlipidemia) during early muscle development may create a growth-adverse environment in which normal muscle growth is stunted. For example, the classic insulin-dependent intracellular signaling pathways which control glucose metabolism are actually multi-user and multi-effect pathways that are utilized by many regulatory proteins (e.g., insulin, IGF-1, leptin, AMPK(energy sensing), and mTOR(amino acid sensing)) to integrate metabolic control and growth control. Therefore it is reasonable that the juvenile onset of insulin resistance, absent leptin signaling, and altered metabolism could reduce early muscle growth in the db/db mouse.

Additional evidence comes from the ob/ob mouse, which, like the db/db mouse, is leptin-signaling deficient and insulin resistant. The ob/ob mouse has normal gastrocnemius weight up to 2 weeks of age, after which ob/ob muscle weight diverges from WT mice, with ob/ob muscle growing much more slowly than the wild-type muscle. (Purchas et al., 1985) Therefore, it is likely that reduced muscle weight in ob/ob mice and db/db mice is due to a failure of physiologic hypertrophy, rather than atrophy of a once healthy muscle. Although this diminished muscle growth could be due to the altered metabolic milieu caused by early insulin resistance (db/db have increased serum insulin levels by 5-7 days of age compared to WT), it could also be caused by the effects of leptin(Hamrick et al., 2010; Sáinz et al., 2009), lipotoxicity, or deficient growth hormone signaling on skeletal muscle growth and metabolism.

Regardless of the mechanism of reduced muscle size, it is reasonable to conclude that the juvenile onset of insulin resistance, diabetes, and decreased muscle size in the
db/db mouse model does not accurately reflect the typical pattern of adult onset diabetes and muscle atrophy of mature muscle seen in human patients with T2DM. We suggest that future studies of muscle and metabolism in leptin-signaling deficient models should take into account this early prediabetic onset of poor muscle growth and verify findings in additional models of T2DM.

2.6.2 Muscle growth and function in TallyHo mice

To expand our findings to a leptin-intact adult onset model of T2DM, we also studied the TallyHo mouse model. The TallyHo mouse is a polygenic mature-onset model of severely hyperglycemic type 2 diabetes with intact leptin signaling, insulin resistance, and deficient muscle-specific glucose uptake.(Kim et al., 2006) We were surprised to find no indication of muscle loss in the TallyHo mice; muscle weight increased with age (Table 2.3) and exercise capacity (running speed and cardiac capacity) was maintained (Figure 2.2) when comparing 2 month old (prediabetes stage) and 6 month old (overt diabetes stage) mice. (Kim et al., 2006) It is possible that studying skeletal muscle in TallyHo mice at older ages or at more time points may demonstrate eventual atrophy. The lack of observed muscle loss in TallyHo mice within the first 6 months of life suggests that insulin resistance and chronic hyperglycemia are insufficient to independently cause rapid marked muscle loss in mice, and that contextual differences such as age of onset of diabetes, absent leptin signaling, degree of hyperinsulinemia, background genetic strain, and differing diabetes-associated comorbidities may determine if muscle loss will occur.
2.6.3 Decreased acute cold tolerance in db/db mice and exacerbation by iBAT removal

Thermogenesis has not been traditionally viewed as an important function of skeletal muscle, especially in mice, where brown adipose tissue plays a large role in facultative thermogenesis. (Cannon and Nedergaard, 2004; Hofmann et al., 2001) However, recent studies by our lab have shown that removal of interscapular BAT in mice (estimated to contain ~75% of total mouse BAT) is insufficient to cause severe cold intolerance, but that iBAT removal in mice with altered skeletal muscle calcium handling (sarcolipin knockout) is sufficient to cause acute cold intolerance. (Bal et al., 2012) This work strongly suggests that skeletal muscle plays a key role in thermogenesis.

Db/db mice have been described previously to exhibit poor cold tolerance. (Trayhurn, 1979) Previous research of db/db cold intolerance has attributed poor thermogenesis to a purported lack of function of the brown adipose tissue in these mice due to blunted adrenergic signaling and decreased mitochondrial heat generation via decreased uncoupling protein (UCP) function. (Masaki et al., 2000; Young et al., 1984) Our data indicating exacerbation of cold intolerance by iBAT removal in db/db mice suggests that BAT function is at least partially intact in db/db mice. Moreover, given the severe cold intolerance of db/db mice, even compared to UCP1−/− mice (BAT thermogenically nonfunctional), it is feasible that the reduced muscle mass of db/db mice may contribute to cold intolerance. Thus decreased muscle function in db/db mice may extend to decreased muscle-based thermogenesis. However, these studies are inconclusive because effects of altered leptin signaling and/or altered metabolism due to
diabetes could theoretically impact thermogenesis. Further research will be necessary to conclusively determine the effects of diabetic metabolism and diabetic muscle loss on the functional role of muscle in thermogenesis. Useful experiments may include rescuing db/db muscle mass and then studying thermogenesis, studying thermogenesis in leptin-intact models of muscle loss in diabetes, and comparing thermogenesis in normal-muscle and reduced-muscle models of T2DM.

2.6.4 Cardiac function in db/db mice

Decreased maximum running speed in db/db mice may be caused by limitations in both skeletal and cardiac muscle. *In vivo* treadmill stress testing demonstrated no significant decrease in maximum cardiopulmonary capacity (VO₂max) in db/db mice, suggesting that cardiac capacity is not a major contributor to limited running speeds in this model.

Despite no change in VO₂max, *ex vivo* Langendorff heart function studies demonstrated a trend for decreased isoproterenol-dependent heart function in db/db mice. (Figures 2.6A-E, Table 2.2) However, a confounding factor in interpreting this trend is that the return to functional baseline during the washout between isoproterenol doses was dissimilar between WT and db/db mice. That is, basal db/db heart rates (after 30 minute washout with isoproterenol-free Krebs-Henseleit buffer) decreased more quickly than WT over the duration of the experiment (Figure 2.6F). Due to the typical decline of cardiac function during Langendorff experiments, it is necessary to normalize isoproterenol-dependent function to the most recent assessment of “basal” cardiac function (Isoproterenol Function % = 100 x (Isoproterenol Function/Basal Function).
Because the return to baseline function was less than WT (decreased function) in db/db hearts after the washout period, and baseline function serves as the denominator in determining % change in function, the decreased basal function of hearts from db/db mice may artificially increase or decrease the apparent effects of isoproterenol in db/db hearts.

Previous literature demonstrates that basal cardiac ejection fraction of db/db mice with overt diabetes is very mildly decreased compared to WT.(Bugger and Abel, 2009) Despite this near-normal resting in vivo cardiac function, ex vivo db/db heart function is very sensitive to which energy substrates are available; Langendorff-prepared heart studies show severely decreased function when perfused with palmitate-containing KH buffer, but WT-level function when perfused with palmitate-free KH buffer.(Hafstad et al., 2006) Taken together, our studies and previous research suggest that cardiac function is not a major deterrent to exercise capacity in the db/db mouse. However, further work must be done to directly characterize under what metabolic and exercise conditions heart function may be compromised in the db/db mouse, and in T2DM models in general. Future in vivo experiments involving palmitate and/or isoproterenol infusion during cardiac MRI or echocardiography may shed additional light on the effects of diabetes on cardiac function.

2.6.5 Effects of chronic exercise on db/db muscle

When muscle loss in patients interferes with physical function, chronic exercise is a treatment commonly prescribed to increase muscle function, muscle mass, and to improve glucose tolerance. Although chronic exercise of db/db mice improved glucose
tolerance, cardiac capacity (VO$_2$max), and running speeds, only a mild increase in final muscle weight was observed in exercised compared to non-exercised db/db mice, indicating that exercise is insufficient to rescue muscle growth in the db/db mouse model. This suggests that decreased routine muscle use by db/db mice, as demonstrated by decreased movements over 24 hours (Figure 2.6), is not the basis for the reduced muscle size of db/db mice. Moreover, these findings do not suggest a significant hypertrophic effect of exercise on muscle in mice with T2DM (similar exercise studies in WT mice have shown a 12-18% increase in muscle mass)(Kemi et al., 2002). These findings support the current clinical use of exercise to improve glucose tolerance, muscle function, and exercise capacity. However, further research is necessary to clarify how exercise impacts muscle loss in diabetes, and conversely how the catabolic muscle environment in diabetes impacts the ability to benefit from exercise.

### 2.6.6 Summary of conclusions

Changes in muscle weight and function in response to the onset of T2DM were inconsistent between the db/db and TallyHo mouse models, suggesting that insulin resistance and chronic hyperglycemia alone are insufficient to consistently cause rapid muscle loss. This indicates that contextual differences, such as age of onset of diabetes, genetic background, degree of compensatory hyperinsulinemia, and extent of comorbidities, determine the fate of muscle health in response to diabetes. Moreover, the reduced muscle size in db/db mice before the onset of overt hyperglycemia and diabetes suggests that the altered muscle metabolic milieu caused by insulin resistance and decreased leptin signaling may be toxic to early muscle growth. These findings highlight
the ability of altered metabolism to impair muscle growth and function, as well as the need for future research to discover better models of insulin resistance that more accurately reflect the mature onset of T2DM and muscle loss seen in humans.
Tables and Figures for Chapter 2

Table 2.1 Clinical Studies of Muscle Mass and Function in Insulin Resistant Individuals

<table>
<thead>
<tr>
<th>Study Name</th>
<th>Type/Population</th>
<th>Measurements</th>
<th>Significant Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Unni et al., 2009)</td>
<td>Cross-sectional study, India;</td>
<td>• Lean body mass (DEXA)</td>
<td>• Insulin sensitivity associated with lean muscle mass, even in lower-BMI individuals.</td>
</tr>
<tr>
<td></td>
<td>51 young men (ages 18-35)</td>
<td>• Hyperinsulinemic/Euglycemic clamp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Hand grip dynamometry</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Indirect calorimetry</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Korean Sarcopenic Obesity Study (Kim et al.,</td>
<td>Cross-sectional study, Korea;</td>
<td>• Lean body mass (dual x-ray absorptiometry – DEXA)</td>
<td>• Sarcopenia was defined as SMI&lt; 2 standard deviations below population average.</td>
</tr>
<tr>
<td>2010)</td>
<td>414 with type 2 diabetes, 396</td>
<td>• Sarcopenia measured using skeletal muscle index (SMI)</td>
<td>15.7% of people with diabetes and 6.9% of controls were categorized as sarcopenic.</td>
</tr>
<tr>
<td></td>
<td>control individuals</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>The Health, Aging, and Composition Study</td>
<td>6 year prospective longitudinal study;</td>
<td>Changes in regional lean muscle mass (DEXA)</td>
<td>• Muscle loss per year was greatest in undiagnosed (and untreated) diabetes (~435 ± 79 g/year), then diagnosed diabetes (~293 ± 72 g/year), and lowest in non-diabetes (~193 ± 22 g/year).</td>
</tr>
<tr>
<td>(Park et al., 2009)</td>
<td>1290 controls, 125 undiagnosed diabetes, 214 diagnosed diabetes.</td>
<td>Changes in CT scan at Years 1 and 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>The Health, Aging, and Composition study</td>
<td>3 year prospective longitudinal;</td>
<td>Regional lean muscle mass (DEXA)</td>
<td>~50% more rapid decline in knee extensor strength in diabetes (-13.5% vs. -9.0%).</td>
</tr>
<tr>
<td>(Park et al., 2007)</td>
<td>1535 non-diabetes and 305 type 2 diabetes (70-79 years old)</td>
<td>Isokinetic knee extension dynamometry</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isometric hand grip dynamometry</td>
<td>~60% more rapid loss in muscle quality (force/lean muscle mass) in diabetics (-10.0% vs -6.2%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• No statistical difference in decline of hand grip strength.</td>
</tr>
</tbody>
</table>

Continued
<table>
<thead>
<tr>
<th>Study (Year)</th>
<th>Design and Sample</th>
<th>Methods</th>
<th>Findings</th>
</tr>
</thead>
</table>
| Park et al. 2006  
The Health Aging, and Composition Study (Park et al., 2006) | Cross sectional study; 2133 control and 485 individuals with diabetes (70-79 years old) | Regional lean muscle mass (DEXA)  
Isokinetic knee extension dynamometry  
Isometric hand grip dynamometry | Men and women with T2DM exhibited mildly increased muscle mass compared to non-diabetics, and lower (men) or unchanged (women) muscle strength.  
Muscle quality (force/lean muscle mass) was decreased by ~8% in upper and lower extremity muscles in type 2 diabetic individuals. |
| Andersen et al., 2004 | Cross sectional; 36 diabetes and 36 control (matched for sex, age, weight, height, and physical activity) | Muscle strength dynamometry of elbow, knee, wrist, and ankle flexors and extensors.  
Motor and sensory nerve function | Decreased ankle flexor and extensor, and knee flexor strength in type 2 diabetics.  
No significant difference in elbow or wrist strength.  
Negative correlation between degree of neuropathy and muscle strength.  
Study weakened by small study group and cross-sectional nature |
Table 2.2 Changes in muscle fiber types in humans with type 2 diabetes mellitus

<table>
<thead>
<tr>
<th>Study</th>
<th>Population</th>
<th>Oxidative Fibers</th>
<th>Glycolytic Fibers</th>
<th>Oxidative Enzyme Activity</th>
<th>Glycolytic Enzyme Activity</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oberbach et al. (25)</td>
<td>15 control (56.8 ±7.9 years old), 10 diabetic (58.7 ± 6.4 years old)</td>
<td>~16% decrease in fraction of oxidative fibers</td>
<td>~49% increase in fraction of glycolytic fibers</td>
<td>Increased in all fiber types, average of 47.5% increase.</td>
<td>Increased in slow oxidative and fast oxidative fibers only, overall average of 16.3% decrease.</td>
<td>Different mean age study populations may confound interpretation of results.</td>
</tr>
<tr>
<td>He et al. (26)</td>
<td>22 lean(35±1 years old) and 20 type 2 diabetes subjects (52 ± 2 years old)</td>
<td>Trend towards decreased oxidative fibers</td>
<td>Trend towards increased glycolytic fibers</td>
<td>Decreased in muscle homogenate</td>
<td>No significant difference in muscle homogenate</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3 Body, muscle, and tibia size in db/db and TallyHo mice with prediabetes and overt diabetes.

<table>
<thead>
<tr>
<th>Age</th>
<th>Treatment</th>
<th>Body (g)</th>
<th>Ventricles (mg)</th>
<th>Gastrocnemius (mg)</th>
<th>Tibialis Anterior (mg)</th>
<th>Extensor Digitorum Longus (mg)</th>
<th>Soleus (mg)</th>
<th>Tibia Length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 week old TallyHo (n=4)</td>
<td></td>
<td>31.7±0.9</td>
<td>108.8±0.6</td>
<td>114.1±2.8</td>
<td>44.4±1</td>
<td>8.1±0.7</td>
<td>6.2±0.2</td>
<td>17.4±0.3</td>
</tr>
<tr>
<td>24 week old TallyHo (n=6)</td>
<td></td>
<td>37.7±1.6#</td>
<td>172.3±9.1#</td>
<td>150.3±4.5#</td>
<td>53.6±0.9#</td>
<td>12.2±0.3#</td>
<td>9.5±0.4#</td>
<td>19.2±0.2#</td>
</tr>
<tr>
<td>6 week control (n=4)</td>
<td></td>
<td>20.2±0.4</td>
<td>97.5±4.2</td>
<td>91.1±6.1</td>
<td>32.7±0.9</td>
<td>6±0.3</td>
<td>4.7±0.3</td>
<td>7±0.7</td>
</tr>
<tr>
<td>6 week old db/db (n=4)</td>
<td></td>
<td>30.9±1.6*</td>
<td>86.7±4.3</td>
<td>60.5±8.4*</td>
<td>27.8±0.9*</td>
<td>5.1±0.3</td>
<td>5.1±0.3</td>
<td>6.3±1</td>
</tr>
<tr>
<td>12 week old C57BLKS control (n=4)</td>
<td></td>
<td>25.4±0.8</td>
<td>117.2±4</td>
<td>128.4±3.6</td>
<td>42.9±2.3</td>
<td>10.4±0.7</td>
<td>6.4±0.5</td>
<td>18.7±0.3</td>
</tr>
<tr>
<td>12 week old db/db (n=4)</td>
<td></td>
<td>48.9±1.2*</td>
<td>117.6±4.9</td>
<td>71.9±2.5*</td>
<td>26.5±1.9*</td>
<td>6.8±0.5*</td>
<td>6.3±0.6</td>
<td>18.4±0.2</td>
</tr>
<tr>
<td>13 week old C57BLKS control (n=4)</td>
<td></td>
<td>21.5±0.40</td>
<td>n.m.</td>
<td>132±3.7</td>
<td>n.m.</td>
<td>n.m.</td>
<td>n.m.</td>
<td>n.m.</td>
</tr>
<tr>
<td>13 week old db/db sedentary (n=8)</td>
<td></td>
<td>47.7±2.5*</td>
<td>n.m.</td>
<td>63.3±1.4*</td>
<td>30.3±0.9*</td>
<td>n.m.</td>
<td>n.m.</td>
<td>n.m.</td>
</tr>
<tr>
<td>13 week old db/db after 8 weeks exercise (n=8)</td>
<td></td>
<td>46.8±1.4*</td>
<td>n.m.</td>
<td>77±3* †</td>
<td>28.6±0*</td>
<td>n.m.</td>
<td>n.m.</td>
<td>n.m.</td>
</tr>
</tbody>
</table>

* = p<0.05 compared to age-matched WT
† = p<0.05 compared to age-matched sedentary db/db
# = p<0.05 compared to 9 week old TallyHo
n.m. = not measured
Table 2.4 Baseline functional parameters in isolated hearts without isoproterenol stimulation

<table>
<thead>
<tr>
<th>Baseline Parameters in isolated hearts</th>
<th>C57BL (n=4)</th>
<th>db/db (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary flow (ml/min/g)</td>
<td>16.45 ± 0.9</td>
<td>18.55 ± 1.3</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>317 ± 5</td>
<td>322 ± 12</td>
</tr>
<tr>
<td>LVDP (mmHg)</td>
<td>110 ± 6</td>
<td>117 ± 17</td>
</tr>
<tr>
<td>minus dP/dt (mmHg/s)</td>
<td>2041 ± 108</td>
<td>2026 ± 248</td>
</tr>
<tr>
<td>plus dP/dt (mmHg/s)</td>
<td>2720 ± 308</td>
<td>2696 ± 324</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>123 ± 3</td>
<td>116 ± 6</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>27 ± 1</td>
<td>49 ± 3*</td>
</tr>
<tr>
<td>HW/BW Ratio (g%)</td>
<td>0.46 ± 0.02</td>
<td>0.24 ± 0.01*</td>
</tr>
</tbody>
</table>
Figure 2.1 – Development of obesity, glucose intolerance, and decreased exercise capacity in db/db male mice – (A) Db/db mice gain body weight more quickly than WT beginning at 5 weeks of age, (B) glucose tolerance progressively deteriorates in db/db mice from 6 to 12 weeks of age, (C) 12 week old db/db mice present with elevated fasting serum cholesterol and triglycerides, (D) Maximum cardiopulmonary capacity (VO2max) is not significantly altered in 5 week or 12 week old db/db diabetic mice compared to age-matched WT, (E) the maximum running speed achieved during the VO2max test is significantly lower in db/db mice compared to WT, both at 5 weeks and 12 weeks of age, (F) forelimb grip strength is significantly reduced in 12 week old db/db mice. (Error bars = Standard error of mean, * = p<0.05 compared to WT)
Figure 2.2 – Development of obesity and glucose intolerance but maintenance of exercise capacity in TallyHo mice  

A) TallyHo mice increase in body weight from 8 to 24 weeks of age, (B) Glucose tolerance is impaired in 8 week old TallyHo and further deteriorates in the 22 week old TallyHo mice, (C) Maximum cardiopulmonary capacity (VO2max) increases with age in TallyHo mice between 7 and 22 weeks of age, (D) The maximum running speed recorded during the VO2max test by 7 week old TallyHo mice is still achieved by the 22 week old TallyHo mice with overt diabetes. (Error bars = Standard error of mean, * = p<0.05 compared to WT)
Figure 2.3 – Reduced muscle size of db/db mice and histology of db/db and TallyHo mice muscle – (A) Apparent muscle size is severely decreased in db/db mice relative to age-matched controls at 6 and 12 weeks of age. Hematoxylin and eosin staining does not demonstrate an increase in centrally located nuclei in db/db or (B) TallyHo tibialis anterior muscle. (C) Masson’s trichrome staining does not demonstrate a visibly apparent increase in collagen staining (blue) of in tibialis anterior muscle sections from overtly diabetic db/db or Tallyho mice. (Arrows indicated apparent differences in anterior-posterior diameter of lower leg)
Figure 2.4 – Chronic interval training treadmill exercise of db/db mice for 8 weeks moderately improves glucose tolerance and muscle phenotype. (A) Basal physical activity levels are decreased in 10 week old db/db mice compared to lean WT controls. (B) Gross and histological appearance of reduced muscle size is not visibly improved by exercise. (C) Weight gain is not altered by chronic exercise of db/db mice. (D) Exercise moderately increases glucose tolerance in exercised db/db mice compared to sedentary db/db controls. (E) Maximum running speed and (F) maximum cardiac capacity (VO₂max) of exercised db/db mice are increased post-exercise compared with non-exercised sedentary db/db controls.
**Figure 2.5 Acute cold tolerance is decreased in db/db mice**  Db/db mice are intolerant to acute cold exposure (6°C). Surgical removal of interscapular brown adipose tissue further reduces the mean cold endurance time of db/db mice, but is not statistically significant due to large standard error. (p>0.05, two-sided student t-test).
Figure 2.6 Ex vivo Langendorff heart function of db/db mice with isoproterenol stimulation (A-E) Isoproterenol increased heart rate, rate of pressure development (+dp/dt), rate of relaxation (-dp/dt), coronary blood flow, and left ventricular diastolic pressure (LVDP) in WT and db/db perfused hearts. However, no statistically significant different in individual dose comparisons of WT vs. db/db function were found. (F) Basal cardiac function in db/db hearts declined more rapidly than WT hearts, as measured during the 30 minute washout phase between escalating doses of isoproterenol.
Chapter 3: How insulin resistance affects pathways regulating protein synthesis and protein degradation

3.1 Introduction

A key limitation of developing therapeutic interventions for muscle loss in T2DM is the lack of a clearly defined mechanism or signaling pathway to target. Many diabetes-associated conditions exist that may affect muscle growth and cause muscle loss. These candidate pathways include: decreased insulin signaling, increased glucocorticoids (Filipovský et al., 1996; Reynolds and Walker, 2003; Rosmond et al., 1998; Stolk et al., 1996), decreased testosterone (Alibhai et al., 2009, 2009; Ding et al., 2006; Yialamas et al., 2007), increased serum myostatin (Allen et al., 2008, 2010; Hittel et al., 2009, 2010; Jespersen et al., 2011), decreases in growth hormone/IGF-1 signaling (Teppala and Shankar, 2010; Yalow et al., 1965), increased obesity, increased inflammatory cytokines, peripheral neuropathy, decreased physical activity, decreased peripheral vasodilation, lipotoxicity, and altered diets. These pathways are not consistently perturbed in all individuals with T2DM, but are altered heterogeneously both horizontally across the population and longitudinally in the temporal progression of T2DM. Thus, with the current knowledge in the field, it is difficult to appropriately predict and treat muscle loss in T2DM because it is unclear which of these pathways are necessary and/or sufficient to cause muscle loss in diabetes.
To address this confounding web of possible pathways, we chose to focus on the reductionist approach: that the growth or atrophy of muscle is determined by net protein balance. We studied factors that change net protein balance by measuring markers and pathways involved with both protein synthesis and protein degradation in three mouse models of T2DM.

3.2 Regulation of protein synthesis

Protein synthesis occurs in multimeric protein complexes known as ribosomes; ribosomal translational rates and processivity are directly regulated at the initiation, elongation, termination phases, and ribosome recycling phases of protein synthesis. The initiation step is highly regulated and is considered the rate limiting step in protein synthesis under most conditions (Jackson et al., 2010). A rate-limiting step in ribosomal initiation is the binding of eukaryotic initiation factor 4E (eIF4E) to the 5’ mRNA cap. eIF4E binds cooperatively with eIF4A (RNA helicase) and eIF4G (scaffold protein) to unwind mRNA secondary structure and enable binding with the 43S pre-initiation ribosomal complex. eIF4E activity is increased by Ser209 phosphorylation. (Jackson et al., 2010; Shveygert et al., 2010). Additional examples of ribosomal regulation include mRNA-ribosome binding (Pain, 1996), amino acid availability (Anthony et al., 2001), and ribosome protein phosphorylation (Ruvinsky et al., 2005). Of relevance to our studies, the S6 ribosomal protein is a component of the 40S ribosome and is activated by phosphorylation at several residues (Krieg et al., 1988). S6 phosphorylation increases protein synthesis and is associated with increased cell size and metabolism. These proteins that comprise protein synthesis machinery are regulated by kinases that integrate
intracellular conditions (such as energy and amino acid availability) and extracellular signaling (such as testosterone, IGF, and insulin) to control synthetic rates of protein.

3.3 Regulation of protein degradation

Protein degradation occurs via several different pathways: ubiquitin-dependent proteasomal, autophagy-lysosomal, and caspase-mediated protein degradation. Of these different types of protein degradation, we focused our degradation studies on the proteasomal degradation pathway because 1) ATP-dependent proteasomal protein degradation is responsible for the 80% of insulin-dependent changes in total muscle protein degradation in T1DM mice (Price et al., 1996), 2) insulin plays a major direct signaling role in regulating proteasomal degradation via the IR-IRS-1-Akt-FOXO1/3-E3 Ligase pathway (Stitt et al., 2004), and 3.) E3 ubiquitin ligases (key enzymes in proteasomal protein degradation) are associated with and necessary for many types of muscle atrophy to occur.

Proteins destined for proteasomal protein degradation are labeled for destruction by covalent ligation of the ubiquitin protein (8.5 kDa) to lysine residues of the target protein via isopeptide bonds. (Glickman and Ciechanover, 2002) This ubiquitin-substrate protein linkage reaction is catalyzed by E3 ligases, a large family of 100s of enzymes, which selectively bind specific protein motifs. (Li et al., 2008) E3 ligases can bind selectively to post-translationally modified proteins, misfolded proteins, unmodified proteins, and proteins which expose binding sites by a shift in protein shape (as may occur by allosteric regulation). (Ciechanover, 1998; Glickman and Ciechanover, 2002) Alternatively, certain intracellular conditions appear to cause less-selective general
ubiquitination of proteins. Ubiquitination does not always lead to proteasomal degradation; monoubiquitination of proteins is a reversible event that does not consistently lead to proteasomal degradation and polyubiquitination of target proteins with K63-linked ubiquitin chains appears to function in cell signaling. (Sigismund et al., 2004) Polyubiquitination with K48-linked ubiquitin chains is non-reversible and is consistently causes trafficking of the target protein to the proteasome for subsequent degradation. (Komander, 2009)

To understand the context of our experiments of protein synthesis and degradation, I will summarize what is known about the relationship between insulin signaling, diabetes, protein synthesis, and proteasomal protein degradation in muscle from T2DM humans and animal models.

3.4 Insulin signaling regulates protein synthesis and protein degradation

Insulin is an endocrine hormone secreted by the beta cells of the pancreas in response to increased levels of glucose in the blood. Insulin binds to the heterotetrameric insulin receptor which, upon insulin binding, autophosphorylates several regulatory tyrosine residues. This insulin receptor tyrosine phosphorylation enables the binding and activation of proteins from the insulin receptor substrate family (IRS-1, IRS-2, IRS-3, IRS-4), which in turn activate a complex cascade of signaling pathways that result in eventual downstream activation of proteins which promote glucose uptake and metabolism, protein translation initiation, amino acid uptake, and protein synthesis (Fujita et al., 2006), while inhibiting protein degradation pathways (Figure 3.1).
3.4.1 Healthy insulin signaling promotes protein accretion

Healthy insulin signaling increases protein synthesis by regulation of specific growth promoting pathways including Akt, mTOR, p70S6K, S6 ribosomal protein, 4E-BP1, GSK3-beta, and eEF2. (Hillier et al., 2000; Taniguchi et al., 2006) In addition, intact insulin signaling decreases protein degradation by Akt-mediated phosphorylation of FOXO1/3 transcription factors. This insulin-dependent FOXO1/3 phosphorylation deactivates FOXO1/3 transcriptional activation of key E3 ubiquitin ligases. E3 ubiquitin ligases perform the typically rate-limiting step of ubiquitin-substrate ligation that labels substrate proteins for trafficking to and degradation by the proteasome. In addition to the acute effects of insulin on protein degradation, chronic increased insulin signaling also affects protein levels of several key components of the protein degradation pathways; MAFBx/Atrogin-1 and proteasome C2 decline, whereas MuRF-1 is largely unchanged (Greenhaff et al., 2008).

3.4.2 Deficient insulin signaling promotes net protein loss

In insulin resistant muscles, despite normal or elevated blood insulin levels, insulin-dependent activation of downstream pathways is suppressed. Although molecular mechanisms responsible for insulin resistance vary on an individual basis, a large proportion of insulin resistance is believed to be mediated by inhibitory phosphorylation of serine residues of the IRS family. IRS serine phosphorylation causes decreased insulin-dependent IRS activity, and thereby decreases insulin-dependent stimulation of both metabolism and growth. This decreased stimulation of anabolic signaling pathways
and disinhibition of protein catabolism can logically contribute to loss of muscle size and strength in individuals with insulin resistant muscle.

3.5 Protein synthesis and degradation in T2DM muscle

3.5.1 Several major signaling pathways which regulate protein synthesis in muscle are affected by insulin resistance

Protein synthesis is regulated by several hormonal and nutrient-sensitive pathways, including AMPK, ATP, insulin, IGF-1, amino acid availability, and mTOR signaling. For example, energy (ATP) availability and amino acid availability does not directly activate protein synthesis, but potentiates signaling pathways (e.g., Akt-mTOR-p70S6K-S6 Ribosomal protein) in a way that permits hormonal signaling (e.g., insulin or IGF-1) to increase protein synthesis and cause muscle hypertrophy. Relevant to our study of T2DM, insulin resistance blunts insulin-dependent phosphorylation of Akt (a nodal upstream regulator of protein synthesis), and several downstream members of the protein synthesis pathway. Similarly, Akt activation leads to FOXO1/3 phosphorylation, which leads to decreased transcription of E3 ubiquitin ligases which promote proteasomal protein degradation; by blunting insulin-dependent Akt activation, it is logical that insulin resistance should increase proteasomal protein degradation.

3.5.2 Meal-dependent protein synthesis is not altered in studies of humans with T2DM

Despite the blunting of insulin-dependent signaling in insulin resistant muscle, two separate clinical studies failed to show a reduction in energy-substrate-induced
muscle protein synthesis using carbohydrate or protein infusion in humans with type 2 diabetes (Manders et al., 2008). However, this does not conclusively indicate that insulin-dependent protein synthesis occurs normally in insulin resistant muscle. Indeed, these human studies used infusion of carbohydrates or protein, which may allow for compensatory hyperinsulinemia; increased insulin secretion in patients with T2DM may compensate for insulin resistance and lead to meal-dependent protein synthesis rates in patients with diabetes that are similar to healthy individuals. In contrast, our approach focuses on the effects similar insulin doses on pathways regulating protein synthesis, and does not allow for compensatory hyperinsulinemia to mask the effects of insulin resistance on insulin-dependent protein synthesis. Studies comparing the effects of equivalent doses of insulin (rather than carbohydrate) on muscle protein synthesis rates in insulin resistant and insulin sensitive individuals have not been reported.

3.5.3 Protein degradation is increased in muscle of patients with type 2 diabetes

Increased rates of protein degradation have been shown in humans with diabetes, as well as in ex vivo studies in T2DM db/db mice (Price et al., 1996; Wang et al., 2006). In addition, rosiglitazone treatment of db/db mice was shown to normalize protein degradation rates (Wang et al., 2006). Increased proteasomal protein degradation has been reported in streptozotocin-induced T1DM rats; however, specific effects of insulin resistance on proteasomal protein degradation have not been reported (Price et al., 1996)
3.6 Experimental hypothesis and rationale

To test if deficient insulin signaling contributes directly to decreased muscle size in T2DM, we studied how insulin-dependent protein synthesis and proteasomal protein degradation pathways are changed in T2DM muscle from db/db mice. Our hypothesis was that deficient insulin signaling in muscle is a key contributor to reduced protein balance and increased muscle loss in T2DM. Our rationale was that insulin-dependent signaling contributes to suppress proteasomal protein degradation and activate protein synthesis. In addition, insulin deficiency (as seen in T1DM) is sufficient to cause muscle loss and insulin replacement rescues T1DM muscle loss, further suggesting a crucial role of insulin signaling in T2DM muscle loss, as well.

We also tested the secondary hypothesis that proteasome dysfunction may contribute to muscle insulin resistance by short-term administration of a pharmacological proteasome inhibitor (MG-132) and measuring glucose tolerance in WT and db/db mice. Our rationale was that in vitro studies have shown that key nodes in insulin-dependent signaling (e.g., IRS-1/IRS-2) are rapidly regulated by proteasomal degradation.(Rui et al., 2002; Shi et al., 2011; Sun et al., 1999)

3.7 Methods:

3.7.1 Animals

Male db/db (BKS.Cg-Dock7m +/+ Leprdb/J), age-matched WT controls (C57BLKS/J), and TallyHo (TallyHo/JngJ) mice were purchased from Jackson Laboratories (Bar
Harbor, Maine) and maintained as described in Chapter 2. All animal experiments were approved by the The Ohio State University IACUC.

3.7.2 Insulin acute response test
To assess tissue-specific acute responses to insulin, basal tail blood glucose was assessed and then recombinant human insulin (Humulin, 0.2U/g body weight, intraperitoneal, Eli Lilly Inc., Indianapolis, IN) or Dulbecco’s phosphate-buffered saline (DPBS) was administered to select mice. Ten minutes after injection, tail blood glucose was assessed and the mice were immediately euthanized and tissues removed.

3.7.3 Chronic exercise
Mice were exercised from 5-13 weeks of age using the interval training method as described previously (Stølen et al., 2009), with slight modifications. Mice were treadmill exercised 80 minutes per day, 5 days per week, for 8 weeks. Training was performed on a 20º incline, alternating 4 minutes high speed and 2 minutes low speed (high = 80-90% of speed at which VO_2 max was reached, slow = 50% of speed at which VO_2 max was reached). The VO_2 max tests for the exercised mice and their controls were performed as described above but with the following changes to speed/incline in order to determine appropriate training speeds for running on an incline: (speed, duration, incline) - (0 m/min, 5 min, 0º), (6 m/min, 5 min, 0º), (6 m/min, 2 min, 20º), (+2 m/min, each 2 min thereafter, 20º).
3.7.4 MG-132 Effect on Glucose Tolerance

To determine the effect of acute proteasome inhibition on glucose tolerance, 26.5 hour pretreatment of age-matched WT and db/db mice with MG-132 (a pharmacological proteasome inhibitor) was used prior to a glucose tolerance test, as depicted in Figure 3.2).

MG-132 was administered by intraperitoneal bolus injections at a dose of 7.5mg MG-132/kg body weight/per injection (at times Day 1 1000, Day 1 1630, Day 2 1000). The mice were fasted from food for the last 2.5 hours(Day 2 1000-1230) of the test to achieve similar basal glucose levels. Starting blood glucose levels were measured using tail blood in a TRUEtrack® blood glucose monitoring kit (Walgreens, Columbus, OH). The maximum limit of the glucometer was 600 mg/dL; glucose levels greater than 600 mg/dL (reading as “HI”) were treated as 600 mg/dL for facility of quantitative data comparison. An injection of glucose (2 g/kg, intraperitoneal, Sigma-Aldrich), dissolved in Dulbecco’s phosphate-buffered saline (DPBS, Invitrogen, Carlsbad, CA), was given to each mouse and blood glucose measurements were obtained at 30, 60, 90, 120, and 240 minutes post injection. Glucose tolerance tests were compared by calculating the integrated area under the curve (AUC) of the blood glucose level for the 240 minutes of the test.

3.7.5 Protein quantification using western blotting

Western blotting was carried out using standard techniques, as described previously.(Bal et al., 2012) Protein concentrations of total homogenate were determined by the Bradford method and equivalent amounts of protein were loaded in each lane of the polyacrylamide gel. After electrophoresis, proteins were transferred to 0.45 µM
nitrocellulose membranes and stained with Ponceau stain (0.1% w/v Ponceau Red in 5% acetic acid) to visualize protein loading. Membranes were washed with Tris-buffered saline containing 0.5% Tween-20 (TBS-T) and blocked for non-specific binding (RT, 2 hrs, 5% (w/v) non-fat milk in Tris-buffered saline containing 0.5% Tween-20 (TBS-T)). Membranes were probed with primary antibodies for either 3 hours at RT or overnight at 4ºC, at the following dilutions: (SERCA1a (1:5000, custom-made antibody), SERCA2a (1:5000, custom-made antibody), alpha-actin (1:25,000), CSQ (1:5000), phospho-Akt (1:2000, pSer473, Clone D9E XP®, Cell Signaling Technology, Danvers, MA), Akt (1:2000), MuRF-1 (1:1000, AP2041, ECM Biosciences, Versailles, KY), Atrogin-1 (1:1000, MP3401, ECM Biosciences), p4EBP-1 (1:5000, pThr37/46, Clone 236B4, Cell Signaling Technology), p70S6K (1:5000, pThr389, Clone 108D2, Cell Signaling Technology), pS6 (1:5000, pSer235/236, Clone D57.2.2E XP®, Cell Signaling Technology), phospho-mTOR (1:2000, 2971S, pSer2448, Cell Signaling Technology), pFOXO1 (1:1000, pSer256, Cell Signaling Technology, anti-pan ubiquitin (1:1000, Clone FK2, Cell Signaling Technology), and anti-poly-ubiquitin, Lys48-Specific (1:5000, Clone Apu2, Millipore, Temecula, CA)). After washing with 0.05% TBS-T, blots were probed with the appropriate HRP-linked secondary antibody for 1 hour at RT, and then washed again with 0.05% TBS-T, developed with enhanced chemiluminescent substrate (SuperSignal West Dura Chemiluminescent Substrate, Thermo Fisher Scientific, Rockport, IL), and exposed to film. Blots were scanned (HP Imaging) and relative protein quantitation were performed by densitometry using the middle third of each band,
normalizing by the relative intensity of the loading control bands (alpha-actin or ponceau stain), using Image J analysis software (NIH). (Rasband, 1997)

3.8 Results

3.8.1 Decreased insulin-dependent phosphorylation of regulators of protein synthesis in db/db muscle

To better understand the mechanisms causing decreased muscle size in the db/db model of T2DM, insulin-dependent regulation of protein synthetic pathways was assessed. A single intraperitoneal injection of insulin in overnight-fasted mice acutely increased phosphorylation of proteins key to activating protein synthesis (Akt, mTOR, 4EBP-1, p70S6K, and S6 proteins). (Figure 3.3) Basal levels of phosphorylation of these proteins was generally slightly greater in WT mice, with the exception of 4EBP-1, which demonstrated greater basal phosphorylation in db/db muscle compared to muscle from WT mice (Figure 3.4). Upon stimulation with insulin, WT mice displayed a robust increase in phosphorylation of these protein synthetic pathways in tibialis anterior, diaphragm, and ventricular muscle, with the exception of mTOR in the diaphragm. In db/db mice, insulin-dependent phosphorylation of these regulators of protein synthesis, although evident, was markedly reduced in slow/mixed (diaphragm), fast (tibialis anterior), and cardiac muscle. (Figure 3.3) This data expands upon previous literature (Shao et al., 2000) and demonstrates that insulin-dependent activation of pathways regulating protein synthesis is blunted in muscle of adult db/db mice.
3.8.2 Increased accumulation of polyubiquitinated proteins in muscle from
young and old db/db mice

We next studied the role of proteasomal protein degradation in decreased muscle
size and function in db/db mice. Previous literature has shown protein degradation rates
and 26S proteasome function to be elevated in muscle from 9 week old db/db
mice. (Wang et al., 2006) We examined how increased proteasomal protein degradation,
as observed by accumulation of polyubiquitinated proteins (Wing et al., 1995), progresses
with decreased muscle size and function in prediabetic and overtly diabetic mice.
Western blotting of whole muscle homogenates with an anti-k48-polyubiquitin specific
antibody revealed a clear increase in the overall quantity of polyubiquitin-conjugated
proteins over the observed range of 25-250 kD in db/db muscle. Accumulation of
polyubiquitinated proteins was already significantly increased in fast, slow, and cardiac
muscles from prediabetic db/db mice (gastrocnemius, soleus, and ventricle) and further
increased in these muscles from the overtly diabetic db/db mice (Figure 3.4 A-C).

3.8.3 Decreased insulin-dependent FOXO1 activation and increased
expression of MuRF-1, Atrogin-1 in db/db muscle

MuRF-1 and Atrogin-1 are well-studied E3 ubiquitin ligases which catalyze a rate
limiting step of protein ubiquitination which labels the substrate protein for subsequent
proteasomal protein degradation. Of the large family of E3 ligases, MuRF-1 and Atrogin-
1 are notable for their role in being associated with and partially necessary for many
types of muscle atrophy. (Bodine et al., 2001) Protein levels of E3 ubiquitin ligases Murf-
1 and Atrogin-1 were clearly increased in fast (tibialis anterior), slow/mixed (diaphragm),
and cardiac muscle from 12 week old db/db mice compared to age-matched WT mice (Figure 3.4F).

FOXO family signaling promotes transcription of Atrogin-1 and MuRF-1. Insulin-dependent phosphorylation of FOXO1 is associated with deactivation of transcriptional activity by translocation of FOXO1 out of the nucleus. We found that insulin-dependent phosphorylation of FOXO1 was blunted in the tibialis anterior, diaphragm, and the cardiac muscles of 12 week old db/db mice compared to WT controls (Figure 3.4D).

3.8.4 Increased accumulation of polyubiquitininated proteins is not found in muscle from TallyHo and High Fat Diet-Fed (HFD) models of insulin resistant diabetes

To test if the accumulation of polyubiquitininated proteins is a feature common to diabetes in general, or just specific to db/db mice, we tested two additional models of insulin resistant diabetes. (Figure 3.4E) We found that the levels of polyubiquitininated proteins were not significantly different in TallyHo with overt diabetes (24wo) compared to TallyHo mice before the onset of diabetes (9wo). In addition, no change in accumulation of polyubiquitininated proteins was evident in the HFD-fed obese mice compared to lean controls. These findings suggest that increased muscle accumulation of polyubiquitininated proteins is not a common feature to all models of insulin resistance or diabetes mellitus.
3.8.5 Chronic exercise reduces levels of ubiquitinated proteins and increases Akt phosphorylation in db/db mice

On the biochemical level, chronic forced treadmill exercise for 8 weeks was sufficient to reduce accumulation of polyubiquitinated proteins in db/db mice to levels similar to those seen in WT sedentary controls (Figure 3.5A). This exercise rescue of ubiquitination was accompanied by a reduction in Atrogin-1 and MuRF-1 protein levels (Figure 3.5B). Although total Akt protein levels are decreased in db/db mice, exercise did not rescue the Akt protein levels, but did increase the level of Akt phosphorylation (Figure 3.5C).

3.8.6 Proteasomal inhibition affects glucose tolerance

Inhibition of the proteasome with MG-132 was sufficient to mildly improve glucose tolerance in WT mice (54445±4198 WT saline vs. 4267±2800 WT MG-132, AUC mg x min/dL, p<0.05) (Figure 3.6). However, MG-132 treatment of db/db mice worsened glucose tolerance (94661±12154 db/db saline vs. 130323±5696 db/db MG-132, AUC mg x min/dL, p<0.05). In addition, mean starting blood glucose of MG-132-treated db/db mice appeared increased compared to saline-treated db/db but the difference was not statistically significant due to high inter-animal variability. MG-132 pretreatment had no effect on starting WT glucose levels. (Figure 3.6) In the week following this test, none of the WT mice or saline treated db/db mice died, but 2 of the 4 MG-132-treated db/db mice spontaneously died 2-4 days, suggesting increased MG-132 toxicity in db/db mice.
3.9 Discussion

3.9.1 Insulin-dependent muscle protein synthesis is decreased in db/db mice

Muscle atrophy is generally caused by decreased protein synthesis and/or increased protein degradation, causing net protein loss. (Jackman and Kandarian, 2004) Several human studies indicate that in diabetes, while meal-induced protein synthesis is normal, protein degradation is increased and drives net protein loss, although the findings are inconsistent (Type 1 diabetes (Dice et al., 1978; Pain et al., 1983; Price et al., 1996; Smith et al., 1989) and type 2 diabetes (Bell et al., 2006; Wang et al., 2006)). To understand the basis for decreased muscle size in db/db mice, we studied biochemical markers of protein synthesis and proteasomal degradation. In the 12 week old db/db mouse, we found that acute insulin-dependent phosphorylation of key regulators of protein synthesis (Akt, mTOR, p70S6K, S6, 4EBP-1) was blunted universally in slow, mixed, fast, and cardiac muscle types (Figure 3.3). This resistance to insulin-stimulated phosphorylation indicates that decreased insulin-dependent protein synthesis may contribute to decreased muscle size in the db/db mouse. These insulin-induced phosphorylation results may differ from human studies (Manders et al., 2008) measuring meal-induced protein synthesis because the use of carbohydrate or protein infusion as the stimulus allows for compensatory hyperinsulinemia in insulin resistant individuals that may be sufficient to normalize protein synthesis. In contrast, the direct intraperitoneal administration of insulin studied here in db/db mice compares responses to equivalent increases in insulin.
3.9.2 Markers of protein degradation are increased in db/db mice but not TallyHo or HFD-fed mice

The rate of muscle protein degradation is increased in db/db mice, as measured previously by *ex vivo* tyrosine release (Wang et al., 2006). This finding is supported by our observations in db/db mice of decreased insulin-dependent FOXO1 phosphorylation, increased atrophy-associated E3 ubiquitin ligase expression (i.e. MuRF-1, Atrogin-1) and increased accumulation of K48-polyubiquitin-conjugated proteins (Figure 3.4). Wang et al. (2006) observed increased proteasomal chymotrypic-like peptidase function in muscle from 9 week old db/db mice, suggesting that the increase in ubiquitin-conjugated protein is caused by an increased rate of ubiquitination, rather than by decreased proteasomal function. Moreover, accumulation of ubiquitin-conjugated proteins has been closely linked with increased muscle protein degradation rates during starvation and denervation muscle atrophy. (Wing et al., 1995) This increase of ubiquitinated proteins was already evident in slow, fast, and cardiac muscle from young db/db mice with prediabetes and further increased in older db/db mice with overt diabetes, suggesting that increased muscle protein degradation is a global db/db muscle phenomenon and contributes to the poor muscle growth present in skeletal muscle from db/db mice.

Increased accumulation of ubiquitinated proteins was not evident in gastrocnemius muscle of diet-induced obese mice or Tallyho mice, indicating that insulin resistance and overt type 2 diabetes are not sufficient to cause accumulation of ubiquitinated proteins in mice. Chronic exercise was sufficient to reduce MuRF-1,
Atrogin-1, and accumulation of ubiquitinated proteins in the muscle of db/db mice. This reduction in markers of protein degradation may be due to increased Akt Ser473 phosphorylation (Figure 3.5C) and activity, which may reflect increased insulin sensitivity or increased insulin or growth hormone signaling in response to exercise.

3.9.3 Factors other than insulin resistance likely contribute to accelerated sarcopenia in diabetes

Our data strongly suggests that insulin resistance prevents normal insulin-dependent activation of protein synthesis and suppression of protein degradation. Because type 2 diabetes mellitus is characterized by insulin resistance, it is tempting to assign sole pathophysiological blame for accelerated sarcopenia in diabetes to suppressed insulin-dependent growth pathways. However, it is unlikely that insulin resistance is acting alone; db/db, TallyHo, and HFD mice are all insulin resistant, but only the db/db mouse demonstrated decreased muscle size in our experiments.

Type 2 diabetes is also associated with disruptions of many other hormonal and cytokine signaling pathways that have a powerful effect on regulating muscle growth. These insulin resistance associated changes include increased circulating glucocorticoids (Filipovský et al., 1996; Reynolds and Walker, 2003; Rosmond et al., 1998; Stolk et al., 1996), decreased testosterone (Alibhai et al., 2009, 2009; Ding et al., 2006; Yialamas et al., 2007), increased serum myostatin (Allen et al., 2008, 2010; Hittel et al., 2009, 2010; Jespersen et al., 2011), decreases in growth hormone/IGF-1 signaling (Teppala and Shankar, 2010; Yalow et al., 1965), increased obesity, increased inflammatory cytokines, peripheral neuropathy, decreased physical activity, decreased
peripheral vasodilation, lipotoxicity, and altered diets. Due to these many diabetes-associated disruptions, it is difficult to ascertain whether poor muscle health in type 2 diabetes is the primary cause of insulin resistance, or a direct effect of insulin resistance. Alternatively, sarcopenia in diabetes could be a secondary or tertiary effect of insulin resistance.
**Figure 3.1**– Insulin signaling promotes protein synthesis and inhibits protein degradation. Insulin resistance prevents this hypertrophic signaling. (+ = activates downstream pathway, - = suppresses downstream pathway)
Figure 3.2 – Timeline of MG-132 administration and fasting to test the effects of proteasome inhibition on glucose tolerance.
Figure 3.3 – Reduced insulin-dependent phosphorylation of regulators of protein synthesis in muscle from db/db mice – (A) Fast (tibialis anterior), (B) slow (diaphragm), and (C) cardiac muscle from 12 week old db/db mice with overt diabetes exhibit a reduced response to a single \textit{in vivo} bolus injection of insulin (0.2 U/g body weight, recombinant human insulin, intraperitoneal, 10 minutes) compared to lean WT controls, as evident in decreased insulin-dependent phosphorylation of key promoters of protein synthesis (Akt, mTOR, 4EBP1, p70S6K, and S6). Skeletal alpha-actin and the ponceau stain for cardiac tissue are loading controls.
Figure 3.4 – Markers of protein degradation are increased in db/db mice and increase in concert with the diabetes progression but are not increased in TallyHo and HFD mice

Levels of ubiquitin-conjugated proteins in muscle increase progressively in young and older db/db muscle compared to age-matched WT; this trend is evident in muscle that is predominantly (A) fast/mixed twitch (gastrocnemius), (B) slow (soleus), and (C) cardiac. (D) Db/db muscle is resistant to insulin-dependent phosphorylation (associated with deactivation) of the transcription factor FOXO1. Among the targets of FOXO1 transcriptional activation are MuRF-1 and Atrogin-1 E3 ubiquitin ligases which have (F) increased protein expression in fast and slow 12 week old db/db skeletal muscle compared to age-matched WT controls. (E) Accumulation of ubiquitin-conjugated proteins is not increased with onset of the diabetes phenotype in TallyHo mice or HFD-induced obese mice.
Figure 3.5 Exercise reduces markers of protein degradation and increases pAkt in db/db mice

Chronic exercise was sufficient to reduce (A) K48 polyubiquitinated protein levels, (B) Atrogin-1, and MuRF-1 levels. (C) Total Akt levels are decreased in db/db mice compared to WT mice, and although exercise does not rescue total Akt levels, activating phosphorylation of Akt (pSer473) is increased by exercise. (Error bars = Standard error of mean, * = p≤0.05 compared to WT)
Figure 3.6 – Proteasomal Inhibition with MG-132 alters glucose tolerance in WT and db/db mice Glucose tolerance was improved by proteasome inhibition in db/db mice, as demonstrated by decreased area under the curve (AUC). Wild-type mice showed a decrease in peak glucose level, but no significant difference in total AUC. (Error bars = Standard error of mean, * = p ≤ 0.05 compared to saline-treated matched genotype)
Chapter 4: Discussion

The purpose of this final chapter is to discuss our research findings in context of the current literature on diabetes and muscle growth/atrophy. I will also address the limitations of our research and suggest future directions that our findings indicate.

4.1 Onset and progression of muscle loss in T2DM

4.1.1 Current understanding

Longitudinal clinical research studies in the elderly have convincingly demonstrated that average muscle mass is lower in elderly individuals with type 2 diabetes compared to individuals without diabetes (Park et al., 2006), the rate of loss of muscle mass is greater in subjects with T2DM than those without T2DM (Park et al., 2007), and diabetes is associated with increased risk for falls, fractures, and disability (to which decreased muscle function may contribute) (Gregg et al., 2000; Maurer et al., 2005; Strotmeyer ES, 2005).

4.1.2 Gaps in knowledge

When does diabetes begin to affect skeletal muscle? The effects of T2DM on muscle have been studied almost exclusively in the elderly (60-80 year old individuals) and that have pre-existing overt T2DM. It is unknown at what time point muscle size in individuals with diabetes diverges from individuals without diabetes. Two human studies
suggest that conditions that precede diabetes may affect muscle growth and maintenance. The first study was a small cross-sectional study which showed that this phenomenon exists in young adults (ages 18-35 yo) (Unni et al., 2009). The second study was a large longitudinal study in which muscle mass was followed for 6 years in a large elderly population which included many individuals with T2DM. In this study, individuals that were already diagnosed with diabetes at the beginning of the study experienced accelerated muscle loss, compared to those without diabetes.(Park et al., 2007) Individuals who did not have diabetes at the start of the study, but were newly diagnosed with T2DM at the end of the study, were found to have lost muscle mass at a much greater rate than those with previously diagnosed diabetes. This suggests that either treatment of diabetes in the previously diagnosed group ameliorates muscle loss in T2DM or prediabetes or early diabetes may be associated with especially rapid muscle loss. Thus, it is unknown if muscle loss is a precondition that contributes to diabetes, associated with prediabetes, or only occurs in consequence to severe chronic T2DM. Most major comorbidities of T2DM are associated with both longer duration of diabetes and lack of glucose control; the general assumption for muscle loss in T2DM has been that diabetes precedes and causes muscle loss.

4.1.3 Insight provided by our research

Contrary to this dogma that diabetes precedes and causes the onset of muscle atrophy (Wang et al., 2006), we found that muscle mass in the db/db mouse model of T2DM did not exhibit longitudinal muscle loss, but rather exhibits a juvenile onset of severely decreased rate of muscle growth. Upon correlating our findings with the
literature, our data is supported by previous reports that the ob/ob mouse (leptin deficient), which exhibits a similar metabolic phenotype to the db/db mouse, exhibits decreased muscle mass at 2-3 weeks of age compared to lean controls (Purchas et al., 1985). This suggests that the juvenile onset of insulin resistance found in db/db mice (onset of insulin resistance at 7-10 days of age, (Coleman and Hummel, 1974)) and rapid progression to severe insulin resistance and T2DM by 8 weeks of age creates an environment incompatible with normal muscle growth in these mice (Chick et al., 1970).

If the db/db mouse is an accurate model of how diabetes affects muscle, these findings suggest that chronic overt diabetes and associated comorbidities of uncontrolled diabetes (such as diabetic peripheral neuropathy (Sima and Robertson, 1978) and diabetic nephropathy (Chow et al., 2004)), are not necessary for decreased muscle weight and function. If the db/db muscle phenotype is representative of muscle loss in human diabetes, this early decreased muscle size would suggest that insulin resistance and/or prediabetes may be sufficient to cause muscle atrophy prior to the onset of overt T2DM.

Our understanding is further advanced and complicated by our finding that TallyHo mice, despite severe T2DM, do not demonstrate significant loss of muscle weight or exercise capacity within the first 6 months of life. This suggests that insulin resistance and severe type 2 diabetes alone are insufficient alone to consistently cause marked muscle loss in mice. It is unclear if this difference in phenotype is due to the juvenile onset of T2DM in the db/db mouse versus the mature onset of T2DM in the TallyHo mouse, or due to differences in the genetic background and etiology of T2DM. If the TallyHo mouse is representative of human T2DM, these findings do suggest that
growth pathways, other than insulin signaling, are dominant in determining if diabetes will cause muscle loss or inhibit muscle growth.

### 4.1.4 Limitations

The value of animals studies in T2DM lies in the ability to extrapolate animal findings to probe a disease mechanism without risking human lives, better understand human disease by careful study of disease pathogenesis in animals models, and testing novel interventions before testing in humans (Cefalu, 2006). However, all animal studies are limited by how closely the disease state in animals correlates with human disease (van der Worp et al., 2010).

The human implications of the data we generated with the mouse models of diabetes are limited for several reasons. First, unlike most humans with T2DM, the etiology of diabetes for the db/db and TallyHo mouse models is purely genetic. The cause of human T2DM is rarely monogenic and typically has both genetic (~30-70%) and environmental (30-70%) etiological components, with a complex interaction of environment and multiple genes contributing (Doria et al., 2008). This dissimilarity in etiology is likely the reason that research findings in rodent models of type 2 diabetes have struggled to achieve broad applicability to human T2DM. For example, although the spontaneous discovery of severe obesity and T2DM in leptin signaling mutant mice was once heralded as a clear mechanism for diabetes, exhaustive studies have shown that leptin signaling mutations in humans are very rare, and have been only found in a few families worldwide (Clément et al., 1998; Fischer-Posovszky et al., 2010; Hummel et al., 1966; Ingalls et al., 1950; Montague et al., 1997). Thus, despite our efforts to utilize
mouse models that best represent the typical human metabolic phenotype of T2DM, because of the non-humanoid etiologies of diabetes, it is difficult to accurately extrapolate the findings of these mouse models to human diabetes.

Second, the ages of onset of T2DM in the db/db, TallyHo, and HFD mouse models are relatively younger than the typical onset of T2DM in humans. These mouse models develop overt T2DM during the first quartile of their 2 year lifespan: ~2 months of age (db/db), ~3 months of age (HFD), or 4 months of age (TallyHo). Conversely, in humans, the onset of T2DM usually occurs after the age of 40 (mean 65-75yo), which is during the the second half of the life span of humans (Laakso and Pyörälä, 1985). The effect of this age discrepancy on data interpretation becomes most evident in the ob/ob (leptin deficient) and db/db (leptin receptor deficient) mouse models, as the db/db mice are insulin resistant as young as 1 week old, and ob/ob muscle mass is decreased compared to WT at 2-3 weeks of age (Purchas et al., 1985). Thus, the db/db muscle data may possibly best represent the effect of T2DM on neonatal or adolescent muscle growth; given that pediatric T2DM accounts for a small proportion of T2DM, it is difficult to determine how well the pattern and mechanism of muscle loss in db/db mice describes adult human muscle loss.

Third, the data suggests that db/db and ob/ob mice exhibit reduced muscle size, whereas most other models of T2DM exhibit near normal growth patterns. The presence of a muscle phenotype principally in models of ablated leptin signaling suggests that leptin signaling plays a key role in determining muscle growth. This is supported by the observation that although the ob/ob and db/db models are severe models of obesity and
diabetes, other models, such as the TallyHo model, are also obese and severely hyperglycemic, but do not exhibit a drastic reduction in skeletal muscle size/function. Supportin evidence from the literature indicates leptin treatment of ob/ob mice is sufficient to decrease protein degradation and improve the muscle phenotype. (Sáinz et al., 2009) What remains unclear is that when leptin signaling is rescued in ob/ob mice, the metabolism of the whole body improves, making it difficult to ascertain the primary cause for improved muscle growth observed in this leptin rescue experiment by Sainz et al.

Finally, due to the limited duration of our longitudinal study (up to 4 months and 6 months of age for db/db and TallyHo mice, respectively), we cannot rule out the possibility that eventual true muscle loss would have been seen in these mouse models.

4.1.5 Future experiments

Given how poorly understood the course of muscle loss in T2DM is in humans, it is difficult to confidently extrapolate findings in mouse models of T2DM and assert that they are representative of the human disease. However, our research challenges the current thinking that muscle loss and dysfunction is only a consequence of chronic T2DM and suggests that further human-based research must be done to establish some of these basic characteristics of the disease: When does altered muscle growth and/or atrophy begin in relation to the onset of prediabetes and T2DM? Could muscle loss be a contributor to the development of insulin resistance? Is muscle loss attenuated by strict glucose control (e.g., HbA1C < 7.0%)? What are the major independent variables (e.g., obesity, muscle use, smoking) that are associated with muscle loss in T2DM? Answering
these basic questions would allow for more timely clinical diagnosis and possibly earlier
treatment to prevent progression of both T2DM and muscle loss. Moreover, addressing
these questions would allow the translational scientist to identify specific models of
T2DM that are representative of muscle loss in humans with T2DM. Greater confidence
in the accuracy of the models of T2DM muscle loss would facilitate study of molecular
mechanisms and therapeutic development for this condition.

Further research is also necessary to determine how T2DM in children and adolescents
may impact muscle growth, exercise capacity, and muscle adaptation to exercise.

Whereas previously, only 1-2% of children with diabetes had T2DM, recent reports
indicate that 8-45% of children with newly diagnosed diabetes have T2DM, depending
on the study population (American Diabetes Association, 2000; Fagot-Campagna et al.,
2000; Hotu et al., 2004). Both the increasing prevalence of T2DM among adolescents
and the positive correlation between insulin sensitivity and muscle mass in young adults
(18-35 yo) support the importance of future studies of muscle growth, function, and
metabolism in youth with T2DM (Unni et al., 2009).

4.2 Muscle protein synthesis and
degradation in type 2 diabetes

4.2.1 Current Knowledge

The mechanisms responsible for accelerated muscle loss and altered muscle
growth in individuals with T2DM are unclear. Disturbances in many anabolic and
catabolic signaling pathways are associated with diabetes (e.g., testosterone, myostatin,
IGF-1, growth hormone, insulin, and TNF-α), although different individuals present with
different combinations of these alterations.
The clearest evidence of specific upstream mechanisms causing muscle loss in T2DM actually comes from the streptozotocin-induced T1DM mouse; rapid muscle loss occurs upon induction of insulin deficiency with streptozotocin treatment (toxic to pancreatic islets). In these T1DM mice, muscle loss is prevented and reversed by exogenous insulin administration, indicating a powerful role of insulin signaling in muscle loss. Moreover, removal of glucocorticoids (by surgical ablation of the adrenal glands) is sufficient to prevent muscle loss in these T1DM mice. These findings suggest that insulin signaling is sufficient to prevent T1DM muscle loss and that elevated glucocorticoids are necessary for muscle loss in T1DM.

From the reductionist approach focused on net protein balance, several human and animal studies show that protein degradation rates are increased in skeletal muscle from individuals with T2DM, compared to individuals without diabetes. Proteasomal degradation is the dominant type of protein degradation in T1DM mice. Ubiquitin E3 ligase expression (MuRF-1 and Atrogin-1) and proteasome function are increased in T2DM db/db mice, compared to lean WT controls, suggesting that the proteasome also likely plays a large role in T2DM protein degradation.

Amino acid and carbohydrate-induced protein synthesis rates are similar in humans with and without T2DM(Bell et al., 2006; Manders et al., 2008). However, it is unclear if basal (i.e. non fed-state) or total protein synthesis rates are changed in T2DM. In other words, measuring only protein synthesis during a fed state creates an incomplete picture of net daily protein synthesis. There is abundant evidence that insulin-dependent activation of Akt (by phosphorylation) is blunted in muscle from mice with T2DM; Akt
is a nodal point in both glucose metabolism and protein synthesis. Therefore, it is logical that reduced Akt activation could decrease protein synthesis.

4.2.2 Gaps in Knowledge

In terms of known mechanisms of muscle loss in T2DM, beyond increased muscle protein degradation and proteasome activity, little is known about how T2DM accelerates muscle loss. The findings in T1DM provide insight but cannot be applied directly to T2DM mice for several reasons. For example, T1DM and T2DM have very different etiological mechanisms, comorbidities, and consequences of disease. Moreover, although insulin treatment in T1DM is sufficient to essentially reverse the muscle phenotype; it is plausible that the insulin rescue of muscle in T1DM is not directly due to insulin signaling but rather to the improved metabolic phenotype. Finally, although adrenal gland ablation prevents muscle atrophy in T1DM mice, and it would be informative to attempt the same in T2DM, this experiment would still not be very specific; adrenal ablation eliminates synthesis of glucocorticoids, mineralcorticoids, and several sex hormones.

Thus, the gaps in knowledge in our understanding of the mechanism of T2DM include the following questions:

- Does insulin resistance directly cause muscle loss or poor muscle growth?
- Is insulin-dependent protein synthesis decreased in T2DM muscle?
- Is protein degradation altered in T2DM muscle?
- How do protein degradation and synthesis abnormalities relate to the development of metabolic and muscle phenotypes of diabetes?
• How does T2DM affect different types of muscles?
• What upstream pathways are necessary and/or sufficient to cause poor muscle growth and/or atrophy in T2DM?

4.2.3 Insights from our research

4.2.3.1 What does the age of onset of T2DM and muscle loss indicate about the pathophysiology of muscle loss in diabetes?

A key finding in db/db mice was that reduced skeletal muscle size and running ability is already present at 5 weeks of age, before the onset of chronic hyperglycemia (Figure 1). This indicates that many of the conditions and signaling pathways associated with overt diabetes, chronic hyperglycemia, and comorbidities which accompany chronic diabetes (such as diabetic peripheral neuropathy (Sima and Robertson, 1978), diabetic nephropathy (Chow et al., 2004), chronic atherosclerosis, absolute insulin deficiency, and accumulation of advanced glycation end-products (AGE) ) are not necessary for decreased muscle weight and function in this model. Moreover, if the db/db muscle phenotype is representative of muscle loss in human diabetes, this early decreased muscle size would suggest that insulin resistance alone or other components of the prediabetes may be sufficient to cause muscle atrophy prior to the onset of overt T2DM.

To see if our findings would be consistent in a leptin-intact adult onset model of T2DM, we also studied the polygenic mature-onset T2DM TallyHo mouse model. The TallyHo mouse is a model of severely hyperglycemic type 2 diabetes with intact leptin signaling, insulin resistance, and deficient muscle-specific glucose uptake. (Kim et al.,
We were surprised to find no indication of muscle loss in the TallyHo mice; muscle weight increased with age (Table 1) and exercise capacity was maintained (Figure 2) when comparing 2 month old (have prediabetes) and 6 month old (have overt diabetes) mice. Although we cannot exclude that muscle mass and function could be affected in older TallyHo mice, the lack of observed muscle loss in TallyHo mice within the first 6 months of life suggests that insulin resistance and chronic hyperglycemia alone are insufficient to cause rapid muscle loss in mice. Moreover, the observed differences between the two models suggest that additional factors such as age of onset of diabetes, poor leptin signaling, degree of hyperinsulinemia, genetic strain, and differing diabetes-associated comorbidities may dictate the effects of diabetes on muscle growth and maintenance.

4.2.3.2 Insulin-dependent protein synthesis is severely blunted in db/db mice

To better understand the basis for decreased muscle size in db/db mice, we studied biochemical markers of protein synthesis. Our findings of blunted insulin-dependent phosphorylation of regulators of protein synthesis (Akt, mTOR, p70S6K, S6, 4EBP-1) in slow, mixed, fast, and cardiac muscle types of 12 week old db/db mice suggest that insulin resistance may decrease insulin-dependent protein synthesis and contribute to decreased muscle size (Figure 4). These findings may differ from human studies measuring meal-induced protein synthesis because the the human studies used carbohydrate or protein ingestion to induce protein synthesis, which allows for compensatory hyperinsulinemia; this hyperinsulinemia, while insufficient to normalize blood glucose levels, may be sufficient to normalize protein synthesis in insulin resistant
individuals. (Bell et al., 2006; Manders et al., 2008) In contrast, our studies used direct intraperitoneal administration of insulin, comparing responses of WT and db/db mice to equivalent increases in insulin. Thus, our findings suggest that the ratio of serum insulin to insulin-dependent protein synthesis is decreased in T2DM muscle.

4.2.3.3 Markers of protein degradation in insulin resistant mice

Previous studies in db/db mice showed that the rate of muscle protein degradation is increased, as measured by ex vivo tyrosine release (Wang et al., 2006). This finding is further supported by our observations in db/db mice of decreased insulin-dependent FOXO1 phosphorylation, increased atrophy-associated E3 ubiquitin ligase expression (i.e. MuRF-1, Atrogin-1) and increased accumulation of K48-polyubiquitin-conjugated proteins (Figure 5). Wang et al. (Wang et al., 2006) observed increased proteasomal chymotrypic-like peptidase function in muscle from 9 week old db/db mice, suggesting that the increase we observed in ubiquitin-conjugated protein is caused by an increased rate of ubiquitination, rather than by decreased proteasomal function. Moreover, accumulation of ubiquitin-conjugated proteins has been closely linked with increased muscle protein degradation rates during starvation and denervation muscle atrophy. (Wing et al., 1995) This increase of ubiquitinated proteins was already evident in slow, fast, and cardiac muscle from young db/db mice with prediabetes and further increased in db/db mice with overt diabetes, suggesting that increased muscle protein degradation is a global db/db muscle phenomenon and contributes to the poor muscle growth present in skeletal muscle from db/db mice. On the other hand, accumulation of ubiquitinated proteins was not evident in gastrocnemius muscle of HFD fed mice or
Tallyho mice, suggesting that factors other than insulin resistance and chronic hyperglycemia may be responsible for increased levels of ubiquitinated proteins.

In summary, our data suggests that increased protein degradation and decreased protein synthesis both contribute to poor muscle growth in db/db mice. However, markers of protein degradation were not increased in TallyHo mice or HFD-fed mice, which correlates with the absence of decreased muscle size in these mice. The early onset of decreased muscle size in db/db mice and lack of muscle atrophy in TallyHo and db/db mice suggest that overt diabetes and chronic hyperglycemia are not necessary nor sufficient to cause muscle atrophy in T2DM.

**4.2.4 Limitations**

The reduced muscle mass seen in ob/ob and db/db mice appears to be due to a failure of physiologic hypertrophy, rather than atrophy of a fully developed muscle, thus it is unknown if our studies of protein synthesis and degradation represent the response of human muscle to T2DM. Although this diminished muscle growth could be due to the altered metabolic milieu caused by early insulin resistance (db/db have increased serum insulin levels by ~10 days of age(Coleman and Hummel, 1974)), it could also be caused by the combined effects of leptin(Hamrick et al., 2010; Sáinz et al., 2009), lipotoxicity(Turpin et al., 2006, 2009), or deficient growth hormone signaling on skeletal muscle growth and metabolism.

Our findings are also limited because we only measured protein synthesis and degradation indirectly – by looking at protein markers strongly associated with these processes. Thus, without direct measurement of protein synthesis rates and degradation
rates under different conditions, it is uncertain how closely actual synthesis and degradation rates correlate with the observed changes in protein biochemistry. Additional studies using radioisotopes to study rates of muscle protein synthesis and degradation *in vivo* in different states (e.g., acute lipid infusion in healthy individual, fed and fasted states of individuals with prediabetes, fed and fasted states of individuals with recent diagnosis of T2DM, fed and fasted states of individuals with poorly controlled chronic T2DM, young and old individuals with T2DM, acute exercise in individuals with T2DM, and chronic exercise in individuals with T2DM) would be useful to accurately characterize how diabetes affects muscle maintenance and growth.

### 4.2.5 Future Directions

Through studying these various animal models, I have come to the conclusion that it is best to study diseases in human tissue whenever possible when trying to define basic characteristics of a disease such as onset, natural progression, and associated pathways. In a disease like T2DM with huge populations, gradual onset of disease, and a persistent chronic phase, it would be informative and practical to study the mechanism and course of muscle loss in human subjects. It would be ideal to study and compare basal, fed-state, and insulin-induced protein synthesis and protein degradation rates using radioisotope labeling approaches in human subjects that are insulin-sensitive, have prediabetes, early diabetes, and chronic diabetes. In parallel with these studies, serum samples and muscle biopsies should be obtained to study how the radioisotope labeling correlates with activation of specific protein synthesis-related signaling pathways (e.g., Akt, mTOR, p70S6K, eIF4E). Moreover, additional study of the different types of
protein degradation in these muscle samples could help determine if proteasomal
degradation is truly the dominant form of degradation in T2DM, and how
autophagy/lysosomal, calpain-mediated, and caspase-mediated protein degradation are
changed in these human muscle samples. These studies would clearly delineate which
downstream pathways are truly associated with muscle loss in T2DM, and when
chronologically they are important. This would facilitate appropriately focusing of
research effort and resources on the most relevant pathways in T2DM muscle and the
correct time frame for study and intervention in T2DM muscle loss.

4.3 Exercise in the treatment of muscle loss in type 2 diabetes mellitus

4.3.1 What is known
When muscle loss in patients interferes with physical function, chronic exercise is a
treatment commonly prescribed to increase muscle function, muscle mass, and to
improve glucose tolerance. Compliance with exercise recommendations correlates with
increased muscle glucose uptake and improved glucose tolerance.

4.3.2 Gap in knowledge
Although it is clear that exercise is beneficial to moderate blood glucose levels, it is
unclear how exercise impacts muscle loss in individuals with T2DM. For example, it is
possible that exercise-mediated muscle hypertrophy may be blunted in T2DM due to
either deficient energy stores or non-permissive signaling.
4.3.3 What our research contributes to the field

Although chronic exercise of db/db mice improved glucose tolerance, cardiac capacity (VO$_2$max), and running speeds, only a mild increase in final muscle weight was observed in exercised compared to non-exercised db/db mice, indicating that exercise is insufficient to fully rescue muscle growth in the db/db mouse model. This suggests that decreased muscle use (Figure 6A) is not the basis for the reduced muscle size of db/db mice. Despite the inability to completely rescue muscle growth, exercise was sufficient to increase basal Akt$^{\text{Ser473}}$ phosphorylation, and reduce both MuRF-1 expression and accumulation of polyubiquitinated proteins (Figures 6G-I). This suggests that decreased protein degradation and increased protein synthesis was responsible for the mild increase in muscle weight in the exercised db/db mice. Moreover, these findings support the current clinical use of exercise to improve glucose tolerance, muscle function, and exercise capacity. Our findings do however support the idea that muscle hypertrophy in response to exercise may be blunted in T2DM; previous studies employing similar interval treadmill training with WT mice have shown 10-15% increases in muscle mass in WT mice. Obviously, further research detailing exercise-dependent effects on fiber cross-sectional area and maximal force generation and fatigue resistance of muscle fibers must be done to validate this initial finding.

4.3.4 Limitations

Our study was limited by the depth of our functional experiments to test the muscular benefits of exercise, as well as by the models. True gains or loss of muscle
ability would be best measured directly using *in situ, ex vivo* intact muscle, and *ex vivo* permeabilized muscle studies.

The other major limitation of our study is the feasibility of exercise as an effective therapeutic. It is true that exercise is the most commonly prescribed therapy to treat muscle loss and sarcopenia. Indeed, when performed regularly, both resistance and endurance exercise training have proven to improve muscle fiber size and function, resulting in improvements in clinical measures of gait speed, ability to climb stairs, and levels of spontaneous activity. However, even the most health conscious individual is painfully aware of the physical discomfort, time constraints, financial costs, and mental challenges that oppose maintaining a regular exercise routine. (Chao et al., 2000) In general, these exercise-impeding factors become more prevalent among the elderly population. In addition, other medical conditions and musculoskeletal injuries that limit or prevent exercise are increasingly common among people with diabetes and/or advanced age. Indeed, less than 10% of sedentary adults begin an exercise program each year; of those adults participating in a regular exercise routine, 50% discontinue exercise each year. (Bouchard et al., 1990; Dishman et al., 1985). Thus, although regular exercise is fairly effective in preventing sarcopenia, it is apparent that, from the patient perspective, the cost to benefit ratio is insufficient to induce adequate patient compliance. Valuable research into increasing patient compliance with exercise programs is underway; however, additional strategies are necessary to ameliorate the impact of diabetes-accelerated sarcopenia.
4.3.5 Future Directions

Further research is needed in humans with type 2 diabetes to determine how endurance training and resistance training each affect protein synthesis, protein degradation, and muscle loss. It would be valuable as well to study which of the current diabetes therapies works best in conjunction with exercise to preserve or increase muscle mass and function.

In addition to exercise, additional strategies are needed to treat diabetic muscle loss. While muscle deterioration does not acutely threaten life, the chronic loss of muscle mass and function is life-threatening. The challenge is to identify treatment strategies that are low-risk yet effective. The most practical approach to identify new low-risk treatments is to focus research to understand how currently available, well characterized interventions may be utilized to reduce muscle loss in diabetes.

The search for candidate interventions should be focused around the principle that the loss of muscle mass in individuals with diabetes occurs when protein synthesis rates are less than protein degradation rates. Therefore, appropriate therapeutic interventions to limit or reverse muscle atrophy must either increase protein synthesis, decrease protein degradation, or both.(Greenlund and Nair, 2003) These interventions may occur in the form of nutrition, exercise, or pharmacologic intervention (Figure 4).

4.3.5.1 Diet

For sarcopenic individuals, standard dietary recommendations to improve net protein balance include increased protein intake or increased leucine intake. Leucine is
known to directly interact with mTOR signaling to permit mTOR activation and signaling for protein synthesis.

For individuals with T2DM, foods with reduced glycemic load are believed to help reduce the severity of glucose peaks following meals. In addition, for individuals with obesity-associated T2DM, caloric restriction and weight loss have been shown to be effective to increasing insulin sensitivity.

For individuals with muscle loss in T2DM, because of the high prevalence of kidney disease of diabetes, caution must be taken in ingesting high-protein diets to combat muscle loss. Amino acids in the blood are transferred in the renal tubules and active transport is used to remove amino acids from the tubules and back into the blood; increased blood amino acid levels increases metabolic stress on the kidneys. Further research must be done to determine the risk: benefit ratio of high-protein diet to sarcopenia associated with diabetes. It is feasible that a leucine-enriched normal-protein diet may be optimal to stimulate muscle protein synthesis in diabetes without putting excessive metabolic demands on the kidneys.

**4.3.5.2 Sex hormone therapy**

Hormone replacement therapy in individuals who are hypogonadal has been shown to be effective in increasing muscle mass and insulin sensitivity. Use of testosterone analogs to treat muscle loss is not routine in normogonadal individuals due to the many androgenic side effects of hypertestosteronism in these individuals. Development of testosterone analogs such as nandrolone decanoate, that are not metabolized into the highly androgenic dihydroxytestosterone, has allowed for promotion
of muscle growth while reducing the typical androgenic side effects of elevated testosterone. The efficacy of nandrolone in treating sarcopenia may be limited by the requirement of resistance exercise to accompany drug administration. This is demonstrated clearly in nandrolone treatment in aged rats, which increases muscle mass and strength only upon functional overload. Further work must be done to characterize the safety and efficacy of nandrolone treatment of individuals with muscle loss due to diabetes.

### 4.3.5.3 Chronic selective beta agonists

Beta adrenergic agonists have been shown to cause muscle hypertrophy. For example, clenbuterol has been shown to inhibit proteasomal protein degradation and cause muscle hypertrophy in mice. Clenbuterol treatment is sufficient to moderately attenuate hindlimb unweighting-induced muscle atrophy. (Yimlamai et al., 2005) Pilot clinical trials in patients with spinal and bulbar muscle atrophy demonstrated increased walking distance and forced vital capacity (Querin et al., 2013).

### 4.4 Summary

In summary, our studies in the db/db and TallyHo mouse models indicate that the effects of type 2 diabetes on muscle mass and function are model-dependent. The reduced muscle size in db/db mice before the onset of overt hyperglycemia and diabetes suggests that the db/db mouse model is an extreme model of diabetes in which the altered metabolic milieu caused by insulin resistance and defective leptin signaling may be toxic to early muscle growth. The lack of decreased muscle mass and exercise capacity in the
TallyHo mice suggests that insulin resistance and chronic hyperglycemia are insufficient to independently cause rapidly decreased muscle size and function. It is likely that additional factors such as genetic background, hyperinsulinemia, lipotoxicity, leptin signaling, age of onset, and comorbidities of diabetes, influence whether the metabolic milieu is toxic to or supportive of growth and maintenance of skeletal muscle in T2DM. Further research is needed to further define the relevant pathways responsible for muscle loss in T2DM and to identify feasible therapeutic approaches.
Figure 4.1 Treatment strategies for sarcopenia – Nutrition, exercise, and drug interventions are all potentially useful in preventing and treating muscle atrophy. The key is to identify which treatments are most effective within the context of specific types of atrophy.


Accelerated Loss of Skeletal Muscle Strength in Older Adults With Type 2 Diabetes The Health, Aging, and Body Composition Study. Diabetes Care 30, 1507–1512.


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